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Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells

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Opacity proteins (Opa) of *Neisseria gonorrhoeae*, a family of variant outer membrane proteins implicated in pathogenesis, are subject to phase variation. In strain MS11, 11 different *opa* gene alleles have been identified, the expression of which can be turned on and off independently. Using a reverse genetic approach, we demonstrate that a single Opa protein variant of strain MS11, Opa_{50}, enables gonococci to invade epithelial cells. The remaining variant Opa proteins show no, or very little, specificity for epithelial cells but instead confer interaction with human polymorphonuclear neutrophils (PMNs). Thus, depending on the *opa* allele expressed, gonococci are capable of invading epithelial cells or of interacting with human leukocytes. The respective properties of Opa proteins are maintained independent of the gonococcal strain; thus, the specificity for epithelial cells or leukocytes is intrinsic to Opa proteins. Significant homology exists in the surface exposed variable regions of two invasion supporting Opa proteins from independent strains. Efficient epithelial cell invasion is favoured by high level Opa production, however, a 10-fold reduction still allows significant invasion by gonococci. In contrast, recombinant *Escherichia coli* expressing Opa proteins adhered or invaded poorly under similar experimental conditions, thus indicating that additional factors besides Opa are required in the Opa-mediated interaction with human cells.

Key words: adherence/cellular invasion/gonococci/phagocytosis/phase and antigenic variation

Introduction

Bacterial pathogens can be distinguished based upon their preference for either extra- or intracellular life-styles (for review see Isberg, 1991). Bacteria of the first group colonize epithelial tissues by binding to matrix proteins or to inert cell receptors in contrast to invasive organisms which adhere to cellular receptors triggering directly or indirectly a phagocytotic uptake process. Once inside epithelial cells, different bacterial species again follow different routes: they may, for example, escape the phagocytic vacuole to multiply within the cytosol or remain within this vacuole and/or transcytose through the infected cell.

Given that each pathogenic species normally follows a predetermined route, *Neisseria gonorrhoeae*, the etiologic agent of gonorrhoea in humans, is quite unique in that individual bacteria can choose between alternative life-styles. The key to this phenomenon is the phase and structural variation of functionally important surface molecules (Robertson and Meyer, 1992). Early work of Ward *et al.* (1974) and McGee *et al.* (1981) using Fallopian tube organ cultures indicated that gonococci initially attach to epithelial cells probably via their pili and then adhere more tightly in order to penetrate and transcytose through a tight layer of epithelial cells. Recent investigations with cell cultures suggest that adherence to, and invasion of epithelial cells by gonococci require the co-operation of multiple factors. Structural variation of pili can dramatically influence the initial binding to epithelial cells (Lambden *et al.*, 1980; Virji *et al.*, 1991; Rudel *et al.*, 1992). For the subsequent tight interaction, pili appear to be obsolete and even inhibit the invasion process (Shaw and Falkow, 1988; Makino *et al.*, 1991a). Conversely, members of the Opa protein family, not required for the initial adherence step, must be expressed in order to allow the invasion of epithelial cells (Makino *et al.*, 1991a; Weel *et al.*, 1991). Thus, depending on the current array of surface parameters individual gonococci behave differently, at least with regard to their preferred extra- or intracellular environment.

The genetic mechanisms underlying gonococcal surface variability have been well characterized for a number of essential factors, including pilin (the major pilus subunit, PilE) and PilC (Jonsson *et al.*, 1991; for review see Robertson and Meyer, 1992). In the case of Opa proteins we have previously shown that a pentameric coding repeat (CR) sequence, which specifies the hydrophobic core regions of the inner membrane transport signals of the Opa pro-proteins, is variable in length (Stern *et al.*, 1986). Spontaneous variations in the number of CR repeat units, which occur independently of RecA (Belland *et al.*, 1989; Murphy *et al.*, 1989), determine the reading frame and thereby the expression phase of individual *opa* genes (Stern *et al.*, 1986). In *N.gonorrhoeae* MS11, a total of 11 variant *opa* genes have been characterized and genetically mapped (Bhat *et al.*, 1991; Bihlmaier *et al.*, 1991), all of which can be turned on and off independently.

Opa proteins are prominent components of the outer membrane and have been implicated in a number of adherence functions. These variable proteins not only allow gonococci to invade epithelial cells (Makino *et al.*, 1991a; Weel *et al.*, 1991) but also mediate interactions with human neutrophils (King and Swanson 1978; Virji and Heckels, 1986; Fischer and Rest, 1988; Belland *et al.*, 1992), and the inter-gonococcal adhesion that causes the typical opaque colony morphology (Blake, 1985). It is possible that Opas

act as carbohydrate-specific adhesins, recognizing moieties also present in the gonococcal LPS (Blake, 1985).

A major difficulty encountered in the analysis of Opa protein function is their variable expression. Previous efforts therefore focussed on the production of recombinant Opa proteins in *Escherichia coli* (Palmer *et al.*, 1989), providing information regarding the interaction of Opa proteins with human polymorphonuclear leukocytes (PMNs) (Belland *et al.*, 1992) and epithelial cells (Simon and Rest, 1992). Yet, it has not been possible to dissect functionally the whole repertoire of Opa proteins that can be produced by a single strain, particularly not in the natural background of *N.gonorrhoeae*. We have therefore used a reverse genetic approach to assess the cellular tropisms associated with individual Opa proteins of strain MS11 and other *Neisseria* strains. We demonstrate here that certain Opa proteins are specific for human epithelial cells, allowing gonococci to enter these cells, whereas other Opa proteins promote selective interaction with human polymorphonuclear leukocytes. Our data question the role of Opa as an autonomous invasin and instead suggest that additional factors are required for the invasion of epithelial cells by *N.gonorrhoeae*.

Results

Cloning and expression of opa genes in E.coli

In order to achieve invariant expression of distinct Opa proteins we modified the variable CR region of the *opa* genes, such that the repetitive character of the CR region was interrupted by nucleotide substitutions which were neutral with regard to the encoded amino acids. This was accomplished by a PCR strategy outlined in Figure 1. Two consecutive PCR reactions were used to amplify each of the *opa* genes contained in *N.gonorrhoeae* strain MS11 and, more importantly, to arrest them in the ON (i.e. in-frame) expression phase. Previous work indicated that high level expression of Opa protein is non-permissive for *E.coli*, therefore the amplified genes were placed under control of the repressible P_{trc} promoter by cloning in plasmid pTrc99A (Amann *et al.*, 1988; Figure 2A). Clones were identified using Opa-specific antiserum, after IPTG induction of *E.coli* DH5 transformants. Positive transformants were further analysed by immunoblotting and restriction analysis

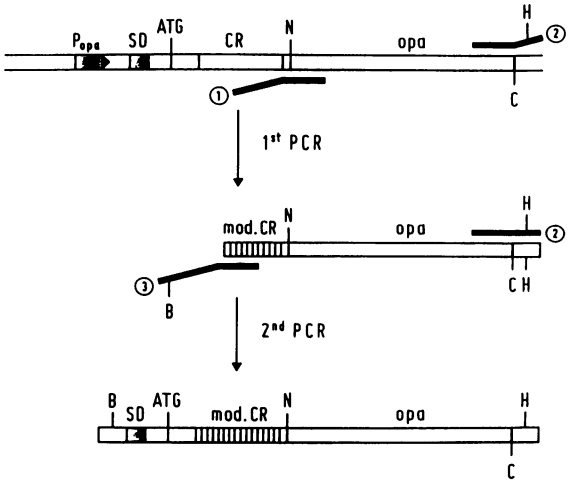

Fig. 1. PCR strategy used for the cloning and invariant expression of *opa* genes. Each *opa* gene carries a coding repeat (CR) specifying part of the Opa signal sequence. It consists of a variable number of pentameric (CTCTT) repeat units which determine translation and hence the ON/OFF expression phase of an *opa* gene (Stern *et al.*, 1986). The illustrated PCR protocol (for details see Materials and methods) is designed to generate *opa* genes comprising an unchanged amino acid sequence in the context of an invariant CR region arrested in the ON phase. Two consecutive PCRs using different primer combinations (1/2 or 3/2) give rise to a complete *opa* gene including a modified CR and a ribosome binding site in front (SD). (N) and (C) refer to the N- and C-termini of the mature Opa. Upon cleavage with the restriction nucleases *Bam*HI (B) and *Hin*dIII (H) the fragment can be inserted into the *E.coli* expression vector pTrc99A and/or Hermes vectors (Figure 2).

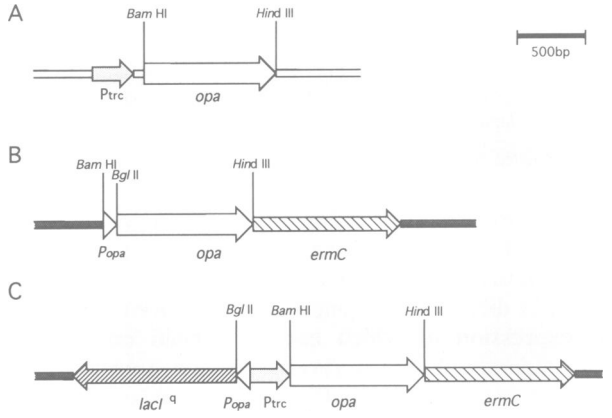

Fig. 2. Essential features of the *opa* constructs used in this study. (**A**) flanking regions of *opa* genes cloned in pTrc99A (Amann *et al.*, 1988). The cloned genes can be excised from the pTrc99A constructs by cleavage with *Bam*HI and *Hin*dIII and inserted into Hermes-6a (**B**) or Hermes-8 (**C**). In Hermes-6a, *opa* genes are placed under control of the P_{opa} promoter which results in high level Opa production not compatible with propagation in *E.coli*; after ligation with an *opa* gene this vector must therefore be directly transferred into gonococci. In Hermes-8 the *opa* genes are controlled by the inducible P_{trc} promoter in connection with the *lacI*^{q} repressor gene product. Panels B and C include the so-called 'shuttle boxes' of the Hermes vectors that are transferred via homologous recombination in the (dark shaded) flanking regions into the conjugative *N.gonorrhoeae* plasmid p*tetM*25.2.

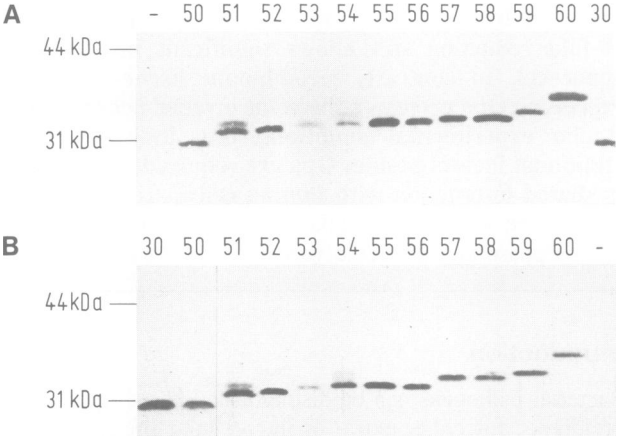

Fig. 3. Immunoblot showing recombinant Opa proteins produced in *E.coli* and *N.gonorrhoeae* MS11. (**A**) Whole Opa repertoire of strain MS11 (Opa_{50}–Opa_{60}) expressed in *E.coli* DH5 containing pTrc99A(Opa) plasmids; controls include DH5 (pTrc99A) and MS11-B2.1 (Opa_{30}). (**B**) Constitutive expression of Opa_{50}–Opa_{60} in *N.gonorrhoeae* N279 [pTH6a(Opa$^+$)]; controls include N279 [pTH6a(Opa$^-$)] and MS11-B2.1 (Opa_{30}). Although Opa_{53} was produced at normal levels it was always poorly labelled.

of the cloned inserts. In accordance with previous work (Bhat *et al.*, 1991; Bihlmaier *et al.*, 1991) a set of 11 variant *opa* genes, consecutively termed $opa_{50}-opa_{60}$, were obtained and could be expressed in *E.coli* (Figure 3A). They were produced at levels similar to the previously described β-lactamase−Opa fusion (Palmer *et al.*, 1989), but carry a native N-terminus. As judged by their heat-modifiability and accessibility to α-chymotrypsin (data not shown), the recombinant Opa proteins were properly exported and inserted into the *E.coli* outer membrane.

To define possible differences in the DNA sequences of the *opa* genes derived from different lines of strain MS11 (Bhat *et al.*, 1991), representatives of each of the 11 cloned *opa* genes were sequenced. Using oligonucleotide probes the nucleotide sequences obtained could be unambiguously assigned to the chromosomally mapped *opa* loci A−K (Bihlmaier *et al.*, 1991; Kupsch, 1991). The deduced Opa amino acid sequence of strain MS11-F3 used in this study (Figure 4) reveals a number of differences with regard to previously published Opa sequences of MS11 variant 4.8 (Bhat *et al.*, 1991); a comparison with nomenclatures used by others is given in Table I.

Invariant expression of opa genes in N.gonorrhoeae

Our previous work suggested that expression of at least one distinct Opa protein of strain MS11, Opa_{30}, could lead to invasive *N.gonorrhoeae* MS11 (Makino *et al.*, 1991a). This locus was always found to be turned ON in invasive MS11 variants selected for in the presence of gentamicin; however, we could not exclude that other *opa* loci in MS11 might have equal invasive potential but had escaped our attention. In order to test this hypothesis, we mutated the chromosomal *opaC* locus encoding Opa_{30}. The $opaC_{30}$ locus cloned on plasmid pOPB11 was disrupted by insertion of the *cat* gene and transferred back into strain MS11-B1 via transformation, with subsequent gene replacement. The genetic disruption of *opaC* was confirmed by Southern hybridization (data not shown). The resulting strain, termed N279, produced S-pilin and was non-piliated, conditions that facilitate invasion of non-polarized Chang epithelial cells (Makino *et al.*, 1991a).

Strain N279 was complemented with cloned *opa* genes using the Hermes shuttle system (E.M.Kupsch and T.F.Meyer, manuscript in preparation). The Hermes shuttle vectors allow the incorporation of a cloned gene by gene replacement into the conjugative *Neisseria* plasmid p*tetM*25.2 (Knapp *et al.*, 1987) and the subsequent mobilization of the hybrid p*tetM*25.2-Hermes plasmids into any other *Neisseria* strain. In these experiments the PCR amplified *opa* genes, opa_{50} to opa_{60} of strain MS11, were transferred from the pTrc99A vector into the shuttle vectors Hermes-6a and Hermes-8 (Figure 2B and C). In Hermes-6a the *opa* genes were constitutively transcribed by the P_{opa} promoter, while expression was IPTG-inducible in Hermes-8. The *opa* genes cloned in the Hermes vectors were transformed into strain N219 and allowed to recombine with the native p*tetM*25.2 plasmid to yield the pTH6a(Opa^+) and pTH8(Opa^+) series of plasmids which were mobilized into N279 (*opaC::cat*). Following this procedure, each of the 11 *opa* genes could be expressed in the native *Neisseria* strain without being subject to phase variability (Figure 3B).

Assessment of Opa_{50}-dependent epithelial cell invasion

The efficiency of epithelial cell invasion by recombinant *E.coli* and *N.gonorrhoeae* MS11 was determined using the human Chang conjunctiva infection model (Weel and van

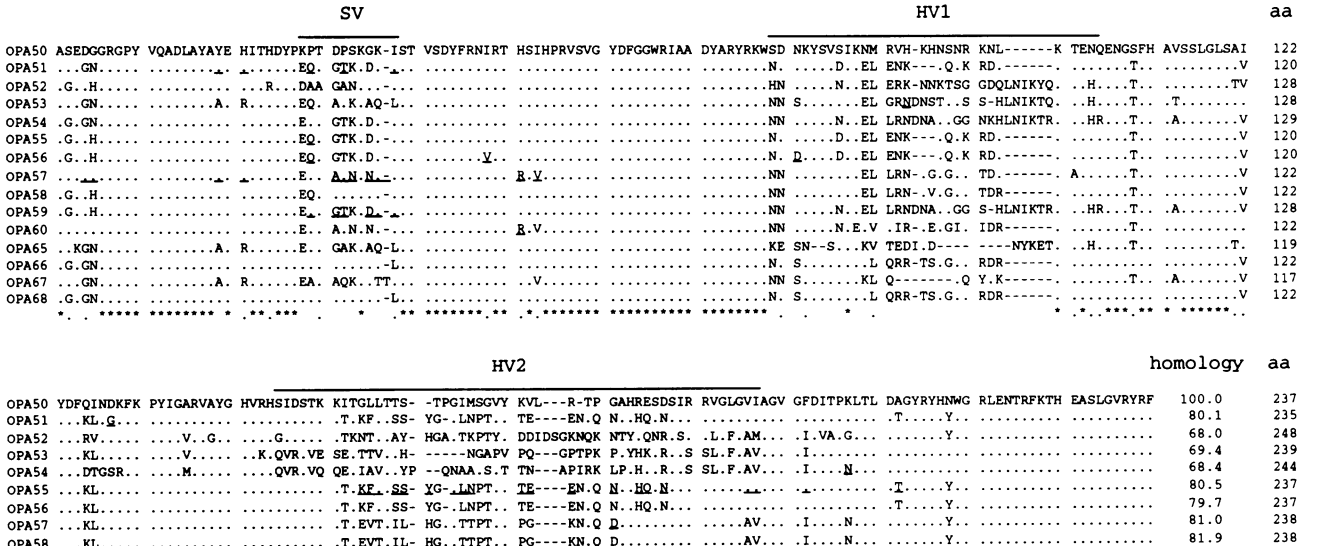

Fig. 4. Amino acid sequences of Opa proteins used in this study. Amino acid sequence alignment of the MS11 $Opa_{50}-Opa_{60}$ proteins and the VP1 $Opa_{65}-Opa_{68}$ proteins. Sequences were deduced from the nucleotide sequences of PCR amplified *opa* genes. Changes with regard to previously published sequences of MS11 variant 4.8 (Bhat *et al.*, 1991) are underlined. The percentage identities relate to Opa_{50} as standard; identical amino acids are indicated by full points; spaces have been introduced (-) to maximize the alignment. Similarities and identities between all amino acid sequences are indicated below the sequences by points and asterisks, respectively. Numbers at the right border indicate the number of amino acids (aa) of each sequence. The corresponding nucleotide sequences are deposited in the EMBL/Genbank database under the accession numbers Z18927−Z18942.

Putten, 1988) in combination with the gentamicin procedure for the selective detection of intracellular bacteria (Isberg *et al.*, 1987; Makino *et al.*, 1991a). Under assay conditions used for the assessment of gonococcal invasion, *E.coli* DH5 or YA21.6 producing Opa_{50} (see Figure 3A) interacted poorly with epithelial cells (data not shown); we therefore focussed our attention on the investigation of recombinant *N.gonorrhoeae*. Strain MS11-B2 (P^- Opa^-) and its isogenic counterpart, B2.1 (P^- Opa_{30}^+) served as negative and positive standards in all invasion assays, showing average invasion rates of $\sim 10^3$ and 10^5 internalized gonococci/ml culture, respectively (Figure 5, v+/−). The *opaC::cat* mutant N279 (Figure 5, c−) showed a 5- to 10-fold lower

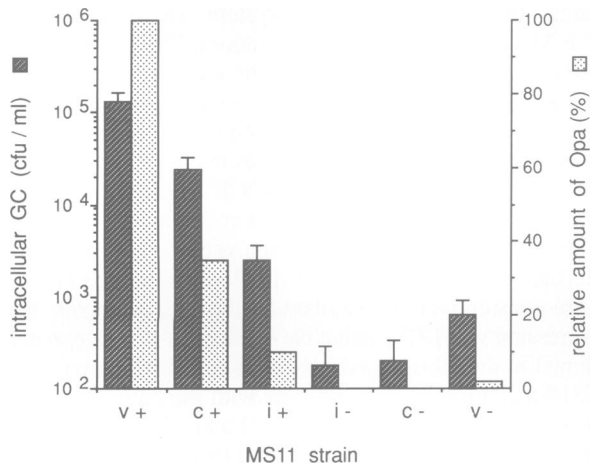

Fig. 5. Relationship between cellular invasion and the level of Opa production. MS11 derivatives either showing phase variable (v), constitutive (c), or inducible (i) *opa* gene expression, which lead to different levels of Opa_{30} or Opa_{50} production and, consequently, different invasion potentials. (v+/−) B2.1 and B2, respectively; (c+/−) N279 carrying pTH6a(Opa_{50}) or pTH6a, respectively; (i+/−) N279 [pTH8(Opa_{50})] in the presence or absence of IPTG. Intracellular gonococci (GC) were determined in the gentamicin assay after a 4 h infection period and confirmed by microscopical examination in four separate experiments.

Table I. Opa proteins involved in this study

a	b	c	d	e	f	g
MS11	Opa_{50}	Opa_{30}	OpaC	A	0	+
MS11	Opa_{51}		OpaB	B	6	−
MS11	Opa_{52}	Opa_{31}	OpaG	C	0	−
MS11	Opa_{53}		OpaA	D	1	−
MS11	Opa_{54}		OpaI	E	1	−
MS11	Opa_{55}		OpaE	F	21	−
MS11	Opa_{56}		OpaF	G	3	−
MS11	Opa_{57}		OpaK	H	13	−
MS11	Opa_{58}		OpaJ	I	0	−
MS11	Opa_{59}		OpaD	J	6	−
MS11	Opa_{60}		OpaH	K	1	−
VP1	Opa_{65}					ND
VP1	Opa_{66}					ND
VP1	Opa_{67}					ND
VP1	Opa_{68}	$Opa_{27.5}$				+
F62-SF	OpaG1					−

(a) Original strain; (b) designation of recombinant genetically defined Opa proteins used in this study (see Materials and methods for explanation of nomenclature); (c) designation of phase variable proteins studied previously (Makino *et al.*, 1991a); (d) Opa nomenclature used by Bhat *et al.* (1991) for DNA and protein sequences and by Bihlmaier *et al.* (1991) for the designation of chromosomal loci which differs from the nomenclature (e) recently used by Belland *et al.* (1992). (f) indicates the numbers of amino acid changes in the Opa proteins investigated here, as compared with previously published sequences of a different MS11 lineage (Bhat *et al.*, 1991). (g) +/− refers to the ability to promote epithelial cell invasion as determined in this study; ND, not determined.

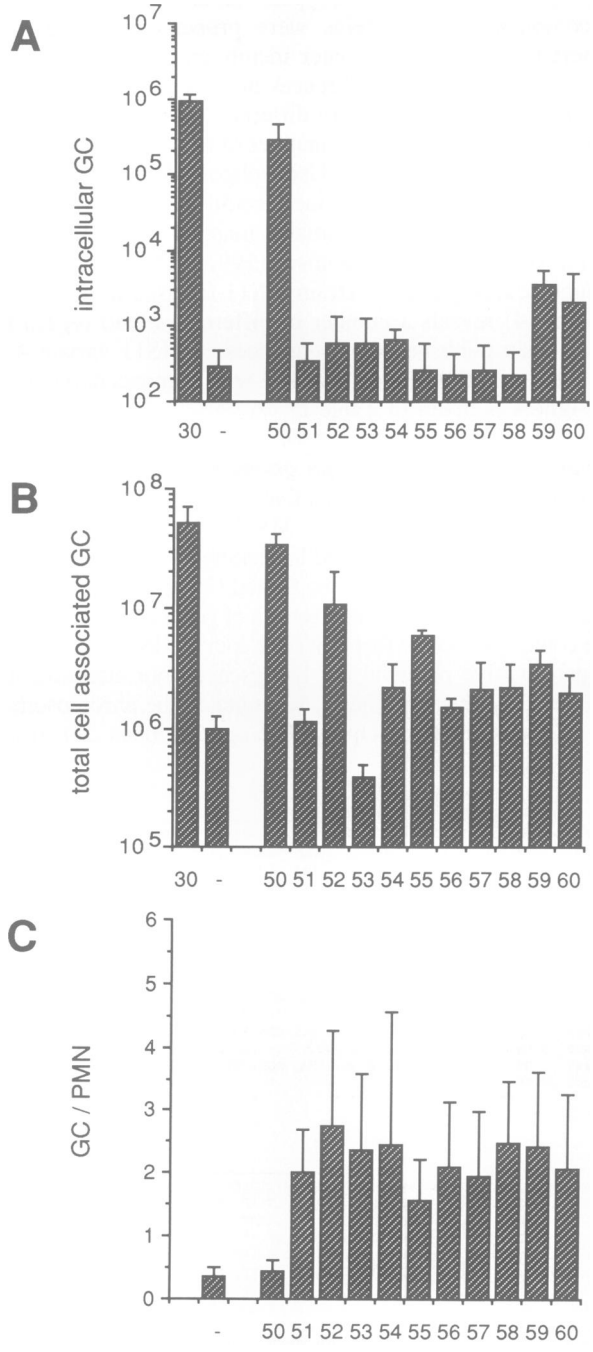

Fig. 6. Opa-mediated interactions of *N.gonorrhoeae* MS11 with epithelial cells and leukocytes. (**A**) Invasion of human Chang conjunctiva epithelial cells. The data present an average of six independent experiments performed according to the standard procedure with a 6 h infection period. (**B**) Opa-mediated epithelial cell adherence determined as total Chang cell-associated (adherent and internalized) gonococci. (**C**) Interaction with human leukocytes expressed as the ratio of total cell associated gonococci (GC) per leukocyte (PMN); this graph summarizes four to seven independent experiments. In all graphs, numbers along the horizontal axis refer to the Opa proteins produced by B2.1 (Opa_{30}) and recombinant N279 carrying pTH6a(Opa^-) and pTH6a(Opa_{50-60}), respectively.

invasion rate when compared with the natural Opa$^-$ variant B2. In agreement with previous observations (Makino et al., 1991a), this difference is explained by the spontaneous generation of invasive Opa$_{30}^+$ variants from B2 during the time of infection while N279 is unable to turn on Opa$_{30}$ synthesis. Complementation of N279 with plasmid pTH6a(Opa$_{50}$), encoding the recombinant analogue of Opa$_{30}$ (Table I), however, led to an almost complete restoration of the invasion activity (Figure 5, c+). Thus, strain N279 carrying an inactivated chromosomal *opaC* locus provides a perfect genetic background, in combination with pTH6a(Opa), for the assessment of Opa function in the absence of intrinsic invasion activity (Figure 5, c+/−).

The difference in the efficiency of invasion observed between the natural Opa$_{30}$ and the recombinant Opa$_{50}$ strains is probably due to the lower production in the latter (Figure 5), because both proteins are most likely structurally identical (see Table I). This view is supported by the even lower invasion potential of the IPTG-induced N279 [pTH8(Opa$_{50}$)] strain: under these conditions only ~10% of the wild type Opa level is produced (Figure 5, i+). No Opa protein and no invasion of this strain is detected under repressed conditions (i−). In summary, these data suggest a linear dependence of the gonococcal invasion efficiency on the amount of Opa$_{30/50}$ produced.

Epithelial cell invasion using genetically defined gonococcal Opa variants

Figure 6A shows a summary evaluation of invasion experiments using genetically defined Opa protein variants of strain N279 [pTH6a(Opa)]. The results obtained after a 6 h infection period of Chang epithelial cells are striking in that only a single Opa protein of strain MS11, Opa$_{50}$, was capable of promoting significant invasion. The invasion rate of the Opa$_{50}$ strain was about three orders of magnitude higher than that of the Opa$^-$ negative control. In contrast, the invasion potential of the other Opa$^+$ strains (Opa$_{51}$−Opa$_{60}$) was marginal, except for a minor activity associated with Opa$_{59}$ and Opa$_{60}$. As already shown by the previous experiment using a slightly shorter 4 h infection time (Figure 5), the natural MS11 variant B2.1 (Opa$_{30}$) is ~5-fold more invasive compared with N279 (Opa$_{50}$) probably due to the higher level of Opa synthesis.

Table II. Invasiveness of trans-complemented *N.gonorrhoeae* strains

Strain	Opa$_{50}$	Opa$_{53}$	Opa$_{68}$
F62*	+	−	+
FA1035*	++	−	+
FA1095*	+	−	+
R16*	ND	−	+
III*	+	−	+
Va*	++	−	+
VP2*	++	−	+
N279	++	−	++

Strains indicated by asterisks were P$^-$ Opa$^-$ Rif^r derivatives of the original isolates; all strains were complemented with pTH6a(Opa$^+$) plasmids encoding the indicated Opa protein. Invasion of Chang conjunctiva cells was determined using both gentamicin selection and microscopical examination; results shown are based on comparison with strain N279 [pTH6a(Opa$_{50}$)]: similar level of invasion (++), 5- to 10-fold reduction (+), no significant invasion (−). ND, not determined.

The epithelial cell adherence of the different Opa proteins varied less dramatically (Figure 6B). Consistent with the invasion experiment, Opa$_{30}$ and Opa$_{50}$ conferred an optimum adherence. However, Opa$_{52}$, the presumed equivalent of Opa$_{31}$ previously found to confer intermediate invasiveness (Makino *et al.*, 1991a), also led to relatively strong adherence but not to invasion. We assume that the increased uptake of Opa$_{31}$-positive gonococci in our previous work was due to an ON switch in the Opa$_{30}$ expression subsequent to Opa$_{31}$-mediated adherence. Because of the *opaC*$_{30}$ mutation in the experiment presented here this possibility is excluded, explaining the lack of significant invasion potential of Opa$_{52}$. Thus, recombinant Opa$_{50}$ and its natural equivalent Opa$_{30}$ are the only Opa proteins of strain MS11 associated with the invasion of Chang conjunctiva cells.

Interaction of Opa proteins with human leukocytes

The fact that only one out of the 11 variant Opa proteins of strain MS11 was associated with epithelial cell invasion was intriguing, particularly since several *N.gonorrhoeae* strains tested in this laboratory were able to invade Chang epithelial cells, and this property was correlated with the expression of distinct Opa proteins (T.Kuroki, J.P.M.van Putten and T.F.Meyer, unpublished data). Thus, expression of proteins with Opa$_{30/50}$-like functions appeared to be an essential attribute of this species, but the significance of the vast majority of Opa proteins which could not confer invasiveness remained obscure. Since previous work from several laboratories suggested a role for Opa in the interaction with human leukocytes (King and Swanson, 1978; Virji and Heckels, 1986; Fischer and Rest, 1988), we tested possible tropisms of our recombinant MS11 strains for this cell type.

Leukocytes were isolated from fresh human blood and allowed to adhere to glass cover slips. The adherent leukocytes were then infected at a ratio of 20 gonococci per cell. After a 1 h incubation period the non-adherent gonococci were removed and the infected cells were stained. The total leukocyte-associated gonococci were counted by microscopy and the number of gonococci per leukocyte calculated. Figure 6C shows the data from several independent experiments using blood from four different donors: each of the *opa* alleles of strain MS11 that did not lead to invasion of epithelial cells showed an ~10-fold more efficient interaction compared with the Opa$^-$ control strain. It appeared from this microscopical survey that some variant Opa strains were preferentially engulfed while others only adhered to these cells. Importantly, the Opa$_{50}$ strain that invaded epithelial cells did not adhere to PMNs. This experiment therefore demonstrates that gonococci expressing defined Opa proteins display distinct cell tropisms, with Opa$_{50}$ being associated with epithelial cells and Opa$_{51}$−Opa$_{60}$ with human PMNs.

Function of Opa proteins in heterologous Neisseria strains

With regard to the observed tropism of Opa$_{50}$ for epithelial cells, the question arose whether this property was restricted to *N.gonorrhoeae* strain MS11 or whether it was also expressed in heterologous backgrounds provided by independent gonococcal isolates. If the first hypothesis was true this would suggest that Opa must interact with another strain-specific factor in order to elicit invasive activity. To

clarify this question we performed complementation studies with several independent gonococcal strains and cloned *opa* genes. To begin with, genetically defined derivatives (Rif^r Opa^- $PilE^-$ P^-) were derived from these strains, which were then complemented by conjugation with recombinant pTH6a(Opa) plasmids encoding either no Opa, Opa_{50} or Opa_{53}. Corresponding strains were constructed producing Opa_{65}–Opa_{68} of *N.gonorrhoeae* VP1 (Figure 4, Table I) and OpaG1 of strain F62-SF, respectively. The gene coding for the latter protein was originally cloned by Palmer *et al.* (1989) as a β-lactamase fusion in plasmid pLPG1; the *opaG1* sequence was amplified from this plasmid using a one step PCR protocol (see Materials and methods) and inserted into Hermes-6a.

Table II summarizes a typical invasion experiment using the Chang epithelial cell line. Of the various Opa proteins investigated only two, Opa_{50} from strain MS11 and Opa_{68} from strain VP1, promote epithelial cell invasion. This experiment reveals that Opa proteins display a dominant phenotype, i.e. a given Opa protein either confers epithelial cell invasion in any gonococcal background, or not at all.

We have previously noted that strain VP1 invades human epithelial cell lines ~10 times more efficiently than strain MS11, providing expression of the invasion promoting Opas is turned ON (Makino *et al.*, 1991a). However, Opa_{68}, promoting strong invasion of strain VP1, does not confer a higher level of invasion on strain MS11 compared with the native Opa_{50}. There is also no significant difference between the two Opa proteins when expressed in other heterologous strains; rather each strain has intrinsic invasion potential, providing Opa_{50} or Opa_{68} are synthesized. We were unable to express the MS11 Opa_{50} in the VP1 background, since it is neither competent for transformation nor able to act as a recipient in conjugation. However, the available data suggest that other factors besides Opa contribute to the invasive behaviour of an individual strain.

Structural homology of two invasion-promoting Opas

Having identified and cloned two invasion-promoting Opa proteins from different strains, MS11 and VP1, we asked whether this particular function has a common structural basis. The deduced amino acid sequences of Opa_{50} and Opa_{68} reveal several differences in the semi- and hypervariable regions (SV, HV1 and HV2) of the proteins (Figure 4). According to the predicted outer membrane topology for the Opas (Meyer *et al.*, 1986; van der Ley, 1988; Bhat *et al.*, 1991), the variable regions constitute three of the four surface exposed loop regions. By comparison with other Opa proteins unable to confer gonococcal invasion, Opa_{50} shows closest homology with Opa_{68} (Figure 4). However, as shown by the dendrogram analysis of whole Opa proteins (Figure 7A), Opa_{68} is more closely related to some Opa proteins other than Opa_{50}, thus indicating a complex structure–function relationship with respect to the invasion property of Opa proteins. To extend this study, dendrograms of the isolated variable sequences are shown (Figure 7B–D). While the two hypervariable regions HV1 and HV2 do not reveal very close homology between Opa_{50} and Opa_{68}, it is interesting that their SV regions are nearly identical, suggesting that the SV region contributes to the Opa tropism. Another feature common to Opa_{50} and Opa_{68} is the similar array of positively charged residues and the absence of multiple negative charges in the variable regions (Figure 4).

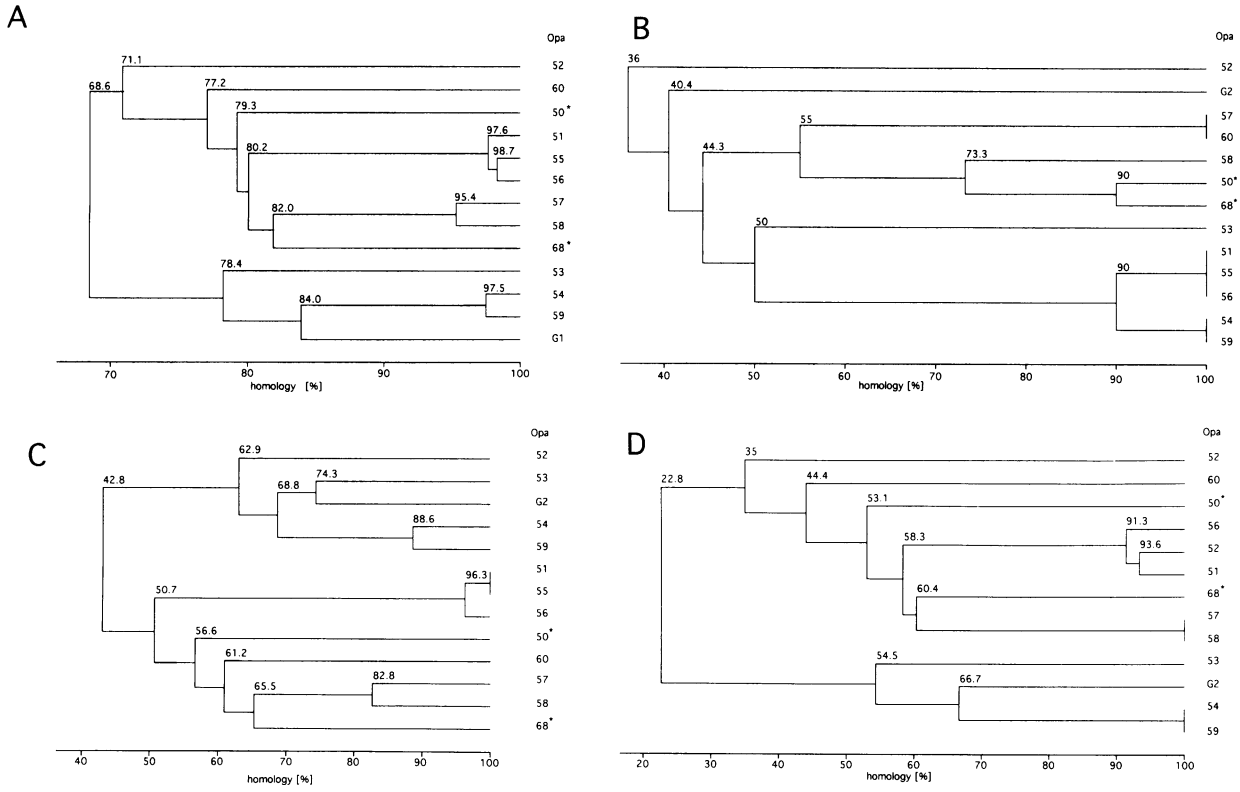

Fig. 7. Dendrogram analyses of Opa proteins. (**A**) Comparison of complete sequences as shown in Figure 4; (**B–D**) comparisons of the semi-variable (SV) and hyper-variable (HV1 and HV2) regions. Numbers indicate the percentage identity between single or groups of Opa proteins. Asterisks denote the two Opa proteins supporting invasion of epithelial cells. (In panels B and D, G2 should read G1.)

Invasion into different epithelial target cell lines

Several Opa-negative and -positive gonococcal strains were chosen to assess which Opa was required for the invasion of human epithelial cells other than the Chang conjunctiva cell line. We tested the Hec-1B endometrium carcinoma and the ME-180 cervix carcinoma cell lines recently used in related studies (Shaw and Falkow, 1988; Chen *et al.*, 1991; Simon and Rest, 1992). As shown in Table III, similar results were obtained with the three cell lines. Opa_{50} and Opa_{68} strains were strongly invasive when compared with Opa^-, Opa_{53}, and OpaG1 strains. The only divergence from this common theme is the observation of an elevated uptake of gonococci by ME-180 cells even if no Opa proteins or proteins other than Opa_{50} and Opa_{68} were synthesized. Such increased residual uptake was even more pronounced using the PC-3 human prostate adenocarcinoma cell line (S.Makino and T.F.Meyer, unpublished). Whether this phenomenon results from a non-specific endocytic activity associated with some epithelial cell lines, or whether it hints at an alternative adherence and invasion pathway for *N.gonorrhoeae* is currently under investigation.

Discussion

We have demonstrated that the family of variant Opa proteins provides essential determinants for the cellular tropisms of *N.gonorrhoeae*. A distinct Opa protein of strain MS11 allows gonococcal entry into epithelial cells, while the expression of the remaining Opa proteins leads to an enhanced interaction of gonococci with human peripheral blood leukocytes. Thus, Opa proteins display an exclusive specificity for either epithelial cells or PMNs. That only a single Opa protein is specific for epithelial cells may reflect the relative importance of epithelial cells versus professional phagocytes as targets during the normal course of a gonococcal infection. Although there has been considerable controversy in the past about whether or not gonococci are able to survive within professional phagocytes it seems to be largely accepted that at least a fraction of internalized gonococci may resist killing after phagocytic ingestion (for review see Rest and Shafer, 1989). This notion has been strongly supported by the work of Smith and coworkers who correlated the ability of gonococci to survive and replicate within phagocytes with the expression of a 20 kDa gonococcal lipoprotein (Parsons *et al.*, 1986). In this study, different types of interaction of gonococci with phagocytic cells were observed in that some Opa-producing strains preferentially adhered to PMNs while others were also engulfed. Whether the interaction of extracellular gonococci with PMNs serves to modulate target cell function, or whether the primary aim of this interaction is always to induce engulfment of the bacteria, remains to be elucidated. Clearly the data stress the need for more intense studies on the role of Opa-mediated interaction with human leukocytes.

The ability to invade epithelial cells is also an important feature of *N.gonorrhoeae* and several independent isolates tested in our laboratory have the capacity to turn on the production of at least one Opa protein allowing the invasion of human Chang epithelial cells (T.Kuroki, J.van Putten and T.F.Meyer, unpublished data). However, we have previously shown that the survival of gonococci internalized by Chang epithelial cells is limited in time (Makino *et al.*, 1991a; Weel *et al.*, 1991). One might therefore speculate that epithelial cells serve as a transient host for gonococci sufficient to allow transcytosis through epithelial tissues, rather than providing a long term intracellular habitat. Such trans-epithelial passage of gonococci may, in the beginning of an infection, enhance the exposure to leukocytes which later on are predominant players in the disease.

In our assessment of Opa-producing *E.coli* clones we observed only poor Opa-dependent interactions with confluent cultures of Hec-1B, Chang, or ME-180 epithelial cells under the experimental conditions used for the *Neisseria* strains (data not shown). The failure of the recombinant Opa^+ *E.coli* to interact efficiently with human epithelial cells was probably not a matter of Opa functionality nor of

Table III. Invasion of different human epithelial cell lines

Opa	Chang	Hec-1B	ME-180[a]
none	−	−	−
Opa_{50}	++	++	++
Opa_{53}	−	−	−
OpaG1	−	−	−
Opa_{68}	++	++	++

Opa invasion activities of recombinant N279 [pTH6a(Opa^+)] strains were determined using the gentamicin assay and microscopical observation; the data presented are based on comparisons with strain N279 [pTH6a(Opa_{50})] using the Chang cell line: similar level of invasion (++), 5- to 10-fold reduction (+), no significant invasion (−). (a) A general increase of gonococcal uptake (2- to 5-fold) was observed in the case of the ME-180 cell line.

Table IV. List of oligonucleotides

Designation	Strand/Location	Sequence
CG9	$+/P_{opa}$	GATCCGGTTTTTGTTAATCCGCCATATTGTGTTGAAACACCGCCCGGAACCCGATATAATCCGCCCTTCAACA
CG10	$-/P_{opa}$	GATCTGTTGAAGGGCGGATTATATCGGGTTCCGGGCGGTGTTTCAACACAATATGGCGGATTAACAAAAACCG
EMK4	−/3′	GGTCAAAGCTTTCAGAAGCGGTAGCGCACGCCC
EMK6	+/5′	TCTTTGTTATTTAGCAGCTTACTGTTCAGCTCATTACTGTTTTCTTCCGCAGCGCGGGCGGCG
TO1	−/3′	GGTCAAAGCTTCTCGAGTCAGAAGCGGTAGCGCACGCCC
IH2	+/5′	GGCAATGGCCGCGGCCCGTATGTGCAGGCGGAT
TM45	+/5′	GGCGCGGATCCAAGGAGCCGAAAATGAACCCAGCCCCCAAAAAACCTTCTTTGTTATTTAGCAGCTTA
TM46	+/5′	TCTTTGTTATTTAGCAGCTTACTGTTCAGCTCATTACTGTTTTCTTCCGCAGCGCAGGCGGCA
TM56	+/5′	TGTAAAACGACGGCCAGTTCCGCAGCGCAGGCGGCA
TM57	−/centre	CAGGAAACAGCTATGACCACGTGTCCGTAGGCGACGCGC
TM58	+/centre	TGTAAAACGACGGCCAGTTTCAAACCCTATATCGG
TM59	−/3′	CAGGAAACAGCTATGACCTCAGAAGCGGTAGCGCA

the amount of Opa produced. It rather reflects the requirement for additional adherence and invasion determinants of the gonococcus, which must be postulated based on the inhibition of Opa-mediated adherence to epithelial cells in the presence of chloramphenicol and erythromycin (Steeb, 1992; J.P.M.van Putten, personal communication). In fact, additional invasion associated loci have been identified by transposon shuttle mutagenesis of gonococci (A.Kahrs and T.F.Meyer, unpublished data). Their existence questions the suitability of invasion experiments using recombinant Opa$^+$ *E.coli*. Whether neisserial functions besides Opa are also required for the interaction with human leukocytes has not been studied by us. However, recent work by Belland *et al.* (1992) indicates that PMN activation can occur in response to recombinant Opa$^+$ *E.coli*.

The complementation experiments using different gonococcal recipients indicate that the cell type tropisms correlate with the expression of distinct Opa proteins, rather than the neisserial context. In other words, a given Opa protein showed qualitatively the same invasion properties when produced in a variety of different gonococcal strains. Therefore, if the OpaG1 protein of strain F62-SF, which reportedly rendered *E.coli* invasive (Simon and Rest, 1992), is truely an invasin, it ought to confer epithelial cell invasion also in a gonococcal context. This was, however, not the case in our experiments using three different human epithelial cell lines including Hec-1B, ME-180 and Chang cells. Consistent with our result are the structural features of OpaG1 suggesting that it belongs to the class of Opas that promote PMN interaction rather than epithelial cell invasion (Figure 7).

The cloning and characterization of a second Opa protein with the ability to promote gonococcal invasion, Opa_{68} of strain VP1, provided essential information on the structural features associated with invasiveness. Homologies between Opa_{50} and Opa_{68} were found in each of the three variable regions (Figure 7). Most significantly the semi-variable region SV is nearly identical for the two Opas but distinct from all other Opa proteins. The variable regions, together with a fourth invariant loop, are predicted to be surface exposed (Meyer *et al.*, 1986; van der Ley, 1988; Bhat *et al.*, 1991) and probably cooperate to form a receptor binding pocket. Our sequence data are in favour of this notion although they do not fully preclude the existence of a linear binding epitope. To elucidate further the structural requirements for Opa-mediated epithelial cell interaction we intend to do site-specific mutagenesis and to construct chimeric Opa proteins.

Our work assumes an important function for distinct Opa proteins in tight gonococcal adherence which we consider as the initial step of epithelial cell invasion in a series of events involving multiple bacterial and cellular determinants. Tight cellular adherence of pathogenic *Neisseriae* may be supported or possibly replaced by other receptor-mediated mechanisms. In *Neisseria meningitidis* the recently identified Opc protein may have a function in receptor recognition and human cell invasion similar to Opa (Virji *et al.*, 1992). Several additional protein adhesins besides Opa have been identified on the gonococcal cell surface (Paruchuri *et al.*, 1990; Benkirane *et al.*, 1992) and in related species (Nyberg *et al.*, 1990). Furthermore, gonococcal lipopolysaccharide (LPS) may represent a candidate adhesin. Gonococcal LPS is phase variable exposing a variety of carbohydrate moieties (Mandrell *et al.*, 1986). Such structures, often resembling host epitopes (Mandrell, 1992) may be recognized by distinct human cell types that play a role in the gonococcal infection. However, the particular aspect of epithelial cell invasion by *N.gonorrhoeae* assessed in this work was independent of structural changes in LPS: gonococcal LPS mutants selected in the presence of pyocin were not affected in their invasive potential (Makino *et al.*, 1991b). Thus, the expression of Opa proteins *per se*, irrespective of LPS expression, is a crucial determinant for the cellular tropisms of *N.gonorrhoeae*.

Materials and methods

Bacterial strains and plasmids

Neisseria gonorrhoeae MS11 variant A [P$^+$(piliated)] (Meyer *et al.*, 1982, 1984) was the progenitor of all MS11 derivatives used in this work, including variants F3 (P$^+$) and B1 [S(for S-pilin)] (Haas *et al.*, 1987), and B2.1 (P_n- Opa_{30}^+) (Makino et al., 1991a). Strain N219 (Rudel *et al.*, 1992) is a Nal^r derivative of MS11-B1 containing the conjugative plasmid p*tetM*25.2 (Knapp *et al.*, 1987). Strain N279 (S, *opaC::cat*) is MS11-B1 carrying the *opaC::cat* mutation; the chromosomal defect was introduced by allelic replacement using pC20 (kindly supplied and constructed by C.P.Gibbs), which carries a 770 bp promoterless *cat* gene inserted into the *Ssp*I site of the MS11 *opaC* locus cloned in plasmid pOPB11 (A.Stern, unpublished). The *opaG1* gene of strain F62-SF was contained on plasmid pLPG1 (Palmer *et al.*, 1989) kindly provided by G.F.Brooks (San Francisco) and R.Rest (Philadelphia). *N.gonorrhoeae* strains F62 and R16 (E.C.Gotschlich, New York), FA1035 and FA1095 (P.F.Sparling, Chapel Hill), VP2 (J.P.M.van Putten, Amsterdam) and III and Va (R.P.Novick, New York), were transformed to Rif^r using *rif*^{r} chromosomal DNA from strain N431 (Rudel *et al.*, 1992), selected for lack of piliation by removing P$^+$ variants via haemagglutination and screened for lack of Opa synthesis by immunoblotting. Conjugative plasmids pTH6a and pTH8 used for the expression of *opa* genes in *N.gonorrhoeae* resulted from recombination of p*tetM*25.2 with the *E.coli* shuttle vectors Hermes-6a and Hermes-8 (Figure 2; E.M.Kupsch and T.F.Meyer, in preparation). Plasmid pTrc99A (Amann *et al.*, 1988) was used for the expression of *opa* in *E.coli* DH5 (Hanahan, 1983) and YA21.6 (*E.coli* K-12 producing heptose-deficient LPS; Yu *et al.*, 1982).

Opa/opa nomenclature

As a rule, *opa* loci mapped in the chromosome of strain MS11 were specified by capital letters according to Bhat *et al.* (1991) and Bihlmaier *et al.* (1991), i.e. *opaA...opaK*. To account for the fact that chromosomal *opa* loci can undergo recombination and hence are subject to sequence changes (Stern *et al.*, 1986; Connell *et al.*, 1989; Bhat *et al.*, 1991), characterized *opa*/Opa sequences/proteins have been arbitrarily numbered, e.g. opa_{50} or Opa_{50}. To indicate the association of an *opa* sequence (e.g. opa_{50}) with a distinct *opa* locus (e.g. *opaC* of the MS11 variant studied here), this is described as $opaC_{50}$; *opa* genes cloned on a plasmid are not specified by a letter. To discriminate between variable, constitutive and inducible *opa* genotypes the symbols v, c and i, respectively, have been used when necessary. Terminology used by other laboratories is explained in Table I.

Cloning and expression of opa genes

Chromosomal DNA of *N.gonorrhoeae* MS11-F3 and VP1 was isolated according to Stern *et al.* (1986). The first step PCR amplification of strain MS11 *opa* genes was carried out in 100 μl Taq polymerase buffer (Perkin Elmer) containing 50 ng chromosomal DNA, primer pair TM46/EMK4 or EMK6/EMK4 (100 pM of each primer; see Table IV), 1.25 mM of each of the four dNTPs and 3 U Taq polymerase (Perkin Elmer). The samples were heated to 94°C for 3 min and passed through 20 cycles (1 min 92°C, 5 min 50°C, 2 min 72°C) in a Perkin Elmer Cetus thermal cycler. The product of the first step reaction gave rise to a slightly diffuse band in agarose gels corresponding to ~800 bp; after staining with methylene blue the material was excised from the gel and purified using Geneclean (BIO101). A second PCR reaction under essentially the same conditions was performed with 5 ng amplified fragment and primer pair TM45/EMK4. The resulting product was gel-purified, cleaved with *Bam*HI and *Hin*dIII and inserted into the expression vector pTrc99A. The ligated DNA was transformed into *E.coli* DH5 and transformants were screened for the expression of Opa proteins by colony blotting using rabbit anti-Opa serum. Individual clones were

analysed with regard to the size of Opa proteins, the restriction pattern of cloned inserts, and the hybridization of *opa*-specific oligonucleotides (Kupsch, 1991). Representatives of each chromosomal *opa* locus of strain MS11 (Bihlmaier *et al.*, 1991) could be identified and were subjected to complete sequence analysis.

The cloning of *opa* genes from strain VP1 was effectively the same, except that (i) the first PCR reaction was performed with *Eco*RI and *Nde*I fragmented chromosomal DNA, partially enriched for distinct *opa* loci by consecutive agarose gel separations, and (ii) primer pair TM46/TO1 was used for the first reaction and primer pair TM45/TO1 for the second reaction. The resulting PCR products were likewise inserted between the *Bam*HI and *Hin*dIII sites of vector pTrc99A. To construct a corresponding pTrc99A plasmid containing the native *opaG1* gene of strain F62-SF, provided as a gene fusion in plasmid pLPG1, the gene was amplified using primer pair IH2/EMK4. The resulting fragment was cleaved with *Sac*II/*Hin*dIII and inserted into pTrc99A(Opa) digested with the same enzymes, thus reconstituting the incomplete 5′ end of the PCR generated *opaG1* gene with the identical sequence of opa_{53} (compare with Palmer *et al.*, 1989).

The various recombinant *opa* genes cloned in pTrc99A could be excised with *Bam*HI/*Hin*dIII and inserted downstream either of the LacI controlled P_{trc} promoter of Hermes-8 or of the P_{opa} promoter of Hermes-6a (see Figure 2); P_{opa} was constructed from oligonucleotides CG9 and CG10. Due to high-level Opa production, Hermes-6a constructs were not viable in *E.coli* and needed to be transformed directly into *N.gonorrhoeae* MS11-N219 containing the p*tetM*25.2 conjugative plasmid. Upon transformation of Hermes into this strain the genes, contained within the 'shuttle box' of these vectors, were inserted into p*tetM*25.2 by homologous recombination. Using this system we created two series of conjugative plasmids for the expression of defined Opa proteins in *N.gonorrhoeae*, the constitutive pTH6a(Opa) series and the IPTG-inducible pTH8(Opa) series. For the assessment of Opa function the plasmids were transferred into N279 or other *N.gonorrhoeae* strains. Transformation and conjugation of *N.gonorrhoeae* have been described (Rudel *et al.*, 1992).

Sequence analysis of opa genes

The sequence analysis of cloned *opa* genes was performed essentially as described for the *pilE* genes of *N.gonorrhoeae* MS11 (Rudel *et al.*, 1992). Briefly, oligonucleotide pairs complementary to conserved regions located at a distance of ~400 bp within the *opa* genes were synthesized for use in PCR. The plus strand-specific oligonucleotides carried 5′ extensions corresponding to the M13 universal primer (M13), whilst the minus strand-specific oligonucleotides carried 5′ extensions corresponding to the M13 reverse primer 1 (RP1). PCR based on such primer pairs yielded fragments which could be directly sequenced in either direction using fluorescent-labelled sequencing primers following protocols for sequencing in an Applied Biosystems 373A automatic sequencer. Accordingly, *opa* genes were covered by two overlapping fragments generated by primer pairs TM56/TM57 and TM58/TM59 (Table IV). The nucleotide sequences have been deposited in the EMBL/Genbank data libraries (see legend to Figure 4). Computer assisted comparisons of the deduced amino acid sequences were done with CLUSTRAL (Higgins and Sharp, 1989) on an IBM personal computer.

Infection of epithelial cells

Epithelial cell lines used in infection experiments were Chang human conjunctiva (ATCC CCL20.2), Hec-1B human endometrium carcinoma (ATCC HTB133) and ME-180 human cervix carcinoma (ATCC HTB33). Cells were cultured in RPMI medium supplemented with 5% fetal calf serum at 37°C in 5% CO_2 and grown to confluency in 24 well plates (Costar) prior to infection with *N.gonorrhoeae* for use in the gentamicin assay, or on glass cover slips for microscopical evaluation.

The gentamicin assay for quantification of intracellular bacteria was performed according to previously described protocols (Makino *et al.*, 1991a). Briefly, 2×10^5 cells were infected with 2×10^6 bacteria in RPMI medium supplemented with 5% fetal calf serum and 1 U Benzonase (Boehringer Mannheim). Infection was allowed to proceed for 4 or 6 h. Afterwards medium and non-adherent bacteria were removed by repeated washings with PBS, and the infected monolayer was either immediately treated with 1% saponin for 15 min to determine total cell associated bacteria, or incubated with 50 μg/ml gentamicin for a period of 2 h, prior to saponin treatment, in order to kill extracellular adherent bacteria and to determine the intracellular surviving bacteria. Microscopical evaluations were done after an infection period of 4 or 6 h without prior gentamicin incubation. For this purpose the infected monolayers were extensively washed before fixation and staining with crystal violet. In each infection experiment aliquots were taken routinely in order to confirm the production of the desired Opa proteins (see Figure 3). Furthermore, analyses showed that PilC was produced by the strains used in these studies.

Interaction with human leukocytes

Human polymorphonuclear leukocytes (PMNs) were isolated from human venous blood of healthy donors. The heparinized blood was mixed 1:1 with PBS and carefully overlayed on a two step Percoll (Pharmacia) gradient consisting of 72 and 60% Percoll (10 ml each). Centrifugation for 30 min at 400 *g* resulted in separation of blood cells with localization of neutrophils in a cloudy fraction above the sedimented erythrocytes. This fraction was collected and washed once in PBS. Contaminating erythrocytes were lysed hypotonically. 95–99% of neutrophils were viable as determined by Trypan blue exclusion. Purified PMNs were diluted in PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$, i.e. PBS(MgCa), to 2×10^6 cells/ml. 500 μl of the suspensions were added on to glass slides (12 mm diameter) in 24 well plates and pre-incubated at 37°C, 5% CO_2 for 45 min to allow adherence of neutrophils to the glass surface. Gonococci were diluted in PBS(MgCa) to $\sim4\times10^7$ c.f.u./ml (OD_{550} of 0.1). The interaction was initiated by adding 500 μl of the bacterial suspensions to the PMNs and allowed to proceed for 60 min. Non-adherent cells and gonococci were removed by three washing steps with PBS(MgCa) prior to fixation and staining with 0.3% Wright Stain in MeOH (Sigma). Interaction between neutrophils and gonococci was determined microscopically by counting the gonococci associated with at least 100 neutrophils. The number of total PMN-associated gonococci (adherent and internalized) was divided by the number of the counted PMNs.

IPTG induction and analysis of Opa protein expression

The expression of *opa* genes placed under control of the P_{trc} promoters in plasmids pTrc99A, Hermes-8 and pTH8 was induced by the addition of 100 μM IPTG to exponentially growing liquid cultures either 2 or 6 h prior to the harvesting of the recombinant *E.coli* or gonococci, respectively. Aliquots of the inocula from infection experiments were routinely assayed for the expression of Opa proteins by immunoblotting. Bacterial lysates were loaded on SDS gels in the fully denatured form (i.e. after boiling) and the blots were developed using the polyclonal antiserum AK10 raised against denatured Opa protein (Stern *et al.*, 1984), which efficiently reacts with most variant Opas. PilC protein was assayed by immunoblotting as described (Rudel *et al.*, 1992). Surface exposure of recombinant Opa proteins on intact cells was assessed by proteolytic digestion using α-chymotrypsin (Belland *et al.*, 1992).

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