Opacity proteins of Neisseria meningitides: structure-function relationship and vaccine potential

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Functional activity of antibodies against recombinant OpaJ protein from Neisseria meningitidis

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Abstract

The Opacity proteins belong to the major outer membrane proteins of the pathogenic *Neisseria* and are involved in adhesion and invasion. We studied the functional activity of antibodies raised against OpaJ protein from strain H44/76. Recombinant OpaJ protein was obtained from *E. coli* in two different ways: cytoplasmic expression in the form of inclusion bodies followed by purification and refolding, and cell surface expression followed by isolation of outer membrane complexes (OMC). Immunisation with purified protein and QuilA induced high levels of Opa-specific antibodies, while the *E. coli* OMC preparations induced generally lower levels of antibodies. Two chimeric Opa proteins, hybrids between OpaB and OpaJ, were generated to demonstrate that the hypervariable region 2 (HV-2) is immunodominant. Denatured OpaJ with QuilA induced high levels of IgG2a in addition to IgG1, while refolded OpaJ with QuilA induced IgG1 exclusively. These sera did not induce significant complement-mediated killing. However, all sera blocked the interaction of OpaJ expressing bacteria to CEACAM1 transfected cells. In addition, cross-reactive blocking of OpaB expressing bacteria to both CEACAM1 and CEA transfected cells was found for all sera. Sera raised against purified OpaJ and against OpaJ-containing meningococcal OMC also blocked the non-opsonic interaction of Opa-expressing meningococci with human polymorphonuclear leukocytes.

Introduction

The obligate human pathogen *Neisseria meningitidis* primarily colonises the nasopharynx in an asymptomatic manner. Only in a minority of cases does infection become systemic, resulting in life-threatening meningitis and septicaemia. Virulent strains freshly isolated from the blood or cerebrospinal fluid are typically encapsulated. Classification into serogroups is based on structural differences in capsular polysaccharide. While the group A, C, Y and W-135 capsules can be used for effective vaccines, the serogroup B polysaccharide was found to be non-immunogenic, probably due to the presence of identical α2,8-linked N-acetyleneuraminic acid structures in developing fetal brain tissue (14, 17, 24, 48). Therefore, alternative strategies have been followed for the development of a vaccine against the common serogroup B, mainly focusing on the use of outer membrane proteins (OMP) (reviewed by Jódar et al., 2002). Several clinical trials have been conducted with outer membrane vesicle (OMV)-based vaccines containing various combinations of OMP (4, 13). OMV contain the major porin proteins, PorA and PorB, lesser amounts of other proteins including those with high molecular weights of 60 – 100 kDa, and lipopolysaccharide. Although efficacy in these
trials range from 51 – 83 %, improvements are clearly needed, in particular since the induced bactericidal antibodies tend to be highly type-specific.

Additional studies are necessary to identify other OMP that are important for inducing protective antibodies. All current vaccines aim to induce bactericidal serum antibodies, as this is known to correlate with protection against invasive disease (18). However, an alternative strategy would be to induce antibodies, which block the infection at an earlier stage, at the level of mucosal attachment and invasion. Since Opa proteins are involved in adhesion and subsequent invasion into nasopharyngeal epithelial tissue (3, 37, 44) it is tempting to speculate that antibodies against Opa proteins block this crucial step in pathogenesis. Opa proteins are encoded by a family of homologous genes that undergo high-frequency phase and antigenic variation (18, 35). Both heparan sulphate proteoglycans and members of the CEACAM family of cell surface proteins have been identified as receptors used for Opa-mediated attachment to host cells (5, 28, 42, 44). As individual meningococcal strains are highly variable in the particular Opa repertoire expressed at any particular moment, studies on the ability of individual Opa proteins to induce a protective immune response are preferably performed using heterologous expression systems. Additionally, by heterologous expression of Opa protein, responses against other meningococcal surface antigens were prevented. In the present study we describe the results of immunisation experiments in mice with the invasion-associated OpaJ protein from N. meningitidis strain H44/76, expressed in an E. coli background. Expression was achieved both in the outer membrane of E. coli, and as cytoplasmic inclusion bodies from which OpaJ was purified and refolded into a native-like conformation (10). The resulting mouse sera were analysed for total and isotype specific antibody response. Functional activity of the antibodies was investigated, in particular their ability to interfere with Opa-mediated adhesion to CEACAM-expressing host cells.

Results

The Opa repertoire of N.meningitidis strain H44/76

Among the four opa genes from strain H44/76, two could be identified as OpaB 128 and OpaJ 129 (11) which display very different versions of hypervariable region 1 and 2 (Fig. 1). This difference in amino acid sequence resulted in a different binding specificity, with OpaJ recognising only CEACAM1 and OpaB both CEACAM1 and CEA (11). The remaining two genes from H44/76, opaA and opaD encode proteins that are nearly identical to either OpaJ or OpaB, respectively. These four Opa proteins are recognised by two different monoclonal antibodies (mAb), MN20E12.70 and 15-1-P5.5. We determined the minimal epitopes recognised by these mAbs with 18-mer overlapping biotinylated peptides based on HV-1 and HV-2 of OpaB and OpaJ.
The minimal epitope for 15-1-P5.5 was found to be GGPIIQ on HV2 of Opaj, which is consistent with previous results from Hobbs et al. (1998) and for MN20E12.70 we determined the minimal epitope to be GIWQELK on HV-1 of Opab (Fig.1).

- SV - - HV 1 - - HV 2 -

Opab 128  KATQSANNT-MNKSNTVKEELQUNSSGWIWHQKATGAMMT-NNMKYSVNTKELQKNNSSGIWSEUt - - KHQVHSVRKETTTTFSPPAQGATVPQKTIVQG IPTNKPAYHESNISSL

MN20E12.70 epitope

Opaj 129  QATQSANNT-KESSSTKTVKTEELNNSKETFQ---KIQGAVETRKTIVSKPTQGAACGGPIIQGTPSKFPYHESNISSV

15-1-P5.5 epitope

Figure 1. Amino acid sequences of the variable regions of Opab 128 and Opaj 129. Minimal epitopes of monoclonal antibodies MN20E12.70 and 15-1-P5.5 are underlined.

In order to target the Opa proteins to the outer membrane of E. coli, an in frame fusion of opaj and opab was constructed using the promoter and signal-sequence encoding segment of the phoE gene of E. coli, as previously described (10). The Opa expressing E. coli strains were used for immunisation experiments, as test strain for Opa-specific antibodies in ELISA and in an in vitro infection assay.

Immunogenicity of purified refolded and denatured Opaj

After purification of Opaj we immunised mice with the refolded and denatured form of the protein with and without the adjuvant QuilA. Opa-specific antibody titres were determined in whole-cell ELISA with E. coli strain CE1265 expressing Opaj and the homologous strain not expressing any Opa proteins as immobilised antigen. Neither refolded nor denatured Opaj protein without adjuvant evoked a significant humoral murine immune response. However, in the presence of QuilA high Opa-specific antibody titres were obtained (Fig. 2). The antisera from the mice immunised with denatured Opaj plus QuilA induced higher total IgG titres than refolded Opaj plus QuilA, although this difference is not significant \((P = 0.084)\). Similar levels were obtained by the positive control, H44/76 Opaj+ OMC (Fig. 2).

Immunogenicity of Opaj presented in E. coli and N. meningitidis OMC

Besides immunising with purified recombinant Opaj protein, Opaj was also presented as part of outer membrane complexes isolated from either E. coli or N. meningitidis. For E. coli, strain CE1265 with and without the opaj-expression was used for OMC isolation. We screened for Opa-negative and Opaj-positive variants of N. meningitidis strain H44/76 using the characterised mAbs in colony blotting, and used them for OMC isolation.
**Figure 2.** (A) Total IgG response measured in whole-cell-ELISA with *E. coli* strain CE1265 expressing OpaJ (CE1265-opaJ) as immobilised antigen and wild-type CE1265 as negative control (CE1265). The data shown represent the geometric means and standard errors of 8 mice per group. (B) Total IgG response measured in whole-cell-ELISA with H44/76 expressing Opa proteins detectable with 15-1-P5.5 mAb (H44/76 P5.5+) and H44/76 expressing Opa proteins detectable with MN20E12.70 mAb (H44/76 D2+) and H44/76 not expressing any Opa protein as negative control (H44/76 Opa-). (C) Total IgG response measured in ELISA with purified OpaB and OpaJ. The data shown represent the geometric means and standard errors of 8 mice per group. Mice immunised with OMC containing Opa protein giving lower titres than mice immunised with OMC without Opa proteins are defined as non-responders. Two out of eight mice immunised with OMC containing Opa did not respond, the titres from these non-responders were included.
In order to measure only anti-Opa antibodies and to avoid specificity in the analysis of sera from mice immunised with the *E. coli* OMC, microtitre plates were coated with 15-1-P5.5-positive, MN20E12.70-positive or Opa-negative H44/76 variants. All total IgG titres with *E. coli* OMC appeared to be strikingly low, approximately tenfold lower that those obtained with either OpaJ+ meningococcal OMC or purified OpaJ (Fig. 2). Some reaction was also found with the Opa negative H44/76 variant, probably due to residual OpaJ expression. Although all *opa* genes are expected to be out-of-frame in the Opa-negative variants, a high frequency ($10^{-3}$) of independent on and off switching has been observed during growth (19), making it difficult to obtain truly Opa-negative meningococci. Significant differences in the reaction of the sera against the three H44/76 variants could not be identified due to high variation within the groups, caused by a relative high number of non-responders (Fig.2B). The specificity of the antibodies raised by the OpaJ containing *E. coli* OMC was confirmed by an ELISA using purified OpaB and OpaJ as immobilised antigen.

![Diagram](image.png)

**Figure 3.** Antibody reaction against chimeric Opa proteins measured in whole cell ELISA. The construction of the chimeric *opa* genes is depicted in panel A. The antibodies present in the sera of mice, immunised with Opa-negative and OpaJ-positive meningococcal OMC and with purified refolded and denatured OpaJ with QuilA, were tested for reaction with *E. coli* bacteria expressing OpaBj and OpaJb (B). The data shown represent the geometric means and standard errors of 8 mice per group.
Cross-reactivity with OpaB and antibody reaction against chimeric Opa proteins

In order to measure cross-reactivity of anti-OpaJ antibodies with OpaB, whole-cell ELISA against *E. coli* CE1265 expressing OpaB was also performed (Fig.3). Cross-reactivity was generally low as compared to total IgG titres against OpaJ. Very low cross-reactive antibody titres were found in the sera of mice immunised with refolded OpaJ plus QuilA, higher titres were found for denatured OpaJ and the H44/76 OpaJ+ OMC (the titres were 761 and 845 respectively, see Fig.3).

To identify the most immunogenic region of the OpaJ protein, hybrids of the two *opa* genes, *opaB* and *opaJ*, were made and expressed in the outer membrane of *E. coli*. The ability of the immune sera to recognise the chimeric Opa proteins was determined after immunisation with H44/76 Opa+ OMC and refolded/denatured OpaJ with QuilA (Fig.3B). In all cases the highest levels of IgG antibodies raised against OpaJ, were directed against the OpaJb protein containing HV-2 from OpaJ and SV and HV-1 from OpaB (Fig. 3A). A significantly higher titre was found against OpaJb than OpaBj with the antisera raised against purified denatured OpaJ protein. (*P*=0.004). The differences found with the other sera were not significant. Apparently, the antibodies were mainly directed against the HV-2 region of OpaJ, since low cross-reactivity with OpaB was found (Fig. 3B).

Antibody isotype distribution and bactericidal activity of sera

The antibody isotype distribution of the response to OpaJ was determined by ELISA using anti-mouse IgG isotype-specific conjugates. Immunisation with purified refolded and denatured OpaJ plus QuilA induced high levels of IgG1 subclass antibodies, titres were 5853 and 7488 respectively (Fig.4). A significant difference (*P* = 0.028) in the induction of IgG2a subclass antibodies between the immunisations with refolded and denatured OpaJ protein was observed, with much higher titres for the denatured protein. Surprisingly, high titres of the IgG3 subclass were detected in the antisera directed against *E. coli* OMC, but not with purified OpaJ (see Fig.4). The highest subclass titre in the antisera directed against *E. coli* OMC containing OpaJ, was found to be the IgG3 subclass (Fig. 4).

Despite the fact that denatured OpaJ with QuilA induced a significant IgG2a titre, 90 % complement-mediated killing was only found at 5-fold or lower serum dilutions. No differences in bactericidal titres were measured after immunisation with H44/76 Opa− and OpaJ+ OMC, indicating that bactericidal antibodies were primarily directed against other components than OpaJ.
Antibody blocking activity in an \textit{in vitro} infection assay

To determine the blocking activity of anti-Opa antibodies we incubated Opa-expressing \textit{E.coli} bacteria with stably transfected HeLa cells expressing different CEACAM receptors. We found that the OpaJ protein specifically binds to CEACAM1, while OpaB binds to both CEACAM1 and CEA (11). The antisera raised against those formulations of OpaJ giving the highest titres, i.e. H44/76 OpaJ\textsuperscript{+} OMC and refolded/denatured OpaJ plus QuilA, were added to OpaB or OpaJ expressing cells of \textit{E.coli} strain PC2984.
Figure 5. Antibody blocking activity in an in vitro infection assay with sera raised against refolded and denatured OpaJ and meningococcal OMC containing OpaJ. Two dilutions (1/100 and 1/1000) of all sera were tested for blocking the binding between OpaJ and CEACAM1 (A), OpaB and CEACAM1 (B) and OpaB and CEA (C). The data shown represent the geometric means and standard errors of three independent experiments.
before incubation with the transfected HeLa cells. For each group, sera from non-responding mice were left out and the others were pooled. To select for specific anti-Opa blocking activity, the reference (0 % blocking) was defined as the average of the number of colony forming units (cfu) recovered after incubation with a non-binding mAb. The sera raised against Opa-negative H44/76 OMC was used as negative control. The blocking activity was measured as the percentage non-recovered cfu after incubation with sera, reflecting the reduction in HeLa cell association (adhesion and/or invasion) of the Opa-expressing E.coli bacteria. At a 1:100 dilution, over 80-90 % blocking was found for the pooled sera from all three groups (Fig.5). A clear concentration dependent effect was observed since a strong reduction in the blocking activity was found after an additional 10-fold serum dilution. Strikingly, all the sera appeared to have cross-reactive blocking activity, since the sera raised against OpaJ could not only block the binding between OpaJ and CEACAM1, but also between OpaB and either CEACAM1 or CEA (Fig.5).

**Effect of OpaJ-specific antibodies on phagocytosis**

Phagocytosis was measured *in vitro* using *ex vivo* human polymorphonuclear leukocytes (PMNs) and heat-killed meningococci. Both H44/76 Opa- and Opa+ variants expressed comparable amounts of PorA P1.16 but no Opc as measured by FACS analysis. The Opa+ variant expressed Opa proteins reactive with both mAbs MN20E12.70 and 15-1-P5.5, while we confirmed that the Opa- variant did not express any Opa protein (Fig. 6a). It should be noted that in this assay no distinction could be made between different types of interaction of bacteria and PMNs, i.e. only adhesion or also phagocytosis.

In the absence of sera low phagocytosis was found for Opa- but high for Opa+, compatible with the Opa-CEACAM1 interaction. Sera raised against either to OMC or purified OpaJ had little or no effect on phagocytosis of Opa-negative H44/76. However, dramatic reduction of the phagocytic index for Opa-positive bacteria was observed using sera from mice immunised with purified OpaJ or OpaJ containing OMC. The dominant effect measured in this assay is therefore the blocking of Opa-mediated association with PMNs by antibodies against OpaJ protein, rather than opsonic activity of these antibodies (Fig. 6b).
**Fig. 6A** Characterisation by FACS analysis of PorA P1.16, Opc, OpaB and OpaD (MAb MN20E12.70) and OpaA and OpaJ expression (MAb 15-1-P5.5) of the meningococcal H44/76 Opa\(^+\) (filled) and Opa\(^-\) (black line) variants used in the opsonophagocytosis assay. The negative control is indicated with a gray line.

![Graphs](image)

**Figure 6B.** Opsono- and Non-opsonophagocytosis of heat-killed Alexa-488 labelled meningococcal H44/76 Opa\(^+\) (a and c) and Opa\(^+\) variants (b and d) by human PMNs in the presence of antisera raised against purified refolded and denatured OpaJ (a and b) and against H44/76 Opa\(^+\) and Opa\(^-\) OMC (c and d). As a positive control for opsonophagocytosis the two meningococcal H44/76 variants were incubated with PMNs in the presence of mAb HMIN12H2 IgG1 a humanised mAb specific for PorA. The experiments were repeated two times with similar results.
Discussion

The Opa proteins in *N. meningitidis* are encoded by a family of four genes that share a conserved framework interspersed by a semivariable (SV) region and two hyper-variable (HV) regions. Different functions have been attributed to the opacity proteins but their main function is mediating adhesion of meningococci to epithelial cells, which can subsequently lead to host cell invasion (15, 46). Although the Opa proteins have been shown to be able to induce bactericidal antibodies in humans (31), their high degree of sequence variability is a major impediment to vaccine application, as these bactericidal antibodies are generally highly type-specific. However, despite this hyper-variability, the number of receptors specifically targeted by the meningococcal Opa proteins is quite limited, as over 80% recognise human CEACAM1 (15, 32). The variable regions are located within the two largest surface exposed loops, which are potentially involved in interaction with the extra-bacterial environment. It can thus be expected that the sequence variation of these regions might be restricted by the need to preserve the specific receptor binding sites. At present, not much is known about the detailed molecular structure of the Opa-receptor complex. However, it is conceivable that antibodies directed against these specific receptor binding sites might block adhesion mediated by a broad range of distinct Opa proteins. In a previous study, we have described a method to obtain individual Opa proteins in highly purified form and native conformation (10). We now used OpaJ protein from *N. meningitidis* H44/76 obtained in this way to investigate its immunogenicity in mice, and the functional activity and specificity of the resulting antisera.

Purified refolded and denatured OpaJ samples were prepared with and without the addition of the non-toxic adjuvant QuilA, since it is known that purified proteins need adjuvant stimulation in order to evoke an immune response (35). Without QuilA no significant IgG titres were measured in ELISA, whereas in its presence both refolded and denatured OpaJ protein induced high levels of total IgG. In contrast, immunisation with the same OpaJ protein in an *E.coli* background formulated as OMC resulted in approximately tenfold lower titres of Opa-specific IgG, in spite of high expression levels of Opa at the cell surface (10). Although the OMC derived from *E. coli* and *N. meningitidis* contained the same amount of protein, the meningococcal OMC elicited a much stronger response. The reduced immunogenicity of the *E.coli* OMC is not due to the quantity of OpaJ but rather the context or delivery. This might result from interference by immunodominant *E. coli* outer membrane proteins, e.g. OmpA (23). In any case, the heterologous surface expression in *E. coli* was designed as a tool to study the immunogenicity and functional activity of individual Opa proteins and not as alternative antigen presentation system with vaccine applications.
The results of the whole cell ELISA with the chimeric OpaBj and OpaJb proteins indicated that antibodies raised against OpaJ protein, both in OMC and as purified protein, were mainly directed against the HV-2 region. Apparently, the longest loop as predicted in a two-dimensional topology model (29, 39), contains the most immunogenic region. The fact that the highest antibody titre was found against this most variable region explains the low cross-reactivity with the heterologous OpaB, showing an approximately tenfold lower IgG titre.

IgG1 was found to be the dominant isotype in the response to purified OpaJ, as was also described for Opc (26). However, a striking difference was found in the induction of IgG2a between folded and denatured protein, as only denatured OpaJ also induced a relatively high IgG2a titre. This is in contrast to the results of Jansen et al. (2000) who found a higher IgG2a titre with refolded PorA compared to denatured PorA, also in the presence of QuilA. The refolding of OpaJ has been studied extensively (10), but correct folding is not a guarantee for the induction of bactericidal antibodies as many other factors also play a role, for example accessibility or density of epitopes. Recently, an immunomodulatory function has been described for gonococcal Opa proteins. Their binding to CEACAM1 arrests the activation of T lymphocytes (6). Although the sequence divergence between human and murine CEACAM1 is probably too high to allow OpaJ binding to the mouse equivalent, it cannot be excluded that it still displays a similar immunomodulatory function in mice through binding to another receptor, thus explaining the striking difference in isotype distribution between native and denatured OpaJ. In agreement with this interpretation, we have previously found that refolded OpaJ is a more effective CEACAM1 binder than the denatured form (10). Only very low bactericidal activity was found for the sera raised against purified OpaJ, in spite of the high IgG2a titer, which is known to be an effective complement activator. Also, there were no differences in the bactericidal titres of the sera against H44/76 Opa+ and H44/76 Opa- OMC. Apparently, OpaJ protein is not such an effective inducer of bactericidal antibodies as for instance PorA, at least not in mice. This agrees with the results of Sacchi et al. (1995) who found no differences in bactericidal titres in sera from mice immunised with OMV preparations differing in the presence or absence of class 5 proteins (Opa and Opc).

Blocking of the Opa adhesin function might be an alternative protective mechanism to bactericidal action. Therefore, the antibody blocking activity was measured in a highly defined *in vitro* infection assay. This assay has been used to determine the binding specificity of OpaB and OpaJ from *N. meningitidis* H44/76, both expressed on the cell surface of *E. coli*, through their binding to transfected HeLa cells expressing the different CEACAM proteins. Whereas in the infection assay OpaJ only binds to CEACAM1, OpaB recognises both CEACAM1 and CEA (11). The sera with the highest anti-OpaJ
titres, obtained either with purified OpaJ or OpaJ in H44/76 OMC, all displayed clear blocking of the Opaj-CEACAM1 mediated adhesion. Interestingly, cross-reactive blocking of the heterologous OpaB protein with both CEACAM 1 and CEA was also found, in spite of the approximately tenfold lower whole-cell ELISA titres against OpaB compared to OpaJ. Apparently, antibodies directed against epitopes common between these distinct Opa proteins are mainly responsible for the observed functional blocking.

Our in vitro adhesion assay, although highly defined, uses Opa expression in E.coli and CEACAM expression in HeLa cells. We also studied the interaction of meningococci with professional phagocytes, which may be a more natural setting. As expected only Opa expressing meningococci associated with human neutrophils in a non-opsonic manner. An important role for Opa proteins in the interaction of meningococci with human monocytes and neutrophils has been described (16, 30). In our assay we used human neutrophils homozygous for the FcγRIIa-R131 encoding allele, which has a relatively high affinity for mouse IgG1 (41). It might therefore be expected that our anti-OpaJ sera should not only be capable of blocking the Opa-CEACAM1 interaction but also mediate opsonophagocytosis via the human Fc- receptors. However, our results indicated that addition of the sera resulted only in blocking of the non-opsonic interaction between the bacteria and the neutrophils. As the number of neutrophil-associated bacteria was much higher for the Opaj+ strain than for the Opaj-, the observed blocking must work through the interaction between OpaJ and its neutrophil receptor, presumably CEACAM1. With the sera raised against the meningococcal OMC, blocking activity was much higher for the Opaj+ than the Opaj- OMC, again demonstrating the dominant role for Opa in the non-opsonic phagocytosis. Only at higher serum concentrations could blocking-antibodies against other outer membrane components than Opa or Opc (not expressed by our strains) come into play. The difference in phagocytic activity between the sera raised against pure OpaJ and against OMC containing OpaJ is also probably due to the opsonophagocytic activity of antibodies raised against other outer membrane proteins of N. meningitidis.

In conclusion, we demonstrated that purified recombinant OpaJ protein with QuilA is highly immunogenic in mice, while OpaJ presented in E.coli OMC is less immunogenic. Although hardly any bactericidal and opsonophagocytic antibodies were found, OpaJ could induce antibodies with blocking activity, both for homologous and heterologous Opa-CEACAM interactions. This implies the presence of conserved surface-exposed epitopes on Opa proteins with distinct HV-1 and HV-2 regions. If similar cross-reactive blocking antibodies can also be induced with purified Opa protein at mucosal surfaces, this would constitute a novel protective mechanism for meningococcal vaccines by blocking the adhesion to nasopharyngeal epithelium.
Materials and methods

Bacterial strains, plasmids, growth conditions

*N. meningitidis* strain H44/76 (B; 15; P1.7, 16) has been described previously (21). *E. coli* strain CE1265 was previously described by Korteland *et al.* (1985) and strain PC2984 was obtained from NCCB (Phabagen collection, Utrecht, The Netherlands). The expression of OpaJ at the cell surface of *E. coli* strains CE1265 and PC2984 was realised using the expression vector pMR05 (2) as previously described (10). Meningococci were grown overnight on GC agar plates (Difco Laboratories, Detroit, MI, USA) supplemented with 1 % IsoVitaleX at 37 °C in a humidified 5 % CO₂ atmosphere. The *E. coli* strains were grown at 37 °C in Luria-Bertani medium (BIO 101, Carlsbad, CA, USA) or on LB agar (Oxoid, Basingstoke, Hampshire, England) plates supplemented with 25 µg/ml chloramphenicol (Sigma, St.Louis, MO, USA). For phagocytosis experiments, bacteria were heat-killed at 56°C (30 minutes) after they were washed in PBS supplemented with 1 % Bovine Serum Albumin (BSA, fraction V, Boehringer, Germany). 10⁰ heat-killed bacteria in 0.5 ml PBS were labelled with the pH-stable green dye Alexa488 (Molecular Probes, Leiden, The Netherlands), according to the manufacturer's protocol.

Epitope mapping

Biotinylated oligopeptides of 18 residues, with 14 amino acid residues overlap, spanning the HV-1 and HV-2 regions of OpaJ129 and OpaB 128, were synthesized on a 10 µM scale using an automated multiple peptide synthesizer, equipped with a 48-column reaction block (AMS 422, ABIMED Analysen-Technik GmbH, Langenfeld, Germany) as described earlier (7). The peptides were used in a peptide-ELISA, using avidin (Sigma) (5 µg/ml) coated immunolon II 96 wells microtitre plates (Dynatech) incubated for 1 h with 10 µg/ml peptide. Reactivity of the monoclonal antibodies (mAbs) 15-1-P5.5 (kindly provided by Wendell Zollinger, Walter Reed Army Institute of Research, USA)(46) and MN20E12.70 (kindly provided by Betsy Kuipers, RIVM, The Netherlands) was detected using affinity purified goat-anti mouse-immunoglobulins (GAM) (Southern Biotechnology Associates, Birmingham, AL, USA) (1:5000), conjugated to horse radish peroxidase (HRP).

Cloning and expression of OpaJ and OpaB and the construction of chimeric Opa proteins

The genes encoding OpaJ129 and OpaB128 were isolated from H44/76 using Taq polymerase (Amersham, Piscataway, NJ, USA) and general *opa* primers (5'-CTTCTCTTCTCTTCCGCAACG-3' and 5'-TCGGTATCGGGGAA-TCAGAA-3'), cloned into plasmid pCR2.1 (Topo TA cloning Kit, Invitrogen, Carlsbad, CA, USA) and subsequently sequenced using M13-forward and M13-reverse primers (Invitrogen). Plasmids pCR2.1 containing *opaB128* or
opaj129 were used to amplify the DNA sequences encoding the mature OpaB128 or OpaJ129 proteins with Taq polymerase. The primers used (5' - AGCGCCCAIGGCAAGTGAAG-3' and 5'- GGCATCGGGATCCGGGAATT CAG-3') were based on the DNA sequence of opaB128 and opaJ129 of Neisseria meningitidis strains H44/76 (unpublished) and 190/87 (GenBank accession no. AF016285 and AF016285) (28). The primers contained base substitutions (underlined) to introduce Ncol and BamHI cleavage sites, respectively. The PCR product was cloned in plasmid pCR2.1. The Ncol-BamHI fragment was isolated from the resulting plasmid and ligated into the Ncol-BamHI digested expression vector pET11d (New England Biolabs, Inc., Beverly, MA, USA) downstream of the inducible T7 promoter. In the resulting construct, the codon for the first amino acid residue of the mature Opa protein was situated directly downstream of the ATG start codon. The sequence of the insert was checked by DNA sequencing, using the DNA sequencing kit and the ABI Prism 310 genetic analyser, according to the instructions of the manufacturer (ABI Prism, Perkin Elmer Applied Biosystems, Warrington, Great Britain). Plasmids pET11d-opaB128 and pET11d-opaJ129 were used to transform the Escherichia coli strain BL21 (DE3).

The OpaB128 and OpaJ129 expression at the cell surface of E.coli strain CE1265 was realised as previously described (10) using the expression vector pMR05, containing the complete phoE gene (2). PCR reactions were performed on pCR2.1 containing either opaB128 or opaJ129 using Taq polymerase and mutagenic primers (5'-ATAGATCTCGGGGAATCAGAAGCG-3' and 5'-CTTCTCTTCTTCCTCTGCAGC-3') to generate a PstI site between the signal sequence and the mature portion and a BglII site behind the stop codon of opaB128 and opaJ129. The PstI-BglII fragments of opaB128 and opaJ129 were used to replace a PstI-BglII fragment of the phoE gene in pMR05, resulting in an in-frame fusion of opa to the signal peptide of phoE and expression from the phoE promoter. The resulting plasmids pMR05-opaB and pMR05-opaJ were isolated and purified using the Wizard Plus SV Miniprep kit (Promega, Madison, WI, USA) and subsequently digested with PstI and AflIII (Boehringer Mannheim, Germany). The fragments were electrophoretically separated on a 1 % (w/v) agarose gel and isolated with the JETsorb gel extraction kit (Genomed, Bad Oeynhausen, Germany). The PstI/AflIII fragments coding for the SV and HV-1 regions were exchanged and re-ligated using T4 ligase in the appropriate buffer according to the instructions of the manufacturer (Boehringer Mannheim). The resulting plasmids were used for transformation of CE1265 as described previously (10). The sequences of the inserts were checked by DNA sequencing. Surface expression of the two chimeras OpajB and OpajB was analyzed in a colony blotting experiment (39) using mAb 15-1-P5.5 and MN20E12.70.
Refolding and purification of Opa protein

In vitro folded and purified OpaJ protein was prepared as previously described (10). The purification of inclusion bodies was improved. Cultures of the E. coli strain BL21 (DE3) containing either pET11d-opaB128 or pET11d-opaJ129, grown overnight at 37°C, were diluted 1/10 into fresh LB medium supplemented with 0.5% glucose (Fluka, Buchs, Switzerland) and ampicillin (100 μg/ml). When the culture reached an optical density of 660 nm (OD660) of 0.6, isopropyl-β-D-galactopyranoside (IPTG) (Boehringer Mannheim, Germany) was added to a final concentration of 1 mM. After 3 h of incubation, at 37°C, the cells were harvested by centrifugation (5000 g for 15 min at 4°C), the cell pellet was resuspended in 50 mM Tris-HCl and 40 mM EDTA (TE buffer). 0.25 g/ml sucrose (Sigma, St. Louis, MO, USA) and 0.2 mg/ml lysozyme (Boehringer Mannheim, Germany) was added prior to incubation for 1 h at 4°C. For osmotic shock treatment 100 ml TE buffer was added, incubated for 30 min at 4°C and sonicated using a macrotip (model 250 Sonifier, Branson, Danbury, Connecticut, USA) for three time (1.5 min, duty cycle 50%, output 9). The cell suspension was sonicated after addition of 5 ml 10% Brij-35 (Sigma, St. Louis, MO, USA) (v/v) and centrifuged (5000 g, 30 min at 4°C) and sonicated (duty cycle 50%, output 9) two times. Opa protein was dissolved in 8 M urea and 50 mM glycine (pH 8). Ultracentrifugation at 40 000 rpm for 2.5 h at 4°C (Centrikon T 1170, Rotor 45-94, Kontron Instruments, Milano, Italy) was used to remove residual membrane fragments. Opa protein (10 mg/ml) dissolved in 8 M urea and 50 mM glycine (pH 8.0) was diluted 100-fold in refolding buffer containing 328 mM ethanolamine (pH 12), 0.5% SB12 (Fluka, Buchs, Switzerland). An SP-Sepharose-HP column (volume 15 ml) (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) was equilibrated with 10 mM Tris-HCl, 0.5% SB12 (pH 7.5) (bufferA), loaded with approximately 10 mg refolded OpaJ129 and washed twice with buffer A with pH 7.5 and pH 8.5. The proteins were eluted with a linear gradient of NaCl from 0 to 1 M in 120 ml. OpaB was refolded and purified by a similar procedure as also described elsewhere (10). To check folding and purification, SDS-PAGE was performed under semi-native and denaturing conditions (data not shown). The folded and purified proteins were stored at -20°C.

Preparation of samples for immunisation

The denatured OpaJ protein was obtained by heating the folded protein for 30 min at 100°C prior to immunisation. To stimulate the immune response, 20 µg Quillaja saponin A (QuilA) (Isotec, Luleå, Sweden) was added to half of the samples containing purified refolded and denatured OpaJ protein. All purified protein samples were diluted in 10 mM Tris-HCl (pH 8) and 0.2% (w/v) n-dodecyl-N,N-dimethyl-1-ammonio-3-propanesulfonate (SB12, Fluka; purified as described by Dekker et al. (1995). The Opa negative H44/76 and OpaJ positive variants were selected by colony blotting (40) using
mAbs 15-1-P5.5 and MN20E12.70. Outer membrane complexes (OMC) were isolated by sarcosyl extraction according to the protocol described by Davies et al. (1990). The OMC were diluted in 10 mM Tris-HCl (pH 8.0). The protein concentration of all samples was determined with the Pierce protein assay (Pierce, Rockford, Illinois, USA) using BSA as standard.

**Immunisations of animals**

Female BALB/c/Rivm mice at 6–8 weeks of age were used for immunisation. Individual mice within groups of eight of approximately equal weight were immunised subcutaneously with 5 μg purified protein or OMC on days 0, 14 and 28. Mice were terminally bled on day 42, and sera were stored at -20°C.

**ELISA**

Whole-cell-ELISAs were performed according to Abdillahi and Poolman (1987). For ELISA with purified protein, flat-bottomed 96-well microtitre plates were coated overnight at 37°C with 100 μl of a 5 μg/ml purified Opa protein solution in PBS. Antibody titres were measured for each individual serum. The titre is defined as the dilution of the serum where 50 % of the OD_{max} in the assay is reached. Starting dilution of all sera was 1:100. In the total IgG- and cross-reactivity-ELISA, GAM-HRP (1:5000) was used to detect antibody binding. In the subclass-specific ELISA IgG1, IgG2a, IgG2b and IgG 3 GAM-HRP monoclonals (Southern Biotechnology Associates, Birmingham, AL, USA) (1:5000) were used for the detection of the isotype specific antibodies. In the whole-cell-ELISA assays where whole meningococcal cells were used for coating, the Opa expression was tested in colony blotting (40) using mAbs 15-1-P5.5 and MN20E12.70. The colour reaction using 3,3',5,5'-tetramethylbenzidine (Sigma, St. Louis, MO, USA) and H_{2}O_{2} (p.a., Merck, Darmstadt, Germany) was performed as described (1). The geometric means were calculated, to average the antibody titres, found in the sera from mice immunised with the same samples.

**Bactericidal assays**

Serum bactericidal activity was determined as described (33) with some modifications. In short, two-fold dilutions of heat inactivated sera (30 min at 56°C) were incubated with baby rabbit complement (Pel-Freez Biologicals, Rogers, Arkansas, USA) (final concentration 20 % v/v) and 2.5 x 10^2 c.f.u. of bacteria for 60 min at 37°C. Titres are expressed as the final dilution giving at least 90 % killing of the inoculum.

**Blocking assays**

The HeLa-Neo, -CEACAM1 and -CEA transfectants were a gift from F. Grunert (Genovac AG, Freiburg, Germany) and were described previously
The HeLa transfectants were cultured in RPMI 1640 medium (Gibco, manufactured in UK) supplemented with 5% fetal calf serum (FCS) (Gibco, Logan, Utah, USA) and 0.75 mg/ml of geneticin (Calbiochem, biosciences inc., La Jolla, CA, USA). Cells were grown for 2 days to near-confluency in 24-wells tissue culture plates (Greiner, Cellstar, Kremsmünster, Austria) in 1-ml portions of RPMI 1640 medium with 10% FCS. PC2984-pMR05-opaB and PC2984-pMR05-opaJ were grown overnight on LB-agar plates as described above (growth conditions), swapped from the plate and resuspended in phosphate buffered saline (PBS) (SVM, Bilthoven, The Netherlands). The optical density (OD) of the bacterial suspension was measured at 620 nm and adjusted to 1.0. The sera were pooled and 5 μl pooled serum was added to 50 μl bacteria. This suspension was immediately added to the cells and incubated for 4 h at 37°C, in a final volume of 500 μl RPMI supplemented with 10% FCS and 25 μg/ml chloramphenicol. After the incubation of bacteria and sera with the transfected HeLa cells, the cells were washed three times with PBS to remove non-adherent bacteria, lysed with 0.5 ml 1% saponin (Sigma, St.Louis, MO, USA). Saponin fractions were plated on LB-agar plates supplemented with 25 μg/ml chloramphenicol. The incubation with non-binding mAb (15-1-P5.5 in the assays using OpaJ + E. coli and MN20E12.700 in the assays using OpaB + E. coli) was referred to as the condition under which 0% blocking occurred to determine the anti-Opa specific blocking.

**Phagocytosis assays**

To characterise the H44/76 Opa+ and Opa- variants used in this assay, 10^8 heat killed bacteria in 10 μl volume were incubated with 5μl of 10 μg/ml monoclonal antibodies; MN5C11G (α P1.16) (37), B306 (α Opc) (45), MN20E12.700 (α OpaB and OpaD) and 15-1-P5.5 (α OpaA and OpaJ) (47) for 30 minutes at 4°C, washed twice and incubated with CY3 labelled Rabbit anti mouse IgG monoclonal antibodies (Jackson Immunoresearch). After washing, the resulting fluorescence of 10^4 gated bacteria was determined using FACS analysis in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

The phagocytosis assay was performed as described by Vidarsson et al. (2001). In short, human neutrophils (PMN) were isolated using Ficoll (Pharmacia) – Histopaque (Sigma) gradients, followed by hypotonic lysis in water (for 30 seconds at 4 °C) of residual red blood cells. Sera were serially diluted in 1ml polypropylene tubes (Micronics, The Netherlands). 10^5 PMN were added along with 5 x 10^6 Alexa488-labeled bacteria (see above) in a final volume of 100 μl, and incubated at 37°C for 30 min. After washing, samples were resuspended in 300 μl FACS buffer (PBS, supplemented with 1% BSA and 0.1% azide), and fluorescence intensities of PMN were measured by flow cytometry. For all experiments, cells from a FcyRIla R/R131, FcyRIIb-NA1/NA2 individual (PCR-allotyped) were used.
Statistical analysis

ELISA results are expressed as geometric means with the standard error of eight independent observations. The results of the blocking assay are expressed as geometric means with the standard deviation of three independent observations. The data were statistically analysed by a one-tailed Student's t-test, differences were considered significant at $P < 0.05$.

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