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

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CHAPTER 6

CEACAM receptor specificity of neisserial Opa proteins expressed by *E. coli* determines the fate of bacteria during interaction with human neutrophils

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Abstract

The opacity (Opa) proteins mediate non-opsonic binding of *Neisseria meningitidis* and *Neisseria gonorrhoeae* to human neutrophils. Members of the carcinoembryonic antigen (CEA) family of proteins have been identified as the major receptors involved in this process. The CEACAM receptors present on human neutrophils are CEACAM1, CEACAM3, CEACAM6 and CEACAM8. Conflicting results have been published on the fate of bacteria after the non-opsonic Opa-mediated binding to neutrophils. We measured the uptake and survival of *E.coli* expressing OpaB, OpaC, OpaI and OpaJ proteins. The expression of OpaB and OpaJ, both binding to CEACAM1, resulted in killing while the expression of OpaI and OpaC, which bind to CEACAM1, CEACAM3, and CEACAM6, led to significantly reduced killing. However, expression of all Opa proteins resulted in increased numbers of cell-associated bacteria per neutrophil as determined with fluorescence microscopy. Significantly higher numbers of OpaC-expressing bacteria were counted per neutrophil compared to the bacteria expressing CEACAM1-binding Opa proteins, OpaB and OpaJ, in spite of the reduced killing in the case of OpaC. This suggests that CEACAM1 binding leads to internalisation followed by phagocytic killing, while Opa proteins that are also able to bind to CEACAM3 confer resistance to killing and might therefore play an important role in the pathogenesis of both gonococci and meningococci.

Introduction

Neutrophils play an important role in the first-line defence against pathogenic bacteria. These cells are specialised in phagocytosis and killing of invading micro-organisms after rapid recruitment by chemotactic signals. In neisserial infections, polymorphonuclear leukocytes (PMN) are abundantly present at the site of infection due to the massive inflammatory response of the host. In the case of *Neisseria gonorrhoeae*, non-opsonic interactions with PMN leading to efficient phagocytosis have been well studied (19, 21, 49, 50). King and Swanson (1978) observed high-level association of gonococcal variants expressing the so-called 'leukocyte association proteins', which were later identified as the heat-modifiable PII or opacity (Opa) proteins, apparently determining this specific interaction (22, 23, 28, 46, 55). Fischer and Rest (1988) demonstrated that PMN respond differently to gonococci depending upon the particular Opa proteins expressed. They found that most Opa-expressing gonococci were readily killed, but one variant expressed an Opa protein that did not increase adherence and subsequent killing. Although it is now well established that Opa proteins play a dominant role in the interaction of gonococci with neutrophils, there is conflicting evidence on the subsequent...
fate of the bacteria, as it has been suggested that the expression of Opa proteins might also result in intra-phagocytic survival. Gray-Owen et al. (1997a) suggested a scenario in which *N. gonorrhoeae* appears to have evolved the means to penetrate to the interior of phagocytic cells while avoiding their bactericidal effects. Chen et al. (2001) suggested the possibility that Opa-expressing gonococci may be protected from bactericidal attack inside the granulocytes and instead can use these cells as an intracellular niche or as vehicles to reach the next host.

Members of the CEACAM family (CD66), a group of transmembrane proteins belonging to the immunoglobulin superfamily, have been identified as major Opa-receptors (6, 11, 12, 26, 56). The CEACAM receptors present on human neutrophils are CEACAM1, CEACAM3, CEACAM6 and CEACAM8 (47, 51). Among these, many cell types express CEACAM1 and 6, while CEACAM3 and 8 are exclusively expressed by neutrophils (39). CEACAM6 and 8 are attached to the membrane with a glycosylphosphatidylinositol anchor, while CEACAM1 and CEACAM3 contain an intracellular signalling domain potentially involved in phagocytosis by neutrophils.

CEACAM1 (BGP, CD66a) is the most broadly distributed of all CEACAM-family members, being expressed in a variety of tissues and cell-types where it is primarily involved in cell-cell adhesion processes (44). The capacity of individual Opa proteins to bind to CEACAM1 was found to be strictly correlated with their ability to induce an oxidative response by PMN (25). The intracellular domain of CEACAM1 contains an immunoreceptor tyrosine-based inhibition motif (ITIM) (3). The functionality of this inhibitory motif was demonstrated by Chen and co-workers (2001a), who showed that its presence resulted in an inhibition of the calcium influx and the recruitment of phosphatases SHP-1 and SHP-2.

By contrast, CEACAM3 (CGM1, CD66d) expression is restricted to PMN (39), where it was identified as a major gonococcal Opa receptor by Chen and Gotschlich (1996). The physiological function of this protein is unclear because no natural ligand has been found yet. CEACAM3 contains an immunoreceptor tyrosine-based activation motif (ITAM) (14). The same motifs were identified in receptors specific for the Fc portion of IgG (FcγR) and IgE (FceR) (9, 52) but also in the T- and B-cell antigen receptors (43, 58). Ligation of Opa to CEACAM3 triggers tyrosine phosphorylation of the receptor by Src-family kinase(s), which promotes the uptake of bound gonococci by an actin microfilament-dependent process (38).

Although the dominant role of Opa-receptor binding in the interaction between the pathogenic *Neisseriae* and PMN is well established, the subsequent fate of the bacteria remains uncertain. Opa-mediated survival has been suggested, but no direct evidence for this phenomenon has been found up till now. We therefore studied the effect of Opa-mediated neutrophil interaction and its effect on phagocytic killing using well-defined *Escherichia*
coli expression constructs. OpaB and OpaJ derived from meningococcal strain H44/76, which target CEACAM1 exclusively, and OpaC and Opal derived from gonococcal strain MS11, which bind to CEACAM1, CEACAM3 and CEACAM6, were used. These Opa proteins were expressed on the surface of Escherichia coli, to exclude the influence of other neisserial surface proteins whose expression can often be difficult to control due to the common occurrence of phase variation in Neisseria meningitidis.

Results

Killing of *E. coli* bacteria by neutrophils

For the optimal measurement of Opa-CEACAM mediated survival or killing we first studied whether the presence of Ca$^{2+}$ and Mg$^{2+}$ during the incubation influenced the killing capacity of neutrophils. The importance of these divalent cations for phagocytosis and killing is ambiguous, since most but not all (4) *in vitro* phagocytosis studies are performed in Ca$^{2+}$- and Mg$^{2+}$-containing buffers. We therefore determined the Opa-independent uptake and killing of *E. coli* bacteria in two different HBSS buffers, i.e. with and without Ca$^{2+}$ and Mg$^{2+}$.

Significant killing of *E. coli* bacteria (strain PC2984) by neutrophils was found in the buffer with Ca$^{2+}$ and Mg$^{2+}$ (Fig. 1A), while there was absence of killing in HBSS without Ca$^{2+}$ and Mg$^{2+}$ (Fig. 1B). Furthermore a two-fold difference in bacterial growth was noted between the incubations in the two different buffers, with the highest bacterial numbers occurring in the presence of Ca$^{2+}$ and Mg$^{2+}$ (Fig.1A and 1B).

Killing of Opa-expressing *E. coli* 

Because the presence of Ca$^{2+}$ and Mg$^{2+}$ appeared to be essential for the killing capacity of the neutrophils, this was chosen as the optimal condition for testing whether Opa-CEACAM-mediated interaction leads to survival or killing. Therefore further studies were performed using HBSS with Ca$^{2+}$ and Mg$^{2+}$.

Four Opa proteins were studied, two derived from *N. meningitidis* which bind the neutrophil receptor CEACAM1 exclusively (OpaB and OpaJ) and two derived from *N. gonorrhoeae* which bind CEACAM1, CEACAM3 and CEACAM6 (OpaC and Opal) (6, 7, 17). All Opa proteins used in this study were expressed in the same *E. coli* strain PC2984. Outer membrane complexes were isolated to confirm the expression of Opa proteins in the outer membrane by western blotting (data not shown). In contrast to OpaB, OpaC and OpaJ the expression of Opal in strain PC2984 resulted in a two-fold reduced growth rate, (Fig. 2D). The reduction in growth rate correlated with a somewhat reduced amount of the Opal protein expressed in the outer membrane as
Figure 1. Survival of *E.coli* strain PC2984 incubated for 0, 1 and 2 hours with (PMN++) and without (PMN−) neutrophils in HBSS, supplemented with gelatin, with (A) and without Ca\(^{2+}\) and Mg\(^{2+}\) (B). On the x-axis the number of colony forming units (cfu) are indicated. The data shown represent the geometric means and standard errors of two independent experiments performed in duplicate. Differences were considered significant at *P < 0.05* or **P<0.01** as determined with Student’s t-test.

compared to the other three. After 1, 2 and 3 hours of incubation with PMN significant killing of OpaB- and OpaJ-expressing *E.coli* was measured (Fig. 2A and B). The expression of OpaB and OpaJ resulted in increased killing (Fig.1A). After 1 hour 27% of the *E.coli* bacteria without Opa expression were killed whereas 60% of the OpaB- and 61% of the OpaJ-expressing bacteria were killed. After 2 hours the differences between the wild type and OpaB- or OpaJ-expressing bacteria were less pronounced, of the Opa B- bacteria 60% was killed, while 77% and 81% of the OpaB and OpaJ bacteria were killed, respectively.

Strikingly, the expression of OpaC and OpaI resulted in a reduced killing, not statistically significant as compared to the Opa B- bacteria, but strongly significant in comparison to OpaB- or OpaJ-expressing *E.coli* (*P < 0.01*). The differences between the presence and absence of neutrophils were
hardly significant, except for the OpaC-expressing *E. coli* incubated for two hours (Fig. 2C and D). Despite the somewhat reduced expression level and reduced growth rate, the reduction of killing as was found for Opal-expressing *E. coli* strongly resembled the results with OpaC-expressing *E. coli*. After 1 hour 13% and 25% killing of the OpaC and Opal-expressing *E. coli* was measured, while this was 30% and 16% after 2 hours, respectively. After 3 hours the differences between the *E. coli* expressing proteins targeting CEACAM1 alone (OpaB and Opal) and the proteins binding to CEACAM1, CEACAM3 and CEACAM6 (OpaC and Opal) remained. Just 20% of the OpaC+ and 43% of the Opal+ *E. coli* were killed while in contrast 82% of the OpaB+ and 77% of the Opal+ *E. coli* were killed (Fig. 2).

**Figure 2.** Killing of *E. coli* strain PC2984 expressing OpaB (A), Opal (B) OpaC (C) or Opal (D) incubated for 0, 1, 2, and 3 hours with (PMN++) and without (PMN--) neutrophils in Ca2+ and Mg2+ containing HBSS supplemented with gelatin. On the x-axis the number of colony forming units (cfu) are indicated. The data shown represent the geometric means and standard errors of three independent experiments performed in duplicate. Differences were considered significant at *P<0.05* or **P<0.01** as determined with Student's *t* test.
Microscopic analysis of Opa-mediated neutrophil association

In order to determine whether the difference in Opa-mediated killing is due to a difference in cell-association mediated by the different Opa proteins, the incubations were repeated and samples were analysed by fluorescence microscopy. Total numbers of bacteria associated to neutrophils were counted, and no distinction was made between merely adhesive and fully internalised bacteria. Since the strongest differences in killing were recorded within 1 hour (Fig. 1 and 2), we analysed the number of cell-associated bacteria after 30 minutes and after 60 min of incubation. Negative controls were *E.coli* not expressing any Opa protein and *E.coli* expressing OpaBl72-4-6, which is an OpaB mutant not able to bind to CEACAM1 due to three point mutations as previously described (17). The latter mutant was included in order to determine any possible non-specific effects of Opa expression not related to receptor binding.

Clearly visible rods characteristic for the *E.coli* bacteria associated to viable PMN were counted. The OpaB, OpaJ, OpaC and OpaI expression resulted in higher numbers of attached and/or ingested bacteria per cell as compared to both negative controls, i.e. Opa- as well as the OpaB172-4-6-expressing bacteria (Fig. 4). Almost all differences were significant (P < 0.05), except for OpaJ (P < 0.058). In addition, a significantly higher association of OpaC- expressing bacteria was measured as compared to OpaB- (P = 0.034) and OpaJ-expressing bacteria (P = 0.033). The expression of OpaI resulted in a slightly but not significantly higher cell-association compared to the expression of OpaB (P = 0.286) or OpaJ (P = 0.238). After 1 hour no significant differences were found between the two groups of Opa proteins.

Figure 3. Association of Opal-expressing *E.coli* bacteria with neutrophils. Ethidium bromide staining was used to visualise both the bacteria and the characteristic multilobed nuclei of the neutrophils. Panels A and B were made of the same section but with the focus set at different planes.
Discussion

The uptake and killing of non-opsonized bacteria by phagocytes is an important aspect of innate immunity (reviewed by Greenberg et al., 2002). Most bacteria are internalised upon opsonisation by complement or specific antibodies. *Neisseria gonorrhoeae* and *Neisseria meningitidis* are also taken up without prior opsonization through a process mediated by specific Opa-CEACAM interaction (11, 56). Whether the specific binding to neutrophils is beneficial for the survival of the bacteria or an evolutionary adaptation of the human species to rapidly clear these infections is as yet not clear. In this study we investigated the hypothesis that targeting of the CEACAM3 receptor, which is exclusively expressed on the surface of polymorphonuclear leukocytes, might lead to bacterial survival.

An important mechanism of bactericidal action of human neutrophils against *E.coli* is perforation of the bacterial cell envelope (29). This perforation process has been found to be closely correlated with the loss of colony-forming capacity of *E.coli*. Killing was therefore monitored by measuring the number of colony forming units (cfu) recovered after incubation with and without neutrophils. We first determined the optimal *in vitro* conditions by studying killing of *E.coli* bacteria not expressing any Opa protein in two different buffers: with and without Mg\(^{2+}\) and Ca\(^{2+}\), since both conditions have been described for phagocytosis assays. In the buffer lacking these cations no killing of wild type *E.coli* bacteria was detected within two hours, while the presence

![](image)

**Figure 4.** Average number of bacteria associated to neutrophils after incubation for 30 or 60 min in Ca\(^{2+}\) and Mg\(^{2+}\) containing HBSS supplemented with gelatin. The bacteria were counted under the fluorescence microscope as described. The data shown represent the geometric means and standard errors of three independent experiments performed in duplicate.
resulted in significant killing (Fig. 1). Mg$^{2+}$ and Ca$^{2+}$, as also present in blood and interstitial fluid (24, 32, 45), appeared to be necessary for killing of co-incubated *E.coli* (not expressing any ligand) by neutrophils. This effect is most probably due to the effect of calcium ions on the cellular function of neutrophils. Phagocytic uptake into human neutrophils is associated with an increase in the intracellular free calcium concentration (20, 33), which is derived from both intra- and extracellular sources (2, 18) and is the necessary signal for phagolysosome fusion (33) and oxidative activation (37). It has been found by Wilsson *et al.* (1996) that removal of extracellular calcium causes a marked decrease in the ability to kill *Staphylococcus aureus*.

We therefore continued with the calcium-containing buffer to measure the rate of killing of *E.coli* strain PC2984 expressing different Opa proteins. Of the three different CEACAM receptors present on neutrophils, two contain an intracellular signalling domain, namely CEACAM1 and CEACAM3. These receptors are therefore the most important for Opa-mediated phagocytosis (5, 14, 38). We tested Opa proteins targeting both receptors. OpaB and OpaJ target CEACAM1 (17), while OpaC and OpaI target CEACAM1, CEACAM3 and CEACAM6 (6, 7). With the exception of OpaJ, all Opa proteins used in this study also bind to CEA (6, 7, 17), however this is not expected to play a role in this study, since CEA is not expressed on neutrophils (48, 51). Expression of OpaB and OpaJ increased the killing by neutrophils especially within the first hour, although no significant differences in killing were found as compared to the wild type strain not expressing Opa (Fig. 2). However, a highly significant reduction in killing was measured with bacteria that targeted the three CEACAM receptors (Fig. 2C and D) compared to those that bind only CEACAM1.

Since both CEACAM1 and CEACAM3 (but not CEACAM6) receptors have intracellular domains containing either an inhibitory (ITIM) or activation (ITAM) motif, respectively, the resistance to phagocytic killing might be dependent on a simultaneous interaction with both CEACAM1 and CEACAM3. This phenomenon has previously been described for the T-cell receptor (containing an ITAM motif) and CEACAM1 (containing an ITIM motif) leading to arrest of activation and proliferation of the CD4$^+$ T lymphocytes (8, 41). On the other hand, some killing of OpaC- and OpaI-expressing bacteria was still detected, which might indicate that a small fraction of bacteria is taken up via the CEACAM1 route, which was shown to lead to killing (see OpaB and OpaJ). However, some background killing as observed with the negative control (Opa-) can not be excluded, since in this assay we measure both specific (Opa-CEACAM-mediated) and non-specific interaction at the same time. Alternatively, the reduced killing might be caused by the exclusive engagement of CEACAM3 instead of the combination of CEACAM1 and CEACAM3. In this scenario CEACAM1-mediated uptake would result in killing, whereas CEACAM3-mediated uptake would lead to
survival. The different intracellular signaling domains (ITIM and ITAM, respectively) could explain these different responses to Opa-mediated binding. In order to elucidate this, one would require an Opa protein specific for CEACAM3 only, which has not been identified but might be obtainable by site-specific mutagenesis (17).

Our results found with OpaC- and Opal-expressing E.coli might seem to be contradictory to the results of Belland et al. (1992), since they could demonstrate killing of OpaC and Opal expressing E.coli bacteria by neutrophils in stead of resistance to killing as measured in our study (Fig. 2C and D). The differences are probably due to the fact that different buffers were used, since these authors tested the response of human neutrophils to recombinant Opa proteins in a buffered salt solution without calcium ions. In addition to OpaC and Opal they tested another recombinant protein expressed in E.coli, OpaA, for which no killing was found. Later studies showed that this OpaA protein is not capable of binding to any of the CEACAM receptors (7), making the E.coli variant expressing OpaA comparable to our Opa- condition. Indeed we found no killing with the Opa- E.coli when measured in a buffer lacking calcium (Fig.1B). It would thus appear that in the absence of calcium ions, the OpaC and Opal proteins behave as if they recognise CEACAM1 exclusively, giving some killing. However, Opa proteins with this specificity (like our OpaB and Opal) were not included in the study of Belland et al. (1992).

Since phagocytic killing of bacteria is strongly determined by the mode of interaction with the phagocytic cell, we studied the cell association of the Opa-expressing E.coli variants. As expected, lower numbers of cell-associated bacteria not expressing any Opa protein or expressing a non-binding Opa mutant were found as compared to the CEACAM-binding Opa proteins. Remarkably, the rate of association was higher for the bacteria expressing OpaC or Opal, since after 30 minutes more bacteria were associated to neutrophils compared to the OpaB- and Opal-expressing bacteria. The reason for the increased association rate might be that in the former case the avidity for neutrophils is increased because three different receptors are targeted, i.e. CEACAM1, CEACAM3 and CEACAM6. On the other hand, since a reduced number of bacteria are killed (Fig. 2 and 3) the number of non-killed bacteria per cell accumulates. In conclusion, a strong reduction in killing of OpaC- and Opal-expressing E.coli correlated with increased neutrophil association.

No evidence was found for the OpaC- and Opal-mediated killing of neutrophils as described by Chen et al. (2001), who reported the induction of apoptosis by a CEACAM3-binding gonococcal Opa protein. However, they measured the induction of cell-death with gonococci after incubation for 6 hours, while our measurements extended for up to 3 hours.

The ability of gonococci to survive and even grow inside phagocytic
cells has been studied previously (10, 53, 54, 60, 61). Densen et al. (1978) concluded that the failure of neutrophils to kill surface-attached gonococci was a consequence of their failure to enclose virulent gonococci within a phagosome. Parsons et al. (1982) investigated determinants of the survival of N. gonorrhoeae within human neutrophils and suggested that three proteins were associated with resistance to phagocytic killing. Hauck et al. (1998) showed that the binding of gonococci as well as E.coli expressing Opa52 (OpaC) to neutrophils induced a signal that was distinct from an Fcγ-receptor-mediated signal. It was previously observed that gonococci are able to induce reduced levels of superoxide anion as measured by the oxidation of cytochrome c in neutrophils (40). Consistent with these results are the observations of Hauck and co-workers (1997) who found that relatively strong oxidative responses were elicited by JOSKM cells upon infection by commensal Neisseria species as compared to Opa52-expressing gonococci. These indications that Opa52 might be involved in resistance to phagocytic killing clearly agree with our results obtained with OpaC and OpaI.

The indication of acute gonococcal urethritis in males is the presence of gram-negative intracellular diplococci able to survive the massive inflammatory response of the host during infection (46). Neutrophils recruited in response to chemotactic signals may also play an important role in the onset of meningococcal infections, since meningococcal derivatives, for instance LPS, might stimulate the migration of neutrophils to the site of infection (34). However, CEACAM3-mediated neutrophil interaction might be less important for meningococci than for gonococci, since only a single instance of CEACAM3 binding by a meningococcal Opa protein has been reported (57). Submucosal inflammation might lead to neutrophil accumulation and uptake of Opa-expressing bacteria. Circumvention of bactericidal mechanisms might result from binding and uptake by a route that prevents subsequent killing and could be important in translocation of the epithelial barrier.

In this study we have presented evidence that the receptor specificity of individual Opa proteins expressed in E.coli determines the nature and outcome of the bacterial interaction with neutrophils. Remarkably, the CEACAM3-targeting OpaC and I proteins led to increased neutrophil association of bacteria but reduced killing, whereas the reverse was found for the CEACAM1-specific OpaB and J. Apparently, the CEACAM binding specificity of the heterologously expressed Opa proteins determines the fate of the bacteria. This might explain why contradictory evidence has been reported previously concerning the role of Opa expression in relation to phagocytic killing. CEACAM1-binding Opa proteins, which constitute the majority, might normally function in adhesion and invasion of epithelial cells, and the increased uptake and killing by PMN might be an inadvertent consequence of this function. On the other hand, the minority of CEACAM3-binding Opa proteins might have specifically evolved to evade bacterial killing by PMN.
Materials and Methods

Bacterial strains and growth conditions

The *Escherichia coli* strain PC2984, obtained from NCCB (Phabagen collection, Utrecht, The Netherlands), contains a *phoR* mutation leading to constitutive *phoE* expression (36) and has truncated LPS due to a *galU* mutation. The expression of OpaB, OpaJ and OpaB172-4-6, at the cell surface of *E.coli* strain PC2984 was realised using the expression vector pMR05 containing the complete *phoE* gene (1). Fusion constructs were made between the *phoE* promoter and signal sequence and the mature Opa proteins as previously (16, 17). *E.coli* strain PC2984 containing pMR05-opaB, -opaJ or -opaB172-4-6 was grown at 37 °C in Luria-Bertani (LB) 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). The expression of OpaC and Opa1 was realised using the expression vector pEX (kindly provided by Robert J. Belland, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Montana, USA). Fusions were constructed between the *bla* promoter and signal peptide and the mature Opa proteins as previously described (4). *E.coli* strain PC2984 containing pEX-opaC and -opal was grown at 37 °C in LB medium or on LB agar plates supplemented with 100 μg/ml ampicillin (Roche, Mannheim, Germany). The expression of the Opa proteins in the outer membrane of strain PC2984 was tested by western blotting of outer membrane complexes that were isolated according to Davies et al. (1990).

Isolation of human neutrophils

Whole blood was obtained from healthy individuals and collected in sodium heparin tubes (Vacutette, Greiner Bio-One, Germany). Human neutrophils (PMN) were isolated using Ficoll (Pharmacia, Upsalla, Sweden) – Histopaque (Sigma, St.Louis, MO, USA) gradients, followed by hypotonic lysis in water (for 30 seconds at 4 °C) of residual red blood cells, and the remaining granulocytes were checked for viability by trypan blue exclusion. PMN were counted using 1 1/4 x 3 1/4 inch microscope slides with standardised examination chambers (Hycor Biomedical, Irvine, USA).

Non-opsonic phagocytosis assay

Bacteria were inoculated in 20 ml LB with the appropriate antibiotic at an optical density (OD) of 0.08 at 620 nm and grown till OD620: 0.24. Forty microliter of 100 x diluted bacterial suspension, corresponding with 4 x 10^5 cfu of bacteria, as determined by colony counting of appropriate dilutions, was added to 60 μl purified PMN suspension containing 4 x 10^5 PMN to give a 1:1 bacteria-to-PMN ratio. The PMN-bacteria mixtures were incubated in Hank’s Balanced Salt Solution (HBSS) (Gibco, Paisley, Scotland, UK) with or without Ca^{2+} and Mg^{2+} supplemented with 0.1% (w/v) gelatin (Sigma, St.Louis, MO,
USA) in a final volume of 100 µl at 37 °C at 250 rpm for various times. Bacteria without PMN were incubated in parallel as a negative control. After the incubation the cells were lysed with 1% saponin (Sigma, St.Louis, MO, USA). Diluted saponin fractions were plated on LB-agar plates supplemented with the appropriate antibiotic. The killing of bacteria was statistically analysed by a one-tailed Student’s t-test. Differences were considered significant at P < 0.05.

**Fluorescence microscopy**

Bacteria and PMN were incubated for 30 min or 60 min under the same conditions as described above. The bacterium-to-PMN ratio was changed to approximately 10:1 for the optimal read-out. Bacteria not associated with cells were removed by centrifugation at 110 x g for 5 min and the pellet was resuspended in gelatin-HBSS (with Ca$^{2+}$ and Mg$^{2+}$). Bacteria and PMN were visualised by adding ethidium bromide (50 µg/ml) and were viewed directly under a NIKON FXA fluorescence microscope equipped with a mercury lamp using a 100 x objective. In each sample, 50 viable PMN were analysed for cell-associated bacteria. The number of cell-associated bacteria was statistically analysed by a one-tailed Student’s t test. Differences were considered significant at P < 0.05. Fluorescence pictures were digitally acquired with a Leica DC300 camera and processed with AnalySIS software (Soft Imaging System, Munster, Germany). DNA fluorescence is illustrated as red.
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