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Naturally Occurring IgG Antibodies Provide Innate Protection against *Vibrio cholerae* Bacteremia by Recognition of the Outer Membrane Protein U

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Key Words

Vibrio cholerae · Outer membrane vesicles · Outer membrane protein U · Serum resistance · Naturally occurring IgG antibodies · C1q

Abstract

Cholera epidemics are caused by *Vibrio cholerae* serogroups O1 and O139, whereas strains collectively known as non-O1/non-O139 *V. cholerae* are found in cases of extraintestinal infections and bacteremia. The mechanisms and factors influencing the occurrence of bacteremia and survival of *V. cholerae* in normal human serum have remained unclear. We found that naturally occurring IgG recognizing *V. cholerae* outer membrane protein U (OmpU) mediates a serum-killing effect in a complement C1q-dependent manner. Moreover, outer membrane vesicles (OMVs) containing OmpU caused enhanced survival of highly serum-sensitive classical *V. cholerae* in a dose-dependent manner. OMVs from wild-type and *ompU* mutant *V. cholerae* thereby provided a novel means to verify by extracellular transcomplementation the involvement of OmpU. Our data conclusively indicate that loss, or reduced expression, of OmpU imparts resistance to *V. cholerae* towards serum killing. We propose that the difference in OmpU protein levels is a plausible reason for differences in serum resistance and the ability to cause bacteremia observed among *V. cholerae* biotypes. Our findings provide a new perspective on how naturally occurring antibodies, perhaps induced by members of the microbiome, may play a role in the recognition of pathogens and the provocation of innate immune defense against bacteremia.

Introduction

Vibrio cholerae is the causal organism of the potentially life-threatening gastrointestinal disease cholera and is commonly transmitted by contaminated water and food. The cholera-causing serogroups O1 and O139 of *V. cholerae* very rarely cause extraintestinal infections. In contrast, the non-O1 non-O139 *V. cholerae* (NOVC) isolates are commonly associated with septicemia in patients with underlying liver disorders and have a fatality rate exceeding 50% [1].

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Bacterial outer membrane proteins (OMPs) are essential components of the outer membrane of Gram-negative bacteria and are involved in multiple processes, such as nutrient transport, antimicrobial resistance and responses to environmental signals. In *V. cholerae*, outer membrane protein U (OmpU) and OmpT play important roles in bacterial physiology. The expression of OmpU and OmpT is under the control of the ToxR regulon, a master regulator of virulence genes in *V. cholerae*. ToxR negatively regulates the expression of OmpT, while OmpU is positively regulated by ToxR [2]. OmpU plays a role in resistance against bile acids and antimicrobial peptides [3]. However, the potential role of *V. cholerae* OMPs in host immunomodulation is poorly characterized.

In general, pathogenic invasive bacteria are capable of effectively evading immune responses. One of the strategies of invading bacteria is the ability to avoid the bactericidal activity of serum [4]. In earlier studies, it was demonstrated that NOVC can cause bacteremia [5]. The underlying mechanism(s) of *V. cholerae* bacteremia was less studied.

Bacterial pathogens have evolved a number of excreted and membrane-bound proteins that interfere with several steps of the complement cascade in order to survive in the human host. The complement system can be activated by the classical, alternative and mannose-binding lectin (MBL) pathways [6]. The binding of complement regulators to OMPs plays an important role in modulating complement activation among several Gram-negative pathogens [7]. While some OMPs can antagonize complement action, others may be targets for the initiation of complement. OmpC, a major immunogen on the surface of *Escherichia coli*, promotes the deposition of C1q and induction of the antibody-dependent classical complement pathway [8]. The direct activation of the classical pathway or antibody-independent binding of C1q was demonstrated recently for *Streptococcus pneumoniae* [9].

During physiological conditions, human serum contains naturally occurring antibodies of the IgG, IgM and IgA isotypes, which presumably are produced in response to endogenously occurring antigens or after the sporadic introduction of foreign antigens [10]. Several functions have been proposed for natural antibodies, including the neutralization of microbes as a consequence of their cross-reactivity to microbial antigens [11]. In earlier studies, it was shown that naturally occurring antibodies to the endotoxin core of *E. coli* can protect against *Pseudomonas aeruginosa* septicemia [12]. Recently, natural IgG was shown to bind to ficolin and MBL, soluble pattern recognition receptors that interact with pattern-associated molecular patterns at bacterial surfaces. Natural IgG-lectin immunocomplexes mediated bacterial clearance during infection/inflammatory conditions [13, 14].

In this study, we found that natural IgG directly recognizes OmpU of *V. cholerae*, thereby mediating C1q binding on to the bacterial surface via IgG resulting in complement-mediated serum sensitivity of *V. cholerae*. Furthermore, we demonstrated that OmpU on released bacterial outer membrane vesicles (OMVs) may divert IgG binding and the C1q recruitment away from the bacteria, contributing to serum resistance.

Materials and Methods

Bacterial Strains, Culture Conditions and Plasmids

The strains and plasmids used in this study are listed in table 1. Bacterial strains were grown overnight at 37°C in Luria-Bertani (LB) broth. One hundred microliters of overnight culture was inoculated into 10 ml of LB broth in a water bath shaker at 37°C and bacterial growth was monitored by optical density ($OD_{600\,nm}$) measurement. When needed, kanamycin (50 μg/ml) or carbenicillin (100 μg/ml) was added to the culture media.

Construction of the ΔompU Mutant

The *ompU* deletion mutant was constructed using procedures that have been described previously [15]. The oligonucleotide primers used are listed in table 2.

Preparation of Human Sera, Complement C1q (Active) Protein, and C1q and IgG Antisera

Normal human serum (NHS) was pooled from healthy volunteers and stored at –80°C. Heat-inactivated serum (HIS) was prepared by heating NHS at 56°C for 30 min. The NHS and HIS were diluted to 20% with sterile phosphate-buffered saline (PBS) for the serum-killing assay. Purified human C1q protein, C1q antiserum and IgG antiserum were purchased from antibodies-online (Aachen, Germany) and Dakopatts (Dako, Glostrup, Denmark).

This study was carried out in accordance with the recommendations of the local ethical committee (Regionala etikprövningsnämnden i Umeå). Ethics committee approval for the use of rabbit red blood cells for this study was issued with the permit number Dnr: A76-12. The present research has been performed in accordance with the Swedish Act (2003:460) on 'Ethical Review of Research Involving Humans' from the Ministry of Education (issued June 5, 2003; http://www.riksdagen.se/sv/Dokument-Lagar/Lagar/Svenskforfattningssamling/Lag-2003460-om-etikprovning_sfs-2003–460/).

EGTA/Mg^{2+} Treatment of NHS and IdeS Enzyme Treatment

To block classical pathway activation, NHS was treated with 5 mM $MgCl_2$ and 10 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA/Mg^{2+}) for 30 min as described previously [16]. To degrade the IgG in the NHS, IgG-degrading IdeS enzyme was used [17, 18].

Table 1. The strains and plasmids used in this study

Strain/plasmid	Description/relevant characteristics	Reference No./source
E. coli strains		
SM10λpir	*thi thr leu tonA lacY supE recA*:: RP4-2 TC::Mu Km λpir	35
MC4100	*araD*139Δ(*lac*)U169 *strA thi*	36
Δ*ompC*	Δ*ompC* derivative of MC4100	37
V. cholerae strains		
A1552	O1 El Tor, Inaba, Rif^R	30
C6706	O1 El Tor, Inaba, Str^R	38
569B	O1 Classical, Inaba, Rif^R	39
O395	O1 Classical, Inaba, Str^R	40
V:1/05	Non-O1 Non-O139, clinically isolated (2005)	Swedish Institute of Infectious Diseases, Sweden
V:5/04	Non-O1 Non-O139, clinically isolated (2004)	Swedish Institute of Infectious Diseases, Sweden
V:10/04	Non-O1 Non-O139, clinically isolated (2004)	Swedish Institute of Infectious Diseases, Sweden
V:7/04	Non-O1 Non-O139, environmentally isolated (2004)	Swedish Institute of Infectious Diseases, Sweden
Δ*ompU*	Δ*ompU* derivative of A1552	This study
Δ*ompT*	Δ*ompT* derivative of A1552	41
Δ*ompA*	Δ*ompA* derivative of A1552	30
Δ*toxR*	Δ*toxR* derivative of A1552	41
Plasmids		
pBAD18	Arabinose-inducible vector, Cb^R	42
pMMB66HE	IPTG inducible vector, Cb^R	43
pJET1.2	Cloning vector, Cb^R	Fermentas
pBR322	Cloning vector, CbR, TcR	44
pJET-*ompU*	pJET1.2, *ompU* gene from A1552	This study
pMM-*ompU*	pMMB66HE, *ompU* gene from A1552	This study
pMM-*ompU*D114A	pMM-*ompU*, D114A substitution	This study

Table 2. The primers used in this study (recognition sites for restriction enzymes are marked by bold italic letters)

Primer	Sequence (5′ to 3′)	Restriction site	Used for construction of
Primers for deletion mutant construction			
TIS-56	CGC***TCTAGA***AATAAAAAATTTCCCAACATC	*Xba*I	Δ*ompU*
TIS-57	CCCATCCACTAAACTTAAACAGTCCATAAATTTGATTTTTG		Δ*ompU*
TIS-58	TGTTTAAGTTTAGTGGATGGGTTCTAATTGTTGACTTCAGG		Δ*ompU*
TIS-59	CGC***TCTAGA***CAAATCGCCCTCAATCCTAC	*Xba*I	Δ*ompU*
Primers for cloning			
ompUup1	CGC***GTCGAC***GCTTGATGCATCACCTATTTCG	*Sal*I	OmpU clone
ompUdo2	C***GAATTC***GTGAGCAGGCGTTTGGCGTGTG	*Eco*RI	OmpU clone
Primers for site-directed mutagenesis			
ompUmutup	GGTAAAAACGCGTCTAACAACAGCCTA***GTCAAC***CGTTATA-CCTACG	D to A introduce *Hinc*II	OmpU amino acid substitution mutant clone
ompUmutdo	CGTAGGTATAACG***GTTGAC***TAGGCTGTTGTTAGACG-CGTTTTTACC	D to A introduce *Hinc*II	OmpU amino acid substitution mutant clone

Serum-Killing Assay

Bacterial cells were grown to $OD_{600\ nm}$ 2.0 in LB broth and 50 µl of bacterial culture was mixed with 50 µl of 20% NHS or HIS and incubated with the sample at 37°C for 1 h. Colony-forming units (CFU) at $OD_{600\ nm}$ 2.0 were determined with samples of each bacterial strain: A1552 (1.40×10^9 CFU/ml), C6706 (1.46×10^9 CFU/ml), 569B (1.13×10^9 CFU/ml), O395 (1.20×10^9 CFU/ml), V1:05 (1.33×10^9 CFU/ml), V5:04 (1.27×10^9 CFU/ml), V10:04 (1.26×10^9 CFU/ml) and V7:04 (1.47×10^9 CFU/ml). To block the classical pathway activation, 50 µl of 20% NHS was pretreated with

10 mM EGTA and 5 mM $MgCl_2$ for 30 min at 37°C. For IgG cleavage, 50 μl of 20% NHS was treated with 2 μg of IgG-degrading enzyme of *Streptococcus pyogenes* (IdeS) at 37°C for 2 h.

For the serum-killing assay with OMVs, a 50-μl aliquot of wild-type *V. cholerae* 569B strain was mixed with 50 μl of OMVs isolated from the wild-type *V. cholerae* strain A1552 or its *omp* mutant derivatives and 50 μl of 20% NHS or HIS. For the serum-killing assay, after blocking the classical pathway, IdeS-treated or EGTA-$MgCl_2$-treated NHS was used. The reaction mixtures were incubated at 37°C for 1 h. Subsequently, viable cell counts were determined by plating serial dilutions onto LB agar plates. Results of the serum-killing assay are reported as the percent survival, which was calculated by dividing the CFU/ml recovered after NHS or HIS incubation by the CFU/milliliter recovered from the sample without serum incubation.

For the MBL-depleted serum preparation, NHS was passed through a 5-ml column of manose-agarose beads (Sigma-Aldrich, St. Louis, Mo., USA) equilibrated in 10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4 at 4°C, and then used in the serum-killing assay. The alternative pathway has been shown to be inhibited by the absorption of properdin with bentonite [8]. Bentonite (10 mg) was washed with sterile PBS three times and incubated with NHS or HIS at 37°C for 10 min to absorb properdin.

For the neutralization assay, 20% NHS was preincubated with 1:5,000 dilutions of polyclonal rabbit antisera against OmpU [19] or 1:500 dilutions of mouse monoclonal anti-human complement C1q antibody or 1:1,000 dilutions of anti-IgG rabbit anti-serum (Dako) or 1:1,000 dilutions of anti-Hcp polyclonal antiserum [20] at 37°C for 30 min, followed by the serum-killing assay.

For the bacterial washing experiment, samples from cultures at $OD_{600\,nm}$ 2.0 of WT A1552 and WT 569B were washed three times with PBS and followed by serum-killing assay. The results were compared to serum-killing assay with nonwashed bacteria.

Electron Microscopy

The bacterial cells were negatively stained and examined by transmission electron microscopy as described previously [21]. For ultrathin sectioning and ferritin labeling, bacteria were grown on LB agar plates overnight at 37°C, harvested and washed once with cacodylate buffer (0.1 M, pH 7.0). Bacterial cells were fixed with 5% glutaraldehyde in cacodylate buffer for 2 h at room temperature. Fixed bacteria were washed and resuspended in cacodylate buffer containing 1 mg of polycationic ferritin (Sigma-Aldrich) per milliliter. After 30 min at room temperature, samples were diluted 1:10 with the same buffer. The ferritin-labeled bacterial cells were washed three times and fixed with 4% agar. After fixation, the sample was treated for 2 h with 1% osmium tetroxide and then washed three times. Samples were dehydrated in a graded series (30–100%) of ethanol solutions, washed twice with propylene oxide and embedded in Epon by a rapid-embedding method. Thin sections were cut and placed on 300-mesh formvar/carbon grids, stained with uranyl acetate and lead citrate, and examined under a JEOL EX transmission electron microscope operating at an accelerating voltage of 100 kV.

Western Blot Analysis

Bacteria cells were grown to $OD_{600\,nm}$ 2.0 in LB broth in a water bath shaker at 37°C. Cells were harvested by centrifugation at 14,000 rpm for 3 min at 4°C. The pellets were resuspended in 20 mM Tris-HCl buffer, pH 8.0, and used as protein samples. Protein samples were denatured in sample buffer containing 10% glycerol, 0.05% bromophenol blue, 2% SDS, 5% 2-mercaptoethanol and 10 mM Tris-HCl, pH 6.8, and resolved by 13.5% SDS-PAGE with a discontinuous buffer system at a constant voltage of 60 V for the stacking gel and 120 V for the resolving gel [22]. The proteins in the gel were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, Mass., USA) in standard transfer buffer with a Bio-Rad semidry transfer system at 23 V for 35 min. Proteins with known molecular masses (Thermo Scientific, Waltham, Mass., USA) were used as molecular mass markers. After the transfer was completed, the membrane was blocked with 5% skimmed milk in PBS (pH 7.2) containing 0.05% Tween-20 (PBST) at 4°C overnight. The immunoblot membrane was incubated with 1:5,000 dilutions of polyclonal antisera against OmpU [19] as a primary antibody for 1 h. Goat anti-rabbit HRP-conjugated IgG antibody (AgriSera AB, Vännäs, Sweden) was used as a secondary antibody at a final dilution of 1:25,000. The ECL^+ chemiluminescence system (GE Healthcare) was used to detect immunoreaction bands that were recorded using a Fluor-S MultiImager (Bio-Rad, Hercules, Calif., USA) and by autoradiography.

Far Western Analyses

For the detection of indirect binding of C1q on the OmpU by Far Western analysis, following blocking of the immunoblot membrane with 5% milk, the membrane was incubated with 20% NHS for 2 h at room temperature and subsequently the membrane was rinsed with PBST. The complement C1q protein (10 μg/ml) in PBST was overlaid on the membrane and incubated for 2 h at room temperature. After washing four times with PBS, the membrane was incubated with a mouse anti-human complement C1q mAb (Aachen) diluted 1:1,000 in PBST for 1 h. After four further washes in PBST, the membrane was incubated in an anti-rabbit horseradish peroxidase-conjugated pAb preparation (Dako) at a final dilution of 1:10,000 in PBST-M for 1 h. Detection was performed as described above.

Isolation of OMVs from E. coli and V. cholerae

OMVs from *V. cholerae* WT-A1552, *E. coli* WT-MC4100 and their *omp* mutants were isolated from the bacterial cultures as previously described [23]. Briefly, bacteria were inoculated into a 200-ml culture flask containing LB and incubated for 16 h at 37°C with shaking. Bacterial cells were removed from the culture fluid by centrifugation at 5,000 *g* for 30 min. The supernatant was sterile filtered through a 0.22-μm PVDF membrane filter (Millipore). The cell-free supernatant was centrifuged at 100,000 *g* for 2 h at 4°C in a 45 Ti rotor (Beckman) to pellet the vesicles. The OMVs were suspended in 20 mM of Tris-HCl (pH 8.0) and adjusted to equal the total protein concentrations measured by a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The protein concentration of the OMV samples was estimated using a bicinchoninic acid assay kit (Thermo Scientific Pierce, Rockford, Ill., USA). To determine the number of OMVs, the samples (5 mg/ml) were subjected to analysis by Nanosight NS300 (Malvern) and nanoparticle tracking analysis, as described earlier [24]. The OMV sample obtained from the Δ*ompA* mutant contains 1.45×10^{11} particles/μl, whereas the wild-type strain A1552, Δ*ompU*, Δ*ompT* and Δ*toxR* mutant contain 1.51×10^{10}, 1.62×10^{10}, 1.66×10^{10} and 1.49×10^{10} OMVs/μl, respectively.

Construction of ompU and ompU D114A Clone

For the construction of the wild-type *ompU* clone, a fragment of the *ompU* gene (VC0633) was PCR amplified from chromosomal DNA isolated from strain A1552 using the primers *ompUup1* and *ompUdo2* with additional *Eco*RI or *Sal*I restriction endonuclease recognition sites at the ends. The PCR amplification was performed using Kapa HiFi HotStart Polymerase (Kapa Biosystems) according to the manufacturer's manual. The PCR product was cloned into pJET1.2 for sequencing and amplification purposes. A correct plasmid construct, pJET-*ompU*, was digested with *Eco*RI and *Sal*I, and the gel-purified *ompU* fragment was cloned into *Eco*RI and *Sal*I digested pMMB66HE, resulting in plasmid pMM-*ompU*.

For the construction of the *ompU* D114A mutant clone, pJET-*ompU* was used as a template to replace aspartic acid (D114) for alanine (A) in the *ompU* gene sequence by site-directed mutagenesis. The primer pair introducing the alanine residue, *OmpU*mutup and *OmpU*mutdo, creates a *Hinc*II site instead of the bases coding for the aspartic acid. Site-directed mutagenesis was performed using 250 ng of pJET-*ompU* DNA and Kapa HiFi HotStart Polymerase (Kapa Biosystems) in a 25-μl reaction according to the manufacturer's manual (58°C anneal 30 s, 69°C extension 9 min, 30 cycles). The template plasmid was then digested by *Dpn*I (fast digest; Fermenta) for 1 h at 37°C, and afterwards the enzyme was inactivated at 80°C for 5 min followed by transformation into $CaCl_2$-competent DH5α. Successful mutagenesis was verified by sequencing. The mutated *ompU* fragment was excised by digestion with *Eco*RI and *Sal*I, gel-purified, and cloned into *Eco*RI and *Sal*I digested pMMB66HE, resulting in plasmid pMM-*ompU*D114A (Δ*ompU*/D114A).

Determination of anti-OmpU IgG in NHS by ELISA

High-binding 96-well polystyrene plates (Thermo Scientific) were coated with 50 μl of OMVs isolated from *V. cholerae* or *E. coli* and 150 μg/ml BSA in 0.1 M carbonate-bicarbonate buffer, pH 9.5. The plates were incubated for 1 h at 37°C and then overnight at 4°C, and were washed three times in PBST. Nonspecific binding was blocked with 5% milk diluted in PBS for 2 h at 37°C. After washing three times with PBST, 100 μl of 20% NHS from 9 donors in PBST was added into the first well and diluted serially 2-fold. PBS was used instead of 20% NHS as a negative control. The plate was incubated for 2 h at 37°C. After washing three times with PBST, the 1:5,000-diluted peroxidase-conjugated anti-human IgG antibodies (AgriSera AB) were added, followed by 1 h of incubation at 37°C and six additional washes with PBST. Detection was performed using the TMB substrate Kit (Thermo Scientific) as recommended by the manufacturer.

Statistical Analyses

All statistical analyses were performed with Student's t test. $p < 0.05$ was considered significant.

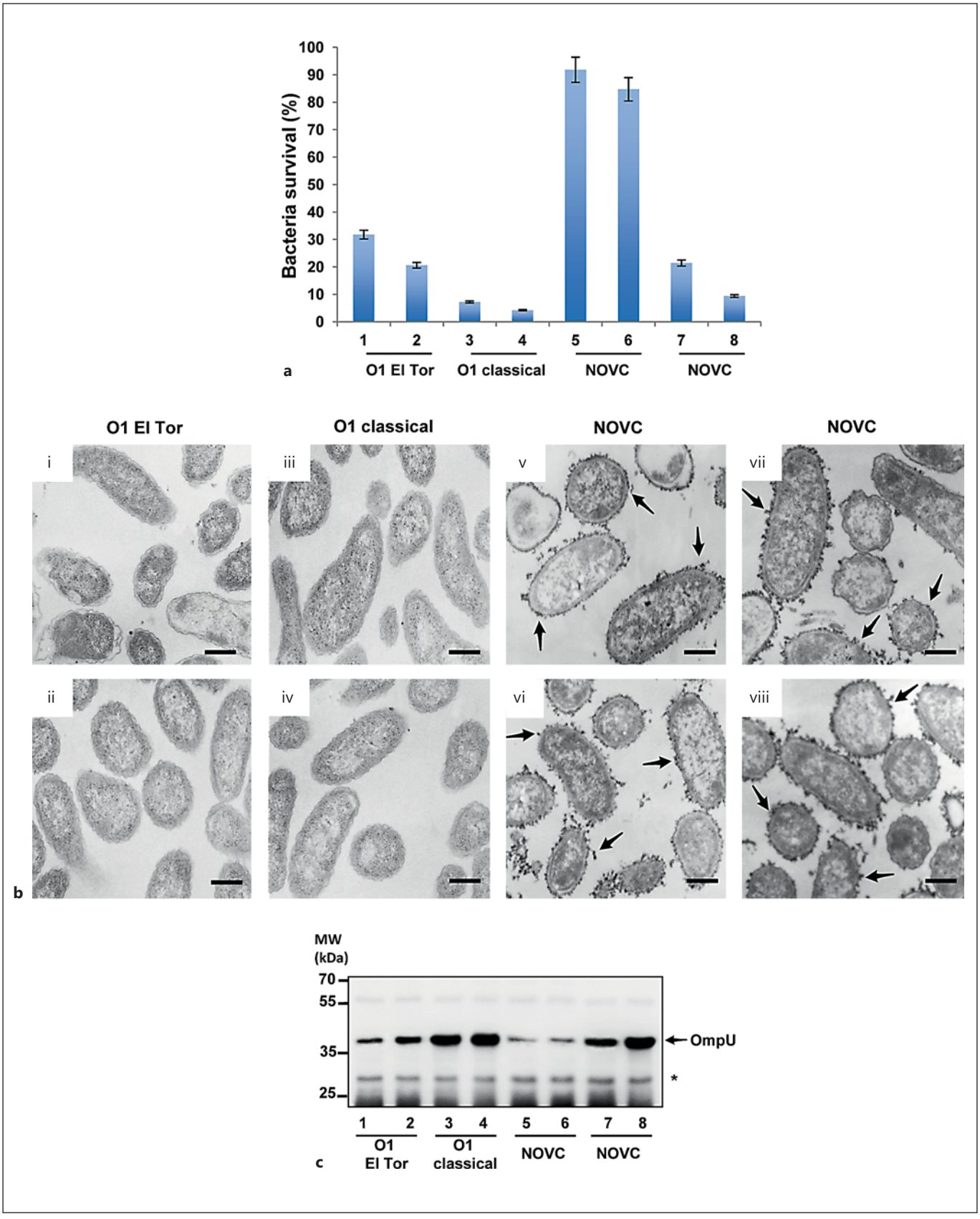
Results

Analysis of Serum Resistance in Different V. cholerae Serogroups

Limited information is available on the phenotypic differences of various *V. cholerae* serogroups in relation to serum resistance. To investigate the levels of serum resistance among isolates of different *V. cholerae* serogroups, serum-killing assays were performed with NHS. We performed serum-killing assays using different concentrations of serum, i.e. 10, 20 and 30%. We found no significant difference in bactericidal activity among these concentrations and, therefore, decided to use a 20% serum concentration for further experiments. Wild-type *V. cholerae* O1 El Tor strains A1552 and C6706, the wild-type O1 classical strains 569B and O395, and the wild-type NOVC strains V:1/05, V:5/04, V:10/04 and V:7/04 were tested for serum sensitivity (fig. 1a). HIS was used as a negative control. The O1 El Tor strains A1552 and C6706 showed 31.8 and 20.6% survival, respectively (fig. 1a, columns 1 and 2), whereas the O1 classical strains 569B and O395 were more susceptible to serum killing, with only 7.2 and 4.2% survival, respectively (fig. 1a, columns 3 and 4). The *V. cholerae* NOVC strains V:1/05 and V:5/04 displayed a quite high resistance level with 91.8 and 84.7% survival, respectively (fig. 1a, columns 5 and 6). In contrast, the wild-type NOVC strains V:10/04 and V7:04 had 21.4 and 9.4% survival, respectively (fig. 1a, columns 7 and 8). All strains showed a similar capacity to survive in the HIS (data not shown). This initial screening suggested that serum resistance among *V. cholerae* varies both within and among different serogroups.

Fig. 1. Serum sensitivity, electron microscopic analysis and Western blot analysis of different *V. cholerae* serogroups. **a** Serum sensitivity of different *V. cholerae* serogroups. O1 El Tor *V. cholerae* strains A1552 and C6706 (columns 1 and 2, respectively), O1 classical *V. cholerae* strains 569B and O395 (columns 3 and 4, respectively), and NOVC strains V:1/05, V:5/04, V:10/04 and V7:04 (columns 5–8, respectively). The survival rate (percentage) is indicated. Values represent the mean ± SD of three independent experiments. **b** Electron micrograph of ultrathin sections of polycationic ferritin-labeled bacterial cells. O1 El Tor strains A1552 and C6706 (i and ii, respectively), O1 classical strains 569B and O139 (iii and iv, respectively), and NOVC strains V:1/05, V:5/04, V:10/04 and V7:04 (v–viii, respectively). The arrows show ferritin particles surrounding the bacterial cells. Scale bars = 250 nm. **c** Immunoblots of whole-cell lysates isolated from different *V. cholerae* serogroups. The immunoblot membrane was probed with polyclonal anti-OmpU. The arrow shows the OmpU reaction band. The asterisk shows nonspecific reaction bands to estimate the amount of sample loading. O1 El Tor strains A1552 and C6706 (lanes 1 and 2, respectively), O1 classical strains 569B and O139 (lanes 3 and 4, respectively) and NOVC strains V:1/05, V:5/04, V:10/04 and V7:04 (lanes 5–8, respectively).

(For figure see next page.)



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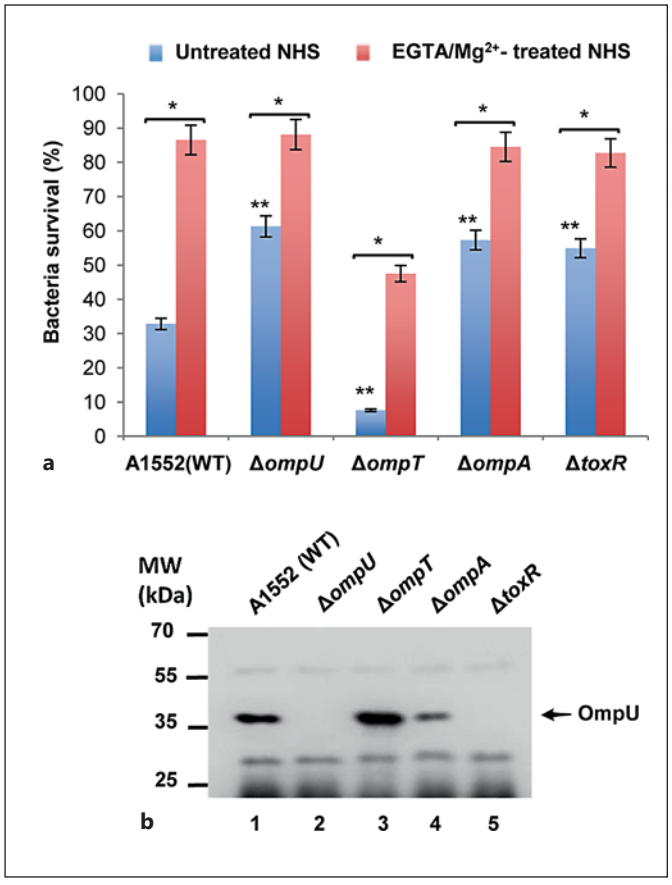

Fig. 2. Serum sensitivity assay of the wild-type (WT) strain *V. cholerae* A1552 and its outer membrane protein-deficient derivatives. **a** The bacterial survival percentage after incubation with untreated NHS is shown with blue columns. The red columns represent the survival percentage of bacterial strains incubated with NHS pretreated with EGTA/Mg^{2+} to inhibit the classical pathway of complement activation (colors refer to the online version only). Values represent the mean ± SD of three independent experiments. The single asterisks indicate EGTA/Mg^{2+}-treated NHS versus untreated NHS ($p < 0.005$). The double asterisks indicate untreated NHS wild-type A1552 versus its mutant derivatives ($p < 0.005$). **b** Immunoblot of whole-cell lysates from the wild-type *V. cholerae* strain A1552 and its mutant derivatives. The arrow indicates immunoblot reactivity to OmpU. The molecular weight markers are shown at the left of the membrane.

Role of OmpU but Not Capsular Polysaccharide Production in V. cholerae Serum Resistance

Encapsulation is an important virulence factor for a number of bacterial species, contributing protection against host defenses including serum killing and phagocytosis [25]. To determine whether the presence of capsular polysaccharide at the surface of *V. cholerae* would correlate to the relative serum resistance, ultrathin sections of bacterial cells of the different strains were stained with polycationic ferritin and examined by transmission electron microscopy (fig. 1b). The moderately serum-resistant *V. cholerae* O1 El Tor strains A1552 and C6706 (fig. 1b, panels i and ii) and the serum-sensitive O1 classical strains 569B and O395 (fig. 1b, panels iii and iv) seemed to lack a capsular layer. In the case of the serum-resistant NOVC strains V:1/05 and V:5/04 (fig. 1b, panels v and vi) and the serum-sensitive NOVC strains V:10/04 and V:7/04 (fig. 1b, panels vii and viii), we observed an electron-dense layer completely surrounding the cells. Therefore, our data indicated that the presence of a capsule per se was not responsible for serum resistance.

Some OMPs have been shown to play a role in resistance to complement-mediated serum killing of Gram-negative bacteria [7]. In order to investigate if the major OMPs could possibly be involved in serum resistance of *V. cholerae*, the OmpU and OmpT protein expression levels were analyzed by Western blot. As shown in figure 1c, OmpU expression was inversely correlated with serum resistance of the different *V. cholerae* serogroups. The most serum-resistant *V. cholerae* strains, V:1/05 and V:5/04 (lanes 5 and 6), expressed the lowest level of OmpU, while much higher levels of OmpU were observed in the most serum-sensitive *V. cholerae* classical strains (lanes 3 and 4). These observations suggest that the level of OmpU expression in *V. cholerae* may determine the level of serum resistance in *V. cholerae*.

OmpU-Mediated Bactericidal Effect of Serum on V. cholerae via the Classical Complement Pathway

To further analyze the role of the OmpU protein in serum resistance of *V. cholerae*, we studied a selection of mutant derivatives of the moderately serum-resistant *V. cholerae* strain A1552. In-frame deletions in the *ompU*, *ompT*, *ompA* and *toxR* genes were constructed in the A1552 strain as described in Materials and Methods. The serum resistance of these mutants was compared with that of the wild-type strain A1552. The survival rates of Δ*ompU*, Δ*ompA* and Δ*toxR* mutants were 61.3, 57.3 and 54.9%, respectively, whereas the wild-type strain A1552 had a 32.8% survival rate (fig. 2a, blue columns). Interestingly, the Δ*ompT* mutant had a clearly lower survival rate (7.6%) when compared with the wild-type A1552 and the other tested mutants (fig. 2a, blue columns). The survival rates of the wild-type strain A1552 and its mutant derivatives were similar in HIS (data not shown), and there was no difference in the growth rate indicating that the mutations did not impair growth (see online suppl. fig. S1; see www.karger.com/doi/10.1159/000443646 for all online

suppl. material). The OmpU expression level was determined in the different strains by immunoblot analysis (fig. 2b). As expected, the Δ*toxR* strain produced no OmpU (fig. 2b, lane 5) since ToxR is a positive regulator of OmpU expression [2]. Interestingly, in the Δ*ompA* strain, OmpU expression was lower than that of the wild type (fig. 2b, lanes 1 and 4), which correlated with reduced serum sensitivity of the Δ*ompA* strain in comparison to the wild type. In addition, we observed that the survival of the Δ*ompA* and Δ*toxR* mutants were similar, although in contrast to the case of the Δ*ompA* mutant there was no detectable level of OmpU in the Δ*toxR* mutant. It might indicate that some additional factor(s) could be involved in serum resistance in the Δ*ompA* mutant. Similarly, the higher serum sensitivity observed for the Δ*ompT* mutant was correlated with an increased level of OmpU protein in that mutant (fig. 2b, lanes 3). By comparing the wild-type strain A1552 and these OMP mutant derivatives, we conclude that there was a strong correlation between higher levels of OmpU and higher serum sensitivity.

Complement activation may proceed through one or more of three pathways: the classical pathway, the MBL pathway or the alternative pathway [6]. To investigate which complement pathway was involved in the OmpU-mediated bactericidal activity of NHS, we performed serum bactericidal assays on the wild-type strain A1552 and its mutant derivatives using serum treated with EGTA/Mg^{2+}, which inhibits the classical complement pathway. The bactericidal activity of EGTA/Mg^{2+}-treated NHS was significantly lower when compared to that of untreated NHS (fig. 2a, compare the blue and red columns). The Δ*ompT* strain showed 47.5% survival in EGTA/Mg^{2+}-treated serum, whereas the survival rates of the wild-type strain A1552 and the Δ*ompU*, Δ*ompA* and Δ*toxR* mutants in EGTA/Mg^{2+}-treated serum were all above 80% (fig. 2a, red columns). In all cases the survival was clearly lower in untreated NHS (fig. 2a, blue columns). In order to assess whether activation of the lectin pathway or the alternative pathway would be involved in OmpU-mediated serum bactericidal activity, serum-killing assays were performed using MBL-depleted NHS or bentonite-absorbed NHS. The alternative pathway was shown to be inhibited by the absorption of properdin with bentonite [8, 26, 27]. The effect on the viability of the wild-type strain A1552 and its mutant derivatives remained similar to that of NHS without such treatments (fig. 3, compare the green and violet columns with the blue column). All strains showed a similar ability to survive in HIS (data not shown). These results suggested that the OmpU-mediated bactericidal

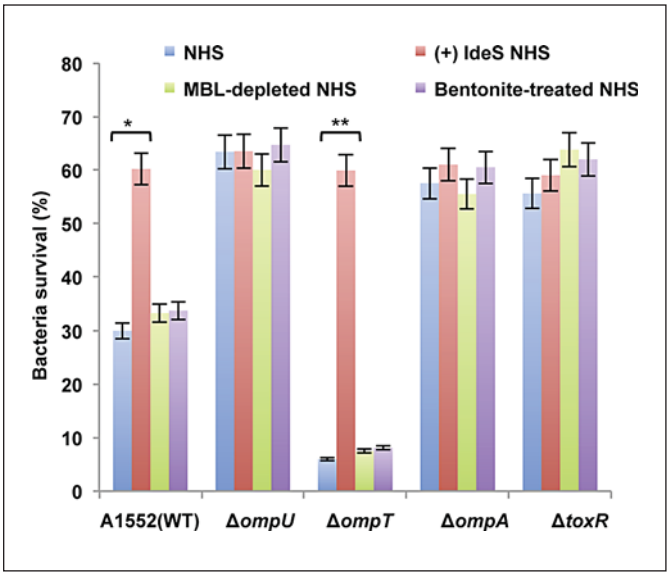

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Fig. 3. Serum sensitivity assay with *V. cholerae* using NHS treated with IgG-degrading enzyme (IdeS), MBL-depleted NHS or bentonite-treated NHS. The blue, red, green and violet columns represent the percentage of bacterial surviving after incubation with NHS, IdeS-treated NHS, MBL-depleted NHS and bentonite-treated NHS, respectively (colors refer to the online version only). Values represent the mean ± SD of three independent experiments. The single asterisk indicates wild-type *V. cholerae* A1552 in NHS without IdeS versus NHS after treatment with IdeS ($p < 0.05$). The double asterisk indicates the Δ*ompT* mutant in NHS without IdeS versus with IdeS ($p < 0.05$).

effect does not involve the lectin pathway or the alternative pathway. Taken together, these findings suggested that the classical complement pathway is the primary reason behind the OmpU-mediated serum killing of *V. cholerae* by NHS.

The classical pathway of the complement system can be triggered by the binding of either IgG or IgM antibody to antigens. To investigate whether IgG was involved in the OmpU-mediated bactericidal effect, the serum-killing assay was performed using NHS with or without treatment with IdeS, an IgG-specific cleavage protease of *S. pyogenes* [17, 18]. When serum was pretreated with IdeS, survival of the wild-type A1552 and the Δ*ompT* mutant were about 60%, i.e. similar to that of the Δ*ompU*, Δ*ompA* and Δ*toxR* mutants in NHS (fig. 3). The Δ*ompU*, Δ*ompA* and Δ*toxR* mutants had similar survival rates in serum with or without IdeS treatment. These data suggested that we need to consider that OmpU and OmpT are involved in antibody-dependent bactericidal activity of complement primarily via IgG and not via IgM.

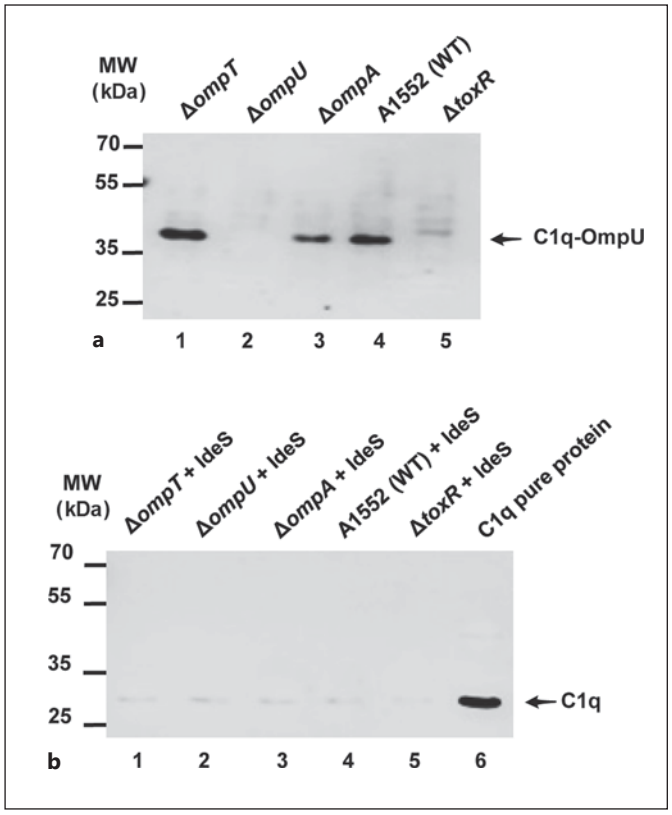

Fig. 4. Far Western analysis to detect C1q binding to the major Omps of *V. cholerae.* **a** SDS-PAGE and immunoblot analysis to detect indirect binding of C1q to OmpU. Lane 1, Δ*ompT*; lane 2, Δ*ompU*; lane 3, Δ*ompA*; lane 4, wild-type A1552; lane 5, Δ*toxR*. The arrow shows the C1q signal on the OmpU protein. Molecular weight markers are shown to the left of the membrane. **b** SDS-PAGE and immunoblot analysis of antibody-dependent binding of C1q on OmpU after IdeS treatment. Lane 1, Δ*ompT*; lane 2, Δ*ompU*; lane 3, Δ*ompA*; lane 4, wild-type A1552; lane 5, Δ*toxR*. Lane 6 was loaded with 100 ng of purified C1q protein. The arrow shows the immunoreaction band of C1q protein. Molecular weight markers are shown to the left of the membrane.

V. cholerae OmpU Involved in C1q Binding via IgG

Antibody-antigen binding exposes a binding site on the antibody for the C1 complex of the complement system. The C1 complex is composed of one C1q molecule, two C1r and two C1s molecules. The C1q molecule was shown to be responsible for binding to the antibody [28]. To define the role of OmpU in serum sensitivity of *V. cholerae,* we performed the Far Western analysis using NHS instead of purified C1q as described in Materials and Methods. The detection using anti-C1q antibody indicated that there was C1q binding as represented by a band on the membrane at the expected position corresponding to the size of OmpU, which was clearly observed in the case of the wild-type strain A1552 (fig. 4a, lane 4). Furthermore, there was no similar C1q binding detected with the samples from the Δ*ompU* or Δ*toxR* mutant strains (fig. 4a, lanes 2 and 5), in line with the hypothesis that there would be an OmpU-dependent C1q binding to *V. cholerae*. Consistently, this analysis indicated a slightly higher binding efficiency of C1q in the case of the sample from the Δ*ompT* mutant that had a higher expression level of OmpU than the wild-type strain (fig. 4a, lane 1). In respect to this lower level of OmpU expressed, the Δ*ompA* mutant appeared to bind somewhat less C1q (fig. 4a, lane 3). From these results we hypothesized that antibodies in NHS that were directed against OmpU would promote the interaction of C1q with the OmpU protein of *V. cholerae*. To further confirm whether the presumed interaction of C1q and OmpU was mediated by some anti-OmpU antibody present in NHS, the ligand overlay assay was performed after depleting the IgG of NHS using the enzyme IdeS. There was no evidence of C1q binding to OmpU in any of the samples from strains tested in the ligand overlay assay after IdeS pretreatment of NHS (fig. 4b).

OMVs Protect V. cholerae against Serum Killing by Binding to the IgG-C1q Complex

Recently, we discovered that OMVs isolated from *V. cholerae* may protect the bacteria against antimicrobial peptides by sequestering and quenching the effect of antimicrobial peptides through binding on the OMV surfaces [29]. In order to test if OMVs could also be involved in the serum resistance of *V. cholerae*, OMVs were isolated from the wild-type strain A1552 and from each of the Δ*ompU*, Δ*ompA*, Δ*ompT* and Δ*toxR* mutants. Using the bicinchoninic acid assay kit, the total protein concentration of each OMV sample was measured. All samples had similar protein concentrations except the OMV sample isolated from the Δ*ompA* mutant [wild-type strain A1552 (5.12 mg/ml), Δ*ompU* (4.61 mg/ml), Δ*ompA* (48.98 mg/ml), Δ*ompT* (4.54 mg/ml) and Δ*toxR* (5.39 mg/ml)]. The protein concentration of the OMV sample obtained from the Δ*ompA* mutant was 10 times higher in comparison with other samples. In our earlier studies, we have shown that the Δ*ompA* mutant released a larger amount of OMVs when compared to the wild-type strain A1552 [30]. Furthermore, the number of OMV particles was measured by nanoparticle tracking analysis using the Nanosight equipment as described in Materials and Methods. Our data show that the Δ*ompA* mutant released 10 times more OMVs in comparison with the other strains. For the serum-killing

protection assay, we used OMV samples in a volume of 50 µl containing 5×10^{10} OMV particles. The classical *V. cholerae* wild-type strain 569B was used in the serum-killing protection assay since this strain showed very low serum resistance among strains of the different *V. cholerae* serogroups tested (fig. 1). As shown in figure 5a, the survival rates of *V. cholerae* strain 569B in NHS and in the presence of OMVs isolated from the wild-type strain A1552 were significantly increased from 6.8 to 30.7% (fig. 5a, panels 1 and 2, blue columns). In addition, the survival rate of the wild-type *V. cholerae* strain 569B was increased to 51% when IdeS-treated NHS was used in the killing assay (fig. 5a, panel 1, red column). When we tested the survival of strain 569B incubated with OMV+IdeS-treated NHS, the survival of 569B was nevertheless not recovered (fig. 5a, panel 2, red column). This effect might be due to both inhibition of IgG-C1q binding on the bacterial cells and IdeS degradation of OMV-associated IgG. It might indicate that some OMV-associated additional factor(s) could be involved. We further tested the role of OmpU in OMV-mediated protection against serum killing of *V. cholerae* using OMVs isolated from the Δ*ompU*, Δ*ompT*, Δ*ompA* and Δ*toxR* mutants. The highest survival rate of strain 569B (44.1%) was observed when the NHS treatment was performed in the presence of OMVs isolated from the Δ*ompT* mutant (fig. 5a, panel 4, blue column). Much lower survival rates (14.2, 14.7 and 18.5%) were observed when the serum treatment was performed in the presence of OMVs isolated from the Δ*ompU*, Δ*ompA* and Δ*toxR* mutants, respectively (fig. 5a, panels 3, 5 and 6, blue columns). These results indicated that OmpU-associated OMVs could efficiently quench the complement activity and we hypothesized that C1q in the NHS would be sequestered through an IgG-C1q complex binding on the surface of the OMVs via the OmpU protein. As described above, the Δ*ompA* mutant showed increased serum resistance at the same level as observed in the case of the Δ*toxR* mutant in comparison with the wild-type A1552. Increased release of OMVs from the Δ*ompA* mutant might contribute to a higher serum resistance of bacteria. In the serum-killing assay, we used a 50-µl bacterial suspension which contains OMVs spontaneously released from the bacterial cells during growth. In order to test if these released OMVs can affect the serum-killing activity, we performed the serum-killing assay using washed *V. cholerae* wild-type strains A1552 and 569B. However, as shown in online supplementary figure S2, there was no difference in survival between washed and unwashed bacteria.

Role of OmpU D114 Amino Acid Residue in the Serum Sensitivity of V. cholerae

In earlier studies, it was shown that a negatively charged residue D116 of *V. cholerae* OmpU influenced the permeation and binding of hydrophilic compounds, including β-lactam antibiotics and Na-deoxycholate bile salt [31]. In the wild-type *V. cholerae* strain A1552 the aspartic acid residue is at position 114 instead of 116. To test if D114 has a role in the OmpU-mediated serum sensitivity of *V. cholerae*, we performed site-directed mutagenesis to generate an *ompU* plasmid clone with a D114A substitution. Serum-killing assays were performed using the Δ*ompU* mutant and the Δ*ompU* mutant harboring either the wild-type *ompU*$^+$ clone (Δ*ompU*/WT), the D114A substitution clone (Δ*ompU*/D114A) or the vector control (Δ*ompU*/Vec). The Δ*ompU* mutant strain, which had a 62.3% serum survival rate, became significantly serum sensitive (14.9% survival) when the wild-type *ompU*$^+$ plasmid clone was introduced (fig. 5b, panels 2 and 4, blue columns), whereas the introduction of the D114A substitution, as in the derivative Δ*ompU*/D114A, did not alter serum sensitivity and it remained at the same level as that of the Δ*ompU* mutant (fig. 5b, panels 2 and 5, blue columns). All strains showed a similar ability to survive in HIS (data not shown). The expression levels of OmpU protein were similar in the Δ*ompU*/WT and the Δ*ompU*/D114A derivatives (online suppl. fig. S3, lanes 4 and 5).

Role of the OmpU-IgG-C1q-Mediated Classical Pathway in Bactericidal Activity

To test the effect of the OmpU-IgG-C1q-mediated activation of the classical pathway on killing OmpU-expressing *V. cholerae*, we performed neutralization assays using anti-C1q, anti-IgG and anti-OmpU antisera. After 1 h of incubation of *V. cholerae* in NHS preincubated with anti-C1q, anti-IgG or anti-OmpU antibodies, survival of the *V. cholerae* wild-type strain A1552 increased significantly (fig. 5b, panel 1; compare the blue column with the red, green and violet columns). The neutralization effect of antibodies recognizing OmpU, IgG or C1q was also observed in the case of the Δ*ompU*/WT derivative (fig. 5b, panel 4; compare the blue column with the red, green and violet columns). On the basis of the results in figure 5b and those reported above, we propose that the serum killing of OmpU-expressing *V. cholerae* was mediated mainly by the anti-OmpU antibody (IgG) and the C1q-dependent classical pathway present in NHS, and that the *ompU* mutant bacteria survived by escaping this complement killing mechanism. The anti-

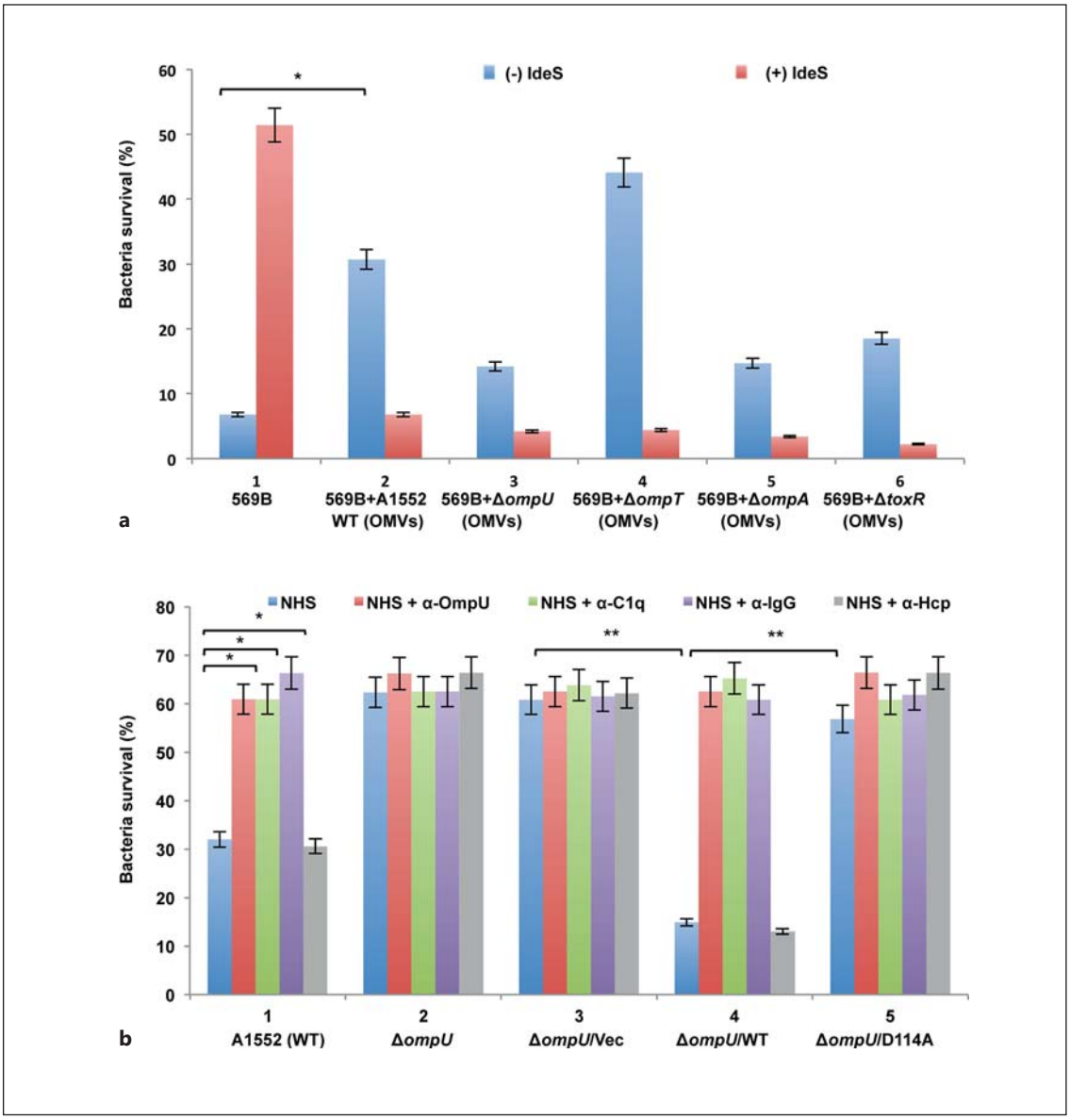

Fig. 5. Serum sensitivity assay with *V. cholerae* monitoring the effects of IdeS, OMVs and antisera raised against OmpU, C1q, IgG or Hcp. **a** Panel 1: blue column, percent survival of *V. cholerae* strain 569B in NHS; red column, percent survival of *V. cholerae* strain 569B in NHS treated with IdeS. Panel 2: blue column, percent survival of 569B in NHS and OMVs from wild-type strain A1552. Panel 3: blue column, percent survival of 569B in NHS with OMVs from the Δ*ompU* mutant. Panel 4: blue column, percent survival of 569B in NHS with OMVs from the Δ*ompT* mutant. Panel 5: blue column, percent survival of 569B in NHS with OMVs from Δ*ompA* mutant. Panel 6: blue column, percent survival of 569B in NHS with OMVs from Δ*toxR* mutant. Panels 2–6: red columns represent percent survival of the same strains in the presence of NHS+OMVs+IdeS (colors refer to the online version only). Values represent the mean ± SD of three independent experiments. The asterisk indicates the survival percentage of *V. cholerae* 569B in NHS treated with OMVs versus NHS without treatment with OMVs ($p < 0.05$). **b** Blue columns represent the survival of *V. cholerae* wild-type strain A1552 and its derivatives in NHS alone. Red columns show the percent survival of A1552 and its derivatives treated in NHS and anti-OmpU antiserum. Green columns represent the survival percentage of A1552 and its derivatives in NHS and anti-C1q antiserum. Violet columns show the percent survival of A1552 and its derivatives in NHS and anti-IgG antibody. Gray columns show the percent survival of A1552 and its derivatives in NHS and anti-Hcp antibody (colors refer to the online version only). The strain genotypes are indicated below the columns. Values represent the mean ± SD of three independent experiments. Single asterisks indicate wild-type *V. cholerae* A1552 in NHS versus NHS pretreated with anti-C1q antibody, anti-IgG antibody or anti-OmpU antibody ($p < 0.05$). Double asterisks indicate Δ*ompU* mutant in NHS versus the Δ*ompU* mutant harboring either vector control or the wild-type *ompU* clone or *ompU*/D114A clone in NHS ($p < 0.05$).

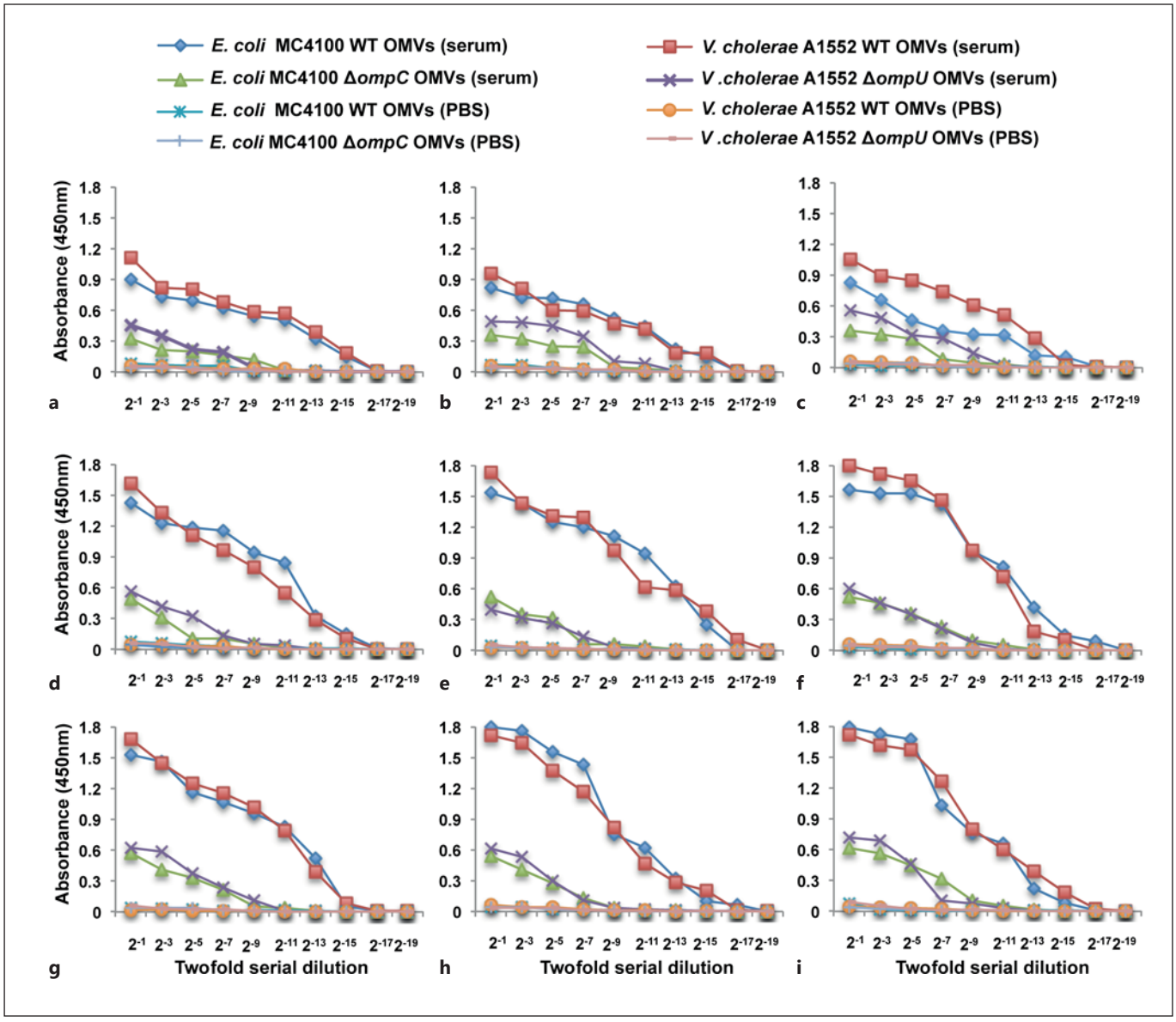

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Fig. 6. a–i Serological analysis of IgG. ELISA to measure the levels of IgG against OmpU of *V. cholerae* and OmpC of *E. coli* in NHS. Sera were isolated from the healthy donors. OMVs isolated from the wild-type *V. cholerae* strain A1552 and its Δ*ompU* mutant, and the wild-type *E. coli* strain MC4100 and its *ompC* mutants were used as antigens for ELISA. Red squares and purple crosses show the IgG titers in sera from the donor against OMVs of the wild-type strain A1552 and that of the Δ*ompU* mutant, respectively. Blue squares and green triangles show the IgG titer in sera from the donor against OMVs of the wild-type *E. coli* strain MC4100 and that of the Δ*ompC* mutant, respectively. Orange circles, pink lines, blue crosses and blue lines represent negative controls (colors refer to the online version only).

serum generated against an unrelated protein, the type VI secretion system protein Hcp, was included as a negative control. As shown in figure 5b (gray columns), anti-Hcp antiserum could not neutralize the serum-killing activity of NHS.

Evidence for Antibodies Recognizing V. cholerae OmpU Protein in Serum of Healthy Humans

Among Gram-negative bacterial species, immunogenic cross-reactions are very common, especially for the antigenic OMPs from various bacteria [32]. The presence of

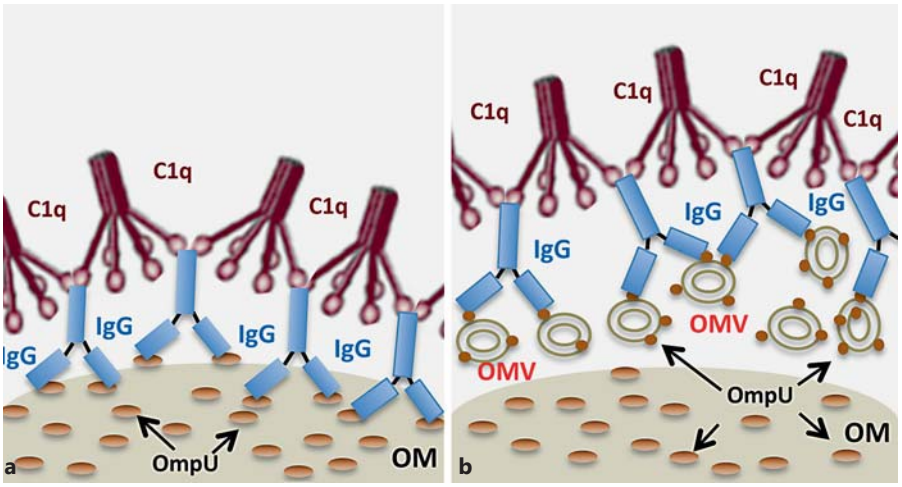

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Fig. 7. An illustration showing the deposition of the OmpU-IgG-C1q complex on a bacterial cell surface and OMV-mediated protection against OmpU-IgG-C1q-dependent serum killing. **a** IgG-C1q complex binding to OmpU protein of bacterial outer membrane leads to a complement-mediated serum killing effect. **b** OmpU-associated OMVs released from the bacterial cell can bind C1q through IgG leading to the inactivation of serum killing. OM = Outer membrane.

anti-OmpU antibodies in NHS might be due to an immunogenic cross-reaction between the OMPs of intestinal commensal bacteria, such as *E. coli* and OmpU of *V. cholerae*. To determine to what extent anti-OmpU antibody is present in NHS, the IgG titers in 9 different serum samples were determined by ELISA. PBS was used as a negative control to test for nonspecific binding of peroxidase-coated IgG to the OMVs. Anti-OmpU IgG was detected in all the sera tested (fig. 6, red squares). When OMVs isolated from the *V. cholerae* Δ*ompU* mutant were used as bate, consistently lower IgG levels were detected (fig. 6, purple crosses). This was presumably due to the presence of other IgG-binding proteins on the surface of the OMVs from the Δ*ompU* mutant.

We considered that a plausible reason for the presence of 'anti-OmpU' antibodies in serum from healthy donors, without any known previous exposure to cholera, would be that these IgG antibodies had been developed against similar proteins in the outer membrane of commensals such as the OmpC protein of *E. coli*, known as a functional homolog of OmpU. We isolated OMVs from the wild-type *E. coli* strain MC4100 and the Δ*ompC* mutant *E. coli* derivative, and used these vesicles as the antigens in ELISA. With all serum samples we observed a higher titer of IgG against the OMVs containing *E. coli* OmpC (fig. 6, compare blue squares and green triangles).

Discussion

In this study, we elucidated a mechanism of how serum resistance may occur in *V. cholerae*. Serum sensitivity and complement-mediated killing of different *V. cholerae* isolates belonging to O1 El Tor, O1 classical and NOVC serogroups were found to differ considerably. Despite not producing cholera toxin, non-O1, non-O139 *V. cholerae* can be clearly pathogenic, causing clinical syndromes ranging from mild diarrheal illness to septicemia, primarily in vulnerable hosts [5].

We suggest that the presence of natural IgG antibodies in NHS, most likely raised against some OmpU homologs of the commensal flora, can contribute to the defense against invading *V. cholerae* pathogens. Such IgG can bind on the surface of bacteria and attract C1q molecules, leading to activation of the complement system and clearance of bacterial invaders in the blood. Furthermore, OMVs released from bacterial cells and containing OmpU can also trap the IgG-C1q complex, thereby protecting the bacterial cells against C1q-IgG-mediated serum killing. An illustration of the OmpU-IgG-C1q-mediated serum-killing effect on bacterial cells and the mechanism by which bacteria can evade this system, by releasing and trapping the complexes, is presented in figure 7. Recently, the recognition of the pattern recognition receptors ficolin and MBL by natural IgG was shown to mediate protection from experimental infection of mice with *P. aeruginosa* [13]. Here we demonstrate a lectin-independent direct immunorecognition of bacterial surface proteins by natural IgGs, mediating activation of the classical complement cascade and serum sensitivity of *V. cholerae.*

Interestingly, the level of OmpU was significantly different among *V. cholerae* isolates. Our findings led to the conclusion that this major outer membrane porin protein was a primary factor in the serum-sensitive phenotype of *V. cholerae*. Decreased expression of OmpU in *V. cholerae* may be advantageous, especially for NOVC *V. chol-*

erae strains associated with invasive infection such as bacteremia. The serum sensitivity of *V. cholerae* was reduced by adding a classical complement pathway blocker (EGTA and $MgCl_2$) to the serum. The results reported here confirm the importance of the classical pathway in serum killing of *V. cholerae* strains and extend the previous knowledge of the interaction between this bacterium and the classical complement pathway. Using defined OMP mutant strains and a mutant deficient in regulating the expression of OMPs, we obtained results clearly consistent with the notion that Clq can bind to OmpU via IgG. The molecular basis of C1q binding to bacterial porins has not been clarified. Earlier studies suggested that residue D116 (D114 in our strain) of OmpU protein might bind to membrane-targeting antibiotics and, thus, confer antibiotic resistance to *V. cholerae* [31]. We anticipated that the homologous pore-exposed residue D114 may control channel properties or ligand binding of the OmpU porins of *V. cholerae*. To test this hypothesis, we engineered a substitution mutation in OmpU at the selected residue D114. To further analyze details of the mechanism of interactions between OmpU and its ligand C1q, the wild-type *ompU* gene was introduced into the highly serum-resistant Δ*ompU* mutant. The resulting strain became very serum sensitive (Δ*ompU*/WT), but introduction of the mutant OmpU (D114A) could not alter serum sensitivity of the Δ*ompU* mutant (Δ*ompU*/D114A).

It has been clearly demonstrated that almost all Gram-negative bacteria release detectable amounts of OMVs during normal growth. The main components of OMVs are major OMPs. We hypothesized that if OmpU-containing OMVs are released, these OMVs might sequester and quench C1q when the bacterial cells are exposed to NHS.

We tested whether OMVs isolated from the wild-type *V. cholerae* strain A1552 can quench C1q present in NHS. Interestingly, OMVs from the *V. cholerae* wild-type strain A1552 could protect the classical serotype strain 569B against the bactericidal effect of NHS while OMVs isolated from the Δ*ompU* mutant could not prevent serum killing. We suggest that OmpU-containing OMVs can efficiently quench the activity of C1q in serum by binding of the complex with IgG and C1q to OmpU on the surface of the OMVs, thus providing serum resistance to the 569B strain.

In our study, a surprisingly high titer of IgG against the OmpU protein of *V. cholerae* was observed in the tested samples of human serum. Detection of the IgG-recognizing OmpC protein of *E. coli*, homolog of *V. cholerae* OmpU, suggested that these antibodies might have developed against the outer membrane of commensal *E. coli* bacteria.

The immune system-microbiota organization can allow the induction of a protective immune response to pathogenic microorganisms and the maintenance of tolerance to inoffensive or harmless antigens [33]. Interestingly, it was recently demonstrated that a novel symbiont molecule, polysaccharide A, PSA, from the human commensal bacteria *Bacteroides fragilis* could protect mice from experimental colitis induced by *Helicobacter hepaticus*, a commensal bacterium with pathogenic potential, by promoting regulatory immune responses [34]. Based on the complexity and abundance of the commensal flora, it can be speculated that opportunities for cross-reaction between commensals and pathogens may be high. The results obtained in this work highlight the potential of *V. cholerae* to utilize a differential expression of OMPs to evade host immunity.

Acknowledgements

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