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A native outer membrane vesicle vaccine confers protection against meningococcal colonization in human CEACAM1 transgenic mice

Rolando Pajon[#1], **Carolyn M. Buckwalter**[#2], **Kay O. Johswich**[2], **Scott D. Gray-Owen**[2], and **Dan M. Granoff**[*,1]

[1]Center for Immunobiology and Vaccine Development, Children's Hospital Oakland Research Institute, Oakland, CA 94609 USA

[2]Department of Molecular Genetics, University of Toronto, Toronto, Ontario Canada

[#] These authors contributed equally to this work.

Abstract

Background—The effect of protein-based meningococcal vaccines on prevention of nasopharyngeal colonization has been difficult to investigate experimentally because a reliable animal colonization model did not exist.

Methods—Human CEACAM1 transgenic mice, which can be colonized by meningococci, were immunized IP with one of two meningococcal native outer membrane vesicle (NOMV) vaccines prepared from mutants with attenuated endotoxin (*lpxL1* knockout) and over-expressed sub-family B Factor H-binding proteins (FHbp). Animals were challenged intranasally two weeks after the third dose with wild-type strain H44/76, or were treated IP with anti-NOMV serum before and during the bacterial challenge.

Results—The NOMV-1 vaccine, prepared from the serogroup B H44/76 mutant, elicited ~40-fold higher serum bactericidal antibody titers against the wild-type H44/76 challenge strain than the NOMV-2 vaccine prepared from a heterologous serogroup W mutant strain with different PorA and FHbp amino acid sequence variants. Compared to aluminum hydroxide-immunized control mice, the efficacy for prevention of any H44/76 colonization was 93% (95% confidence interval, 52-99, P<0.0001) for the NOMV-1 vaccine, and 19% (−3-36, P=0.23) for NOMV-2. NOMV-2-vaccinated mice had a 5.6-fold decrease in geometric mean CFU of bacteria per animal in tracheal washes compared to control mice (P=0.007). The efficacy of passive administration of serum from NOMV-1-vaccinated mice to immunologically naïve mice against colonization was 44% (17-61; P=0.002).

[*]Corresponding author: 5700 Martin Luther King Jr. Way, Oakland, CA 94609 Tel: 510-450-7640; Fax: 510-450-7915; dgranoff@chori.org.

CMB, KOJ and SDG-O report no conflicts.

Conclusions—Both NOMV vaccines protected against meningococcal colonization but there was greater protection by the NOMV-1 vaccine with antigens matched with the challenge strain. Meningococcal vaccines that target protein antigens have potential to decrease colonization.

Keywords

vaccine; Factor H binding protein; FHbp; OMV; PorA; colonization model; *Neisseria meningitidis*

I. Introduction

Neisseria meningitidis is a common inhabitant of the human nasopharyngeal microflora. The organism can be sub-divided into encapsulated and non-encapsulated strains. Non-encapsulated strains are nearly always non-pathogenic with infection limited to the nasopharynx, while encapsulated strains can rarely spread to the bloodstream and cause disease.

Meningococcal polysaccharide-protein conjugate vaccines against capsular serogroups A, C, W and Y confer protection against both invasive meningococcal disease and meningococcal colonization [1]. Following introduction of meningococcal group C polysaccharide conjugate vaccines in the UK, approximately one-third of the overall decrease in serogroup C disease was attributed to herd immunity [1]. In contrast, plain (un-conjugated) meningococcal polysaccharide vaccines appeared to have minimal effect on colonization [2]. The reasons why conjugate vaccines, but not plain polysaccharide vaccines, confer protection against carriage are not known.

Serogroup B capsular polysaccharide is structurally similar to polysaccharides in human tissues [3]. Thus serogroup B vaccine development focused on use of noncapsular antigens such as detergent-treated outer membrane vesicles (dOMV) [4], recombinant proteins [5-7], or a combination of both [8, 9]. Native OMV (NOMV) vaccines with genetically attenuated endotoxin that do not require treatment with detergents to deplete endotoxin are also under investigation [10, 11]. Recently, a serogroup B vaccine containing recombinant Factor H binding protein (FHbp) was licensed in the United States, and a four-component serogroup B vaccine (called 4CMenB) that contains recombinant FHbp, two other recombinant proteins, and dOMV was licensed in Europe, Canada and Australia [12]. Both vaccines elicit broad serum bactericidal responses [8, 9, 13], and are expected to confer protection against invasive disease by the majority of serogroup B strains [14]. However, in a recent study in university students, the 4CMenB vaccine had only a modest effect on decreasing serogroup C and Y carriage [15] (the protein antigens in 4CMenB are also present in strains with other capsular groups), and did not decrease acquisition of serogroup B carriage [15].

The effect of vaccination on nasopharyngeal colonization of *N. meningitidis* has been difficult to investigate experimentally because the receptors important for meningococcal colonization, such as carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), are human-specific [16]. Recently, Johswich et al [16] reported that transgenic mice expressing human CEACAM1 permitted establishment of meningococcal intranasal colonization. Further, human CEACAM1 transgenic mice immunized with a serogroup C polysaccharide-conjugate vaccine were protected against colonization caused

by a *N. meningitidis* serogroup C strain. These results demonstrated the utility of this model for investigation of the effects of vaccination on carriage.

We are investigating the vaccine-potential of meningococcal NOMV vaccines prepared from mutants with genetically attenuated endotoxin and over-expressed FHbp. In mice and infant primates these vaccine elicited broad serum bactericidal antibody responses [11, 17-19]. The purpose of the present study was to investigate the ability of meningococcal NOMV vaccines to confer protection against nasopharyngeal colonization caused by a serogroup B strain.

2. Methods

2.1. Vaccine

The two NOMV vaccines, designated NOMV-1 (prepared from a mutant of serogroup B strain H44/76) and NOMV-2 (prepared from a mutant of serogroup W strain Su 1/06), have been previously described [19, 20]. In brief, endotoxin activity was attenuated by inactivation of the *lpxL1* gene. The group W capsule was deleted by knocking out *cssA-cssEw* as described [20]. Factor H binding protein (FHbp) was over-expressed by chromosomal insertion of two copies of FHbp either ID 1 (H44/76 mutant) or ID 9 (Su 1/06 mutant) with an upstream modified PorA/NadA gene promoter [20]. The FHbp genes contained a single base pair substitution that introduced a serine at amino acid residue 41 instead of arginine (i.e. R41S). This mutation decreased binding of human Factor H (FH) to FHbp [21] and enhanced serum bactericidal antibody responses in human FH transgenic mice [19, 21]. The NOMV vaccines were prepared from membrane blebs released into bacterial culture supernatants and characterized as previously described [20]. By Western blot, the FHbp content of the NOMV vaccines was ~5-fold that of control NOMV vaccines prepared from the respective parental WT strains.

2.2. Ethical statement

All animal experiment procedures were approved by the Animal Ethics Review Committee of the University of Toronto (Permit Numbers: 20008007 and 20008657), which is subject to the ethical and legal requirements under the province of Ontario's Animals for Research Act and the Canadian Council on Animal Care (CCAC). All efforts were made to minimize suffering.

2.3. Transgenic mouse line

The human CEACAM1 mouse line in an FvB background has been described [16, 22]. The transgene is expressed under the control of the human *ceacam1* promoter region, and its overall expression pattern on the mucosa of the olfactory and respiratory epithelium lining and the palate and the nasopharyngeal ducts matches well with humans [16].

2.4. Immunization

The 200 μl NOMV dose contained 2.5 μg of protein suspended in a solution of 3 mg/ml of aluminum hydroxide (Alhydrogel, Invivogen), 10 mM histidine (Sigma), and 150 mM NaCl

(Sigma). A control vaccine containing aluminum hydroxide (Alhydrogel, Invivogen), 10 mM histidine and 150 mM NaCl (Sigma) was prepared without the addition of the NOMV.

On day 0, groups of mice (N=14 to 16), aged 6 to 8 weeks, were administered the NOMV vaccines or aluminum hydroxide by IP injection. The injections were repeated on days 14 and 28. On day 42, the animals were challenged intranasally with the wildtype serogroup B strain H44/76 (Figure 1). Three days later, the animals were sacrificed and nasotracheal washes were obtained for measurement of mucosal antibody and quantification of *N. meningitidis* CFU (See below).

2.5. Measurement of antibodies to NOMV

The ELISA for measurement of mucosal antibodies to meningococcal NOMV was performed as described previously [16]. Diluted samples of nasal lavage fluid were added to the wells, incubated for two hours, and washed. Bound Ig was detected with 1:10,000 dilutions of AP-goat-anti-mouse IgG Fc(γ) or AP-goat-anti-mouse IgA (Abcam).

For measurement of serum antibodies to the NOMV, the ELISA was performed as previously described [19] with the only difference that 5 μg/ml of the NOMV vaccine diluted in PBS were added to the wells and incubated for 15 hrs at 4°C.

2.6. Serum bactericidal antibody activity

The assay was performed as previously described [23] except that the bacteria were grown to mid-exponential phase in Frantz media supplemented with 4mM D,L-Lactate (Sigma), and 2 mM cytidine 5'-monophospho-n-acetyl-neuramic acid (CMP-NANA; Carbosynth) as described by Costa et al [24]. The two test strains were the H44/76 wild type parent of the mutant used to prepare the NOMV-1 vaccine, and a serogroup W strain from Mali (Mali 29/07) that shares the same *porA*, and *fhbp* genes as that of Sudan 1/06 (the wild type parent strain of the mutant used to prepare the NOMV-2 vaccine). The complement source was IgG-depleted human serum [21]. The bactericidal titer was the serum dilution that resulted in a 50% decrease in CFU/ml after 60 minutes of incubation compared with CFU/ml in negative controls wells.

2.7. Mouse colonization

Mouse intranasal infection was conducted as previously described [16]. Mice were anesthetized with Isofluran (Baxter) inhalation. The bacteria were grown to mid-log phase in brain heart infusion broth (Becton Dickinson) supplemented with glucose (0.3 %, Sigma, St. Louis, MO, USA) and deferoxamine mesylate (60 μg/ml) as described [16]. A total of 10 μl inoculum of strain H44/76 and 16 μg/μl of human transferrin (Sigma) were applied to both nares (5 μl per nares for a total of $1x10^7$-$1x10^8$ CFU). 72 hours after the challenge, the animals were sacrificed and CFU were determined by retrograde lavage of the upper airways through the trachea with 0.25 ml of PBS/Mg^{2++}, followed by swabbing of the exposed nasal cavities using aluminum shaft applicators (Puritan Medical Products, Guilford, USA). Swabs were re-suspended into 500 μl of PBS/Mg^{2++}. These samples were plated onto GC agar plates supplemented with IsoVitalex and VCNT inhibitor (Becton Dickinson) to

suppress growth of nasal flora. The data were expressed as the sum of recovered CFU from each mouse.

2.8. Passive protection by antibody against colonization

Sera obtained three days after the bacterial challenge from mice immunized with the NOMV-1 vaccine or $Al(OH)_3$ alone were pooled and tested for passive protection against colonization in immunologically naïve CEACAM1 transgenic mice. Three hrs before the intranasal challenge, groups of human CEACAM1-transgenic mice were given 100 μl IP of the serum pools diluted 1:5 in sterile PBS. The mice received a second injection of the pooled serum diluted 1:5 at 24 h after challenge (Figure 1).

2.9. Statistical analyses

For calculation of geometric means, CFU in nasal washes, and IgG and bactericidal titers were transformed (Log_{10}). The respective geometric means of two independent groups of mice were compared by a T test. The Fisher exact test was used to compare the proportions of treated mice and control mice with positive bacterial cultures in nasotracheal washes. Efficacy was calculated from the formula [colonization rate in the control group - the colonization rate in the vaccinated group]/ [colonization rate in the control group]. A two-tailed p value ≤0.05 was considered statistically significant.

3. Results

3.1. NOMV vaccination elicits serum and mucosal antibody responses and protects against acquisition of carriage

In the first experiment, animals were immunized with the NOMV-1 vaccine prepared from the serogroup B H44/76 mutant and challenged with the H44/76 WT parental strain. After three doses of NOMV-1, the human CEACAM1 transgenic mice developed serum and mucosal IgG anti-NOMV antibody responses (Figure 2, Panels A and B). There were no significant differences in the mucosal IgA anti-NOMV antibody responses between NOMV-1-vaccinated and control mice (data not shown). The animals also developed high serum bactericidal titers measured against the WT H44/76 strain (Figure 2, Panel C). A total of 13 of 14 NOMV-vaccinated animals challenged intranasally with the H44/76 WT strain had sterile cultures of the nasotracheal washes at 72 hrs, compared with 0 of 14 $Al(OH)_3$-vaccinated control mice (Figure 2, Panel D, P<0.0001). The efficacy of NOMV-1 vaccination against colonization was 93% (95% confidence interval, 53-99, Table 1).

In a second experiment, animals were immunized with the NOMV-2 vaccine prepared from the serogroup W mutant strain and challenged with the serogroup B H44/76 WT parental strain. As expected, the serum bactericidal titers against the heterologous serogroup B H44/76 challenge strain were ~10-fold lower than against a serogroup W test strain from Mali (29/07) that had the same PorA (P1.5,2) and sub-family B FHbp amino acid sequence variant (ID 9) as the mutant NOMV-2 vaccine (Figure 3, Panel A). Compared to aluminum hydroxide-treated control mice, the efficacy of the NOMV-2 vaccine for prevention of any H44/76 colonization was 19% (95% CI, −3-36, P=0.23, Table 1). However, the geometric mean CFU per animal in tracheal washes from the NOMV-2 vaccinated mice was 5.6-fold

lower than in the negative control mice immunized with the adjuvant only (geometric mean of 23 vs 130, P=0.007) (Figure 3, Panel B).

3.2. Serum antibodies passively protect against meningococcal serogroup B intranasal colonization

To investigate whether passively administered antibodies confer protection against colonization, immunologically naïve, human CEACAM1 transgenic mice were injected IP with 1:5 dilutions of serum pools from mice previously immunized with either the NOMV-1 vaccine or $Al(OH)_3$ control. (See Figure 1). In each of the three experiments (Figure 4), animals given the anti-NOMV serum and challenged with strain H44/76 had lower CFU in nasotracheal washes than animals given the negative control serum ($P<0.05$ in experiments A and C, and $P=0.10$ in experiment B (two-tailed probability values). When the data from all three experiments were combined, the efficacy of passively administered antibody against colonization was 44% (95% CI, 17-61, P=0.002, Table 1). Thus, serum antibodies contribute to protection against meningococcal colonization.

4. Discussion

The extent to which meningococcal vaccines that target protein antigens can protect against nasopharygenal carriage remains uncertain. In Norway and New Zealand, dOMV vaccines appeared to have had minimal effects on decreasing nasopharygeal carriage [25-27]. In Normandy, France, a dOMV vaccine was reported to decrease carriage but confounding biases may have contributed to the results [28]. The ability of two recently licensed serogroup B vaccines (4CMenB [12] or a bivalent FHbp [29]) to prevent colonization in the population is not known [30].

Several groups investigated the vaccine-potential of meningococcal NOMV vaccines with attenuated endotoxin activity for prevention of invasive meningococcal disease [10, 31-33]. In one experimental study, an NOMV-FHbp vaccine elicited broader serum bactericidal activity in mice against genetically diverse serogroup B strains than a dOMV vaccine used to control a meningococcal outbreak in Norway [18]. The NOMVFHbp vaccine also elicited broader bactericidal activity than the three recombinant proteins used in the 4CMenB vaccine [18]. NOMV-FHbp vaccines such as the one used in experiment 2 of the present study also are being developed for prevention of epidemic meningococcal serogroup A, C and X disease in Sub-Sahara Africa [20, 34, 35]. While promising for prevention of invasive disease, an important gap in knowledge is whether NOMV vaccines will decrease transmission of meningococci in the population.

In the present study we investigated whether meningococcal NOMV vaccines elicited protection against colonization. When the challenge strain had PorA, FHbp and other antigens that exactly matched those of the NOMV-1 vaccine, there was near-complete protection against colonization. Further, the observed protection can be attributed at least in part to serum antibodies in that passively administered post-vaccination serum pools from NOMV-1 vaccinated mice also protected immunologically naïve CEACAM1 transgenic mice against H44/76 colonization. In these experiments, we did not correlate the specific antibody titers with protection against colonization or identity the specific antibodies

responsible for protection. However, in previous studies, antibodies to FHbp and/or PorA were responsible for nearly all of the complement-mediated serum bactericidal activity in NOMV-FHbp-vaccinated mice [17, 18, 36].

In experiment 2, we immunized transgenic mice with a NOMV-2 vaccine prepared from a serogroup W mutant with a heterologous PorA sequence type, and a sub-family B FHbp amino acid sequence variant that was 94% percent identical with the sub-family B FHbp ID 1 in the serogroup B H44/76 challenge strain. While the NOMV-2 vaccine elicited high serum bactericidal antibody responses against strain H44/76, the magnitude of the titers was ~40-fold lower than in experiment 1 where the mice had been immunized with the NOMV-1 vaccine prepared from a mutant of strain H44/76. Perhaps related to these lower serum titers, the NOMV-2 vaccine in experiment 2 had much lower efficacy against any H44/76 colonization (19%) than the NOMV-1 vaccine in experiment 1 (93%). However, in experiment 2, there was a 5.6-fold decrease in the ”density” of colonization in the vaccinated mice compared to control mice immunized with aluminum hydroxide alone.

There is ample evidence that pneumoocccal, *Haemophilus influenzae* type b (Hib) and meningococcal serogroups A and C polysaccharide-protein conjugate vaccines decrease transmission of these organisms in the population [37-41]. For example, in the U.S. Hib conjugate vaccination at 18 to 72 months of age decreased disease incidence in children <18 months of age who at the time were not being vaccinated [42]. However, the effect of Hib conjugate vaccination on colonization was not absolute. For example, Murphy et al reported 65% efficacy against any Hib colonization in children immunized with a Hib conjugate vaccine and exposed to Hib carriers in a day care center [43]. Barbour et al found that of major of effect of Hib conjugate vaccination on colonization may be by decreasing the “density” of bacteria in the nasopharynx, and not by eliminating colonization [44]. Therefore, the 5.6-fold decrease in meningococcal colonization in experiment 2 in mice immunized with the NOMV-2 vaccine provides experimental support for the potential of this vaccine to decrease transmission of meningococci caused by strains with heterologous PorA and FHbp antigens.

In the present study we did not investigate the mechanism by which vaccination conferred protection against colonization. For other pathogens such as *Streptococcus pneumoniae* [45], nontypeable *Haemophilus influenzae* [46], *N. gonorrhoeae [47, 48]*, and *Bordetella pertussis* [49] whole cell vaccines, CD4+ TH17 T-cells appeared to be important for protection against mucosal infections. When these are considered alongside our results, NOMV-mediated protection against meningococcal colonization most likely resulted from eliciting both meningococcal specific antibody-dependent mechanisms to the homologous PorA and FHbp antigens, and antibody-independent mechanisms, which may include specific T-cell subsets and inflammation-recruited neutrophils.

Finally, several possible limitations of our study should be considered. First, we investigated the effect of vaccination on meningococcal colonization in a mouse model. In previous work, mouse models may have falsely predicted the ability of vaccination to protect against colonization in humans or animal models more relevant than mice. For example acellular pertussis vaccination, which protected mice from *Bordertella pertussis* colonization, failed

to protect against colonization in a baboon model [49]. We also investigated only a single time point in the mice, 14 days after a third dose of the NOMV vaccines, to test whether NOMV-induced immunity against colonization was possible. Future studies should consider vaccine dosage and number, the kinetics of response and duration of protection after immunization, but these endpoints were beyond the scope of our study. Another possible limitation of our study was the use of sera from NOMV-1 and aluminum hydroxide-immunized mice in the passive protection experiments that had been obtained three days after the intranasal bacterial challenge, which may have stimulated antibodies or inflammatory mediators. In a separate experiment, mice immunized with the NOMV-2 vaccine and given an intranasal challenge with the bacteria had indistinguishable serum bactericidal titers 3 days after the challenge than immunized mice that had not been challenged (geometric mean titers of 550 and 650, respectively, P=0.93). Since the passive serum protection experiments primarily explored the role of serum antibodies, it seems unlikely that meningococcal nasopharyngeal challenge three days prior to serum collection significantly affected the results.

In summary, development of meningococcal serogroup B vaccines remains a high public health priority. At a population level, vaccines that both prevent disease in individuals and that elicit herd immunity are the most effective for controlling disease and therefore prove cost-effective. Our results suggest that mutant meningococcal NOMV vaccines, which in previous studies elicited broad serum bactericidal antibody responses, also have the potential to prevent colonization.

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Non-standard abbreviations

NOMV	native outer membrane vesicle
dOMV	detergent-treated OMV
CEACAM	carcinoembryonic antigen-related cell adhesion molecule

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Highlights

1. Human CEACAM1 transgenic mice were immunized with native meningococcal OMV vaccines with attenuated endotoxin and over-expressed FHbp.
2. The mice developed serum bactericidal antibodies and mucosal antibodies to the OMV.
3. Mice were protected against nasotracheal serogroup B meningococcal infection.
4. Passively administered serum antibody also conferred protection against colonization.
5. Meningococcal vaccines that target protein antigens may decrease carriage.

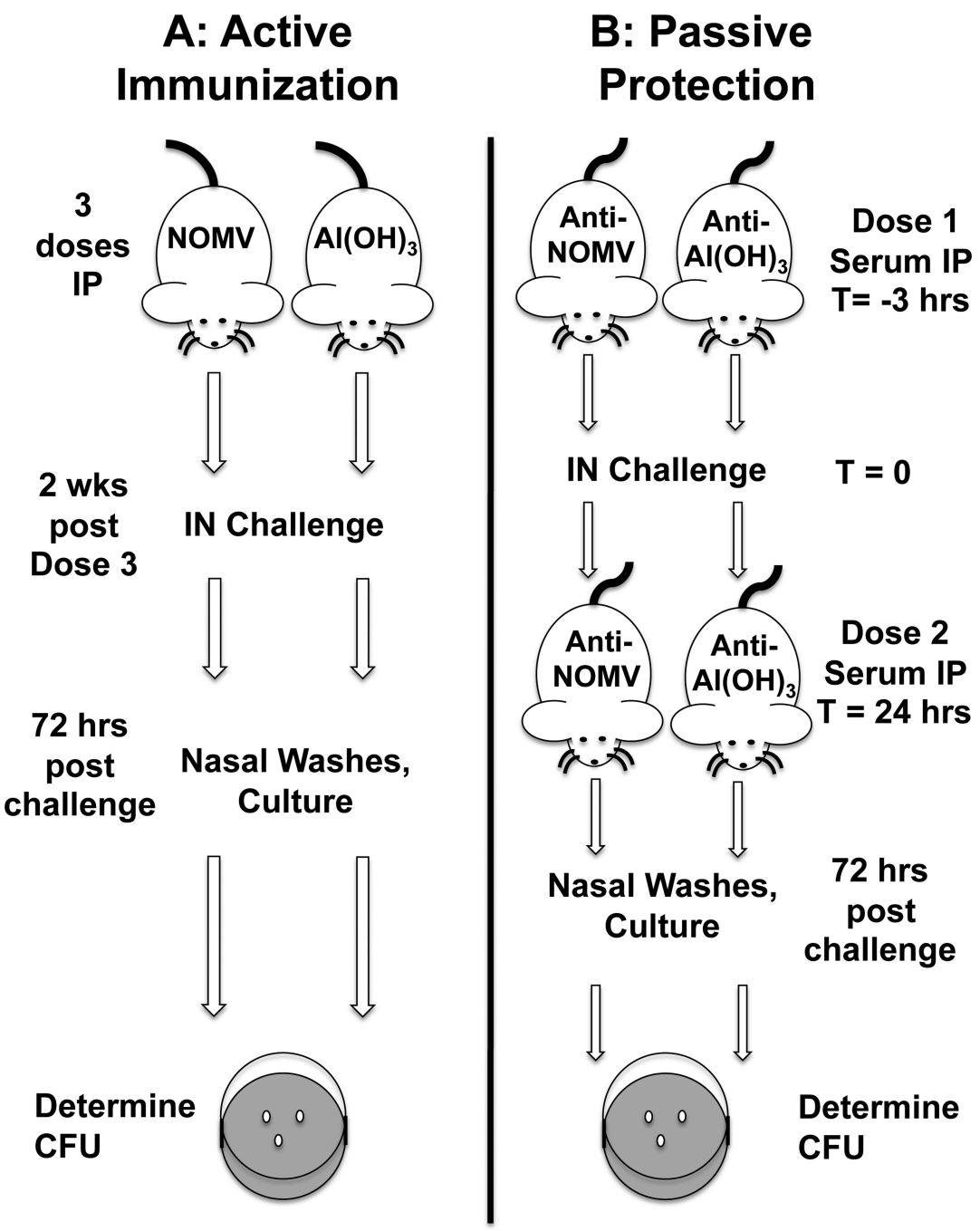

Figure 1. Schema illustrating active and passive immunization protocols

IN, intranasal; IP, intraperitoneal; CFU, colony forming units. In the active immunization, each NOMV dose contained 2.5 μg of protein. In the passive protection, the animals were given 100 μl of 1:5 dilutions of serum pools from NOMV- or aluminum hydroxideimmunized mice.

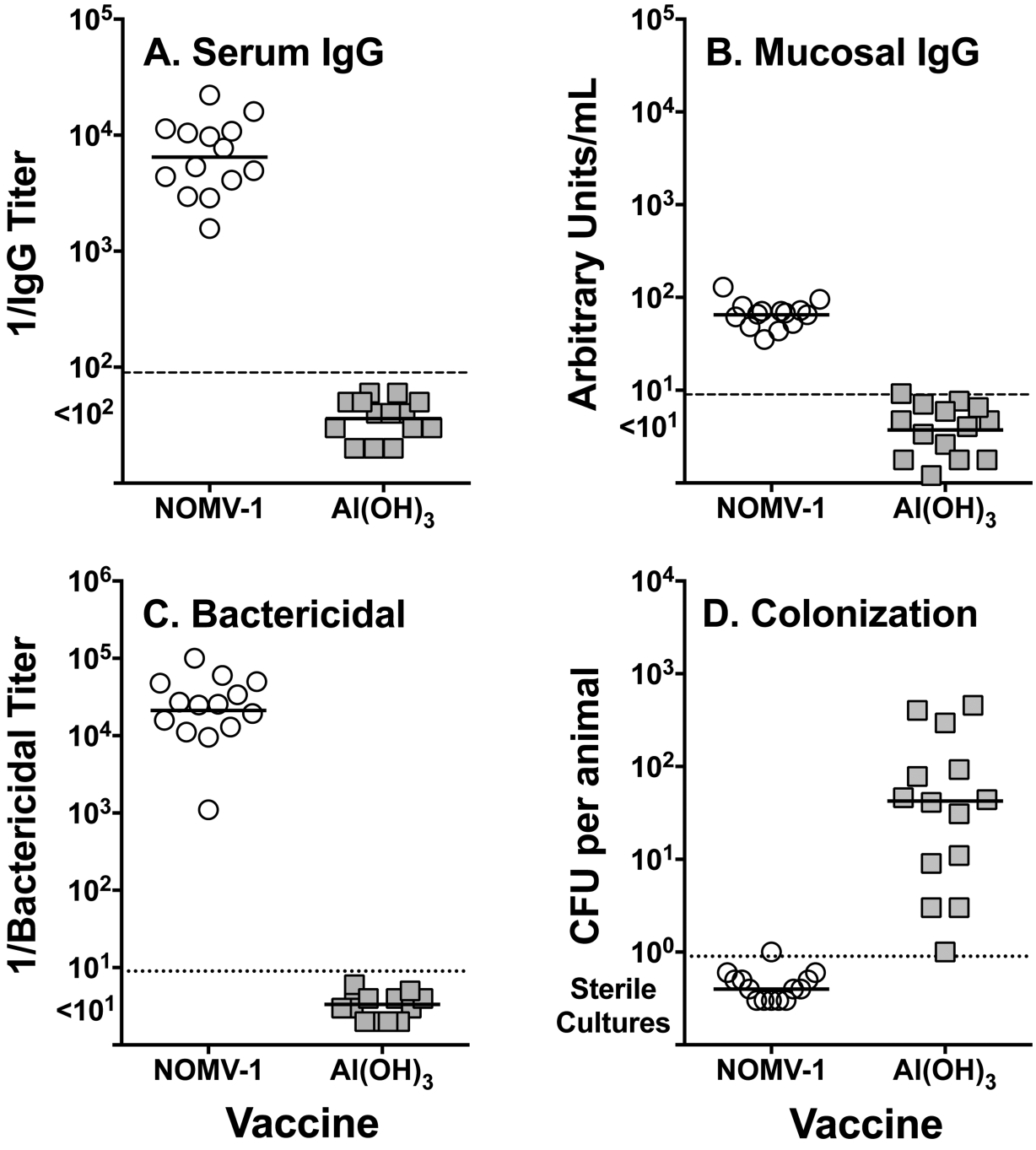

Figure 2. Immunization with a mutant NOMV vaccine elicits mucosal and serum antibody responses and protects against colonization

CEACAM1 transgenic mice immunized with NOMV vaccine (open circles), or Al(OH)$_3$ adjuvant alone (grey squares). Panels A-C, each symbol represents the serum titer of an individual mouse. Panel D, each symbol represents the colony forming units (CFU) of an individual mouse. A. Serum IgG antibody titers to NOMV (ELISA). B. IgG antibody to NOMV in tracheal washes (ELISA). C. Serum bactericidal activity (human complement). D. Colony forming units (CFU) per animal recovered from tracheal washes obtained 72 hrs

after intranasal inoculation with *N. meningitidis* serogroup B strain H44/76. In each panel, the respective differences between the geometric means of the two groups were significant ($P<0.001$).

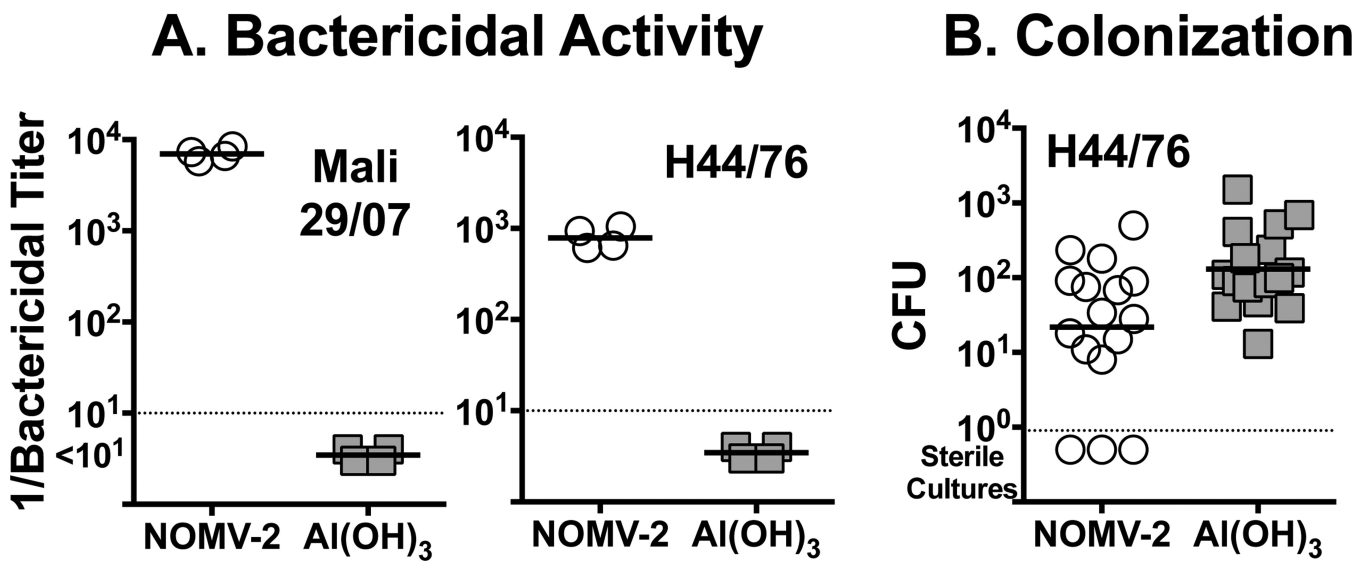

Figure 3. Immunization of CEACAM1 transgenic mice with the NOMV-2 vaccine, prepared from a serogroup W mutant, elicits serum bactericidal antibody responses and protects against colonization by serogroup B strain H44/76 Panel

A. Serum bactericidal activity. Left, a serogroup W test strain (Mali 29/07) with PorA P1.5,2 and FHbp ID9 that matched the NOMV-2 vaccine; Right, serogroup B H44/76 wildtype strain with a heterologous PorA P1. 7,16 and FHbp ID9 to the NOMV2 vaccine. Each symbol represents the titer of pooled serum from four animals. Panel B, Bacterial CFU in tracheal washes obtained 72 hrs after challenge with wild-type serogroup B strain H44/76; each symbol represents CFU of an individual mouse. The respective difference between the geometric means of the CFU in the two groups is significant ($P<0.01$)

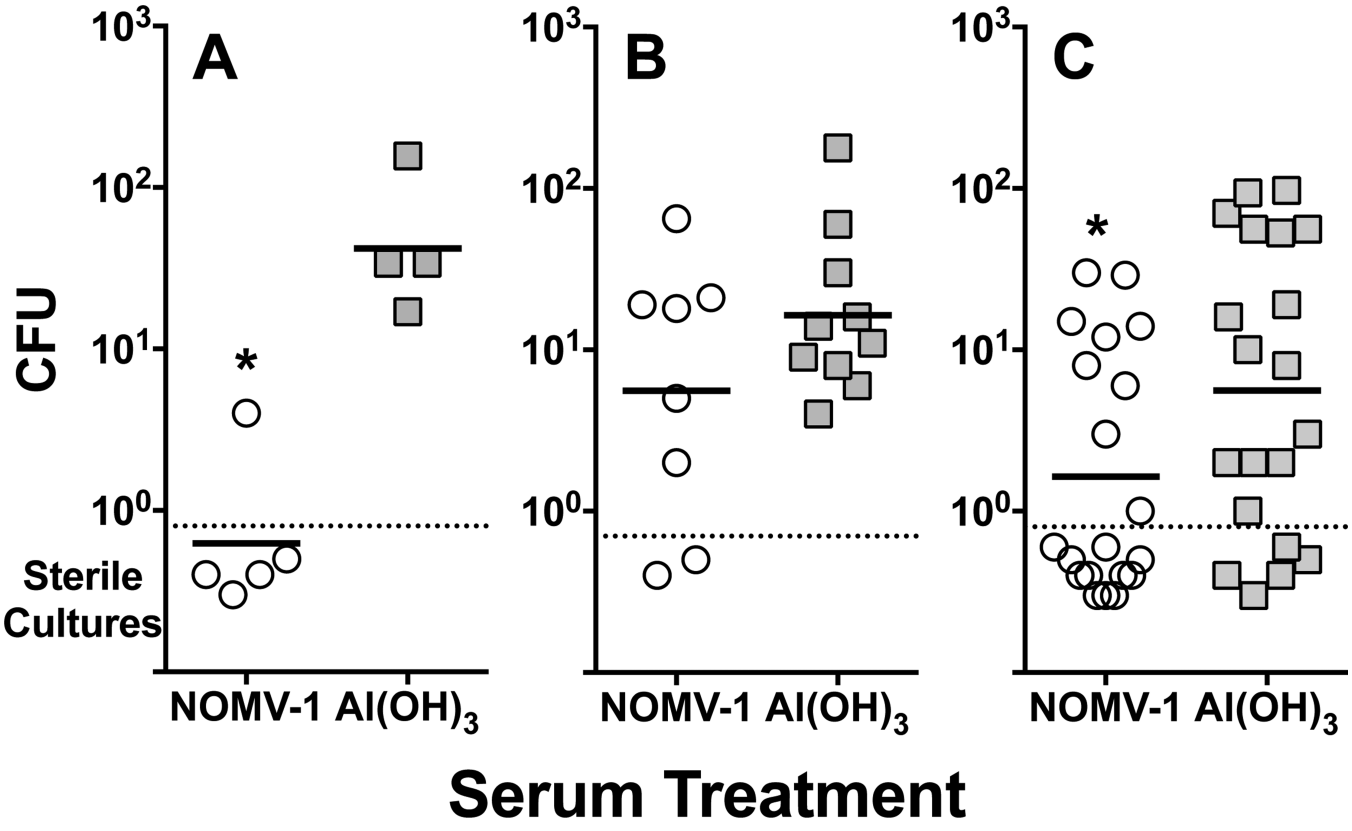

Figure 4. Sera from human CEACAM transgenic mice immunized with a mutant NOMV vaccine confer passive protection against nasotracheal colonization

Mice were challenged intranasally at time 0 with *N. meningitidis* serogroup B strain H44/76 and CFU were determined in nasotracheal washes at 72 hrs. At time -3 hrs and + 24 hrs, the mice were treated IP with serum pools diluted 1:5 from mice immunized with the NOMV vaccine or $Al(OH)_3$. Each panel represents an independent experiment. Each symbol represents the CFU isolated from an individual mouse. Open circles, sera from NOMV-vaccinated animals; grey squares, sera from negative control mice immunized with $Al(OH)_3$. Horizontal bars denote the geometric mean CFU recovered per group. Asterisks denote statistically significant differences. Mice treated with sera from NOMV-vaccinated mice had significantly lower geometric means of the CFU ($P<0.05$ in experiment 1, Panel A, and experiment 3, Panel C; $P=0.1$ in experiment 2, Panel B).

Table 1

Efficacy at preventing any meningococcal colonization by intervention

	% Any Colonization		Efficacy (95% CI)	P Value*
	Control	Vaccinated		
Active NOMV-FHbp immunization, homologous NOMV-1 vaccine	100	7	93 (53 to 99)	<0.0001
Active NOMV-FHbp immunization, heterologous NOMV-2 vaccine	100	81	19** (−3 to 36)	0.23
Passive anti-NOMV-1-FHbp serum treatment	86	48	44 (17 to 61)	0.002

* Fisher exact test

** There was a 5.6-fold decrease in tracheal wash CFU per mouse in the vaccinated group, compared to control mice (P=0.007, see Figure 3).