

Characterization of *Vibrio cholerae* Outer Membrane Vesicles as a Candidate Vaccine for Cholera[∇]

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Outer membrane vesicles (OMVs) offer a new approach for an effective cholera vaccine. We recently demonstrated that immunization of female mice with OMVs induces a long-lasting immune response and results in protection of their neonatal offspring from *Vibrio cholerae* intestinal colonization. This study investigates the induced protective immunity observed after immunization with OMVs in more detail. Analysis of the stomach contents and sera of the neonates revealed significant amounts of anti-OMV immunoglobulins (Igs). Swapping of litters born to immunized and nonvaccinated control mice allowed us to distinguish between prenatal and neonatal uptakes of Igs. Transfer of Igs to neonates via milk was sufficient for complete protection of the neonates from colonization with *V. cholerae*, while prenatal transfer alone reduced colonization only. Detection of IgA and IgG1 in the fecal pellets of intranasally immunized adult mice indicates an induced immune response at the mucosal surface in the gastrointestinal tract, which is the site of colonization by *V. cholerae*. When a protocol with three intranasal immunizations 14 days apart was used, the OMVs proved to be efficacious at doses as low as 0.025 µg per immunization. This is almost equivalent to OMV concentrations found naturally in the supernatants of LB-grown cultures of *V. cholerae*. Heterologous expression of the periplasmic alkaline phosphatase (PhoA) of *Escherichia coli* resulted in the incorporation of PhoA into OMVs derived from *V. cholerae*. Intranasal immunization with OMVs loaded with PhoA induced a specific immune response against this heterologous antigen in mice. The detection of an immune response against this heterologously expressed protein is a promising step toward the potential use of OMVs as antigen delivery vehicles in vaccine design.

Cholera, a life-threatening secretory diarrheal disease, is caused by the gram-negative, motile, curved-rod bacterium *Vibrio cholerae* (38). This disease is transmitted via the fecal-oral route and is characterized by its ability to cause explosive outbreaks, mainly in African and Asian developing countries with poor sanitation. Cholera is an underreported disease largely for political reasons. The reported incidence is 200,000 cases worldwide per year (63); however, the true burden is likely to reach several million cases a year (51).

Although more than 200 serogroups are known, severe cholera outbreaks are caused only by *V. cholerae* strains of the serogroups O1 and (to a lesser extent) O139 (30, 48, 52). These two closely related serogroups are distinguished by their unique lipopolysaccharide composition and structure (10, 32, 37). *V. cholerae* O1 can be further distinguished into the classical and El Tor biotypes by physiological properties, such as polymyxin B resistance and the presence of mannose-sensitive hemagglutinin and hemolysin activity (5). Although O1 classical was responsible for the pandemics in the 19th and early 20th centuries, current outbreaks are principally caused by the El Tor biotype (14, 29).

Cholera symptoms include profuse watery diarrhea frequently accompanied with vomiting. Untreated, the fluid and

electrolyte loss can result in hypotensive shock and death within hours after the onset of the first symptoms (4). Oral or intravenous rehydration therapy is highly effective, but availability in rural areas is limited, and during the explosive outbreaks, medical facilities cannot always cope with the massive numbers of incoming patients. Currently, two variants of killed whole-cell *V. cholerae* O1 cholera vaccines are commercially available (25–27, 57). Despite enormous efforts, a cheap, temperature-stable, and effective cholera vaccine that can be easily administered is currently lacking.

Recent studies revealed the induction of a stable immune response and/or successful protection in mice after immunization with outer membrane vesicles (OMVs) derived from *Neisseria meningitidis*, *Porphyromonas gingivalis*, *Salmonella enterica* serovar Typhimurium, or *Helicobacter pylori* (2, 12, 20, 31, 33, 53). OMVs, which are naturally released by a variety of gram-negative bacterial species, generally resemble the outer membrane and periplasm in composition and are highly immunogenic. In the case of *N. meningitidis*, two related OMV-based vaccines, MenBvac and MeNZB, have been developed and administered to control epidemics in Norway and New Zealand (16, 41). MeNZB vaccine campaign data from New Zealand show an 80% efficacy rate during an epidemic meningococcal serotype B outbreak (<http://hugin.info/134323/R/1068580/181212.pdf>).

On the basis of these positive examples of immunizations with OMVs from other gram-negative pathogens (2, 12, 20, 31, 33, 53) and the report that *V. cholerae* also releases OMVs (39), we recently analyzed the potential of OMVs derived from

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V. cholerae as a new candidate vaccine against cholera (54). In the previously published study, we demonstrated that mice immunized by the intranasal (i.n.), intragastric (i.g.), or intraperitoneal (i.p.) route with purified OMVs derived from *V. cholerae* induced specific, long-lasting, high-titer immune responses against a variety of antigens present in the OMVs (54). When the offspring of immunized female mice were challenged with *V. cholerae* according to the infant mouse model of cholera (36) and a previously described protection model (18), a protective induced immunity of at least 3 months after the last immunization was revealed. Interestingly, the half-maximum total immunoglobulin (Ig) titers as well as the final degrees of protection were similar in all immunized mice, independent of the immunization route. In contrast, the inductions of specific Ig isotypes differed in groups immunized via different routes. IgA levels, for example, which have previously been correlated with protection against *V. cholerae* infections (17, 23, 64), were significantly induced only in i.n. and i.g. immunized mice.

In the present study, we examined the transfer of acquired immunity from the primary immunized female mice to the protected offspring in more detail. Practical utility in the field requires potency and broad application. We investigated these features of the vaccine candidate in more detail. We focused on the i.n. route of immunization and determined the dose-related immune response and the corresponding protection. It has recently been demonstrated that OMVs of other bacterial species can be loaded with foreign proteins expressed within the bacteria and secreted into the periplasm (34, 44). This finding suggests that OMVs may be used as delivery vehicles for heterologous antigens. As a proof of principle, we expressed the periplasmic alkaline phosphatase of *Escherichia coli* in *V. cholerae*, monitored its transport into the OMVs, and analyzed the immunogenic capacity of such a heterologous protein.

MATERIALS AND METHODS

Strains and growth conditions. Unless stated otherwise, *V. cholerae* strain AC53 (a spontaneous Sm^r mutant of *V. cholerae* E7946 [O1 El Tor Inaba], a clinical isolate from Bahrain [ATCC 55056; *hapR*⁺]) was used as a wild-type strain and bacteria were grown in Luria-Bertani (LB) broth at 37°C with aeration (43, 60). AC108 (a spontaneous Sm^r mutant of *V. cholerae* O395 [O1 classical Ogawa], a clinical isolate from India [ATCC 39541; *tcp::TnpHoA::pSKCATmpR::res1-tet-res1* Sm^r Kn^r Tc^r Ap^r]) is a variant of the previously described *TnpHoA* mutant strain KP8.85 (46) and was constructed by mobilization of pSKCATmpR::res1-tet-res1 into KP8.85, followed by homologous recombination of pSKCATmpR into *TnpHoA* on the *V. cholerae* chromosome, as described previously (8). The *tcp* promoter was induced by growing AC108 in LB (pH 6.5, 50 mM NaCl) at 30°C with aeration (58). Supplements were used at the following final concentrations: for streptomycin (Sm), 100 µg/ml; and for kanamycin (Kn), 50 µg/ml.

Preparation of OMVs. OMVs were isolated from *V. cholerae* cultures as described previously (54). Briefly, cultures of AC53 were grown at 37°C in LB, whereas AC108 was grown at 30°C in LB (pH 6.5, 50 mM NaCl) to allow induction of *tcp::TnpHoA* transcriptional fusion. After reaching late exponential phase, bacterial cells were removed by centrifugation (6,000 × g, 15 min, 4°C) and consecutive filtration of the supernatant through 0.45-µm- and 0.22-µm-pore-size filters. One milliliter of the filtrate was plated on an LB agar plate and incubated overnight to confirm the absence of viable bacteria. In all cases, no colonies were observed. Protein degradation was inhibited by addition of protease inhibitor (complete EDTA-free protease inhibitor cocktail, 1 tablet per 1 liter of filtrate; Roche) to the filtrate and storage at 4°C. Within a week, OMVs were purified from the filtrate by ultracentrifugation (4 h, 140,000 × g, 4°C) using a Beckmann SW32Ti or 50.2Ti rotor, washed once with phosphate-buffered saline (PBS), and finally resuspended in 625 µl of PBS. The protein concentra-

tion was determined by a modified Lowry protein assay kit (Pierce) and adjusted to 2.5 µg/µl using PBS. Purified OMVs were stored at -80°C.

Animals. BALB/c mice (Charles River Laboratories or Jackson Laboratories) were used in all experiments. Mice were housed with food and water ad libitum under the care of full-time staff in accordance with the rules of the Department of Lab Animal Medicine at Tufts University and Tufts Medical Center. All animals were acclimated for at least 1 week before use.

Immunization protocol. Sera and stomach contents from neonatal mice were collected during a previously reported immunization study using OMVs derived from *V. cholerae* AC53 (54). Briefly, in that study, 7-week-old female mice were immunized on days 0, 14, and 28 with OMVs via the i.p., i.g., or i.n. route, using the following concentrations: for i.n. treatment, 25 µg in 10 µl PBS (5 µl per naris) for all immunizations; for i.g. treatment, 25 µg in 100 µl PBS for all immunizations; and for i.p. treatment, 1 µg in 50 µl PBS for the initial immunization on day 0 and 0.25 µg in 50 µl PBS on days 14 and 28.

In the present study, unless noted otherwise, mice were immunized via the i.n. route as described above. In order to test the dose-related response to OMVs, different quantities of OMVs, between 0.0025 µg and 25 µg, in PBS were administered via i.n. immunization on days 0, 14, and 28. Mice were anesthetized by inhalation of isoflurane gas prior to all immunizations. A group of nonvaccinated control mice were housed in parallel with vaccinated mice for the duration of each experiment.

Collection of samples. Blood samples from vaccinated and nonvaccinated control mice were collected on days 0, 28, and 38 and by cardiac puncture on day 78 after the first challenge. The collected blood was allowed to clot at room temperature (RT) for 30 min, after which serum was isolated by removing the blood clot with a sterile toothpick, followed by centrifugation (15 min, 3,000 × g). The supernatant was removed and diluted threefold in PBS. After addition of sodium azide to give a final concentration of 0.05%, the serum was stored at -80°C.

Twenty-four hours after i.g. inoculation with *V. cholerae*, the small bowel, blood, and stomach contents of infected neonatal mice were collected. Blood or stomach samples from neonatal mice of the same litter were pooled. Igs were extracted from the stomach contents by adding 300 µl of extraction buffer (PBS, 0.01% sodium azide, 5% fetal calf serum, 1 tablet complete EDTA-free protease inhibitor cocktail [Roche] per ml) per 100 mg of stomach content and vortexing the samples for 15 min at 4°C. Solid material was separated by centrifugation (2 min, 13,000 × g), and the supernatants were stored at -80°C.

The Ig concentrations in fecal pellets of i.n. immunized mice of the OMV dose response experiment were determined. Three to five freshly voided fecal pellets were vacuum dried for 10 min before their weights were recorded. Igs were extracted as described above for the stomach contents by using 1 ml extraction buffer per 100 mg feces.

Challenge. Control and vaccinated female mice were mated with age-matched males at a 2:1 ratio from day 41 to 55 (after initial immunization). Then, mice were separated and females monitored for birth. Five- to six-day-old neonatal mice were separated from their dams for 1 h, anesthetized by inhalation of 2.5% isoflurane gas, and then inoculated i.g. by oral gavage with 50 µl of the appropriate serial dilution of the inoculum. The inoculum was prepared as described previously (54). Neonatal mice were challenged at either one dose approximately 70-fold above the dose at which 50% of mice were colonized (ID₅₀) or two doses approximately 7- and 70-fold above the ID₅₀, which was previously determined to be ~200 CFU for strain AC53 (54). In the cases of two doses, neonatal mice of each litter were split approximately equally for infection with the different doses. Except as noted otherwise, neonatal mice were placed back in the presence of their respective dams after i.g. inoculation. At 24 h postinoculation, mice were euthanized by humane measures consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medicine Association. The small bowel from each neonatal mouse was removed by dissection and mechanically homogenized in 1 ml LB with 20% glycerol. Appropriate dilutions, starting with 100 µl of the homogenate, were plated on LB-plus-Sm plates in duplicate. Thus, the limit of detection was 10 CFU per small bowel.

Quantitation of Igs. Levels of anti-OMV IgA, IgG1, IgG2, and IgM were determined by an enzyme-linked immunosorbent assay (ELISA) using 96-well ELISA microplates (BD Falcon) as described previously (54). Briefly, plates were coated by incubation with the appropriate OMVs (5 µg/ml in PBS) at 4°C for 2 days. Standard curves for each isotype were generated by coating the plates in triplicate with twofold dilutions of the appropriate purified isotype mouse Ig standard (IgA, IgG1, IgG2a, or IgM; BD Biosciences), starting at 1 µg/ml, in PBS. After four washes with 0.05% Tween in PBS (PBS-T), nonspecific binding sites were blocked with 10% heat-inactivated fetal calf serum in PBS (PBS-F) for 1 h at RT. Appropriate fivefold dilutions of the test samples, starting at 1:100 (fecal pellet extract or stomach content extract) or 1:400 (sera) in PBS-F, were

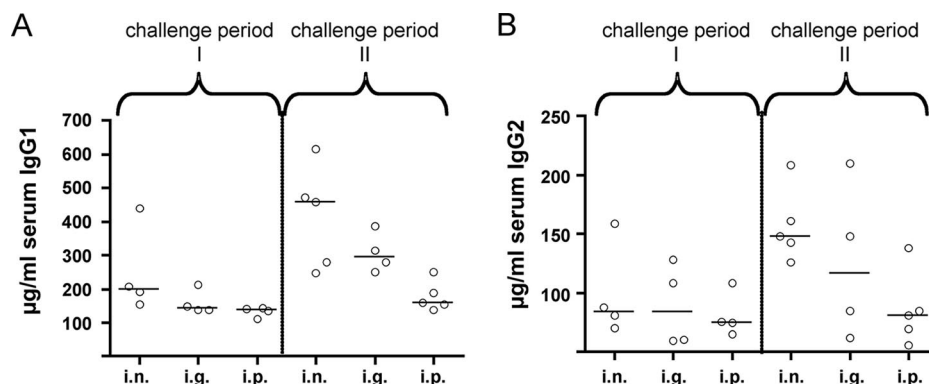


FIG. 1. Ig titers in sera of neonatal mice from dams immunized with OMVs. Shown are the IgG1 (A) and IgG2 (B) titers in sera collected from litters of i.n., i.g., and i.p. immunized female mice at the first or second challenge period. Each circle represents the specific isotype titer for one independent litter. The horizontal bars indicate the median of results from each data set.

applied on the OMV-coated wells in triplicate, whereas PBS-F was used for the wells coated with isotype standards. Plates were incubated for 1 h at RT and washed four times with PBS-T before they were incubated for 1 h at RT with the appropriate alkaline phosphatase-conjugated, affinity-purified goat Igs against mouse IgA (α chain specific; Southern Biotech), IgG1 (γ 1 chain specific; Southern Biotech), IgG2a (γ 2a chain specific; Southern Biotech), or IgM (μ chain specific; Southern Biotech) in PBS-F. After four washes with PBS-T, plates were developed using the BluePhos microwell phosphatase substrate system and stop solution (KPL) according to the manufacturer's manual. Optical densities were read at 620 nm with a Synergy HT plate reader (Biotek, Inc.), and titers were calculated using values from appropriate dilutions of test samples and a log-log regression calculated from at least four dilutions of the isotype standards. The limits of detection were 0.5 μ g/ml in the cases of sera for all isotypes, 0.5 μ g/g in the cases of stomach content extract for all isotypes and fecal pellet extract for IgA, and 0.05 μ g/g in the cases of fecal pellet extract for IgG1.

Half-maximum total Ig titers (IgA, IgG, and IgM) were determined by ELISA as described above, except that alkaline phosphatase-conjugated, affinity-purified goat Igs against mouse (IgM + IgG + IgA, H+L; Southern Biotech) served as a secondary Ig, no Ig standards were used, and at least four fivefold dilutions starting at 1:4 up to 1:400 were used to calculate the half-maximum total Ig titers. In the cases of mice immunized i.n. with OMVs derived from AC108, half-maximum total Ig titers against PhoA and total OMVs were determined separately. Thus, ELISA plates were coated either with OMVs derived from AC108 (as described above) or with purified PhoA (Sigma). Since PhoA cross-reacts with BluePhos microwell phosphatase substrate, we used horseradish peroxidase-conjugated, affinity-purified goat Igs against mouse (IgM plus IgG plus IgA, H+L; Southern Biotech) as secondary Igs in combination with a TMB two-component microwell peroxidase substrate kit and TMB BlueSTOP solution (KPL) according to the manufacturer's manual in these assays. Half-maximum titers were calculated by plotting the log of the reciprocal dilutions of mouse sera against the resulting absorbances, which has a sigmoidal relationship, to determine the reciprocal that gave half of the maximum optical density.

Immunoblot analysis. Equal amounts of protein from purified OMVs or whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 to 15% ready gels, Tris-HCl; Bio-Rad) and transferred onto a nitrocellulose membrane (Invitrogen). The membrane was blocked by incubation in 5% skim milk in PBS buffer with 0.1% Tween (PBS-TW) at 4°C overnight. The primary Ig (anti-PhoA from rabbit; courtesy of A. Wright, Tufts University School of Medicine) was diluted 1:10,000 in 5% skim milk dissolved in PBS-TW, incubated at RT for 4 h, and washed three times for 5 min with PBS-T. The secondary Ig (horseradish peroxidase-conjugated anti-rabbit IgG from goat; GE Healthcare UK Limited) was diluted 1:5,000 in PBS-TW, applied to the membrane, and incubated at RT for 2 h at RT with gentle rocking. The membrane was washed three times for 5 min with PBS-TW, and chemiluminescence detection was performed using the ECL Plus Western blotting detection system (GE Healthcare UK Limited) and exposure to X-ray film (Kodak).

Assay for alkaline phosphatase (PhoA) activity. To determine the PhoA enzymatic activities for the Tcp-PhoA fusion, an alkaline phosphatase assay was performed as described earlier (40), using culture and isolated OMVs of AC108 grown for 8 h at 30°C in LB (pH 6.5, 50 mM NaCl). Five milliliters was used to assay the PhoA activity of the culture, and OMVs were isolated from the re-

maining 995 ml in the cultures as described above. To measure the PhoA activity of the culture, the cells were washed once in 10 mM Tris-HCl, pH 8, 150 mM NaCl and resuspended in 10 mM Tris-HCl, pH 8, 0.1 mM ZnCl₂. After determination of the absorbance at 600 nm (A_{600}), PhoA activity was measured by determining the rate of hydrolysis of *p*-nitrophenyl phosphate by permeabilized cells. We determined the PhoA activities of the isolated OMVs. In order to compare the results, we adjusted the quantity of OMVs in the assay to the quantity of cells used in the PhoA assay by determining the protein concentration of the OMVs per volume of cells in the original culture. The PhoA activities of an AC53 culture grown for 8 h at 37°C in LB and of the isolated OMVs were used as a negative control. The activities were expressed in Miller units, given by $(A_{405} \times 1,000)/(A_{600} \times 1\text{-ml culture} \times 1\text{ min})$ in the cases of whole-cell lysates or by $(A_{405} \times 1,000)/1\text{ min}$ in the cases of purified OMVs derived from the same quantity of cells.

Statistical analysis. Data were analyzed using the Mann-Whitney U test, a Kruskal-Wallis test followed by post hoc Dunn multiple comparisons, or a Wilcoxon signed-rank test. Differences with *P* values of <0.05 were considered significant.

RESULTS

IgG can be detected in milk samples of immunized female mice and sera of corresponding neonatal mice. We recently reported that immunization with OMVs derived from *V. cholerae* induces immunity in mice and protects the corresponding offspring from colonization with *V. cholerae*. This protection of neonatal mice born to i.n., i.g., and i.p. immunized dams against infection with *V. cholerae* indicates a transfer of the acquired immunity from the dams to their offspring. To investigate this transfer in more detail, we analyzed the anti-OMV Ig titers in sera and stomach contents from neonatal mice at two challenge periods. Period I corresponds to 67 to 75 days after the initial immunization and 39 to 47 days after the second and final boost, whereas period II, which represents a long-term immunity study, corresponds to 117 to 130 days after the initial immunization and 89 to 102 days after the second and final boost of the adult female mice (54). Independent of the immunization route, we detected significant levels of IgG1 and IgG2 at both challenge periods in the sera of neonatal mice from immunized dams (Fig. 1). In contrast, IgA and IgM titers were below the limit of detection for all serum samples (data not shown). At the first challenge period, the offspring of i.n., i.g., and i.p. immunized dams had almost comparable median IgG1 and IgG2 titers, with the only exception being that the IgG1 titers for the i.n. immunized group were signif-

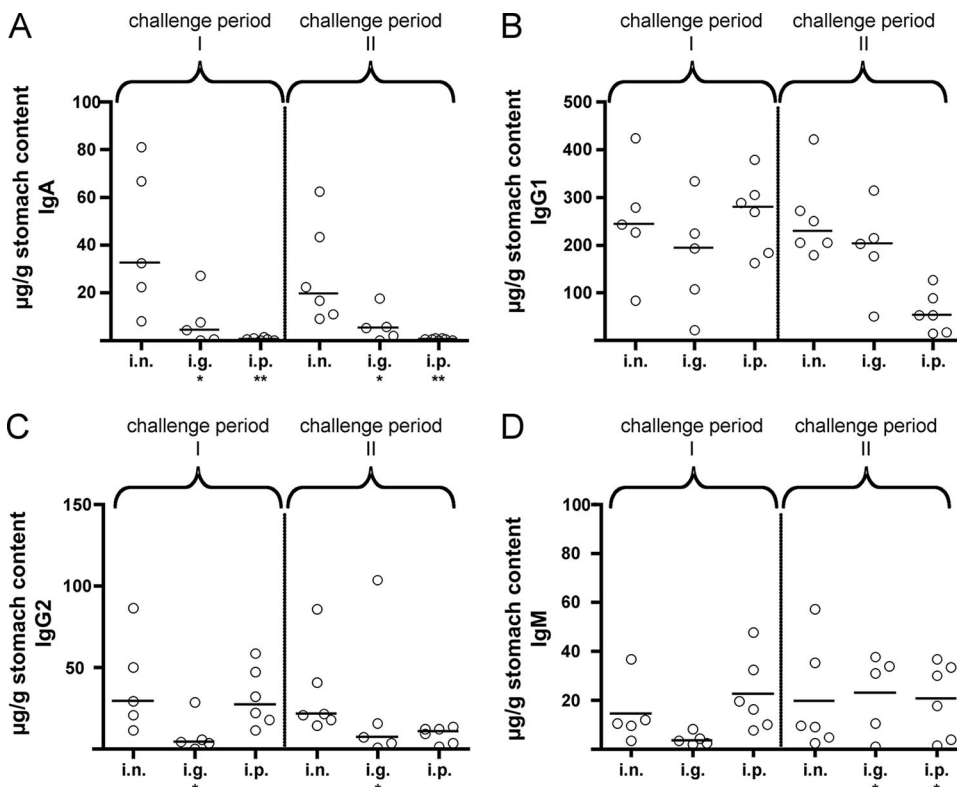


FIG. 2. Ig titers in the stomach contents of neonatal mice from dams immunized with OMVs. Shown are the IgA (A), IgG1 (B), IgG2 (C), and IgM (D) titers in the stomach contents collected from litters born from i.n., i.g., and i.p. immunized female mice at the first or second challenge period. Each circle represents the specific isotype titer for one independent litter. The horizontal bars indicate the median of results from each data set. One asterisk indicates that the result for one sample of the data set was below the detection limit, and two asterisks indicate that the results for all samples were below the limit of detection.

icantly higher than those for the i.p. immunized group ($P < 0.05$; Kruskal-Wallis test and post hoc Dunn multiple comparisons). Median Ig neonatal serum titers increased in the second challenge period in the i.n. and i.g. immunized groups but not in the i.p. immunized group. Thus, at the second challenge period, the highest median IgG1 and IgG2 titers were found in the i.n. immunized group, followed by the i.g. immunized group. At the second challenge period, the IgG1 titers for the i.n. and i.g. immunized groups, as well as the IgG2 titers for the i.n. immunized group, were significantly higher than those for the i.p. immunized group ($P < 0.05$; Kruskal-Wallis test and post hoc Dunn multiple comparisons).

In parallel, we were able to detect IgA, IgG1, IgG2, and IgM in the stomach contents of the neonatal mice from immunized dams (Fig. 2). Since these mice were only 5 to 6 days old at the challenge time point, the stomach mainly contained milk from the dams. The ELISA results for the stomach content samples exhibited more variation than those for the serum samples, and the Ig titers in some samples in the i.g. and i.p. immunized groups were below the limit of detection. Nevertheless, some general trends can be observed. IgG1 was the most abundant isotype in all neonates, independent of the immunization route. The highest levels of IgA were detected in neonatal mice from i.n. immunized dams, whereas in the i.p. immunized group, the IgA titers were below the limit of detection for all samples analyzed. The median Ig titers from the i.n. and i.g.

immunized groups showed either no difference or only a minimal decrease between the first and the second challenge periods. In contrast, the IgG1 and IgG2 titers for the i.p. immunized group showed two- to threefold reductions between the two periods, but only the decrease of IgG1 was significant ($P < 0.05$; Mann-Whitney U test). The median IgM titers did not follow this trend and either increased or stayed stable between the first and second challenge periods. Ig titers in sera and stomach contents were also monitored for the offspring of nonvaccinated control mice, but the titers were below the limit of detection at both challenge periods (data not shown).

In this and in a previous study, we could not detect any major differences in protection and total induced immunity between i.n., i.g., and i.p. immunized mice (54). Thus, in the subsequent experiments described below, we focused on the i.n. immunization route, which provides high and stable Ig titers of all isotypes analyzed. We noted no obvious toxicity of i.n. immunization with OMVs. In all the immunization experiments described herein and in a recently published study (54), we observed morbidity rates of only 6% in the i.n. immunized group and 4% in the nonvaccinated control group. In addition, there were no noticeable differences in abilities to gestate, give birth, and feed offspring between the i.n. immunized mice and the nonvaccinated control group.

Milk provides protection against colonization. The detection of Igs in the sera of the neonatal mice from immunized

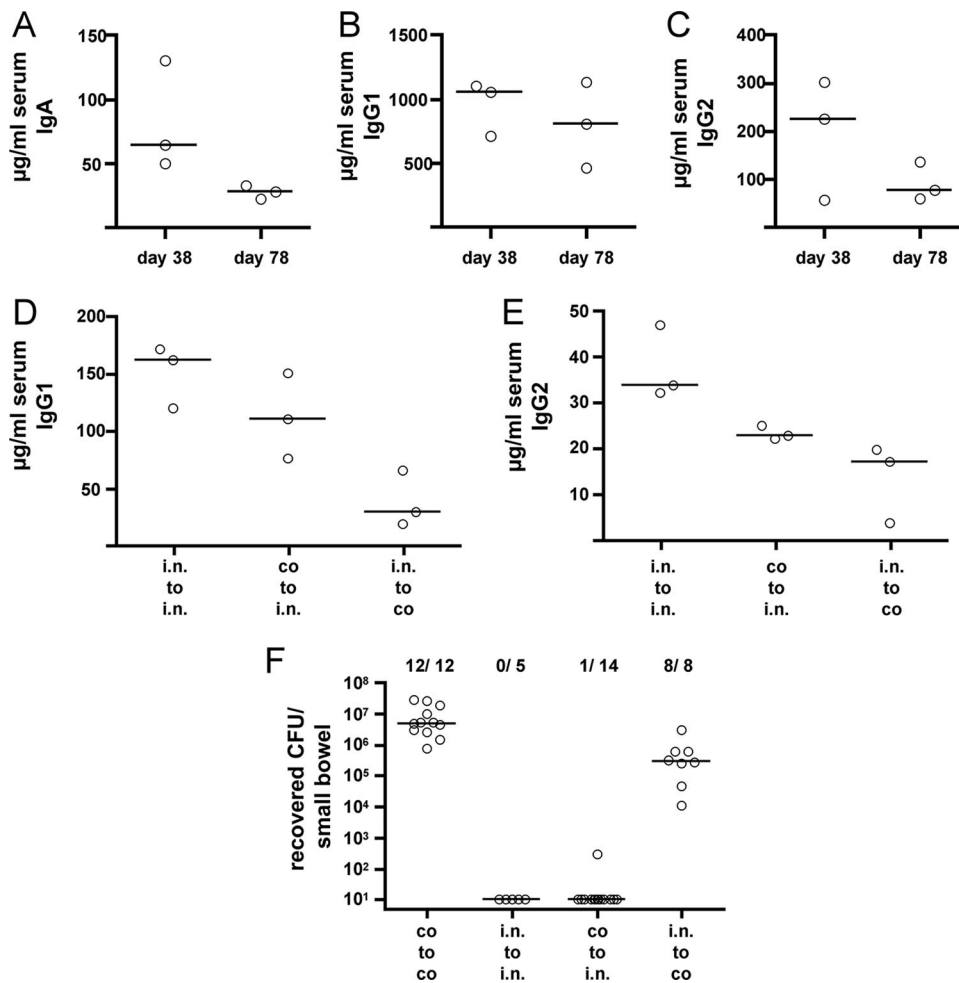


FIG. 3. Ig titers and protection of neonatal mice after exchange of immunized and nonvaccinated dams. Shown are the IgA (A), IgG1 (B), and IgG2 (C) titers in sera collected on day 38 or day 78 from i.n. immunized female mice as well as the IgG1 (D) and IgG2 (E) titers in sera collected from litters born to i.n. immunized mice or nonvaccinated control mice (co). The neonatal mice were either kept with their dams (i.n. to i.n.) or swapped to a dam of the other group (i.n. to co or co to i.n.). Each circle represents the specific isotype titer for one immunized female mouse or independent litter. The horizontal bars indicate the median of results from each data set. Panel F shows the number of recovered CFU per small bowel after challenge with *V. cholerae* for neonatal mice born to the nonvaccinated control mice and kept with their dams (co to co), neonatal mice born to i.n. immunized mice and kept with their dams (i.n. to i.n.), or neonatal mice born to either nonvaccinated control or i.n. immunized mice and then swapped to a dam of the other group (co to i.n. or i.n. to co). Each circle represents the number of CFU for one neonatal mouse. Due to the nature of the experiment (explained in Materials and Methods), the exact number of CFU for the inoculum doses varied from day to day, ranging from 15,100 to 17,000. If no bacteria could be recovered, then the number of CFU was set to the limit of detection (10 CFU/small bowel). The number of mice with detectable colonization/total number of challenged mice is shown above the panel for each group of mice.

dams indicates a transfer of Igs to the offspring. This acquisition of Igs can be achieved by placental transfer and/or via the mother's milk after birth, as supported by the significant amounts of Igs in the stomach contents of neonates fed by lactating immunized dams. In order to elucidate the impacts of both transfer routes, we swapped half of the litters from immunized mice with half of the litters from nonvaccinated control mice within 16 h after birth. The remaining (nonswapped) halves of the litters stayed with their respective mothers as controls. Ig titers in sera of the i.n. immunized and the nonvaccinated control adult female mice were monitored by ELISA. Blood was collected on day 0, 10 days after the last immunization (on day 38), and about 3 to 6 days after the challenge (on day 78). The isotype-specific titers of Ig against OMVs were below detection in all mice on day 0 and for

nonvaccinated control mice during the entire experiment (data not shown). The remaining results for days 38 and 78 are summarized in Fig. 3A to C. All three i.n. immunized mice induced an immune response on day 38, followed by a slight decrease on day 78. The detected levels are comparable to those for earlier i.n. immunizations (54).

In addition, we analyzed the IgG1 and IgG2 titers in the sera of neonatal mice (Fig. 3D and E). Since we swapped parts of the litters between the immunized and nonvaccinated control dams, we had four different groups to analyze: (i) neonatal mice that were born from i.n. immunized mice and given to nonvaccinated control mice, (ii) neonatal mice that were born from nonvaccinated control mice and given to i.n. immunized mice, and (iii and iv) the two sets of neonatal mice that stayed with their respective dams. The highest IgG1 and IgG2 titers

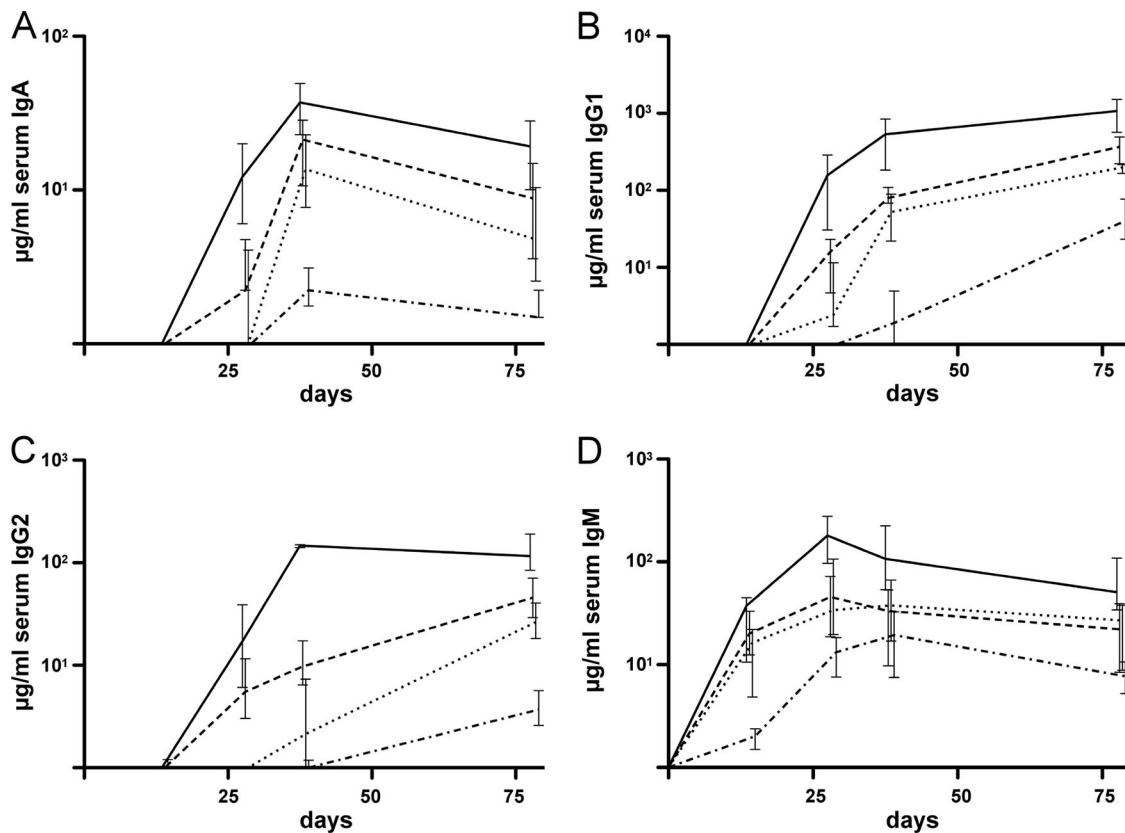


FIG. 4. Ig titers from sera of mice immunized with OMVs. Shown are the median titers over time of IgA (A), IgG1 (B), IgG2 (C), and IgM (D) Igs to OMVs in sera from mice immunized i.n. with 25 µg (solid line), 2.5 µg (dashed line), 0.25 µg (dotted line), or 0.025 µg (dashed and dotted line) OMVs per immunization (n ≥ 5 for each group). The error bars indicate the interquartile range of results from each data set for each time point.

were detected in neonatal mice that stayed with their i.n. immunized dams, followed by neonatal mice swapped from non-vaccinated control to immunized mice. Much lower titers were observed in neonatal mice swapped from immunized mice to nonvaccinated control mice. The IgG1 and IgG2 titers in the sera from neonatal mice that stayed with their nonvaccinated control mice were below the limit of detection (data not shown).

Finally, we analyzed the levels of protection in these neonatal mice by challenge with *V. cholerae*, using an infection dose of ~15,000 CFU per mouse, which is approximately 70-fold above the ID₅₀ (54). The colonization results are presented in Fig. 3F. All neonatal mice that stayed with the nonvaccinated control dams were colonized with 10⁶ to 10⁸ CFU per small bowel. In contrast, all mice that stayed with the i.n. immunized dams demonstrated complete protection. Neonatal mice swapped from control to immunized dams were protected, with only 1 out of 14 challenged mice colonized at a very low level. All mice swapped from immunized to control dams were colonized, but importantly, the level of colonization was significantly lower than that for the neonatal mice that stayed with the nonvaccinated control dams all the time (*P* < 0.05; Kruskal-Wallis test and post hoc Dunn multiple comparisons). Thus, the observed protection depends on the access to milk from immunized dams and to a lesser extent on the IgG1 and IgG2 titers in the neonatal sera.

Dose-related immune response. The immunization doses in our first immunizations were based on recently published protocols for OMVs derived from various gram-negative bacterial species (12, 15, 19, 31, 53). In order to determine if a lower dose of OMVs would be sufficient to induce a protective immune response, we i.n. immunized mice with different quantities of OMVs according to our standard immunization protocol, which consists of an initial immunization followed by two boosters 14 days apart. A total of five different OMV concentrations, ranging from 25 µg to 0.0025 µg per immunization, were examined. Isotype-specific-Ig titers and half-maximum titers in the sera of immunized mice were monitored over time.

The temporal IgA, IgG1, IgG2, and IgM responses for mice immunized with different doses of OMVs are given in Fig. 4. On day 0, the titers of isotype-specific Ig against OMVs were below the limit of detection in all mice. In general, the isotype-specific-Ig titers for mice immunized with 0.025 µg OMVs per immunization or higher doses increased during the vaccination period for all dosage groups, with a peak on day 28 or day 38 for IgM and day 38 for IgA. This peak was followed by a slight decline until day 78. In the cases of IgG1 and IgG2, however, the highest levels were detected on day 78. A comparison of the different groups revealed that the median Ig titers generally increased with increasing dosage, but not necessarily by the same factor. Especially at the endpoint, the median Ig titers for groups receiving the three highest immunization doses differed

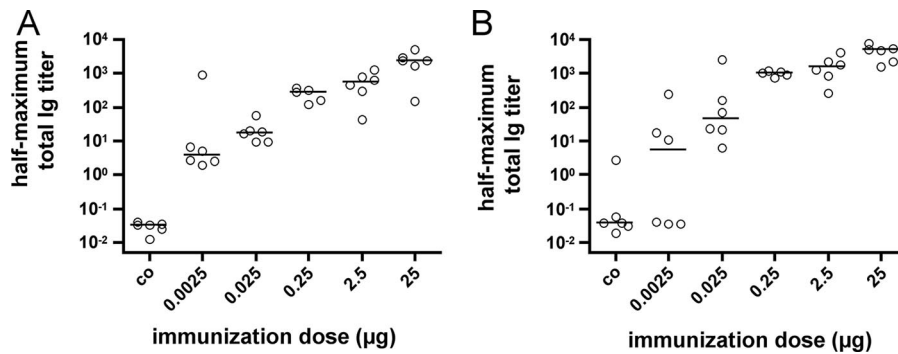


FIG. 5. Half-maximum total Ig titers in sera of mice immunized with OMVs derived from *V. cholerae* and the control group. Results are shown for sera taken on day 38 (A) and days 125 to 135 (B) from mice i.n. immunized with different quantities of OMVs. The OMV dose per immunization is given for each group on the *x* axis. The nonvaccinated control group is indicated by "co." Each circle represents the half-maximum total Ig titer for one mouse. The horizontal bars indicate the median of results from each data set.

only 2- to 3-fold, although the dosage of OMVs between each group changed 10-fold. The median isotype-specific-Ig titers for the nonvaccinated control group and the group receiving 0.0025 µg OMVs per immunization stayed below the limit of detection during the entire experiment (data not shown).

Additionally, we determined the half-maximum total Ig titers against OMVs for the sera collected from immunized mice on day 38 and day 78. The results are shown in Fig. 5A and B. The median half-maximum Ig titers for all immunization groups were at least 2 logs higher than those for the nonvaccinated control mice. In the case of the samples collected on day 38, all median half-maximum Ig titers were significantly higher than those for the nonvaccinated control mice ($P < 0.05$; Kruskal-Wallis test and post hoc Dunn multiple comparisons). Due to the high variation of some samples collected on day 78, only the median half-maximum Ig titers from immunized groups with an immunization dose of 0.025 µg or higher were significantly different from those for the nonvaccinated control mice ($P < 0.05$; Kruskal-Wallis test and post hoc Dunn multiple comparisons). The Ig titers increased with higher immunization dosages, but this increment was saturated at the highest immunization doses. Although the tested immuniza-

tion doses ranged over 4 logs, from 0.0025 to 25 µg per immunization, the median half-maximum Ig titers differed at both time points by only 3 logs. Groups of immunized mice with immunization doses of 0.25 µg or higher had similar median half-maximum Ig titers on day 78, while at lower immunization doses the half-maximum titers for the different groups increased quite steadily.

Thus far, we have examined induced immunity in primary immunized mice only through Ig titers in sera as well as the passive transfer of this acquired immunity to the offspring. In order to determine the induced immune response in the gastrointestinal tracts of our primary immunized mice, we collected fecal pellets on day 78. The determined IgA and IgG1 titers in feces from immunized mice and nonvaccinated control mice are shown in Fig. 6A and B. IgA was detected in most of the fecal pellet samples from mice immunized with 0.025 µg OMVs per immunization and higher doses. In contrast, IgA titers were below the limit of detection in all samples of nonvaccinated control mice and mice immunized with 0.0025 µg OMVs. The IgA titers for the groups with the four highest immunization doses were significantly different from the limit of detection ($P < 0.05$; Wilcoxon signed-rank test). In feces,

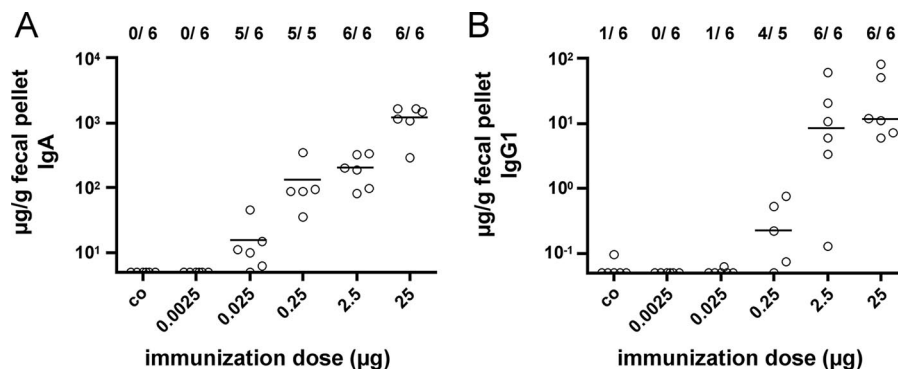


FIG. 6. Ig titers in fecal pellets of mice immunized with different quantities of OMVs derived from *V. cholerae*. Shown are the titers IgA (A) and IgG1 (B) extracted from fecal pellets collected on day 38 from mice i.n. immunized with different quantities of OMVs. The OMV dose per immunization is given for each group on the *x* axis. The nonvaccinated control group is indicated by "co." Each circle represents the specific isotype titer for one mouse. The horizontal bars indicate the median of results from each data set. The number of samples with detectable Ig titers/total number of samples is shown above the panel for each data set. The values for samples with no detectable isotype titers were set to the respective limits of detection, which were 5 µg/g fecal pellet in the case of IgA and 0.05 µg/g fecal pellet in the case of IgG1.

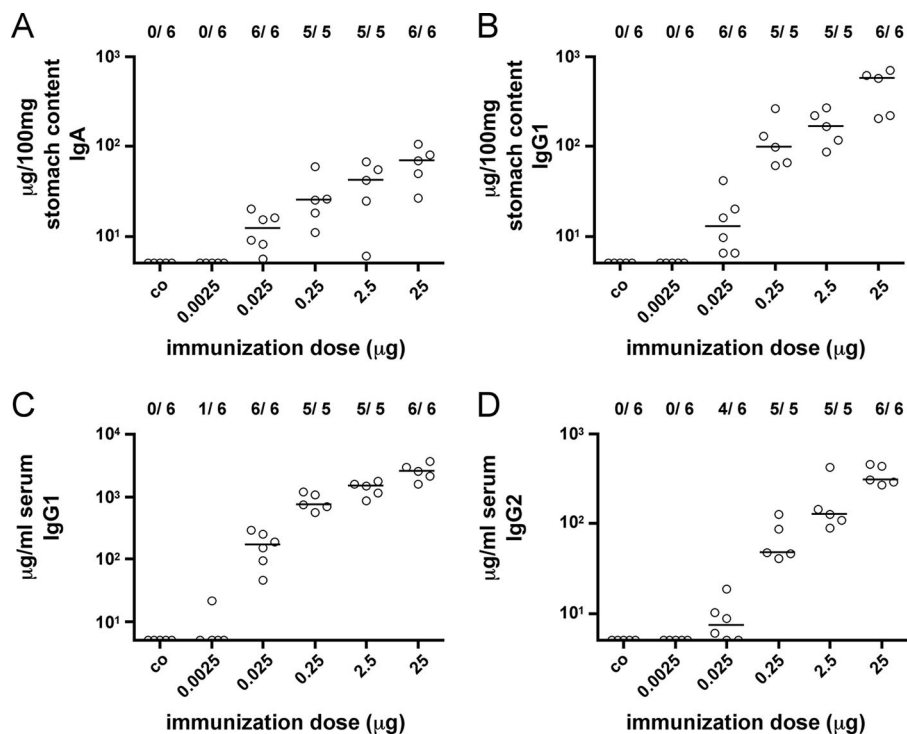


FIG. 7. Ig titers in the stomach contents and sera of neonatal mice from dams immunized with OMVs. Shown are the IgA (A) and IgG1 (B) titers in the stomach contents as well as the IgG1 (C) and IgG2 (D) titers in sera collected from litters born to mice i.n. immunized with different quantities of OMVs. The OMV dose per immunization is given for each group on the x axis. The nonvaccinated control group is indicated by "co." Each circle represents the Ig titer in the pooled sample from neonates of one independent litter. The horizontal bars indicate the median of results from each data set. Values for samples with no detectable isotype titers were set to the limit of detection ($0.5 \mu\text{g}/100 \text{ mg}$ stomach content or $0.5 \mu\text{g}/\text{ml}$ serum). The number of litters with detectable Igs/total number of independent litters is shown above the panel for the respective group.

IgG1 titers were generally 10- to 100-fold lower than IgA titers for the respective groups. Detectable amounts of IgG1 were measured for mice immunized with $0.25 \mu\text{g}$ OMVs per immunization or higher doses, and only the groups with the two highest immunization doses had IgG1 titers significantly different from the limit of detection ($P < 0.05$; Wilcoxon signed-rank test). The vast majority of samples for the nonvaccinated control group and mice immunized with the two lowest OMV doses were below the limit of detection for IgG1. In the cases of detectable titers, IgA and IgG1 titers increased with increasing immunization doses.

Dose-dependent transfer of Igs. We further characterized the transfer of Igs to the offspring by analyzing isotype-specific-Ig titers in the sera and stomach contents of neonatal mice from dams immunized with different quantities of OMVs. We limited our analysis to the two most abundant isotypes in the respective sample. The IgA and IgG1 titers in the stomach contents of neonatal mice are given in Fig. 7A and B, and the IgG1 and IgG2 titers in sera of neonatal mice are shown in Fig. 7C and D. Except for one sample, the Ig titers for the nonvaccinated control mice and the group that received the lowest immunization dose were below the limit of detection in all four ELISAs. Detectable Ig titers were observed in most sera and stomach content samples of neonatal mice from mice immunized with $0.025 \mu\text{g}$ per immunization or higher doses. Again, increases of Ig titers with higher immunization doses were observed for all isotypes tested. In the cases of the stomach

content samples, the IgA and IgG1 titers for the groups with the four highest immunization doses were significantly above the limit of detection ($P < 0.05$; Wilcoxon signed-rank test). Consistent with the data in Fig. 2, IgG1 titers were generally higher than IgA titers for the respective groups. For the serum samples, the IgG1 titers for the groups with the four highest immunization doses and the IgG2 titers for the groups with the three highest immunization doses were significantly above the limit of detection ($P < 0.05$; Wilcoxon signed-rank test). In summary, mice immunized with $0.025 \mu\text{g}$ OMVs per immunization or higher doses transferred detectable amounts of Igs to the offspring.

Dose-related protective immunity. In order to determine if the acquired immunity passively transferred from dam to offspring was protective against colonization of *V. cholerae*, we used a version of the infant mouse model wherein the offspring of immunized dams were challenged and the level of protection was measured by the degree of colonization in the small bowel (18, 36, 54). Continuous transfer of Igs from the immunized dams to the suckling offspring was allowed by placing the infected neonatal mice back with their respective dams for the challenge period of 24 h. The results for the groups immunized with different OMV doses and nonvaccinated control mice are shown in Fig. 8. Neonatal mice were challenged with *V. cholerae* by using $\sim 1,500$ or $15,000$ CFU per mouse, which is about 7- or 70-fold above the recently determined ID_{50} (54). As displayed in Fig. 8A, neonatal mice from nonvaccinated con-

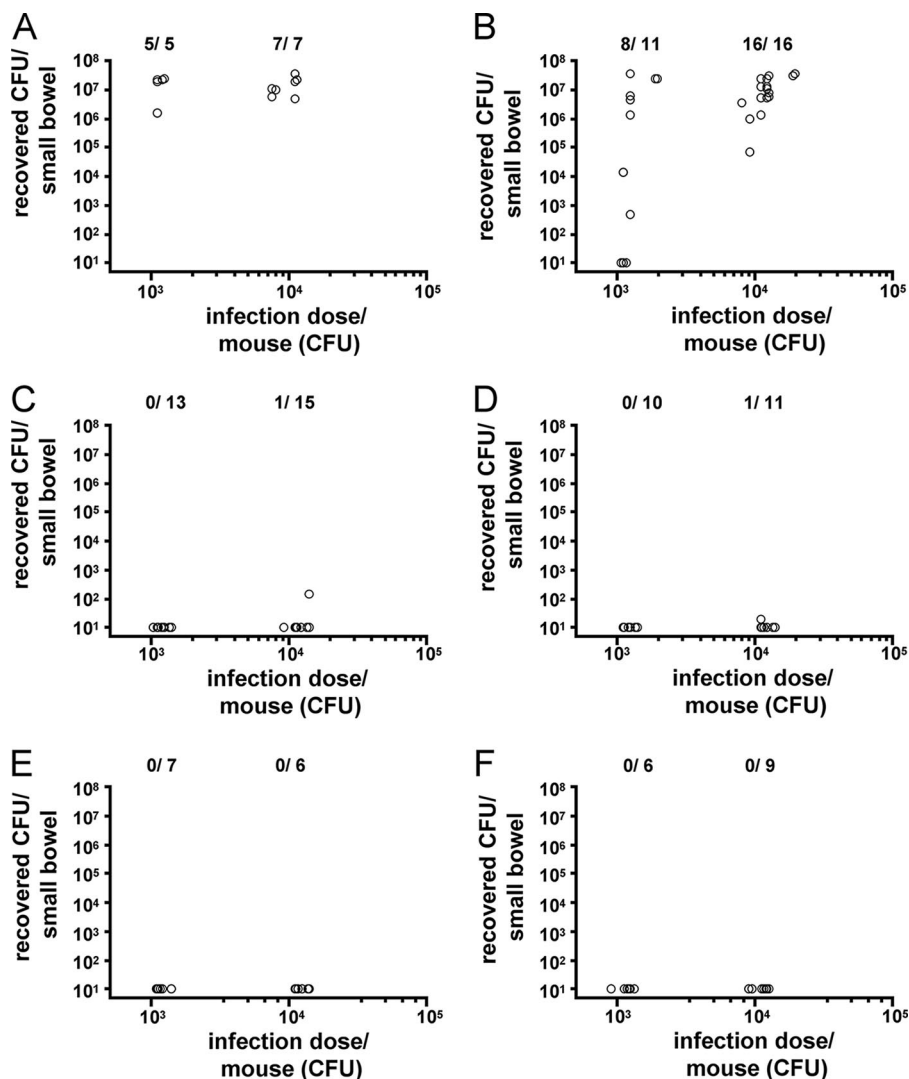


FIG. 8. Protection against colonization with *V. cholerae* for neonatal mice born to dams immunized with different quantities of OMVs. The numbers of recovered CFU per small bowel are shown on the y axis versus the respective inoculum on the x axis. Results are shown for neonatal mice born to dams of the nonvaccinated control group (A) and groups i.n. immunized with 0.0025 μg (B), 0.025 μg (C), 0.25 μg (D), 2.5 μg (E), and 25 μg (F) OMVs per immunization. Each circle represents the number of CFU for one neonatal mouse. Due to the nature of the experiment (explained in Materials and Methods), the exact number of CFU for each inoculum varied from day to day, ranging from 1,030 to 1,950 and 7,600 to 19,500 for the individual 10-fold dilutions of the inoculation mixture. If no bacteria were recovered, then the number of CFU was set to the limit of detection (10 CFU/small bowel). The number of mice with detectable colonization/total number of challenged mice is shown above the panel for the respective inoculum.

control dams were colonized with approximately 10⁷ CFU per small bowel, independent of the infection dose. The colonization of neonatal mice from dams immunized with 0.0025 μg OMVs per immunization exhibited more variation (Fig. 8B). At the lower infection dose, the numbers of recovered bacteria ranged from 0 to 10⁷ CFU per small bowel. At the higher infection dose, all mice were colonized with at least 10⁵ CFU per small bowel. In contrast, neonatal mice from dams immunized with 0.025 μg or 0.25 μg OMVs per immunization were almost completely protected (Fig. 8C and D): no CFU were recovered by challenging the neonatal mice of both groups with the lower infection dose; only one neonatal mouse of each group was colonized with the higher infection dose, but the colonization was at least 10,000-fold lower than that in neona-

tal mice from nonvaccinated control dams. In the cases of neonatal mice from dams immunized with 2.5 μg or 25 μg OMVs per immunization, all mice were completely protected and no CFU were recovered, regardless of the infection dose (Fig. 8E and F). In summary, neonatal mice from dams immunized with 0.025 μg OMVs per immunization or higher doses demonstrated robust protection against colonization, and the ID₅₀ was at least 70-fold higher than that for nonvaccinated control mice.

OMVs can be used as an antigen delivery vehicle. A potential advantage of an OMV-based vaccine is packaging of antigens from different pathogens into OMVs. The transfer into OMVs requires the secretion of these antigens into the periplasm or integration into the outer membrane. To deter-

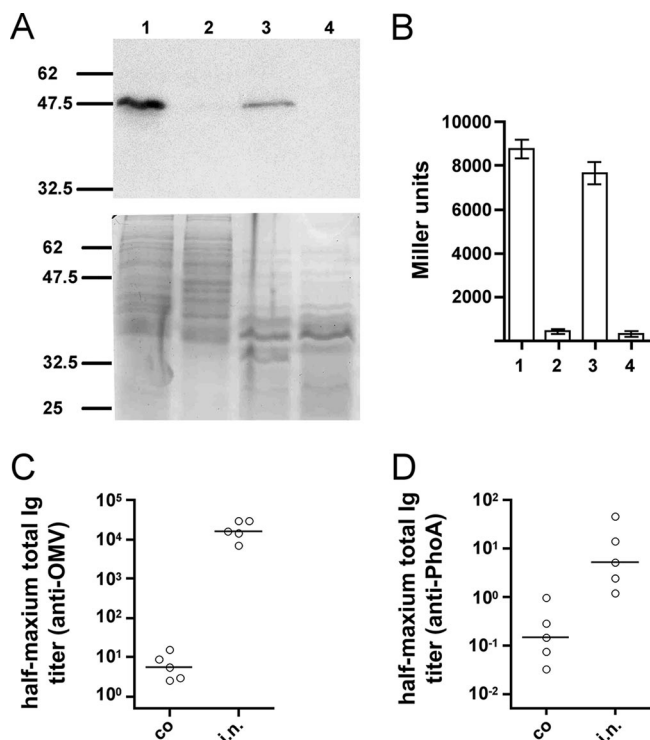


FIG. 9. Heterologous proteins can be loaded into OMVs derived from *V. cholerae* and serve as antigens. Panel A shows an immunoblot against PhoA and Coomassie-stained sodium dodecyl sulfate gel (below), loaded with whole-cell lysates of AC108 (lane 1) or AC53 (lane 2) and OMVs derived from AC108 (lane 3) or AC53 (lane 4). Values at left indicate the molecular masses of the protein standards in kilodaltons. The PhoA activities of the individual samples are given in panel B. Furthermore, the half-maximum total Ig titers against OMVs (C) or PhoA (D) in sera of mice i.n. immunized with OMVs derived from AC108 are shown. The nonvaccinated control group is indicated by "co." Each circle represents the half-maximum total Ig titer for one mouse. The horizontal bars indicate the median of results from each data set.

mine whether such a heterologous protein could be transferred into OMVs derived from *V. cholerae*, we used a variant of a previously described *tcp-phoA* transcriptional fusion strain, AC108 (46). In this strain, expression of periplasmic PhoA from *E. coli* is under the control of the *tcp* promoter, which is highly induced if the *V. cholerae* classical biotype is grown in LB (pH 6.5, 50 mM NaCl) at 30°C with aeration (58). Thus, we purified OMVs derived from the *tcp-phoA* fusion strain grown under inducing conditions. Expression of PhoA as well as transport into the OMVs was verified by immunoblot analysis using whole-cell lysates and purified OMVs in combination with an anti-PhoA Ig (Fig. 9A). No band was detected in whole-cell lysates or OMVs derived from strain AC53, which served as a negative control (Fig. 9A, lanes 2 and 4). The detected band in Fig. 9A, lane 1, is more intense than that in lane 3. Thus, it seems that the whole-cell lysate contained more PhoA than the OMVs derived from AC108, since equal amounts of protein were loaded. However, the whole-cell lysate harbors cytoplasmic and periplasmic PhoA, but only the latter can be transferred into the OMVs. To investigate the transfer efficiency in more detail, we determined the PhoA

activities of all samples. This is more accurate, since only the PhoA in the periplasm and OMVs is active. The results are shown in Fig. 9B. The activity is given in Miller units for a defined quantity of cells or their derived OMVs. The PhoA activity results indicate that whole-cell lysate and OMV samples contain similar amounts of active PhoA. This suggests that PhoA is efficiently transferred into the OMVs. Whole-cell lysates and OMVs derived from strain AC53 exhibited almost no activity.

In order to elucidate if PhoA in these OMVs has immunogenic properties, female mice were immunized i.n. with these OMVs. We used our standard immunization protocol, with an initial immunization on day 0 and two boosters on days 14 and 28, with 25 µg OMVs derived from AC108. Sera were collected on day 38, and the half-maximum titers of Ig against total OMVs and purified PhoA were determined by ELISA for the immunized mice and the nonvaccinated control mice. The results are shown in Fig. 9C and D. All immunized mice exhibited immune responses against both OMVs and PhoA, though the immune response against PhoA alone was approximately 1,000-fold less than the induced immune response against the total OMVs. Nevertheless, in both cases, the median titers for the immunized mice were significantly higher than those for the nonvaccinated control group ($P < 0.05$; Mann-Whitney U test). Thus, mice i.n. immunized with OMVs harboring PhoA induced a specific immune response against this heterologously expressed antigen.

DISCUSSION

Recently, we reported the induction of robust immunity after i.n., i.g., and i.p. immunization of female mice with OMVs derived from *V. cholerae* (54). By combining the infant mouse model of cholera (36) with an adapted version of a previously described challenge model (18), we demonstrated that the majority of neonatal mice from immunized dams were protected against colonization with *V. cholerae*, independent of the route of immunization. The present study provides a more comprehensive analysis of this induced protective immunity as well as the transfer of immunity to the offspring. In addition, we examined herein the potential use of OMVs as a delivery vehicle for heterologous antigens in vaccine design.

First, we investigated the mechanism of the observed protection, which implies a transfer of immunity from the primary immunized female mice to the offspring. Since we were not able to collect breast milk directly, we analyzed the stomach contents from neonates after the challenge experiments. These 5- to 6-day-old neonatal mice were exclusively breast fed by their dams. Therefore, their stomach contents consisted mainly of breast milk. The detection of significant amounts of OMV-specific Igs in the stomach contents of neonates suckling from immunized dams indicates that Igs are transferred from the immunized dams to the offspring via the milk.

We detected similar compositions of anti-OMV Ig isotypes in the stomach contents and the sera of the primary immunized female mice (54). For example, neonates of the i.n. and i.p. immunized groups exhibited high IgA titers in their stomach contents, while the IgA titers were below the detection limit for all neonatal samples in the i.p. immunized group. Furthermore, the stomach contents of neonatal mice from all three

immunization groups revealed comparable IgG1 titers for the first challenge period, but neonatal samples of the i.p. immunized group showed the lowest IgG1 titers at the second challenge period. These observations are consistent with the data revealed from the sera of the primary immunized mice (54). Interestingly, even in the i.n. and i.g. immunized groups, the most abundant isotype in the stomach contents was IgG1, followed by IgA. The recovered stomach content had a paste-like texture with highly insoluble material. Thus, our Ig extraction was most likely incomplete, and we can only speculate about the amounts of Igs remaining in the insoluble fraction of each sample.

Except for IgA in the i.p. immunized group, all Ig isotypes investigated were detected in the majority of the stomach content samples. In contrast, serum samples obtained from the neonatal mice of all immunization groups contained only IgG1 and IgG2 in significant amounts. This result is not surprising, based on previously published work on transfer of Igs to fetal and neonatal mice. In rodents, a small but significant amount of IgG is transferred to the fetus transplacentally during the last week of gestation (3, 9). The bulk of IgG in the neonatal sera, however, is provided after birth by the breast milk and transported across the intestinal epithelium into neonate circulation (3, 13). The major histocompatibility complex class I-related neonatal Fc receptor plays an important role in both transfer across the yolk sac in utero and transport across the intestinal epithelial cells in neonates (1, 28, 49, 55, 56). The transport mediated by the neonatal Fc receptor is highly selective for IgG, although breast milk contains a variety of different isotypes, as shown by the isotypes detected in the stomach contents (13, 50, 62). Consistent with this observation, we detected only IgG1 and IgG2 in the sera of neonates, not IgM or IgA. Although prenatal IgG transfer exists in mice, it has previously been reported that IgG titers in the sera of neonates dramatically rise within the first 10 days after birth (3). This clearly emphasizes the impact of transfer of Igs to neonates via breast milk. However, in humans, Igs from breast milk do not enter neonate circulation in substantial amounts (61). This is consistent with observations that IgG1 was found in such high concentrations in the milk of mice, whereas in humans, secretory IgA is the most abundant isotype in breast milk (6, 21, 22).

By swapping part of the litter between immunized and nonvaccinated control female mice, we were able to distinguish between prenatal and neonatal transfers of Igs. Neonates born from nonvaccinated control mice and given to immunized female mice immediately after birth were able to acquire OMV-specific Igs exclusively from the milk but not via placental transfer. In contrast, neonates swapped the other way acquired OMV-specific Igs via parental transfer only. Independent of their parentage, all neonates suckling from immunized dams were protected against colonization with *V. cholerae*. Particularly, the protection of neonates swapped from nonvaccinated control to immunized dams implies that Igs present in milk are crucial. Thus, protection in our model is mainly correlated with access to breast milk from immunized dams, and this transfer route is sufficient for complete protection. In contrast, the parentage of the neonatal mice played only a minor role in their protection, as indicated by a small but significant reduction in colonization in neonates swapped from immunized to

nonvaccinated control dams, compared to the level in neonates that stayed with their nonvaccinated control dams all the time. These data show that although placental transfer provides some immunity, this is not sufficient to protect against *V. cholerae* colonization. It should be noted that we cannot completely exclude the possibility of Ig uptake via breast milk in this group, since we swapped the mice shortly but not immediately after birth. It is possible that residual Igs were not cleared in the swapped mice.

Overall, our results are consistent with earlier work demonstrating a protective effect against *V. cholerae* infection of neonates caused by the milk of immunized dams (59). However, in contrast to our study design, dams were subcutaneously immunized with heat-killed *V. cholerae* bacteria, and protection was measured by the mortality rate of neonates suckling from immunized or nonimmunized dams. Using the mortality rate might not be as sensitive as determining the actual colonization. However, a significant percentage of 6-day-old neonates were protected when access to milk from immunized dams was limited to the period after oral challenge (59). As indicated by both studies, passive immunization by milk leads to protection against *V. cholerae* infection in neonatal mice.

Interestingly, for humans, a protective effect of breast-feeding against cholera in areas of endemicity has been shown (11, 47, 65), which also implies passive immunization by the mother's milk. The level of this protection correlates with the IgA titer in the mother's milk, and the majority of Igs are directed against *V. cholerae* surface structures and cholera toxin (17, 23). This is not surprising, since secretory IgA is the most abundant isotype in human breast milk (6, 21, 22). These observations strongly argue for a cholera vaccine on the basis of cell surface components in combination with a delivery route that induces high IgA titers. Both requirements are fulfilled by i.n. or i.g. immunization with OMVs. The immunization of adult females would lead to a passive immunization of the newborns via mother's milk and hopefully result in a significant protection in children, which represent the segment of the population most affected by cholera during epidemics.

One limitation of our study is that we cannot challenge the primary immunized adult mice. Adult mouse models for *V. cholerae* colonization have been described but depend on the use of either sterile or antibiotic-treated mice to eliminate the natural gastrointestinal flora (7, 42, 45). The use of sterile mice for an entire immunization project is not suitable, and antibiotic treatment could lead to inflammation and other side effects, which can interfere with colonization. Therefore, we decided not to use the adult mouse model but to determine the immune response in the gastrointestinal tract by using fecal pellet extracts. A recent study of household contacts of cholera patients in Bangladesh revealed a correlation between protection against cholera and levels of serum IgA but not serum IgG titers (24). It can be hypothesized that the serum IgA titers reflect the protective secretory IgA immune response on the mucosal surface in the gastrointestinal tract. In order to elucidate this protective immunity in the intestine, we analyzed the IgA and IgG1 titers in the fecal pellets of our primary immunized mice from the dose response experiment. In contrast to what was found for the stomach contents, IgA was the most abundant isotype in the fecal pellets. We were able to detect IgA titers above the limit of detection in fecal pellets of

mice immunized with 0.025 μg OMVs or higher doses. Consistent with this observation, neonates of these groups were protected against colonization with *V. cholerae*. This result reinforces the use of an immunization route that induces a strong mucosal IgA response. The detection of robust induced IgA responses in the intestinal tracts of our primary immunized mice suggests that these mice would most likely have been protected against a *V. cholerae* infection.

The identification of an induced protective immune response in mice immunized with only 0.025 μg OMVs per immunization is noteworthy. The small differences in induced immunity between the groups with the three highest immunization doses indicate that the system was nearly saturated at these antigen concentrations. On the basis of our OMV preparations, the concentration of OMVs in the supernatant of a *V. cholerae* late-exponential-phase culture is approximately 0.002 $\mu\text{g}/\mu\text{l}$. This is just below the concentration for an immunization dose of 0.025 μg , since we applied volumes of 10 μl for i.n. immunizations. Taken together, the results from the dose response and the simple isolation protocols for OMVs from culture supernatant open a new alternative approach for an affordable and effective cholera vaccine.

Finally, we investigated the potential use of OMVs as a delivery vehicle for heterologously expressed antigens in vaccine design. As reported earlier for other microorganisms, OMVs can be loaded with foreign proteins by simply expressing the heterologous protein with a signal sequence to allow secretion into the periplasm or expressing fusions of the heterologous protein to proteins that are known to be localized in the OMVs (34, 35). We used a *V. cholerae* strain harboring a *TnphoA* insertion in the *tcp* operon. The site of *TnphoA* insertion is undetermined (46) but has resulted in a translational fusion of the N-terminally truncated PhoA protein present on *TnphoA* to a secreted protein such that PhoA is exported to the periplasm and incorporated into OMVs. Expression and efficient localization of PhoA into the OMVs were monitored by immunoblot analysis and alkaline phosphatase activity. Mice i.n. immunized with these PhoA-modified OMVs induced a small but significant specific immune response against PhoA. The relatively low immune response against PhoA might be due to the location of PhoA inside the lumens of the OMVs as opposed to the surfaces. Future studies should investigate if the location of antigens inside or on the OMVs is favorable for their immunogenic potential. On the basis of our encouraging data, one could engineer strains to generate OMVs with desired antigens, which could broaden the application of this OMV vaccine beyond the context of cholera.

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