Intrarectal Immunization with Rotavirus 2/6 Virus-Like Particles Induces an Antiotavirus Immune Response Localized in the Intestinal Mucosa and Protects against Rotavirus Infection in Mice‡

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Rotavirus (RV) is the main etiological agent of severe gastroenteritis in infants, and vaccination seems the most effective way to control the disease. Recombinant rotavirus-like particles composed of the viral protein 6 (VP6) and VP2 (2/6-VLPs) have been reported to induce protective immunity in mice when administered by the intranasal (i.n.) route. In this study, we show that administration of 2/6-VLPs by the intrarectal (i.r.) route together with either cholera toxin (CT) or a CpG-containing oligodeoxynucleotide as the adjuvant protects adult mice against RV infection. Moreover, when CT is used, RV shedding in animals immunized by the i.r. route is even reduced in comparison with that in animals immunized by the i.n. route. Humoral and cellular immune responses induced by these immunization protocols were analyzed. We found that although i.r. immunization with 2/6-VLPs induces lower RV-specific immunoglobulin G (IgG) and IgA levels in serum, intestinal anti-RV IgA production is higher in mice immunized by the i.r. route. Cellular immune response has been evaluated by measuring cytokine production by spleen and Peyer’s patch cells (PPs) after ex vivo restimulation with RV. Mice immunized by the i.n. and i.r. routes display higher gamma interferon production in spleen and PPs, respectively. In conclusion, we demonstrate that i.r. immunization with 2/6-VLPs protects against RV infection in mice and is more efficient than i.n. immunization in inducing an anti-RV immune response in intestinal mucosa.

The induction of immune response at the level of mucosal surfaces is increasingly important in viral vaccine development. Many viruses enter the organism through mucosal tissues, and several of them also replicate in mucosal epithelial cells of the gastrointestinal and respiratory tracts. Although immunization by the parenteral route usually induces a strong systemic immune response but weak or no mucosal response, the induction of an immune response at the mucosal level often requires direct antigen application on a mucosal surface (22). The mucosa-associated lymphoid tissue represents a highly compartmentalized immunological system that functions essentially independently from the systemic immune system (22). Lymphocytes that have been primed in the mucosal inductive sites tend to migrate and exert their effector functions mainly into mucosal tissues, although not necessarily into the mucosa of origin (22). Immunization by the intranasal (i.n.) route stimulates an immune response in the respiratory tract but also in cervicovaginal mucosa, and immunization by the oral route induces antibody production not only in intestine but also in mammary glands (22). However, some compartmentalization also exists within the mucosa-associated lymphoid tissue; for instance, previous studies showed that oral immunization is relatively inefficient for inducing an immune response in the genital tract (22).

Rotavirus (RV) is the main etiological agent of severe gastroenteritis in infants and young children worldwide, causing approximately 440,000 deaths and 1.4 billion episodes of diarrhea per year (43). Vaccination will have a significant impact on the incidence of the disease, and development of an RV vaccine remains a global priority. Since this virus essentially infects and replicates within the enterocytes of small intestine, an anti-RV immune response in the gut seems to be important to provide specific protection against RV infection. To address the safety concerns associated with live vaccines, several immunization strategies using recombinant RV proteins or non-replicating virus-like particles (VLPs) are currently being evaluated by several groups. The coexpression of the viral protein 2 (VP2) and VP6 using a recombinant baculovirus system results in their spontaneous assembly into double-layered VP2/VP6 rotavirus-like particles (2/6-VLPs) (12, 33). 2/6-VLPs have been reported to induce protective immunity in mice when administered by the oral or i.n. route together with cholera toxin (CT) or *Escherichia coli* heat-labile toxin (LT) as the adjuvant (41, 42). Immunization by the i.n. route requires smaller doses of VLPs and elicits higher serum and intestinal anti-RV antibody levels than immunization by the oral route, resulting also in a better protection against RV infection (42). However, the administration of enterotoxin-based adjuvants, such as CT and LT, by the i.n. route has recently been questioned because of the possible neurological effects that may derive from their retrograde axonal transport from the olfactory epithelium into the brain (20). Moreover, a nasal influenza

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vaccine has been withdrawn from the market recently because of the development of Bell’s palsy by some recipients, and the LT present in the formulation is suspected to be responsible for this unwanted effect (39).

The intrarectal (i.r.) route for immunization has been investigated mainly for its ability to induce a local immune response on the rectal mucosa, with the aim of protecting against sexually transmitted diseases. Intrarectal immunization in mice, humans, and nonhuman primates induces high antibody titers in rectal secretions and feces (13, 23, 25, 30). Other studies showed that i.r. immunization of mice with a synthetic human immunodeficiency virus (HIV) peptide induced cytotoxic T lymphocytes (CTLs) in the spleen and in the small intestine, whereas subcutaneous immunization with the same peptide induced a CTL response only in the spleen (5, 6). In macaques, i.r. immunization with an HIV/simian immunodeficiency virus peptide is more effective against i.r. challenge with simian/ human immunodeficiency virus than parenteral vaccination because of a better clearance of the virus from its major replication site in intestinal mucosa (7).

In this study we compared the protection efficacy and the immune response induced by i.r. immunization with 2/6-VPs with those induced by i.n. immunization with the same particles. 2/6-VPs were administered in association with two different adjuvants, CT and a CpG-containing oligodeoxynucleotide (CpG ODN), both known to be active by the mucosal route (18). CT is a widely used mucosal adjuvant which enhances both humoral and cellular immunity when administered by the oral, i.n., and i.r. routes (18, 21). CpG ODNs are synthetic oligodeoxynucleotides which contain unmethylated CpG motifs that mimic bacterial DNA and activate B cells and antigen-presenting cells by triggering Toll-like receptor 9 signaling (29). Our results demonstrate that i.r. immunization with 2/6-VPs shows better efficacy than i.n. immunization for raising an anti-RV immune response in the digestive tract. Moreover, we observed a marked decrease of the viral shedding in mice immunized with 2/6-VPs by the i.r. route and, when immunization is performed with CT, animals are even more protected than they are when immunized by the i.n. route.

MATERIALS AND METHODS

Materials. CT and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO), and CpG ODN 1826 (sequence TCCATGACGTTCCTGACGTT) was synthesized with a phosphorothioate backbone by Eurogentec (Seraing, Belgium). 2/6-VPs containing VP2 and VP6 of the RF strain of bovine RV following oral administration of 100 μl of 1 × 10^7 TCID50 of 13.33% sodium bicarbonate to neutralize stomach acidity. Fecal samples were collected from each mouse at 3, 5, and 8 days postchallenge (d.p.c.) and homogenized 1:10 with PBS-BSA. The suspension was then clarified by centrifugation.

Vaccines. The EDIM strain of murine RV was kindly provided by Javier Buesa (University of Valencia, Spain). Viral stock was prepared by orally infecting newborn mice with serial 10-fold dilutions of the viral stock. The bovine RF strain of RV was propagated in the MA104 cell line in the presence of 1 μg/ml trypsin, as previously described (34). Cell culture supernatant was subjected to three cycles of freeze-thawing, clarified by centrifugation at 400 × g for 15 min, and used directly for ex vivo restimulation of spleen and Peyer’s patch (PP) cells. To pellet and partially purify viral particles, supernatant from infected MA104 cells was layered onto a 40% sucrose cushion and centrifuged at 83,000 × g for 90 min. The pellet was resuspended in 50 mM Tris (pH = 8.0) with 10 mM EDTA in order to remove RV outer capsid proteins. Viral antigen suspension was then diluted in 50 mM carbonate buffer (pH = 9.5), homogenized with a tissue blender to break up aggregates, and used for enzyme-linked immunosorbent assay (ELISA) plate coating.

Animals and treatments. Nine-week-old female BALB/c mice were obtained from Charles River Laboratories (L’Arbresle, France). Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies. Mice were confirmed to be RV antibody free by ELISA prior to immunization. They were immunized every other week for a total of three times with 5 μg of 2/6-VPs together with the indicated doses of CT or CpG ODN 1826 by either the i.r. or the i.n. route. A group of control mice was mock immunized by the i.r. route with 5 μg of OVA and the indicated amount of CT. Mice were deprived of food 24 h before immunization, and during this time grids were placed on the bottoms of cages in order to prevent the animals from ingesting litter. Prior to inoculation, mice were anesthetized by intraperitoneal administration of a mixture of 80 μg of ketamine/kg of body weight (Imalgene; Merial, Lyon, France) and 16 mg of xylazine/kg (Rompun; Bayer, Puteaux, France) diluted in D-PBS in a final volume of 200 μl/mouse. For i.r. immunization, mice were given a final volume of 50 μl split in five doses of 10 μl. Immunization was performed through a 0.5- to 20-μl pipette tip (Eppendorf, Hamburg, Germany) lubricated with a thin film of petroleum ointment and inserted approximately 1 cm in the cul-de-sac. For i.n. immunization, mice were given a final volume of 20 μl split in two doses of 10 μl. Serum and stool samples were collected from each mouse 4 weeks after the last immunization. Blood was taken from retro-orbital plexus under ketamine/xylazine anesthesia, and sera were prepared. Four to 6 weeks after the last immunization, animals were subjected to oral gavage with 100 μl of intestinal homogenate from infected newborn mice containing 1,000 50% diarrhea-inducing doses of murine EDIM RV following oral administration of 100 μl of 13.33% sodium bicarbonate to neutralize stomach acidity. Fecal samples were collected from each mouse at 3, 5, and 8 days postchallenge (d.p.c.) and homogenized 1:10 (wt/vol) in PBS. The suspension was then clarified by centrifugation.

Measurement of RV-specific antibodies by ELISA. Ninety-six-well polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl/well of partially purified RV antigen suspension (prepared as described above) diluted in carbonate buffer. Wells were washed three times with 0.05% (vol/vol) Tween 20 in PBS (PBS-T) and blocked with 200 μl of 3% BSA (Sigma) in PBS (PBS-BSA buffer) for 2 h at 37°C. After the wells were washed, 100 μl/well of serum or stool homogenate diluted in PBS-BSA was added, and plates were incubated for 90 min at 37°C. Subsequently, plates were washed and incubated (90 min at 37°C) with 100 μl/well of biotin-conjugated goat anti-mouse immunoglobulin G (IgG) (Southern Biotechno-
logy, Birmingham, AL) diluted 1:5,000 in PBS-BSA. After the wash, 100 μl of horseradish peroxidase (HRP)-labeled avidin (Southern Biotechnology) diluted 1:14,000 in PBS-BSA was added to each well, and plates were incubated for 1 h at 37°C. Color was developed by adding 100 μl of Sure Blue TMB peroxidase substrate (KPL, Gaithersburg, MD), and optical density (OD) was read at 620 nm. To determine antibody titers, serum or stool homogenates were serially (threefold) diluted and the titers were calculated by determining the inverse of the dilution at an OD of 0.5.

Determination of RV antigen shedding with ELISA. Microtiter plates were coated overnight at 4°C with rat anti-SA11 immune serum diluted in carbonate buffer. Wells were washed with PBS-T and blocked with 200 μl of PBS-BSA for 2 h at 37°C. Wells were washed again and incubated for 90 min at 37°C with 100 μl/well of serial dilutions of stool homogenate in PBS-BSA. After the wells were washed, plates were incubated (90 min at 37°C) with 100 μl/well of rabbit anti-SA11 immune serum diluted in PBS-BSA. After another wash, 100 μl of HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) diluted 1:3,000 in PBS-BSA was added to each well, and plates were incubated for 1 h at 37°C. Color was developed by adding 100 μl of TMB peroxidase substrate and OD was read at 620 nm.

Determination of RV shedding by real-time RT-PCR. Viral RNA was extracted from 20 μl of stool homogenate by using a QIAamp viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Viral RNA was eluted in a final volume of 80 μl of AVE buffer supplied with the kit. A single-step real-time reverse transcription (RT)-PCR was carried out by using TaqMan one-step RT-PCR master mix reagents (Applied Biosystems, Applied Biosystems, Foster City, CA). Amplification was performed using a LightCycler instrument (Roche). Amplification was performed using a LightCycler instrument (Roche).
Intrarectal immunization with 2/6-VLPs induces lower anti-RV antibody levels in serum but higher anti-RV IgA in stools than intranasal immunization. To evaluate the efficacy of i.r. administration of 2/6-VLPs against RV infection, mice were immunized three times at 2-week intervals by the i.r. route with 5 μg of 2/6-VLPs together with either 10 μg of CT or 250 μg of CpG ODN 1826 as the adjuvant. Since i.n. immunization with 2/6-VLPs has been previously reported to protect mice against RV infection (41, 42), we also immunized two groups of mice by the i.n. route with the same dose of 2/6-VLPs together with either 1 μg of CT or 25 μg of CpG ODN 1826 in order to compare the immune responses induced by these two routes of immunization. A fifth group of control animals was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. The dose of 2/6-VLPs was determined based upon preliminary experiments (data not shown). CT was used at doses reported previously (36), and the doses of CpG ODN 1826 were adjusted similarly in order to have a 10-fold increase in the adjuvant dose utilized for i.r. immunization over that utilized for i.n. immunization.

Four weeks after the last immunization, RV-specific IgG and IgA titers were measured in serum. We found that immunization by the i.r. route induced lower IgG levels than immunization by the i.n. route when either CT or CpG ODN 1826 was used as the adjuvant (Fig. 1A). RV-specific IgA titers after i.r. immunization were also lower than those induced by i.n. immunization (Fig. 1B). No RV-specific IgG or IgA was detected in sera from OVA-immunized control mice (Fig. 1A and B). We also analyzed the relative levels of anti-RV IgG belonging to the IgG1 and IgG2a subclasses, since the IgG1/IgG2a ratio is a widely used marker to determine the type of helper T-cell response (Th2 versus Th1). Mice immunized with CT as the adjuvant by either the i.r. or the i.n. route displayed higher IgG1/IgG2a ratios than mice immunized with CpG ODN 1826 (Fig. 1C and D). These data suggest that immunization with CT as the adjuvant induces a Th2-skewed immune response, whereas immunization in the presence of CpG ODNs induces a Th1-skewed immune response, in agreement with previous reports (29, 35).

We also examined fecal antibody levels in stool samples collected 4 weeks after the last immunization. The titers of RV-specific IgA in stools were eightfold higher in mice immunized with 2/6-VLPs and CT by the i.r. route than in mice immunized by the i.n. route with the same antigen and adjuvant (Fig. 2). When CpG ODN 1826 was used as the adjuvant, i.r. immunization induced fecal IgA levels that were only two-fold higher than those induced by i.n. immunization (Fig. 2).

Intrarectal immunization with 2/6-VLPs protects against RV infection in mice. In order to evaluate the protection efficacy of i.r. administration of 2/6-VLPs against RV infection, 6 weeks after the last immunization mice were orally challenged with murine RV strain EDIM, and viral shedding was mea-
sured in stool samples at different time points after infection. At 3 d.p.c., RV antigen was not detected by ELISA in samples obtained from EDIM-challenged mice immunized with 2/6-VLPs and CT by either the i.n. or the i.r. route. When CpG ODN 1826 was used, stool samples were RV antigen negative for 11 of the 14 mice tested in each group (i.n. and i.r.). As expected, RV was detected in stool samples obtained from all OVA-immunized control mice (data not shown). Although these results clearly showed that i.r. immunization with 2/6-VLPs protected mice from RV infection, this evaluation of viral shedding by measuring RV antigen by ELISA in stool samples was not sensitive enough to compare the efficacies of the different immunization protocols. Moreover, RV-specific antibodies present in stool samples of immunized mice were found to compete with antibodies used for RV detection by ELISA (data not shown). Therefore, we chose to evaluate RV shedding by quantifying viral genome using real-time RT-PCR. At 3 d.p.c., RV shedding was reduced in mice immunized with 2/6-VLPs by the i.n. route in comparison with that seen for the control mice; the RV titers decreased by 2.5 and 1.5 logs when CT and CpG ODN 1826, respectively, were used (Fig. 3A). Intrarectal immunization with 2/6-VLPs and CpG ODN 1826 led to a similar reduction in viral shedding (Fig. 3A). However, mice immunized by the i.r. route with 2/6-VLPs

FIG. 1. Serum anti-RV antibody levels induced by i.n. and i.r. immunization with 2/6-VLPs. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs in the presence of 1 μg of CT or 25 μg of CpG ODN 1826 by the i.n. route or with 5 μg of 2/6-VLPs together with 10 μg of CT or 250 μg of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. Four weeks after the last immunization, blood samples were taken and serum anti-RV antibodies were measured by ELISA. The levels of total anti-RV IgG (A) and IgA (B) are shown. The levels of RV-specific IgG1 and IgG2a subclasses in mice immunized with 2/6-VLPs by the i.n. (C) and i.r. (D) routes are also reported (the ratios between IgG1 and IgG2a levels are reported at the bottom of the graph). Data are displayed as mean ± standard error (SE) (six to seven mice/group). **, Significantly higher than levels of the groups of mice immunized by the i.r. route (P < 0.01 by the Kruskal-Wallis test). +, Significantly higher than levels of the VLP-CpG ODN i.r. group (P < 0.05 by the Kruskal-Wallis test).

FIG. 2. Fecal anti-RV IgA titers induced by i.n. and i.r. immunization with 2/6-VLPs. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs together with 1 μg of CT or 25 μg of CpG ODN 1826 by the i.n. route or with 5 μg of 2/6-VLPs together with 10 μg of CT or 250 μg of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. Four weeks after the last immunization, stool samples were collected and anti-RV IgA levels were measured by ELISA. Data are means ± SEs (six to seven mice/group). **, Significantly higher than levels of the VLP-CT i.n. group (P < 0.01 by the Kruskal-Wallis test). +, Significantly higher than levels of the VLP+CpG ODN i.r. group (P < 0.05 by the Kruskal-Wallis test).
and CT displayed a higher protection against RV infection, with a 5-log reduction of the viral shedding (Fig. 3A). Within this group, viral genome was undetectable in fecal samples of 9 out of 13 mice after 40 cycles of real-time RT-PCR ($P < 0.01$ by Fisher’s exact test). Conversely, RV genomes were detected in 12 out of 13 mice immunized intranasally with 2/6-VLPs and CT and in 12 out of 14 mice immunized with 2/6-VLPs and CpG ODN 1826 by either the i.n. or the i.r. route (Fig. 3A). A similar pattern was observed at 5 d.p.c., although the amounts of viral particles in the stool samples were significantly lower than those seen at 3 d.p.c. (Fig. 3B). At 8 d.p.c., RV genome was almost undetectable in fecal samples collected from any group of animals (Fig. 3C).

Intrarectal immunization with 2/6-VLPs induces lower cytokine production in spleen but higher cytokine production in PPs. We also evaluated the cellular immune response elicited by i.r. and i.n. immunization with 2/6-VLPs and the different adjuvants. Splenocytes were harvested from control and immunized mice 4 weeks after the last immunization, and production of IFN-γ and IL-5, which are Th1- and Th2-associated cytokines, respectively, was measured following ex vivo restimulation with RV. Splenocytes from mice immunized with 2/6-VLPs by the i.r. route produced less IFN-γ than splenocytes from mice immunized by the i.n. route (Fig. 4A). IL-5 production was observed only in splenocytes of mice immunized with CT, and mice immunized by the i.n. route displayed higher cytokine production than mice immunized by the i.r. route (Fig. 4B). Similar patterns of cytokine production were observed in culture supernatants of splenocytes obtained from 2/6-VLP-immu-

![FIG. 3. Protection from RV infection in mice immunized with 2/6-VLPs by the i.n and i.r. routes. Mice were immunized three times at 2-week intervals with 5 $\mu$g of 2/6-VLPs together with 1 $\mu$g of CT or 25 $\mu$g of CpG ODN 1826 by the i.n. route or with 5 $\mu$g of 2/6-VLPs together with 10 $\mu$g of CT or 250 $\mu$g of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 $\mu$g of OVA and 10 $\mu$g of CT by the i.r. route. Six weeks after the last immunization, mice were orally challenged with murine RV (strain EDIM), and viral shedding was measured in stool samples at 3 d.p.c. (A), 5 d.p.c. (B), and 8 d.p.c. (C) by quantifying genomic RNA by real-time RT-PCR. Results are given in copies of viral genome per mg of stool. Bars indicate the median RV shedding level for each group of animals (13 to 14 animals/group in two separate experiments), and diamonds indicate the amount of RV shed by each individual animal. The numbers of mice for which RV shedding was undetectable (≤2 copies/mg; indicated by arrows) are also reported at the bottom of the graph. * Significantly lower than levels of the VLP-CT i.n. group ($P < 0.01$ by the Kruskal-Wallis test). §§ Significantly lower than levels of the OVA-CT i.r. group ($P < 0.01$ by the Kruskal-Wallis test). §§§, Significantly lower than levels of the VLP-CT i.n. group ($P < 0.01$ by the Kruskal-Wallis test).

![FIG. 4. Cytokine production by splenocytes of mice immunized with 2/6-VLPs by the i.n and i.r. routes. Mice were immunized three times at 2-week intervals with 5 $\mu$g of 2/6-VLPs together with 1 $\mu$g of CT or 25 $\mu$g of CpG ODN 1826 by the i.n. route or with 5 $\mu$g of 2/6-VLPs together with 10 $\mu$g of CT or 250 $\mu$g of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 $\mu$g of OVA and 10 $\mu$g of CT by the i.r. route. Four weeks after the last immunization, three mice/group were sacrificed and their spleens were collected. Splenocytes obtained from mice of the same group were pooled and cultured in the presence of uninfected (−RV; white columns) or RV-infected (+RV; black columns) MA104 cell supernatant. One week later, IFN-γ (A) and IL-5 (B) levels were measured in culture supernatants by ELISA. Data are means ± SEs of three independent experiments. * Significantly higher than cytokine levels produced by restimulated splenocytes from mice immunized with 2/6-VLPs and the same adjuvant by the i.r. route ($P < 0.05$ by the Kruskal-Wallis test).]
mice were orally challenged with murine RV (strain EDIM). At 3 d.p.c., animals were sacrificed and IFN-γ was measured by ELISA (A). Data are means ± SEs of three independent experiments. * Significantly higher than IFN-γ levels produced by restimulated PP cells of mice immunized with 2/6-VLPs and the same adjuvant by the i.n. route (P < 0.05 by the Kruskal-Wallis test).

Intrarectal immunization with 2/6-VLPs also protects against RV infection when performed with low adjuvant doses. In order to investigate if i.r. immunization was still protective against RV infection at lower adjuvant doses, we immunized three times by the i.r. route, at 2-week intervals, several groups of mice with 5 μg of 2/6-VLPs together with 1, 3, 5, or 10 μg of CT. For comparison, other groups of mice were immunized intranasally with 5 μg of 2/6-VLPs together with 1, 3, or 5 μg of CT, and a group of control mice was left untreated (because of toxicity problems, we were not able to immunize mice with 10 μg of CT by the i.n. route). Four weeks after the last immunization, mice were orally challenged with murine RV, and viral shedding was measured in stool samples at 3 d.p.c. All the immunization protocols significantly reduced RV shedding in comparison with what was seen for the control mice (Fig. 7A). In particular, i.r. immunization with 2/6-VLPs in the presence of only 1, 3, or 5 μg of CT provided a protection against RV infection that was similar to that obtained with 10 μg of CT, with a level of viral shedding undetectable for the majority of the mice (Fig. 7A). The same levels of RV shedding were observed in mice immunized by the i.n. route in the presence of 3 or 5 μg of CT, whereas mice immunized by the i.n. route with only 1 μg of CT shed higher quantities of virus (Fig. 7A).

Cellular and humoral immune responses in the intestinal mucosa were also analyzed for mice immunized with 2/6-VLPs and 3 μg of CT by either the i.n. or the i.r. route by measuring IFN-γ or RV-specific IgA production by PP cells at 3 d.p.c. The levels of IFN-γ production after restimulation (Fig. 7B) and the numbers of RV-specific IgA ASCs (Fig. 7C) were higher in PP cells from mice immunized by the i.r. route than in mice immunized by the i.n. route, indicating that i.r. immunization still induced a stronger intestinal immune response than i.n. immunization, when performed with the same dose of adjuvant.
In the present study, we compared the immunogenicities and protective efficacies of 2/6-VLPs administered by the i.r. and i.n. routes in the presence of CT or CpG ODN as the adjuvant. Administration of antigens through the nasal mucosa is very efficient for the induction of an immune response and we indeed found high levels of anti-RV antibodies in sera of mice immunized with 2/6-VLPs by the i.n. route. In mice immunized by the i.r. route, the titers of RV-specific antibodies were much lower, even if immunizations were performed with higher adjuvant doses. Similar findings were reported in a human study in which i.n. administration of cholera vaccine induced higher serum antibody responses than administration by the i.r. route (31). On the contrary, when we investigated antibody production at the intestinal level, we found that i.r. immunization induces a higher number of RV-specific IgA ASCs in PPs. Similar results were found for the cellular immune responses, since mice immunized by the i.n. route displayed higher spleen IFN-γ production than mice immunized by the i.r. route, whereas mice immunized by the i.r. route displayed higher IFN-γ production by PP cells. Therefore, immunization by the i.r. route induces humoral and cellular immune responses that are localized in the intestinal mucosa. Moreover, mice immunized with 2/6-VLPs by the i.r. route are protected against RV infection, as viral shedding in stool samples was significantly reduced in comparison with that seen for OVA-immunized control animals. Furthermore, when CT is used as the adjuvant, even at low doses, mice immunized by the i.r. route show better protection than mice immunized by the i.n. route, and viral shedding is not even detectable in many of them. Hence, i.r. immunization may represent a valuable strategy for eliciting a protective immunity against intestinal pathogens such as RV.

The high levels of anti-RV IgA found in intestinal mucosa of mice immunized by the i.r. route suggest a role for these antibodies in protection against RV infection. In fact, although 2/6-VLPs do not contain the RV outer layer proteins VP4 and VP7 and therefore do not induce neutralizing antibodies, it is known that VP6-specific IgA can inhibit RV replication. Murine hybridomas producing anti-VP6 IgA implanted in a back-pack model have been reported to protect adult mice from RV infection and clear chronic infection in immunocompromised mice (9). Based upon these observations, it has been postulated that anti-VP6 IgA, during its transcytosis inside enterocytes, might interact with partially decapsidated virions and inhibit viral replication. Consistently, J-chain knockout mice, defective in transcellular transport of IgA and IgM by the polymeric immunoglobulin receptor, are less protected than wild-type mice when immunized with 2/6-VLPs and CT by the i.n. route (45). However, we show that when mice are immunized with CpG ODN 1826, the intestinal production of RV-specific IgA does not correlate with the decrease in RV shedding, so other mechanisms may contribute to the protection conferred by immunization with 2/6-VLPs.

We show that i.r. immunization with 2/6-VLPs also elicits a robust T-cell response in the small intestine. Previous studies have identified a protective T-helper epitope on VP6 (3, 11), and CD4+ T cells were reported to be the only lymphocytes required to prevent RV infection in mice immunized intranasally with VP6 (38). CD4+ effector T cells are classified into two groups based upon cytokines they secrete, which are named Th1 and Th2 and promote cellular and humoral immune responses, respectively (1). The polarization of the immune response towards the Th1 or Th2 phenotype in vivo depends on several factors, and the type of adjuvant used for immunization is one of these. Adjuvants that activate Toll-like receptor signaling, such as CpG ODNs, stimulate IL-12 production by antigen-presenting cells and skew the immune response toward the Th1 phenotype (29). Immunization with CT instead has been reported to inhibit IL-12 synthesis (8) and induce a Th2-polarized or a mixed Th1/Th2 immune response (19, 35). Consistently, we found that lymphocytes from mice immunized with 2/6-VLPs together with CT produced both the Th1-associated cytokine IFN-γ and the Th2-associated IL-5. In addition, isotype analysis of anti-RV IgG antibodies shows that...
in mice immunized in the presence of CT, the IgG1/IgG2a ratio is high, confirming that CT skews the effector response towards Th2. On the contrary, lymphocytes from mice immunized with CpG ODN 1826 produced only IFN-γ towards Th2. On the contrary, lymphocytes from mice immunized in the presence of CT, the IgG1/IgG2a ratio is high, confirming that CT skews the effector response towards Th1. The Th1-associated cytokine IFN-γ is well known for its important immunomodulatory and also antiviral activity but does not seem to play a major role in the response against RV. Indeed, IFN-γ−/− mice clear RV infection as efficiently as wild-type mice and are equally protected by i.n. immunization with VP6 and attenuated LT (2, 17, 47). Conversely, cytokines produced by Th2 lymphocytes may enhance the humoral anti-RV response, for instance, intestinal IgA production. IL-5 is known to induce maturation of IgA-committed B cells into IgA-producing plasma cells (4).

It is known that CD8+ T cells also play an important role in RV clearance in mice (15, 37). VP6 is one of the targets of CTLs, and several CD8+ T-cell epitopes are harbored by this protein (16, 24), indicating that the anti-RV immune response induced by immunization with 2/6-VLPs may be partly CTL mediated. Indeed, both CT and CpG ODNs have been reported to facilitate the development of antigen-specific CTLs (6, 29); therefore, the role played by these lymphocytes in our immunization protocols needs further investigation.

It seems that the local microenvironment where T and B cells are primed with antigen determines their homing preferences; for instance, the activation of naïve lymphocytes in lymphoid tissues draining the intestinal mucosa leads to the generation of effector and memory cells that migrate selectively into the gut. This tissue-selective trafficking of lymphocytes is related to the expression of specific adhesion molecules and chemokine receptors that recognize ligands which are selectively expressed in these tissues. The homing of T cells and IgA-producing plasma cells to the small intestine depends on the expression of the integrin αβ7, and the chemokine receptor CCR9 (32). The αβ7 integrin interacts with the mucosal address cell adhesion molecule MadCAM-1, which is expressed on intestinal endothelium, whereas CCR9 is the receptor for chemokine CCL25, which is abundantly produced by epithelial and endothelial cells of small bowel (32). Immunization by the oral and i.r. routes in humans has been shown to induce ASCs which mostly express αβ7 integrin, whereas only a minor fraction of them express l-selectin, a homing receptor that confers tissue specificity for peripheral lymph nodes (27, 44). ASCs induced by i.n. immunization display a mixed pattern of these adhesion molecules, and most of them express both αβ7 and l-selectin (27, 44). More recently, a study with mice showed that i.n. immunization with 2/6-VLPs induced a high frequency of RV-specific B cells in the respiratory lymphoid tissue and spleen, but only a minor fraction of them expressed the αβ7 integrin (40). Taken together, these results may explain why the administration of antigens by the i.n. route evokes an immune response that is mainly systemic but rather weak in the gut (32). On the other hand, our data suggest that lymphocytes activated by i.n. immunization migrate to the intestine, and further studies are required to determine which pattern of adhesion molecules is expressed by T and B cells primed in the rectal mucosa.

Studies conducted with humans and nonhuman primates showed that i.r. immunization, in the absence of infection, could induce high ASC numbers in rectum but low ASC numbers in duodenum and jejunum, suggesting that the compartmentalization of the immune system may differ among species (14, 26). However, i.r. immunization in macaques with the HIV/simian immunodeficiency virus peptide has been reported to clear viral load not only in colon but also in jejunum after challenge with simian/human immunodeficiency virus (7). It...
should also be noted that in mice immunized with 2/6-VLPs by the i.r. route, the numbers of RV-specific ASCs in PPs are relatively low in the absence of infection, but they rapidly increase after RV administration, suggesting that at the intestinal level i.r. immunization primes an immune response that can be boosted by the challenge. Similarly, i.r. immunization with recombinant urease from Helicobacter pylori has been reported to induce detectable numbers of antigen-specific ASCs in stomach only after bacterial challenge (28).

In conclusion, we have shown here that in mice, i.r. immunization with 2/6-VLPs induces a powerful anti-RV immune response in gut and protects against RV infection. Therefore, i.r. immunization may be an efficient way to direct the immune response to sites defined by their source of infection: B cells after oral and rectal immunizations to sites defined by their vascularization. Infect. Immun. 66:7580–7586.


