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Shing C. Chen

University of Massachusetts Medical School

Et al.

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Protective Immunity Induced by Oral Immunization with a Rotavirus DNA Vaccine Encapsulated in Microparticles

SHING C. CHEN,1 DAVID H. JONES,2 ELLEN F. FYNAN,1,3 GRAHAM H. FARRAR,2 J. CHRISTOPHER S. CLEGG,2 HARRY B. GREENBERG,4 AND JOHN E. HERRMANN1*

Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, Massachusetts 01655; Center for Applied Microbiology and Research, Salisbury SP4 OJG, United Kingdom; Department of Biology, Worcester State College, Worcester, Massachusetts 01602; and Division of Gastroenterology, Stanford University School of Medicine, Stanford, California 94304

DNA vaccines are usually given by intramuscular injection or by gene gun delivery of DNA-coated particles into the epidermis. Induction of mucosal immunity by targeting DNA vaccines to mucosal surfaces may offer advantages, and an oral vaccine could be effective for controlling infections of the gut mucosa. In a murine model, we obtained protective immune responses after oral immunization with a rotavirus VP6 DNA vaccine encapsulated in poly(lactide-coglycolide) (PLG) microparticles. One dose of vaccine given to BALB/c mice elicited both rotavirus-specific serum antibodies and intestinal immunoglobulin A (IgA). After challenge at 12 weeks postimmunization with homologous rotavirus, fecal rotavirus antigen was significantly reduced compared with controls. Earlier and higher fecal rotavirus-specific IgA responses were noted during the peak period of viral shedding, suggesting that protection was due to specific mucosal immune responses. The results that we obtained with PLG-encapsulated rotavirus VP6 DNA are the first to demonstrate protection against an infectious agent elicited after oral administration of a DNA vaccine.

Group A rotavirus infections cause an estimated 870,000 deaths each year in developing countries (12). They also cause 55,000 to 70,000 hospitalizations per year in the United States, with an estimated cost of more than $1 billion (12). Because of the widespread nature of rotavirus disease, development of vaccines is considered key to their control (1, 12). Although progress has been made in the development of live oral rotavirus vaccines (32), improved vaccines are still needed, particularly in many developing countries where the need is the greatest (1, 12, 22, 33) but where the live oral vaccines have been less effective (25, 26). Development of killed rotavirus vaccines and subunit vaccines may be possible (1), but these types of vaccines do not provide endogenously synthesized proteins and generally do not elicit cytotoxic T-lymphocyte (CTL) responses (13) that may be important in controlling rotavirus infection. The use of DNA encoding specific viral proteins allows for the expression of immunizing proteins by host cells that take up inoculated DNA. This results in the presentation of normally processed proteins to the immune system, which is important for raising immune responses against the native forms of proteins (11, 36). Expression of the immunogen in host cells also results in the immunogen having access to class I major histocompatibility complex presentation, which is necessary for eliciting CD8+ CTL responses.

Rotavirus virions have a three-layered protein capsid. The protein-coated RNA core is coated by VP6, a protein that is antigenically conserved among group A rotaviruses but does not elicit antibodies that neutralize rotavirus in vitro. The two outer capsid surface proteins, VP4 and VP7, elicit neutralizing antibodies. In prior studies, we found that DNA vaccines encoding VP4, VP7, or VP6 were protective when administered by gene gun delivery of the DNA to the epidermis (3, 15, 16). Direct gene gun inoculation to the anal mucosa required five-fold less DNA (0.5 rather than 2.5 μg per mouse) to give the same level of protection (17), suggesting that targeting mucosal tissue enhances the generation of protective immunity. Both inoculation routes resulted in enhanced intestinal immunoglobulin A (IgA) responses after rotavirus challenge, but neither induced detectable intestinal IgA prior to challenge. Protective immune responses against rotavirus infections have been correlated with production of rotavirus-specific fecal IgA in vivo in human and porcine studies as well as in the murine model (4, 10, 27, 34, 38). Thus, induction of intestinal IgA may be an important correlate in the development of rotavirus vaccines.

Targeting of rotaviruses to the gut-associated lymphoid tissue by oral administration of an aqueous-based system of microencapsulated noninfectious rotaviruses generated serum IgG and intestinal IgA antibody responses (24). This finding suggests that mucosal targeting of DNAs expressing rotavirus proteins might also generate immune responses. Recently, a method for encapsulation of plasmid DNA which permits the DNA to be orally administered has been developed. Plasmid DNA encoding insect luciferase was encapsulated in poly(lactide-coglycolide) (PLG) microparticles and oral administration of these PLG microparticles stimulated serum IgG, IgM, and IgA antibodies to luciferase (21). Luciferase-specific IgA was also detected in stool samples, indicating a mucosal response. In this study, we examined the ability of a PLG-encapsulated rotavirus VP6 DNA vaccine to induce serum and mucosal antibody responses and to protect against rotavirus infection after challenge of adult mice.
MATERIALS AND METHODS

Virus and mice. Epizootic diarrhea of infant mice (EDIM) rotavirus strain EW (P10[16], G3) was used for preparation of cDNA encoding VP6 and for virus challenge of mice. The virus challenge stock was prepared by passaging virus from intestinal homogenates of EDIM rotavirus-infected infant mice in adult mice. For virus challenge was a stool sample diluted in saline. The 50% infective dose (ID₅₀) of the stock virus was the 50% shedding dose as determined by detection of rotavirus antigen shed in feces of infected mice. The mice used for vaccine studies were obtained from rotavirus-free colonies (Charles River Laboratories, Portage, Mich.) at 6 to 8 weeks of age and were housed in plastic microisolater cages before and after immunization. The model developed by Ward et al. for BALB/c mice (35) was used to measure protective immunity. In this model, the endpoint is infection rather than illness, because illness is generally limited to infant mice aged 15 days or younger. The adult mouse (6 weeks or older) becomes infected and sheds virus in feces for approximately 1 week postinfection. Protection after virus challenge was defined as significant reduction in rotavirus antigen shedding in feces.

Encapsulated DNA vaccine. The plasmid encoding rotavirus VP6 DNA (Fig. 1) was prepared by insertion of murine rotavirus VP6 cDNA into the CMV intron A TPA expression vector provided by J. Mullins, University of Washington (plasmid JW4303) (37). This vector uses sequences from the cytomegalovirus (CMV) immediate-early promoter to drive transcription and sequences from bovine growth hormone genes to provide polyadenylation signals. To prepare VP6 DNA vaccine by cohesive end ligation, the TPA leader sequence was removed by treatment with restriction endonucleases HinIII and BamHI. The HinIII site was changed to a BamHI site, and the gene for VP6 (GenBank accession no. U36474) was inserted as a BamHI-BamHI fragment. The gene had been inserted in the BamHI site of plasmid Bluescript KS− and was released by BamHI digestion prior to insertion into plasmid JW4303. Newly constructed plasmids in the correct orientation were identified by restriction endonuclease digestion. Expression of rotavirus VP6 in transfected COS cells was confirmed by indirect immunofluorescent staining with monoclonal antibody to VP6. The monoclonal antibody had been prepared against a rotavirus SA-11 strain (5). The control DNA vaccine was the plasmid without the viral cDNA insert.

Plasmid DNA was encapsulated in PLG microparticles by the solvent extraction technique as previously described (20, 21). In brief, the DNA was emulsified with PLG dissolved in dichloromethane, and this water-in-oil emulsion was emulsified with aqueous polyvinyl alcohol (an emulsion stabilizer) to form a (water-in-oil)-in-water double emulsion. This double emulsion was added to a large quantity of water to dissipate the dichloromethane, which resulted in the microdroplets hardening to form microparticles. These were harvested by centrifugation, washed several times to remove the polyvinyl alcohol and residual solvent, and finally lyophilized. The microparticles containing DNA had a mean diameter of 0.5 μm. To test for DNA content, the microparticles were dissolved in 0.1 M NaOH at 100°C for 10 min. The A₂₆₀ was measured, and DNA was calculated from a standard curve. Incorporation of DNA into microparticles was 1.76 to 2.7 μg of DNA per mg of PLG for the VP6 DNA vaccine and 1.75 to 3.61 μg per mg of PLG for the plasmid control.

Immunization of mice. Three groups of BALB/c mice were inoculated orally (by gavage) with PLG-encapsulated plasmid DNA encoding murine rotavirus VP6 (n = 13 mice total) or control plasmid DNA (n = 10 mice total). The microparticles were suspended in a solution of 0.1 M sodium bicarbonate in distilled water (pH 8.5) and given at 0.5 ml/mouse. The DNA dose administered was approximately 50 μg per mouse.

Antigen and antibody testing. For monitoring viral antigen shedding in mouse feces, we used a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) in microtiter plates as previously described (14). For evaluating serum antibody responses, an indirect ELISA for total antibody (IgG, IgM, and IgA) (3, 10, 15, 16) was used with EDIM rotavirus-coated wells. Intestinal IgA antibodies to EDIM virus were determined by use of IgA-specific peroxidase-labeled antoglobulin in an indirect ELISA (3, 10, 15, 16). Five percent (wt/vol) stool suspensions in 0.01 M phosphate-buffered saline (PBS; pH 7.1) were further diluted 1:4 (final dilution of 1:80) and used for assays of fecal IgA.

Statistical analyses. Statistical analyses were performed using a nonparametric Wilcoxon two-sample test for ranked data and analysis of variance and the Student-Newman-Keuls test for multiple comparison of the differences among experimental groups.

RESULTS

Serum antibodies. Inoculated mice were examined for serum antibodies (IgG, IgM, and IgA) every 2 weeks for 12 weeks. A single immunization was sufficient to elicit a serum antibody response by 4 weeks after inoculation, with peak titers being reached by 6 weeks (Fig. 2). Twofold dilutions were tested starting at a 1:100 dilution.

Protection against rotavirus challenge. Mice were challenged with 100 ID₅₀ of EDIM rotavirus at 12 weeks postimmunization to determine if the immunizations had provided protection. The challenge virus used was given by oral gavage. Protection was assessed by testing for the reduction of rotavirus antigen shedding in stools. Significant reductions (P < 0.0002) in virus antigen shed were noted on days 2, 3, and 4 (Fig. 3).

Rotavirus-specific IgA in stools. Immunized mice were examined for intestinal rotavirus-specific IgA before virus challenge at 4, 6, 8, 10, and 12 weeks postimmunization. The results of the tests for detection of rotavirus-specific IgA in stools are shown in Fig. 4. Significant production of fecal IgA (P < 0.003) was detected in the PLG-encapsulated VP6 DNA-vaccinated animals at 6, 8, 10, and 12 weeks, suggesting that rotavirus antigen was expressed and a mucosal antibody response had been induced. In previous studies using gene gun delivery, we did not detect rotavirus-specific IgA in the stools of DNA-immunized animals until after they were challenged with live rotavirus (3, 15, 16).

To determine if oral immunization also enhanced IgA responses after virus challenge, intestinal IgA was measured by an IgA rotavirus-specific ELISA. The mice orally inoculated with the PLG-VP6 DNA vaccine gave higher and earlier A₄₉₂ values (P < 0.01) at 0, 1, 3, and 5 days postchallenge than mice

FIG. 1. Diagram of the pCMVIA vector and the virus cDNA insert. SV 40 Ori, simian virus 40 origin of replication; CMV Pro, CMV immediate-early promoter, intron A (largest CMV intron); BGH, bovine growth hormone gene (provides polyadenylation [PA] signals).

FIG. 2. Rotavirus-specific serum ELISA antibodies mice from BALB/c mice that had been orally inoculated (by gavage) with PLG-encapsulated VP6 DNA vaccine (n = 13) or with PLG-encapsulated control plasmid DNA (n = 10). Serum was collected at the times indicated and tested by an ELISA for total antibody (IgG, IgM, and IgA) every 2 weeks for 12 weeks. Results are expressed as geometric mean titers ± standard error.
that had been orally inoculated with PLG-control plasmid DNA (Fig. 5).

**DISCUSSION**

Our studies with PLG-encapsulated DNA vaccines presented here used VP6 DNA. We selected the VP6 DNA vaccine for these studies because of the broad implications a VP6-based vaccine has for the prevention of rotavirus infections. VP6 is an antigenically conserved protein among all group A rotaviruses of both animal and human origin. Thus, serotype specificity may not be a problem with VP6-based vaccines. The protection was not as complete as that we reported previously with gene gun immunizations, where rotavirus antigen was not detected in the stools of mice immunized with VP6 DNA vaccine (3, 15, 16). This could be related to vaccine dose. The dose of 50 μg per mouse used was based on doses used for expression of other antigens and may not be optimal for our system, and lower doses of VP6 DNA vaccine given by gene gun also resulted in partial protection (17). Dose-response studies will determine the optimal vaccine dose. Compared with gene gun delivery used in our previous studies (3, 15, 16), more DNA is required when encapsulated and given orally (50 μg, compared with two doses of 2.5 μg given by gene gun). Although it may also be possible to induce immune responses by oral administration of naked DNA in a saline solution, encapsulated material protects the DNA from degradation by nucleases. Oral administration of naked DNA encoding luciferase did induce immune responses, but PLG encapsulation enhanced the responses (22). We expect to compare naked DNA with encapsulated DNA in future experiments.

Protective immunity was measured by reduction of rotavirus antigen shed after challenge, because adult mice (mice older than 2 weeks) do not develop diarrhea following rotavirus infection. However, as pointed out by others, protection from rotavirus infection may be a more stringent measure of protection than protection from disease, because infection can occur in the absence of disease (31). In studies with murine rotaviruses given orally to mice, protection against rotavirus challenge is associated with rotavirus-specific fecal IgA (10, 35). We have also found that fecal IgA antibodies were rapidly induced in mice immunized with rotavirus DNA vaccines, but only after they were virus challenged (3, 15, 16). The serum antibodies did not neutralize rotavirus in vitro; thus, it is unlikely that traditional virus neutralization is involved in the protection found.

The mechanism of protection seen with the VP6 DNA vaccine and also with VP6-based virus-like particles (31) are not known. Among potential mechanisms of protection are cell-mediated immunity and IgA-mediated intracellular neutralization of virus that is undergoing assembly. In studies with rotavirus VP2, 6 virus-like particles (31), protective immunity was obtained by coadministration of cholera toxin, which is known.
to enhance both mucosal antibody responses and CTL responses, and it is possible that either or both types of immune responses are involved. IgA-mediated intracellular viral neutralization has been shown for Sendai virus and influenza virus (28, 29), and studies with IgA monoclonal antibodies to VP6 suggest that IgA-mediated intracellular neutralization may also occur with rotaviruses (2). Based on these findings and our demonstration of enhanced IgA responses in VP6 DNA-vaccinated mice both before and after virus challenge, IgA-mediated intracellular neutralization in the intestinal mucosa may be a factor in the protective immunity that we have obtained. We expect to test DNA vaccines in immunodeficient mice to help determine the relative importance of CTL responses and intestinal IgA in the protection obtained with VP6 DNA vaccines.

Determination of the cell or cells targeted by the encapsulated DNA and the ultimate fate of the DNA was beyond the scope of this study, but it is likely that the cells involved are similar to those that have been shown to be involved in the uptake of PLG microparticles. Following oral administration to mice, PLG microparticles 1 to 10 μm in diameter were taken up into the Peyer’s patches of the gut-associated lymphoid tissue. Those particles ≥5 μm that were taken up remained localized for up to 35 days, whereas those particles <5 μm were disseminated within macrophages, mesenteric lymph nodes, blood circulation, and spleen (8, 9). PLG microparticles are not selectively targeted to M cells, but nonspecific binding to M cells and subsequent transcytosis has been shown in rabbits (18, 19). PLG microparticles <5 μm have also been shown to cross the intestinal mucosa at the site of Peyer’s patches in rats (6). The DNA-containing PLG microparticles used in our study had a mean diameter of 0.5 μm. It has been presumed that PLG microparticles containing antigen bind to and are transported by M cells in a manner similar to that found with empty PLG microparticles (30). Supporting this assumption, uptake of bovine serum albumin encapsulated in PLG microparticles by Peyer’s patches has been shown in a rat model (7).

The use of DNA vaccines is a new approach to immunization that may provide more effective rotavirus vaccines. It has been suggested that this approach and the virus-like particle approach may make a third generation of rotavirus vaccines (33). DNA vaccines encapsulated in PLG microparticles combine the advantages of DNA-based vaccination with the ease of administration by the oral route and concomitant induction of mucosal immune responses. The results that we obtained with PLG-encapsulated rotavirus VP6 DNA are the first to demonstrate protection against an infectious agent elicited after oral administration of a DNA vaccine.

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