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Enhancement of VP6 immunogenicity and protective efficacy against rotavirus by VP2 in a genetic immunization

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ABSTRACT

VP2/VP6 virus like particles (VLPs) are very effective in inducing protection against the rotavirus infection in animal models. Individually, VP6 can also induce protection. However, there is no information about the immunogenicity of VP2. The aim of this work was to evaluate the efficacy of DNA vaccines codifying for VP2 or VP6, alone or combined, to induce protection against the rotavirus infection. Murine rotavirus *VP2* and *VP6* genes were cloned into the pcDNA3 vector. Adult BALB/c mice were inoculated three times by intramuscular (i.m.) injections with 100 or 200 μg of pcDNA3-VP2 or pcDNA3-VP6 alone or co-administered with 100 μg of pcDNA3-VP2/100 μg of pcDNA3-VP6. Two weeks after the last inoculation, mice were challenged with the wild type murine rotavirus strain epizootic diarrhea of infant mice ($EDIM_{wt}$). We found that both plasmids, pcDNA3-VP2 and pcDNA3-VP6, were able to induce rotavirus-specific serum antibodies, but not intestinal rotavirus-specific IgA; only 200 μg of pcDNA3-VP6 induced 35% protection against the infection. A similar level of protection was found when mice were co-administered with 100 μg of pcDNA3-VP2/100 μg of pcDNA3-VP6 (1:1 ratio). However, the best protection (up to 58%) occurred when mice were inoculated with 10 μg of pcDNA3-VP2/100 μg of pcDNA3-VP6 (1:10 ratio). These results indicate that the DNA plasmid expressing VP6 is a better vaccine candidate that the one expressing VP2. However, when co-expressed, VP2 potentiates the immunogenicity and protective efficacy of VP6.

1. Introduction

Rotaviruses are the single most important cause of severe dehydrating infantile gastroenteritis in the world. Since the introduction of two vaccines (Rotarix, GSK and Rotateq, Merk) based on attenuated rotavirus in more than 100 countries in 2006, the death estimates had decreased from 500,000 to 250,000 by 2013 [1]. Although the protection against severe gastroenteritis induced by the vaccines was above 80% in high and medium-income countries, it was only between 39% and 70% in some low-income countries in Africa and Asia [2]. Thus, it is important to develop new generation vaccines that induce long-lasting heterotypic protection, especially in low-income countries.

Rotavirus belongs to the Reoviridae family, and it infects and destroys the enterocytes at the villus tips of the small intestine. The virion is composed of three concentric layers of protein that contain a genome of 11 double-stranded RNA (dsRNA) segments. The outer layer is constituted by two proteins, VP4 and VP7, which among other properties, are the targets of neutralizing antibodies and define the virus P and G serotypes, respectively. Twenty seven G-types and 35 P-types have been described; however, only 5 G serotypes (G1, G2, G3, G4 and G9), and 3 P genotypes [P(4), P(6) and (P8)] have been commonly associated to human illnesses [3]. VP7 forms trimers that constitute the "floor" of the outer layer, on which VP4 dimers are anchored and arranged as spicules. The middle layer is made up of VP6 trimers; this is the most abundant protein, constituting 50% of the total mass of the virion, and it is also the most conserved (87–99% base homology) [4,5]. VP6 carries

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antigen determinants that are common to all group A rotaviruses; it also contains epitopes that have been used to classify the group A viruses into subgroups [5]. VP2 forms the innermost layer; it surrounds the viral genome, and small amounts of viral guanylyltransferase VP3, and viral polymerase VP1. VP2 is the second most conserved protein in the virion (81–91% base homology) [4].

Previous studies have indicated that VP4, VP6 and VP7 can be the targets of protective immune responses. Both VP4 and VP7 are able to induce neutralizing antibodies [6]. V6 is highly immunogenic, and it contains B and T cell antigenic determinants shared by various rotavirus types in group A [7]. Recombinant proteins, peptides and DNA vaccines administered to mice via mucosal and parenteral routes can induce T-cell-dependent protection against an oral rotavirus challenge [8–10]. VP6 can also prime mice for a T helper (Th)-dependent enhanced neutralizing antibody response against VP7 and VP4, which can contribute to reduce viral replication [11].

The co-expression of VP2 and VP6 results in the production of double-layered VLPs in insect cells [12]. VP7 and VP4 can be added to generate VP2/VP6/VP7 and VP2/VP6/VP7/VP4 VLPs, respectively. All these VLPs have been used as efficient vaccines against rotavirus infection in mice [13–15]. However, the cost of producing these VLPs in culture is high, so they may not be the best vaccine option in medium- and low-income countries. An alternative for a new anti-rotavirus generation vaccine is the use of DNA plasmids, which are potentially cheaper, more stable at high temperatures, and easier to produce. Besides, it has been shown that they can induce protective immune responses in different viral models that involve both the humoral and the cellular components [16], including the rotavirus infectious model. Mice immunized parenterally and orally with plasmids expressing rotavirus proteins have shown different levels of protection in the rotavirus infectious model. Chen et al. [17] showed that mice inoculated epidermally with plasmids codifying for VP4, VP7 or VP6 induced protection against a rotavirus challenge. Plasmids codifying for VP6 through i.m., oral and intranasal (i.n.) routes have shown different degrees of protection, and the mucosal routes have been the most effective [10,18,19].

Ideally, a DNA vaccine should incorporate the most conserved rotavirus antigens, such as VP6 and VP2, in order to generate an efficient heterotypic response that protects against different circulating serotypes. VP6 is highly immunogenic, and it can induce a heterotypic protection [7]. However, there are no reports on the immunogenicity of VP2. In this study, we compared the immunogenicity of DNA vaccines codifying for VP6 or VP2, individually or co-administrated by the i.m. route in mice. As expected, we found that VP6 induced high levels of serum antibodies and up to 35% protection, whereas VP2 induced low levels of serum antibodies and no protection. Nevertheless, VP2 could enhance the immunogenicity and protective efficacy of VP6 when both plasmids were co-administered.

2. Material and methods

2.1. Mice

Rotavirus-free female BALB/c mice (H-2^d), between six and eight weeks of age, purchased from Harlam (Mexico), were used throughout this study. During the experiments, the mice were kept in the animal houses at the Biotechnology Institute (UNAM) and the Center for Research on Infectious Diseases (INSP), Cuernavaca, Morelos, Mexico.

2.2. Virus

Mice were challenged with wild-type murine rotavirus $EDIM_{wt}$ [Mu/G3P10(16)], kindly donated by Dr. Richard Ward (Children's Hospital Medical Center, Cincinnati, OH), that was grown *in vivo* in neonatal mice (3–5 days old). Pups were orally infected, and their intestines were collected 48 h later to generate homogenates [20]. Aliquots of the homogenates were stored at −80 °C until they were used. Tissue culture-adapted murine rotavirus EDIM and simian rotavirus RRV were used as lysates of infected MA-104 cells (African monkey kidney-derived epithelial cells; ATCC CRL-2378.1) [21] or as cesium chloride purified virus [21,22].

2.3. Plasmids

The *VP2* and *VP6* genes from murine rotaviruses EDIM and Ew, respectively, were cloned into the plasmid pcDNA3 (Invitrogen) under the CMV promoter from Citomegalovirus. CMV is a strong promoter for eukaryotic genes. $EDIM_{wt}$ and Ew viruses are very similar since they come from the original EDIM isolate [23]. In fact, $EDIM_{wt}$ VP6 differs only in two amino acids out of a total of 397 when compared with Ew VP6.

The complete rotavirus Ew VP6 coding sequence (GeneBank accession number U36474.1) was kindly donated by Dr. H. Greenberg (Department of Microbiology and Immunology, Stanford University, USA) in a pBluescript plasmid. The *VP6* gen was cut using restriction sites *Kpn* I and *Xba* I (corresponding to the 5′ and 3′ ends, respectively) and inserted into the pcDNA-3 plasmid using the same restriction sites. The *VP2* gene was obtained from mRNA of MA-104 cells infected with tissue culture-adapted murine rotavirus EDIM. Briefly, the total mRNA isolated from infected MA-104 cells was subjected to a RT-PCR reaction, using oligonucleotides CAGACCCGGGTACCTATTAAAGGCTCAAT and CAGACCCG GGCCGCGGTCATATCTCCACAGTG, which are complementary to the 5′ consensus sequence of rotavirus genes and to the 3′ consensus sequence of various rotavirus *VP2* genes, respectively. The product of the amplification was cut with restriction enzyme *Sma* I and inserted into the same site in the plasmid pGEM 3Z (Promega). The VP2 sequence was excised from this plasmid with restriction enzymes *BamH* I and *Eco R* I; the *BamH* I site was blunt with Klenow, and the fragment was inserted into plasmid pcDNA3 using restriction sites *EcoR* I and *Eco R* V. The *VP2* gene was sequenced using an automated capillary fluorescent sequencer (ABI PRISM, Applied Biosystems) (Biotechnology Institute, UNAM) (Supplementary Fig. 1).

Plasmids were purified from transformed *E. coli* DH5α cells using the plasmid purification system Gigaprep Endofree (QiaGen), resulting in endotoxin-free plasmids. The plasmids were stored in ultra-pure water at −20 °C until they were used. Before inoculation, enough NaCl was added to the plasmid solution in order to have an isotonic concentration (0.9% w/v).

2.4. Immunizations

Groups of four to five mice were inoculated i.m. in the quadriceps with the different plasmids at the indicated dose in each experiment in a volume of 50 μL for each quadriceps (100 μL per mouse). Three inoculations were performed at days 0, 21 and 36. Control mice were inoculated with empty plasmid. Two weeks after the last inoculation, mice were challenged orally with murine rotavirus $EDIM_{wt}$. To do this, the gastric acid was first neutralized administering 100 μL of 1.3% (w/v) sodium bicarbonate by proximal esophageal intubation. After 10 min, mice were inoculated with 100 μL of serum-free modified Eagle's medium (MEM) containing 100 times 50% infectious dose (ID_{50}) of murine rotavirus $EDIM_{wt}$; the stool samples were collected daily up to day 8, and

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