INTRANASAL IMMUNIZATION OF MICE WITH VP2 DNA OF HUMAN ROTAVIRUS A INDUCES CELLULAR AND HUMORAL IMMUNITY

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Summary. – DNA vaccination using a plasmid encoding Human rotavirus A (HuRV-A) inner capsid VP2 was examined in a mouse model. BALB/c mice were immunized intranasally (i.n.) with a VP2 DNA vaccine that induced cellular and humoral immune response to HuRV-A. The increased levels of cytokines IFN-γ and IL-4 and the production of anti-VP2 IgG antibodies were detected. In addition, splenocyte proliferation detected by MTT test was enhanced in the mice treated with the vaccine. These results may encourage the development of a HuRV-A DNA vaccine derived from the inner layer of viral capsid that can be administered i.n.

Key words: Human rotavirus A; VP2 protein; immunogenicity; DNA vaccine; mouse

Introduction

HuRV-A belonging to the genus *Rotavirus,* the family *Reoviridae* is the most important pathogen causing severe diarrhea in infants and young children (Wilhelmi *et al*., 2003; Kapikian *et al*., 2001). Each year this rotavirus causes an estimated 111 million episodes of diarrhea requiring only home care, 25 million clinic visits, 2 million hospitalizations, and average 440,000 deaths in children under 5 years of age (Parashar *et al*., 2003). Worldwide, more than 70% of serious diarrhea cases in children are caused by the rotavirus infection due to the lacking immunity in the early stage of life (Adler *et al*., 2005; Simpson *et al*., 2003). Therefore, vaccination appears to be the only feasible method of eliminating this annual scourge.

Morphologically and biologically, the triple-layered rotavirus particle is composed of (i) an outer protein layer including of VP4 and VP7, (ii) a middle protein layer of VP6, (iii) an inner protein layer composed of VP1, VP2, VP3, and (iv) 11 segments of double-stranded genomic RNA that code for 6 structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and 6 nonstructural proteins. It has been shown that the innermost capsid layer is composed of 120 molecules of a VP2 protein (102 K), which encloses the genomic dsRNA (Kapikian *et al*., 2001; Labbe *et al*., 1991; Estes and Cohen, 1989).

Use of VP2 as a scaffolding protein permit the formation of virus-like particles (VLPs) of different protein composition formed by the expression of different combinations of VP2, VP4, VP6, and VP7 genes for immunological studies (Crawford *et al*., 1994). VLPs can be triple-layered, double-layered or single-layered core-like particles.

Small animal models have been developed to evaluate rotavirus vaccine strategies (Conner *et al*., 1996; Ward *et al*., 1990). Vaccines to prevent rotavirus disease evaluated in human trials over the past 20 years have all been live attenuated rotaviruses that are administered orally to mimic natural infection. The use of DNA vaccines is a new approach to immunization that may provide more effective rotavirus vaccines (Garcia-Diaz *et al*., 2004). Like live vaccines and recombinant vectors, DNA vaccination has

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Abbreviations: HuRV-A = Human rotavirus A; i.n. = intranasally; SI = stimulation index; VLP(s) = virus-like particle(s)

been shown to result in the presentation of antigenic determinants in association with MHC molecules. This vaccination approach appears to be safe, a vaccine can be prepared rapidly, and DNA stability is not affected by high environmental temperatures (Krishnan *et al*., 1995). Furthermore, DNA vaccines reduce virulence and enhance antigen load (Ogra *et al*., 2001). Some studies showed the protection of mice against the rotavirus infection by DNA vaccination (Yang *et al*., 2001; Chen *et al*., 1998; Herrmann *et al*., 1996). VLPs and DNA vaccines may constitute a third generation of the rotavirus vaccines (Chen *et al*., 1998). Previous studies reported that the rotavirus 2/6 (composed of VP2 and VP6) and 2/6/7 (composed of VP2, VP6 and VP7) double and triple layered particles could induce an active protection against HuRV-A (Ciarlet *et al*., 1998; McNeal *et al*., 1998; O'Neal *et al*., 1997, 1998).

In this study, we report the i.n. immunization of adult BALB/c mice by the DNA vaccine encoding the HuRV-A VP2 protein. Mucosal immunization induced humoral and cellular responses that were examined by levels of cytokines IFN-γ and IL-4 in the splenocytes and levels of IgG in the serum of immunized mice.

Materials and Methods

Viruses and cells. HuRV-A**,** strain IR, used in this study was isolated from a child diarrheal stool. The rotaviral origin was confirmed by the standard examination (Pourasgari *et al*., 2007a). The isolated virus was cultivated in the confluent MA104 cells (fetal rhesus monkey kidney cells) maintained in DMEM without serum and with addition of 5 µg/ml trypsin (Ahmadian and Shahrabadi, 1999).

Recombinant plasmid. Recombinant plasmid pcDNA3-VP2 used in this study contained the HuRV-A VP2 cDNA inserted into pcDNA3.1(+) vector (Pourasgari *et al*., 2007a). The expression of VP2 in pcDNA3-VP2 can be driven by human cytomegalovirus promoter located upstream. The VP2 sequence was deposited in GenBank database under Acc. No. DQ480724.

Immunization of mice with VP2 gene. Before i.n. administration, the plasmid DNA was mixed with dendrosome (a dendritic spheroidal nanoparticles gene porter) solution at a DNA:lipid (w/w) ratio of 1:5, incubated at room temperature for 15 mins (Pourasgari *et al*., 2007b), and gently inhaled by mice with an aerosol-producer chamber. In order to evaluate the immunogenicity of VP2 gene, 18 adult BALB/c mice (8-week-old) were divided into four groups. A group of 6 adult BALB/c mice were administered i.n. with 75 µg of pcDNA3-VP2 prepared with dendrosome (pcDNA3-VP2 group). The remaining three groups (containing 4 mice in each group) were negative control groups, e.g. first group received equal doses of pcDNA3.1(+) lacking the insert and dendrosome solution (pcDNA3.1 group), second group received only dendrosome (dendrosome group) and third group received a sample with no plasmid and no dendrosome. All animals were boosted twice at 2-week intervals and were housed in plastic microisolator cages.

Splenocyte cultures. In order to assess the production of anti-VP2 cellular responses, the lymphoid cells cultures were prepared from the spleen of the immunized mice using previously described techniques (Coffin and Clark, 2001). For *in vitro* cell re-stimulation, the cell culture was prepared in the 96-well plates in the presence or absence of 5 µg/well of purified rotavirus. The plates without the rotavirus were used as a negative control. The cells were incubated at 37ºC with 5% CO_2 for 3 days.

Splenocyte proliferation assay. The MTT assay was used for measurement of the cell proliferation. The stimulated splenocytes from the mice were treated by 25 µl MTT and incubated 4 hrs at 37ºC with 5% CO_2 (Zhang *et al*., 2008). After incubation and adding of dimethylsulfoxide, the viable cells were detected by measuring A_{590}. The cell viability was expressed as stimulation index (SI) value equaled A_{590} stimulated cells/A_{590} control cells.

Assay of cytokines. The supernatants of spleen lymphoid cell cultures from all examined groups of mice were collected 3 days after incubation at 37ºC with 5% CO_2 for cytokine detection. The concentration of IL-4 and IFN-γ were analyzed in the supernatant of lymphoid cell cultures from the spleen of immunized mice 10 days after the final VP2 DNA booster. Cytokine levels in the cell supernatant were assayed by mouse IL-4 and IFN-γ Immunoassays kits (R & D Systems) according to the protocols provided by the manufacturer.

ELISA of IgG antibodies. Anti-HuRV-A IgG antibody titers in serum samples were determined by ELISA as previously described (Colomina *et al*., 1998). The rotavirus preparation used as the coating antigen was purified by CsCl gradient centrifugation from the virus-infected MA104 cells (Offit *et al*., 1983). Blank wells coated with PBS instead of the virus were included in the test. Serum samples from immunized mice including pre-immunization (control) serum were serially diluted with PBS-0.1% Tween 20 containing 1% BSA, starting from 1:20 to 1:500 and added to the wells. After incubation for 2 hrs at 37ºC, the plates were washed and peroxidase-conjugated goat anti-mouse IgG (Sigma) in 1% BSA-PBS-T were added and incubated for 2 hrs at 37ºC. After three washes the color reaction was developed using TMB (tetramethyl benzidin) and H_2O_2 and stopped with 2 N H_2SO_4. Absorbance was determined at 492 nm (A_{492}).

Statistical analysis. Statistical analysis was carried out using SPSS software. Analysis of variance was used to determine the significance of the differences in the antibody responses and in the level of cytokines among the mouse groups. All possible pairwise comparisons were performed using the Mann-Whitney U-test (Garcia-Diaz *et al*., 2004).

Results

The examination of splenocyte proliferation from control mice didn't show detectable proliferation after virus stimulation. On the other hand, the proliferation of splenocytes isolated from vaccinated mice was significantly higher in comparison to the control group ($P < 0.001$, Fig. 1). In order to determine the type of immune responses, IFN-γ and IL-4 released from the stimulated splenocytes were measured. The results indicated a significant increase in levels of both of IFN-γ and IL-4 in the VP2-specific vaccinated mice ($P < 0.05$) (Fig. 2 and 3), when compared to the control mice.

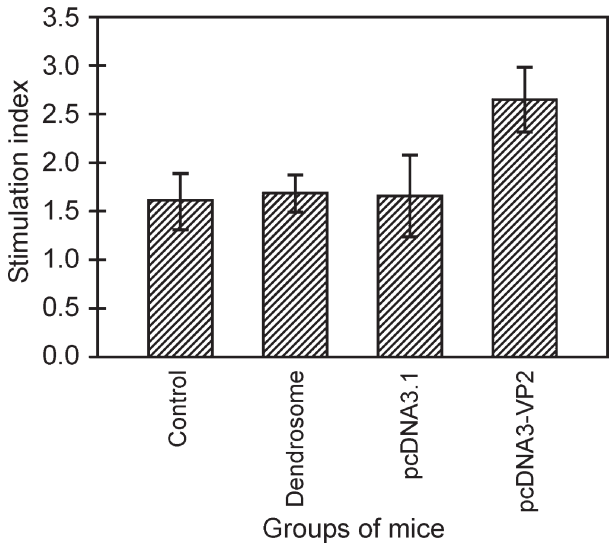

Fig. 1

Effect of immunization with VP2 DNA vaccine on splenocyte proliferation

Statistical analysis showed $P < 0.05$ in all groups.

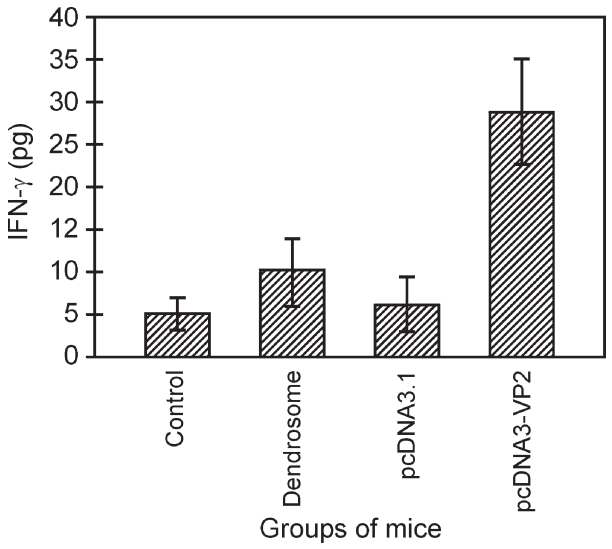

Fig. 2

Induction of IFN-γ by VP2 DNA vaccine

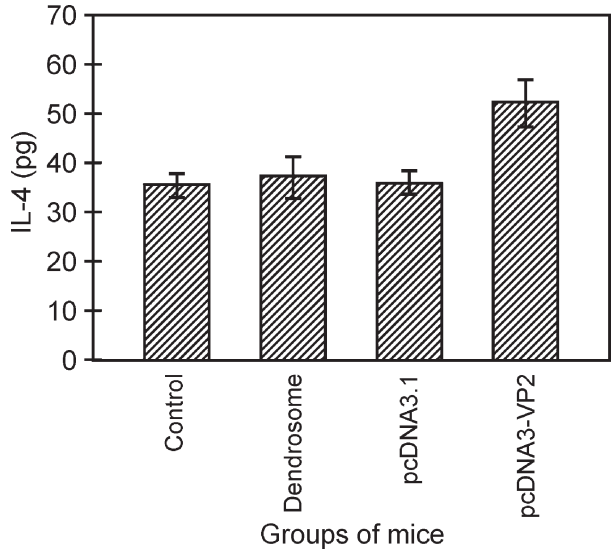

Fig. 3

Induction of IL-4 by VP2 DNA vaccine

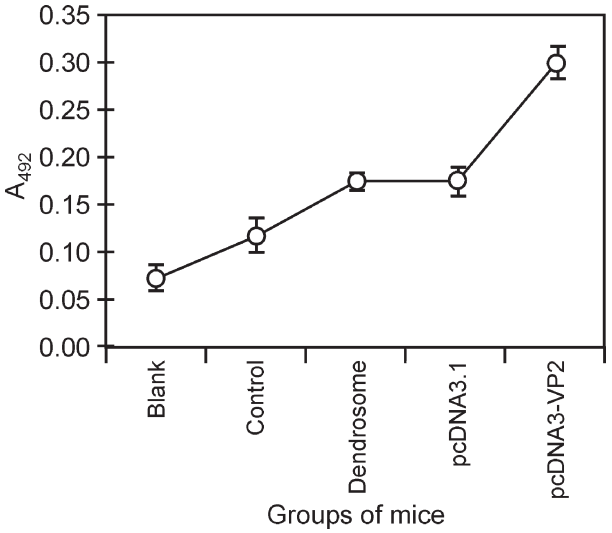

Fig. 4

Induction of IgG antibodies by VP2 DNA vaccine

The IgG titers of sera corresponding to the tested group of mice are depicted.

We evaluated the antibody response in serum of vaccinated and control mice collected 10 days after the second booster. The results indicated a significant increase in the level of total IgG in the serum of mice inoculated with pcDNA3.1(+) and dendrosome comparing with the control groups. VP2-specific serum IgG antibodies were significantly enhanced in mice after i.n. immunization with DNA vaccine containing pcDNA3-VP2 ($P < 0.05$). These results indicated that the DNA vaccine expressing the VP2 proteins induced strong antibody response in the mice (Fig. 4).

Discussion

Due to the absence of efficient anti-rotavirus drugs, efforts have been made to design vaccines to prevent the disease (Coste *et al*., 2000). Different strategies of immunization have been developed to elicit protective immune response based on either live attenuated viruses or subunit component from the rotaviruses, or RNA silencing methods for the rotavirus gene expression (Arias *et al*., 2004). Among the different vaccine candidates, the subunit vaccines are more attractive in a vaccine development. The mechanism by which the natural rotavirus infection or immunization in turn elicits protection remains poorly understood (Ward, 2003). In general, it appears that serum IgA is a good predictor of clinical protection after natural infection, but there is no or only a poor correlation between serum IgA and vaccination with rhesus-human rotavirus reassortant-tetravalent vaccine (RRV-TV) or other rotavirus vaccines (Ward, 2003; Heath *et al*., 1997; Madore *et al*., 1992). Nevertheless, also serum IgG is a good predictor of clinical protection, because it can cross the placenta and create immunity in infants.

Some previous studies showed that HuRV-A double-layered particles or heterologous 2/6-VLPs could induce active protection against rotavirus infection in mice or rabbits (Agnello *et al*., 2006; Ciarlet *et al*., 1998; McNeal *et al*., 1998; O'Neal *et al*., 1998, 1997). Comparing the immunogenicity and protective efficacy of homologous and heterologous 2/6-VLPs revealed that both types of 2/6-VLPs were immunogenic in mice and different levels of protective efficacy could be achieved depending on the dose, route, or co-administration with the adjuvants (Bertolotti-Ciarlet *et al*., 2003). The intramuscular and intranasal routes of immunization afforded the highest levels of protection, while the oral route offered low levels of protection against virus challenge. Also, intrarectal immunization of mice showed induction of a powerful anti-rotavirus immune response in gut and protected animals against rotavirus infection. Up till now there has been no immunization study about core-like particles including VP2 used as an antigen. For the first time, we report here the immunization of mice with HuRV-A VP2 gene administered intranasally.

In this study, we have demonstrated immunization of the BALB/c mice with the pcDNA3-VP2 plasmid. VP2 is one of the major HuRV-A structural proteins and its primary structure has been deduced from the gene sequence analysis (Zeng *et al*., 1994). VP2 gene has about 2700 bp in length. In our previous studies, the expression of the gene was confirmed in a human lung cell line using dendrosome gene porter (Pourasgari *et al*., 2007a,b). Ten days after the second boost, the proliferation of splenocytes was increased, what showed the ability of VP2 expressed in lung macrophages to induce the proliferation of lymphocytes.

Cellular immune response featuring CD4 and Th1 cells is an important response to the viral infections, since the viruses replicate inside the cells. Nevertheless, humoral immune response activated during CD4 and Th2 cells response is also important against HuRV-A. The virus replicates in gut cells and since the humoral response is efficient in this region, the antibodies inhibit the virus replication and release. We have also detected the production of an anti-VP2 IgG antibody response after i.n. inoculation of the pcDNA3-VP2 plasmid in mice. The importance of IgG production is reinforced by the fact that IgG is able to cross the placenta. So, after vaccination the immunogenic molecules can be transferred from mother to her offsprings.

In conclusion, we have shown that i.n. immunization with VP2 DNA induced an anti-HuRV-A immune response in the serum and spleen of the mice. Therefore, i.n. immunization may be an efficient way for DNA vaccination not only against HuRV-A, but also against other viruses that replicate in the mucosal surface. However, more studies are necessary to conclude that the VP2 gene is suitable as a DNA vaccine against HuRV-A infection.

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