Oral immunization with rotavirus VP7 expressed in transgenic potatoes induced high titers of mucosal neutralizing IgA

Yu-Zhang Wu, Jin-Tao Li,* Zhi-Rong Mou, Lei Fei, Bing Ni, Miao Geng, Zheng-Cai Jia, Wei Zhou, Li-Yun Zou, and Yan Tang

Institute of Immunology, Third Military Medical University, District Shapingba, Chongqing 400038, People’s Republic of China

Received 23 December 2002; returned to author for revision 12 February 2003; accepted 6 March 2003

Abstract

Rotaviruses (RV) are a common cause of severe diarrhea in young children, resulting in nearly one million deaths worldwide annually. Rotavirus VP7 was the rotavirus neutralizing protein. Previous study reported that VP7 DNA vaccine can induce high levels of IgG in mice but cannot protect mice against challenge (Choi, A.H., Basu, M., Rae, M.N., McNeal, M.M., Ward, R.L., 1998. Virology 250, 230 –240). We found that rotavirus VP7 could maintain its neutralizing immunity when it was transformed into the potato genome. Mice immunized with the transformed tubers successfully elicited serum IgG and mucosal IgA specific for VP7. The mucosal IgA titer was as high as 1000, while serum IgG titer was only 600. Neutralizing assays indicated that IgA could neutralize rotavirus. These results indicate the potential usefulness of plants for production and delivery of edible rotavirus vaccines.

© 2003 Elsevier Science (USA). All rights reserved.

Introduction

Human rotaviruses (HRV) are the common cause of severe diarrhea in young children and are estimated to cause nearly one million deaths worldwide annually (Avendano et al., 1993). RV are responsible for 6% of deaths among children under 5 years of age and for 25% of deaths due to diarrheal disease in developing countries. In developed countries, where HRV-acute gastroenteritis is usually mild (Glass et al., 1996), they still cause enormous socioeconomic costs (Berner et al., 1999; Johansen et al., 1999). Thus there is an urgent need to develop a rotavirus vaccine. Several rotavirus vaccine candidates have been evaluated in clinical trials with various degrees of success. The licensure of the first rotavirus vaccine, a tetravalent rhesus-based rotavirus vaccine, in the United States in 1998, marked a significant advance in preventing the morbidity associated with rotavirus diarrhea. However it was withdrawn in less than 1 year after being associated with intussusception, a rare form of bowel blockage found most frequently in young children (Coste et al., 2000). These data suggested that safety should be considered above all while developing an RV vaccine. In recent years, experiments demonstrated that a different vector-expressing HRV glycoprotein VP7 was capable of stimulating protective immunity and plays a key role in protection (Andrew et al., 1992; Hoshino et al., 1985; Wang et al., 1999). Previous studies in naturally infected children have shown that intestinal antibodies, especially IgA, are pivotal in protection from rotavirus reinfection (Giammarioli et al., 1996). Oral immunization is an efficient method of inducing high levels of mucosal IgA injection or mainline (Choi et al., 1998) and thus an RV vaccine may focus on an edible vaccine.

The utilization of plants as bioreactors for the production of foreign proteins have been increasingly used to acquire experimental immunogens. They are potentially an inexpensive source of antigens that could be parenterally administered or to be used as edible vaccines. To date, several viral antigens have been produced in transgenic plants, which possessed immunogenicity in animals (Haq et al., 1995; Mason et al., 1992; Tacket et al., 1998). As yet, there are no national data about human rotavirus antigen gene expressed...
in transgenic plants, although there are reports of murine (epitope of NSP4) or bovine (VP6) rotavirus (Matsumura et al., 2002; Yu and Langridge, 2001). So, using the VP7 as the antigen in developing edible transgenic plants RV vaccine is a reasonable, worthwhile work and may represent a strategy for the development of safe, effective vaccines against rotavirus-induced diarrhea. We report here the human rotavirus group A antigen gene VP7 expressed in transgenic potato and its immunogenicity in mice.

**Results**

**Production and genetic analysis of transformed plants**

The whole length of human group A rotavirus serotype G1 VP7 gene with endoplasmic reticulum sequence SEKDEL was cloned into the plant expression vector PB1121 (Fig. 1A). We transformed the whole length of human group A rotavirus VP7 gene into potato genome. Twenty different transgenic plants with the ability of growing in the presence of kanamycin were produced. The presence of the VP7 gene was detected by Southern blot. A single band of the target gene was detected in all the VP7 transformants but no band was detected in the mock-transformants (Fig. 1B). The expression of the VP7 mRNA in the transgenic plants was detected by RT-PCR. A band of 1062 bp, which was amplified with specific primers for VP7 gene, was obtained in all the kanamycin-resistant plants tested, while it was consistently absent in mock-transformed plants (Fig. 1C).
The VP7 protein in 20 different transformed plants were detected by ELISA. Each ELISA plate included positive controls containing 1 to 50 ng of recombinant VP7 protein. The amount of protein in each of the extracts was determined by the Bio-Rad protein assay. The protein levels of VP7, ranging from 0.18 to 3.84 \( \mu \text{g}/\text{mg} \) of soluble protein, were observed in most transformants (Fig. 2). The maximum level of VP7 protein in the leaves reached up to 3.84 \( \mu \text{g}/\text{mg} \) of total soluble protein. Four transgenic plants (plant nos. 4, 8, 11, 19) with higher VP7 protein were selected for later Western blot analysis.

**VP7 protein expressed in transformants tubers**

Transformed potato tuber extract proteins were analyzed by Western blot. The molecular weight of the VP7 protein expressed by the transgenic potato plants corresponded to that of native VP7, which has an apparent molecular mass of 38 kDa. The reaction of the recombinant polypeptides found in the transformants to specific antibodies also supported the hypothesis that in those constructs the translation started at the authentic initiation codon described for the VP7 found in the virions (Fig. 1D). This also indicated that recombinant VP7 did not undergo major posttranslational processing in the plant system as other researchers reported.

**Antibody titers in animal responded to the plant-expressed VP7**

Anti-VP7 titers in the sera of the animals fed with mock-transformed potato tubers were not detectable 67 days after immunization (Fig. 3A). By contrast, at this time the mice immunized with VP7-derived potato tubers showed high antibody titers (Fig. 3A), which did not increase after the last boost. Intestinal VP7-specific and total IgA levels in the mice of Fig. 3B were assayed by ELISA. At the same time, the feces, the urine, and the saliva were also collected. All of the samples were stored at \(-20^\circ\text{C}\) until enzyme-linked immunosorbent assay (ELISA) detection. Before analysis, feces pellets were dissolved in PBS with 2 \( \mu \text{g} \) ml\(^{-1}\) sodium azide and serially diluted with PBS. Urine and saliva were also serially diluted with PBS and detected by ELISA. Of the mice that had a positive serum antibody response at a titer of 600, 18/19 (with CT) and 19/20 (with CT-B) also had high VP7-specific intestinal IgA (Fig. 3B). However, urine or saliva IgA titers were significantly lower \( (P < 0.005) \) than feces' IgA titers. Specific IgA titers ranged from 150 to 1000 for CT and from 157 to 995 for CT-B.

**Fig. 3. Measurement of anti-VP7 antibody titers responses in mice after oral immunization with transgenic potato tubers.** (A) Kinetics of serum IgG antibody titers against HRV-VP7 at different times (from left to right); 1–7 represent mice blood collected on 0, 7, 14, 21, 35, 42, and 67 days. (B) Kinetics of feces IgA antibody titers against HRV-VP7 at different times (from left to right); columns from left to right represent mice blood collected on 0, 7, 14, 21, 35, 42, and 67 days. (C) Mucosal IgA antibody titers against HRV-VP7. Columns 1–4 represent group 1, 2, 3, and 4, respectively. Columns 5–8 had the same representation. Antibody titers were determined by immunoperoxidase ELISA method. The antibody titer was determined as the reciprocal of the highest dilution of the samples that generated a color signal above the background levels. Group 1: 2 g transformed potato tubers (containing 84 \( \mu \text{g} \) VP7) + 10 \( \mu \text{g} \) CT; group 2: 1 g transformed potato tubers + 10 \( \mu \text{g} \) CT; group 3: 2 g transformed potato tubers + 10 \( \mu \text{g} \) CT-B; group 4: 1 g transformed potato tubers + 10 \( \mu \text{g} \) CT-B; group C: control group mice fed with mock-transformed potato tubers.
VP7-specific IgA was detected in the mucosal of mice fed with mock transformants.

**Neutralize assay**

To examine the functional activity of the antirotavirus antibodies detected after oral administration of plant-derived VP7 protein, in vitro rotavirus neutralization assays were performed using sera or feces collected from day 42 (Figs. 3A and B). Sera derived from mice included with control transgenic plants and VP7 transgenic plants showed no neutralization activity against rotavirus Wa (Fig. 4). Significant levels of neutralizing antibodies were, however, detected in feces collected from mice fed with transgenic plants (Fig. 4). While feces collected from the control group did not detect neutralizing activity against to rotavirus Wa, these results confirmed that transgenic plants could induce functionally active antirotavirus intestinal antibodies.

**Comparing the effect of CT with CT-B as mucosal adjuvant**

Mice orally immunized with four doses of transgenic potato tubers with adjuvant cholera toxin (CT) or cholera toxin subunit B (CT-B) both elicited VP7-specific IgG in sera and IgA in mucosal system when examined on day 42. There was no significant difference ($P > 0.05$) of VP7-specific IgG or IgA between immunization groups with CT or CT-B as mucosal adjuvant.

**Discussion**

The mucosal immune system and its predominant effector, secretory immunoglobulin A (IgA), provide the initial immunologic barriers against most pathogens that invade the body at a mucosal surface (Johansen and Svensson, 1997). This is especially true for viruses, since resistance to infection has strongly correlated with the presence of specific IgA antibody in mucosal secretions. The characteristics and functions of mucosal IgA appear ideally designed to maintain the integrity of the mucosal epithelium while defending the body against foreign pathogens and antigens (Herrmann et al., 1999). Thus selecting a suitable antigen to immunize high IgA response is considerable in designing a mucosal system pathogen such as rotavirus. There of the rotavirus structure proteins VP4, VP7, and VP6 that were used in rotavirus vaccine strategies could independently induce neutralization antibodies (Choi et al., 1998). Antibodies to VP4 and VP7 can passively protect mice against rotavirus challenge (Both et al., 1993). Vaccine strategies based on VP6 were also tested. An interesting note was that IgA were always lower than IgG in the animal immunization test in previous reports, whereas our results first showed higher IgA in mice intestinal feces immunized with VP7-transformed potatoes and the neutralizing activity against rotavirus existed in mucosal antibodies, IgA, but not in humoral antibodies, the sera. These data demonstrated the main role of VP7 in protecting body from rotavirus reinfection was to stimulate the mucosal system of the body to secrete IgA. Our results proved that VP7 gene was expressed accurately in the plant to maintain its glycopein immunity and also indicated that plant was a suitable express system for producing a foreign gene such as viral immunogen.

Both CT and *Escherichia coli* heat-labile enterotoxin (LT) were capable of providing adjuvant activity to antigens that are coadministered orally in many cases (Rask et al., 2000). Although CT and the closely related LT can act as powerful mucosal adjuvants, their use in humans is hampered by their high toxicity. Both CT and LT consist of a homopentamer of cell-binding B subunits associated with a single toxic active A subunit. The B subunits of CT (CTB) and LT (LTB) can be used both as carrier molecules and as mucosal adjuvants (Sun et al., 2000), the efficacy of which in the latter case depends on the route of mucosal administration. Our data in this article showed that the immune responses and levels of neutralizing rotavirus were enhanced equally with CTB or CT as a mucosal adjuvant. These would be worthwhile for further study of construction of multivalent transgenic plants vaccine.

In conclusion, we demonstrated here that rotavirus antigen VP7 was successfully transformed into potato, and in animal tests, VP7 maintained its immunogen. The neutralizing activity against rotavirus of VP7 mainly depended on antibodies IgA but not IgG. Plant is a candidate express system for researching rotavirus vaccine and CTB has an equally mucosal adjuvant effect compared to CT.

**Materials and methods**

**Cloning VP7 gene and construction of the recombinant plasmid**

For the construction of plant expression vector PBI-VP7, the whole VP7 gene was cloned as the following. The total RNA was isolated from a Chinese child’s feces and used as
the RT-PCR template to amplify the full-length VP7 gene; the primers were as follows: P1 5’-GGCTTTAAAAAGAGAGAATTTCGTCG-3’ (forward) and P2 5’-GT-CACATCATAAATTCTAATAG-3’ (reverse). The amplified VP7 gene was sequenced as 1062 nucleotides (nt) long and 100% homogenous with group A human rotavirus serotype G1 VP7 gene, (GenBank Accession No. 10242241), and performed PCR again using the same forward primer above and the reverse primer as P3 5’-TCATAGTTCCGTCTTTTTCAGATACTCTATAATAAAACGCA-3’ (reverse) to adding an endoplasmic reticulum sequence SEKDEL. The latter PCR products were cloned into the PUC-T vector (Sangon). The resulting clone, PUC-T/VP7, was digested with BamHI and SacI and subcloned into the binary plasmid vector pBI121 (Clontech) to yield pBI-VP7.

Southern blot and RT-PCR analysis of VP7 gene transferred in transformants

Agrobacterium tumefaciens LBA4404 containing either the PBI-VP7 vector or the PBI121 mock vector were used to transform potato plants (Solanum tuberosum cv. taiwan-hong). The presence of the VP7 gene in the rooted plants was investigated by Southern blot as described elsewhere (Mason et al., 1992). Randomly 32P-labeled probe corresponding to the VP7 ORF was generated using Klenow polymerase from the NEBlot kit (New England Biolabs) and was used in Southern hybridizations. To analyze VP7 mRNA in transgenic potato leaves, RT-PCR assay was performed by using the primers above (P1 and P2) to amplify the VP7 gene. Total RNA from individual transformant fresh leaves were extracted by plant RNA extract kit (Takara). The RT-PCR was performed with a TaKaRa One-step RT-PCR kit (TaKaRa, Japan).

ELISA detection of the protein expression level of the VP7 in transformatant leaves

The presence of recombinant protein in the plants harboring the VP7 gene was tested by ELISA. Protein extracts obtained by homogenization of transformants leaves in PBS plus 0.05% Tween 20 were centrifuged at 10,000 g for 3 min and bound to 96-well microtiter plates (Hyclone) for 16 to 24 h at 4°C. Then the plates were blocked with 5% defatted milk in PBS containing 0.05% Tween 20. Polyclonal antibody antirtovirus protein and horseradish peroxydase IgG were added in subsequent steps. The plates were developed by the addition of δ-phenylenediamine-H2O2 in citrate buffer (pH5).

Western blot of VP7 expressed in transformant tubers

The expression of the VP7 protein in the transgenic plants was analyzed by Western blot. Potato tuber extracts were prepared by grinding 100 mg of freshly harvested potato tuber slices in the presence of liquid nitrogen and the resulting powder was resuspended in SDS–PAGE sample buffer (50 mM Tris, pH 7.5, 1 mM PMSF, 8 M urea, 1% SDS, 2 mM DTT, and 2% mercaptoethanol), boiled for 10 min, electrophoresed in 12% SDS–PAGE, and blotted to a PVDF membrane (Roche). The membrane was blocked overnight with PBS containing 0.05% Tween 20 (PBST), 5% skim milk (all subsequent steps were performed using this buffer), and incubated with a goat anti-human rotavirus polyclone antibody (ABI129, Chemicon) for 2 h at 37°C. The membrane was then washed in PBST and incubated with an alkaline phosphatase labeled anti-goat Ig mouse antiserum for 1 h at 37°C. After washing three times the reaction was developed by the addition of the substrate chemiluminescence (chemiluminescence detecting kit, Roche).

Immunogenicity test in mice

Plant no. 4, which expressed high levels of VP7, was used to produce tubers in a greenhouse and later the tubers were used to feed mice. The VP7 protein levels in the tubers was 4.2 μg g−1 by ELISA detection. To investigate the induction of protective immune responses to plant-derived VP7, 40 mice were equally divided into four groups. Each group was directly fed potato tuber slices containing 42 μg of double of VP7 on day 0,7,14, and 42, with 10 μg of CT or CT-B (Sigma) as mucosal adjuvant. Mock-transformed potatoes plus the same adjuvant were fed to the control group. The mice were fasted overnight and caged separately (one mouse in one cage) before being fed potato tubers covered with adjuvants. The potato slices were left in the cage until all of them were consumed or left overnight, whichever was earlier. For the duration of the immunization, mice were fed regular animal food and separated by groups. Before the first immunization and after the second and the later boosters, mice were tail-bled. Antibody in the serum against HRV-VP7 was tested by ELISA, using recombinant VP7 as the antigen, of serial dilutions of sera from immunized and control mice. Differences between the groups were evaluated by using the Student’s t-test, where P < 0.05 was considered statistically significant.

Neutralization assay

To determine neutralizing anti-HRV antibodies in sera and stools, sera and stools obtained from mice were tested by a microneutralization assay as described previously (Matson et al., 1993). Briefly, sera and stools from mice were heat-inactivated at 56°C for 30 min and incubated with Wa strain rotavirus. A reduction in the number of HRV-infected cells of greater than 60% with respect to the number in control wells was considered to indicate neutralization. Neutralizing titers were expressed as the reciprocal of the highest dilution of sera or stools yielding neutralization, as described.
Acknowledgments

Y.-Z. Wu and J.-T. Li contributed equally to this study. This work was supported by National Key Basic Research Program of China (2001CB510001) and National Natural Science Foundation of China (NSFC 30170882). We are grateful to Prof. Yan Pei for supplying PBI121 plasmids, Agrobacterium tumefaciens LBA4404 and Taiwanhong potato plant. We also sincerely thank Prof. Fang Zhao-Yin for giving human group A rotavirus cDNA and Dr. Elschner for kindly giving us rotavirus Wa for later neutralize assay.

References


