

Rice-based oral antibody fragment prophylaxis and therapy against rotavirus infection

Daisuke Tokuhara,^{1,2} Beatriz Álvarez,³ Mio Mejima,¹ Tomoko Hiroiwa,¹ Yuko Takahashi,¹ Shiho Kurokawa,¹ Masaharu Kuroda,⁴ Masaaki Oyama,⁵ Hiroko Kozuka-Hata,⁵ Tomonori Nochi,¹ Hiroshi Sagara,⁵ Farah Aladin,⁶ Harold Marcotte,³ Leon G.J. Frenken,⁷ Miren Iturriza-Gómara,⁸ Hiroshi Kiyono,^{1,9} Lennart Hammarström,³ and Yoshikazu Yuki^{1,9}

¹Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, University of Tokyo, Tokyo, Japan.

²Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka, Japan. ³Division of Clinical Immunology, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden. ⁴Crop Development Division, NARO Agriculture Research Center, Niigata, Japan. ⁵Medical Proteomics Laboratory, The Institute of Medical Science, University of Tokyo, Tokyo, Japan. ⁶Enteric Virus Unit, Virus Reference Department, Health Protection Agency, London, United Kingdom. ⁷Unilever Research and Development Vlaardingen BV, Vlaardingen, Netherlands. ⁸Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom.

⁹International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, University of Tokyo, Tokyo, Japan.

Rotavirus-induced diarrhea is a life-threatening disease in immunocompromised individuals and in children in developing countries. We have developed a system for prophylaxis and therapy against rotavirus disease using transgenic rice expressing the neutralizing variable domain of a rotavirus-specific llama heavy-chain antibody fragment (MucoRice-ARP1). MucoRice-ARP1 was produced at high levels in rice seeds using an overexpression system and RNAi technology to suppress the production of major rice endogenous storage proteins. Orally administered MucoRice-ARP1 markedly decreased the viral load in immunocompetent and immunodeficient mice. The antibody retained *in vitro* neutralizing activity after long-term storage (> 1 yr) and boiling and conferred protection in mice even after heat treatment at 94°C for 30 minutes. High-yield, water-soluble, and purification-free MucoRice-ARP1 thus forms the basis for orally administered prophylaxis and therapy against rotavirus infections.

Introduction

Rotavirus is the leading cause of diarrhea in infants and young children worldwide, causing more than 114 million episodes of diarrhea annually in children under the age of 5 (1–4), 80% of which occur in developing countries (4). More than 600,000 children die annually from rotavirus (RV) infection, and although most RV-related deaths occur in low income countries, in developed countries, thousands of children under 5 years of age are hospitalized every year, including approximately 50,000–80,000 in Japan and 60,000 in the US (2, 3), causing a huge financial burden (2, 5). Currently, there are 2 licensed RV vaccines (Rotarix and RotaTeq) that have been reported to be highly effective (>85%) in reducing severe RV-induced diarrhea in developed countries (6–8). More recent clinical trials in developing countries in Africa and Asia (9–11) have also demonstrated a potential impact of these vaccines, but with a markedly lower efficacy, ranging from 39.3% (RotaTeq in Sub-Saharan Africa) to 61.2% (Rotarix in Malawi and South Africa) (9, 10). Both vaccines have proven to be safe in clinical trials and post-marketing studies, but presently, they

Conflict of interest: ARP1 has been patented by Unilever and was provided under a material transfer agreement for research relating to the potential to apply the technology to food products. Leon G.J. Frenken owns shares in Unilever. Miren Iturriza-Gómara receives research support provided by GlaxoSmithKline and Sanofi Pasteur MSD.

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are licensed for use only within a very narrow age window (>6 weeks and ≤26 weeks of age) to avoid the risk of intussusception.

Vaccines are often associated with poor efficacy in immunocompromised individuals (12–14). Such individuals may even acquire infections derived from the use of live vaccines. Cases of chronic diarrhea induced by the attenuated RotaTeq vaccine have indeed been recently reported (12–14), and the current RV vaccines are therefore not recommended for use in immunodeficient infants.

The lag time between vaccine administration and induction of an immune response, as well as poor efficacy in immunocompromised individuals, can be critical in highly endemic regions and outbreak situations, such as those occurring in hospital settings (15–18). Furthermore, severely immunocompromised individuals do not mount protective immunity when infected with RV and thus suffer chronic infection (19–22), which can subsequently lead to extraintestinal spread with severe clinical consequences (19–21). There is still a need for alternative strategies that can be used in situations where the available vaccines are not indicated and/or to complement vaccination strategies in situations where efficacy of vaccination alone may not be sufficient.

Passive immunotherapy is, at present, the only available intervention that can provide immediate protection. It may thus represent the prophylaxis of choice in highly endemic areas where vaccine responses may be poor or in selected groups of patients such as hospitalized children, immunocompromised individuals, and older infants and children in whom vaccination is contraindicated. It has been previously shown that oral administration of polyclonal antibodies from various sources can be used successfully both as

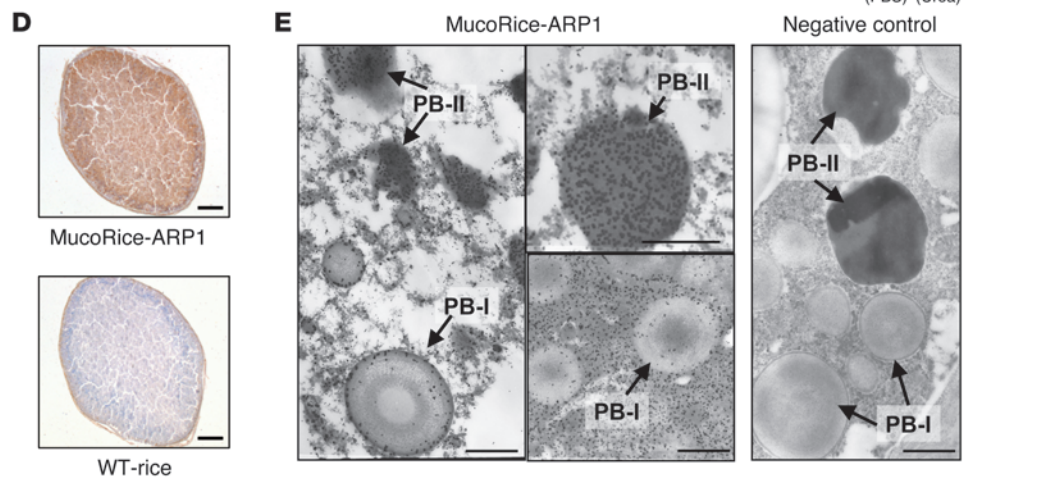
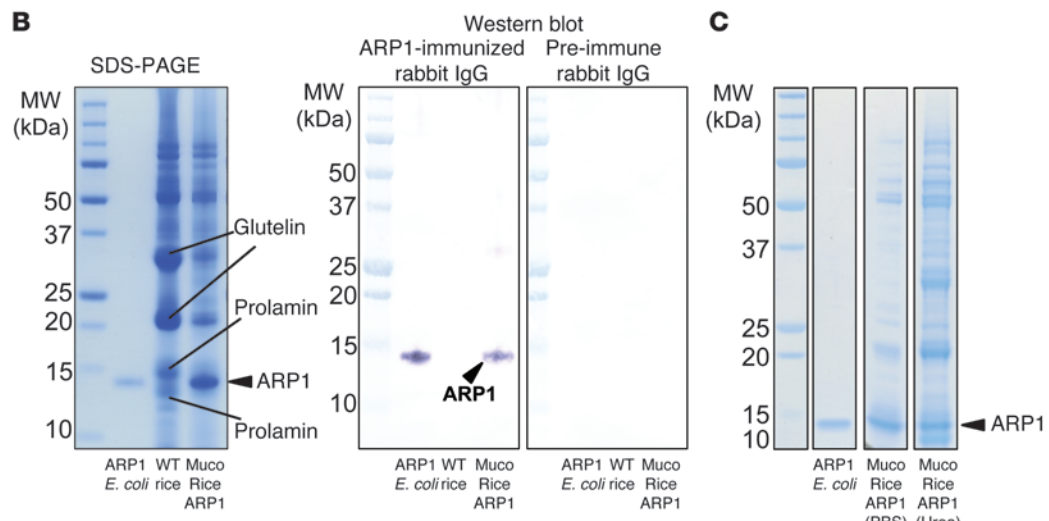
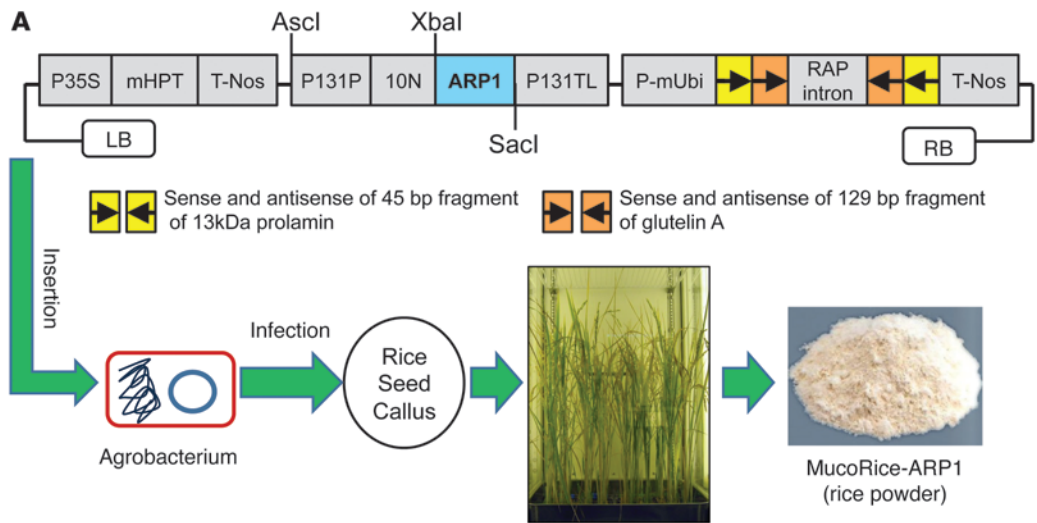




Figure 1

Expression and localization of water-soluble ARP1 in transgenic rice. (A) Inserted plasmid for overexpression of ARP1 in rice seeds. P35S, CaMV35S promoter; mHPT, modified hygromycin phosphotransferase; P131P, 13-kDa prolamin promoter; 10N, signal sequence of 10-kDa prolamin; P131TL, 13-kDa prolamin terminator; T-Nos, nos terminator; RAP intron, rice aspartic protease intron; P-mUbi, ubiquitin promoter; LB, T-DNA left border; RB, T-DNA right border. (B) Production of MucoRice-ARP1. SDS-PAGE showed predominant expression of the transgenic protein with a molecular weight of approximately 12 kDa (arrowhead). Original rice proteins (arrows; 22- to 23-kDa and 34- to 37-kDa subunits of glutelin and 13-kDa prolamin) in nontransformed WT rice were markedly suppressed in MucoRice-ARP1. Western blotting revealed that a transgenic protein of 12 kDa was specifically detected using the anti-ARP1 antibody. ARP1 *E. coli*, ARP1 purified from *E. coli*. (C) Solubility of MucoRice-ARP1. MucoRice-ARP1 (PBS), extracts of MucoRice-ARP1 in PBS; MucoRice-ARP1 (urea), extracts of MucoRice-ARP1 in 8 M urea. (D) Immunohistochemistry showed that ARP1 had accumulated throughout the whole MucoRice-ARP1 seed, whereas it was not detected in a WT rice seed. Scale bars: 1 mm. (E) Immune electron microscopy showed that ARP1 is observed as black spots (left and middle panels). ARP1 is predominantly localized in the PB-II and the interspace between the PB-I and PB-II (left and middle panels). ARP1 is also slightly found at the surface of PB-I (left panel and lower middle panel). ARP1 is not detected in a WT rice seed used as a negative control (right panel). Scale bars: 1 μ m.

prophylaxis and as therapy in children with RV-induced diarrhea (23, 24). However, the production and purification of the antibodies is costly, making it necessary to find alternative approaches.

The variable domain of llama heavy chain antibodies (VHH) consists of a single domain and constitutes the smallest naturally occurring antigen-binding domain known to date (25). In addition to its high binding capacity, VHH has several attractive features, such as resistance to pepsin, acid environment, and heat (26, 27). Furthermore, because of its small size and simple form, it is easy to produce VHH as a recombinant protein with an intact spatial structure (28). An anti-RV VHH (ARP1) produced in yeast and capable of protecting mouse pups against RV-induced diarrhea has been described previously (29). Modified lactobacilli-producing surface-anchored ARP1 were generated and shown to be effective in the same mouse pup model (30). Furthermore, orally administered ARP1 produced by yeast was recently found to be safe and effective in reducing severity of RV induced diarrhea in children in a clinical trial conducted in Bangladesh (S.A. Sarker, M. Jäkel, S. Sultana, N.H. Alam, P.K. Bardhan, M.A. Salam, W. Theis, L. Hammarström, L.G.J. Frenken., unpublished observations). Western blots using a variety of RV strains and recombinant VP6 suggest that ARP1 binds to polymeric VP6 (31). VP6 is the most conserved protein in the RV capsid and contains the group and subgroup determining epitopes (31).

We previously reported that a rice-based vaccine containing the cholera toxin B subunit (MucoRice-CTB) was effective against cholera (32, 33). Recently, we also introduced RNAi technology in an attempt to suppress internal storage rice protein production in order to enhance accumulation of foreign proteins in rice seed (34). In this study, we extended this system further to include a rice-based, orally administered product against RV by producing rice expressing an antibody fragment, ARP1 (MucoRice-ARP1). MucoRice-ARP1 rice powder or rice water offer what we believe are novel approaches to the prevention and treatment of RV-induced diarrhea, which may be used to reduce the medical and economic

burden in both developed and developing countries, complement current vaccine-based prophylaxis, and in situations where live attenuated vaccines are contraindicated.

Results

Development of rice-based water soluble ARP1 (MucoRice-ARP1) with a high expression level. Using a binary vector (pZH2Bik45G1B) (35), described in Figure 1A, a codon-optimized ARP1 gene combined with a RNAi suppression cassette was transfected into rice plants (Nippon-Bare) using *Agrobacterium tumefaciens*-mediated transformation. As shown in Figure 1B, the expression of the prolamin (13 kDa) and glutelin (22–23 kDa and 34–37 kDa) was suppressed and the 12 kDa ARP1 protein was predominantly expressed in the transgenic rice (MucoRice-ARP1). When accumulation of the ARP1 protein in the MucoRice-ARP1 seed was examined by SDS-PAGE and Western blot, using a rabbit anti-ARP1 polyclonal antibody, 1 band (12 kDa) was detected under denaturing conditions (Figure 1B). ARP1 was released from the MucoRice-ARP1 rice powder after PBS addition at room temperature (rice water) as shown in Figure 1C. Using densitometric analysis, with purified yeast-derived ARP1 as a standard, the yield of soluble ARP1 (which is 95% of the total yield) was found to reach an average of 170 μ g per seed, representing 11.9% of the total seed protein (0.85% of seed weight). In contrast, transgenic rice, without RNAi suppression of prolamin and glutelin, yielded a low amount of ARP1 (on average 14 μ g / seed) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70266DS1).

Localization of ARP1 protein in a MucoRice-ARP1 seed. Immunohistochemistry staining showed that MucoRice-ARP1 is expressed throughout the entire seed (Figure 1D). Storage proteins of WT rice seeds generally accumulate into 2 different types of protein bodies (PB), PB-I and PB-II, which contain prolamin and glutelin as their major components, respectively (36). PB-I is resistant to digestive enzyme whereas PB-II is easily digested in the gut (37). When the sections were examined by immune electron microscopy, ARP1 was predominantly localized in PB-II and the cytoplasm between PB-I and PB-II (Figure 1E).

Mass spectrometric analyses of MucoRice-ARP1. Using Q-STAR and Orbitrap mass spectrometry, we determined the full sequence of the 123 amino acids of ARP1 and the N-terminal sequence (serine-arginine) derived from the restriction enzyme site (*Xba*I) necessary for introduction of the ARP1 encoding gene into the binary vector (Supplemental Tables 1 and 2).

Heat stability and long-term room temperature stability of MucoRice-ARP1. MucoRice-ARP1 showed a binding to rhesus RV (RRV) particles similar to that of yeast-derived ARP1 (BAC BV, Naarden, the Netherlands) (Supplemental Figure 2). In order to determine the heat stability of MucoRice-ARP1, ARP1 (from rice and yeast) and bovine hyperimmune colostrum (HBC) (38) were boiled for 10, 20, and 30 minutes. After 30 minutes of boiling, a loss in the binding capacity to RV using ELISA of between 33% and 57% was observed in the ARP1 samples. It is worth noting that rice water containing MucoRice-ARP1 (protein solution from MucoRice-ARP1 powder in PBS) showed a higher heat resistance (33% loss in the binding capacity after 30 minutes of boiling) than the purified ARP1 samples from MucoRice-ARP1 (57% loss) and yeast (46% loss). In contrast, the HBC sample completely lost its binding activity already after 10 minutes of boiling (Supplemental Figure 3).

Furthermore, MucoRice-ARP1, stored at room temperature (>1 year), had a neutralizing activity against RV in vitro equal to

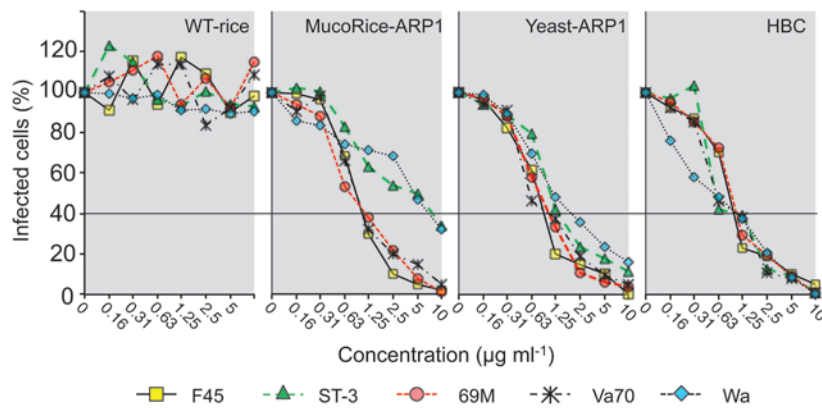


Figure 2

Neutralizing activity of MucoRice-ARP1 against human RV strains. Neutralizing activity of ARP1 samples from different origins and HBC against the human RV strains Wa (G1P[8]), ST-3 (G4P[6]), 69M (G8P[10]), F45 (G9P[8]) and Va70 (G4P[8]) using 2-fold dilutions of MucoRice-ARP1 containing rice water (shown as MucoRice-ARP1), WT rice (used as a negative control), ARP1 purified from yeast (shown as yeast-ARP1), and HBC. Lines indicate the percentage of infected cells related to control wells infected in the absence of added proteins. The horizontal line at 40% infected cells indicates a reduction of 60% in the infectivity of the RV strains.

that of freshly harvested MucoRice-ARP1 when applied as rice water (Supplemental Figure 4).

MucoRice-ARP1 neutralizes human RV strains of different serotypes in vitro. Neutralization assays, preventing infection in MA104 cells, were carried out to determine whether MucoRice-ARP1 was able to detect human RV strains of different serotypes (Figure 2). MucoRice-ARP1 containing rice water neutralized the human RV strains Wa G1P[8], ST-3 G4P[6], 69M G8P[10], F45 G9P[8] and Va70 G4P[8] in a dose-dependent manner. A reduction of more than 60% in the infectivity of the RV strains F45, 69M, and Va70 was observed in vitro using the same dose of MucoRice-ARP1 and purified yeast-derived ARP1 (1.25 µg ml⁻¹). For neutralization of the RV strains ST-3 and Wa, 4 times the amount of rice water of MucoRice-ARP1 (10 µg ml⁻¹) was needed as compared with purified yeast-derived ARP1 (2.5 µg ml⁻¹) (Figure 2).

MucoRice-ARP1 reduced diarrhea in a mouse pup model of RV infection. All 4-day-old mice infected with RRV and given PBS or WT rice developed diarrhea (Figure 3, A–C) whereas none of the noninfected control mice (fed PBS or WT rice) did (Figure 3, A–C). When MucoRice-ARP1 (containing 8.5 µg of ARP1) was prophylactically given to neonatal mice, the percentage of animals with diarrhea on day 2 after RRV inoculation was significantly lower ($P = 0.007$) than in mice receiving PBS or WT rice (Figure 3A). In addition, disease severity in mice treated with MucoRice-ARP1 was significantly lower ($P = 0.022$ and $P = 0.041$) than in mice given PBS or WT rice.

Heat treated MucoRice-ARP1 (for 10 minutes or 30 minutes at 94°C) used prophylactically against RRV-induced diarrhea also significantly ($P = 0.035$) reduced the percentage of animals with diarrhea on day 2 in comparison with mice receiving PBS or WT rice. In addition, disease severity in mice treated with heat-treated MucoRice-ARP1 was significantly lower ($P = 0.041$) than in mice given PBS or WT rice. No significant difference was observed between heat-treated and non-heat treated MucoRice-ARP1 in their ability to reduce the percentage of mice with diarrhea and disease severity (Figure 3A).

As shown in Figure 3B, long-term (>1 year) stored MucoRice-ARP1 (8.5 µg ARP1) applied prophylactically significantly reduced the incidence of diarrhea and disease severity ($P = 0.011$ and $P = 0.008$, respectively on day 2) compared with that of mice receiving PBS and was as effective as freshly harvested MucoRice-ARP1 ($P = 0.297$ and $P = 0.267$, respectively on day 2).

When MucoRice-ARP1 was given therapeutically, 9 hours after RRV inoculation, the percentage of mice with the virus-induced diarrhea and disease severity was also significantly lower

($P = 0.007$ and $P = 0.041$, respectively on day 2) than in the control mice receiving PBS or WT rice (Figure 3C).

Histopathological changes and virus loads in the small intestine of neonatal murine pups. No lesions were seen in any of the control mice receiving PBS and sacrificed on day 3 (Figure 4A). In the RRV-infected neonatal mice that received WT rice water solution or PBS, all of the examined mice showed typical histological signs of RV infection, with accumulation of large vacuoles in the enterocytes lining the surface of the villous tips in the ileum (Figure 4, B and D). Some vacuoles were also detected in the duodenum and the jejunum (data not shown). RV-infected mice treated prophylactically with the MucoRice-ARP1 preparation showed no pathological changes (Figure 4C). When VP7 mRNA-specific RT-PCR was performed, the level of virus load tended to correlate with the histopathological changes. Thus, the virus load in the intestines of RRV-inoculated mice that received MucoRice-ARP1 was significantly reduced compared with that of mice given PBS ($n = 6$, $P = 0.002$) or WT rice ($P < 0.01$) ($n = 6$, $P = 0.002$) (Figure 4E).

Stability of MucoRice-ARP1 in the neonatal mice gut. Intragastrically administered MucoRice-ARP1 containing rice water (8.5 µg of soluble ARP1) was detected in the small intestine of the mice (4 days after birth) for at least 6 hours (Supplemental Figure 5). In some mice (40%, $n = 5$), MucoRice-ARP1 could be detected even 9 hours after intragastric administration.

MucoRice-ARP1 reduces the viral shedding in SCID mice. Following challenge with RRV, watery diarrhea was observed in all SCID neonatal pups. MucoRice-ARP1 (containing 8.5 µg of ARP1), given therapeutically to SCID pups 9 hours after RRV inoculation, significantly reduced the percentage of mice with diarrhea ($P = 0.022$) and disease severity ($P = 0.038$) compared with SCID pups given PBS or WT rice (Supplemental Figure 6).

Adult SCID mice did not develop overt diarrhea. However, the mice suffered a chronic infection lasting more than 6 weeks, as recognized by viral shedding in feces (Figure 5). Oral administration of 200 mg of MucoRice-ARP1 powder (containing 1.7 mg of soluble ARP1) twice daily for 7 days markedly reduced the number of viral VP7 RNA copies in feces on day 3 and day 9 ($n = 6$, $P = 0.002$ and 0.003) compared with that in mice given PBS or WT rice. No difference was, however, observed after the cessation of MucoRice-ARP1 administration (day 14) (Figure 5). No reduction in viral shedding was observed in RRV-inoculated SCID adult mice treated with WT rice powder compared with mice receiving PBS. These data indicate that MucoRice-ARP1 can transiently, but effectively, decrease the shedding of RV even in immunocompromised adult mice.

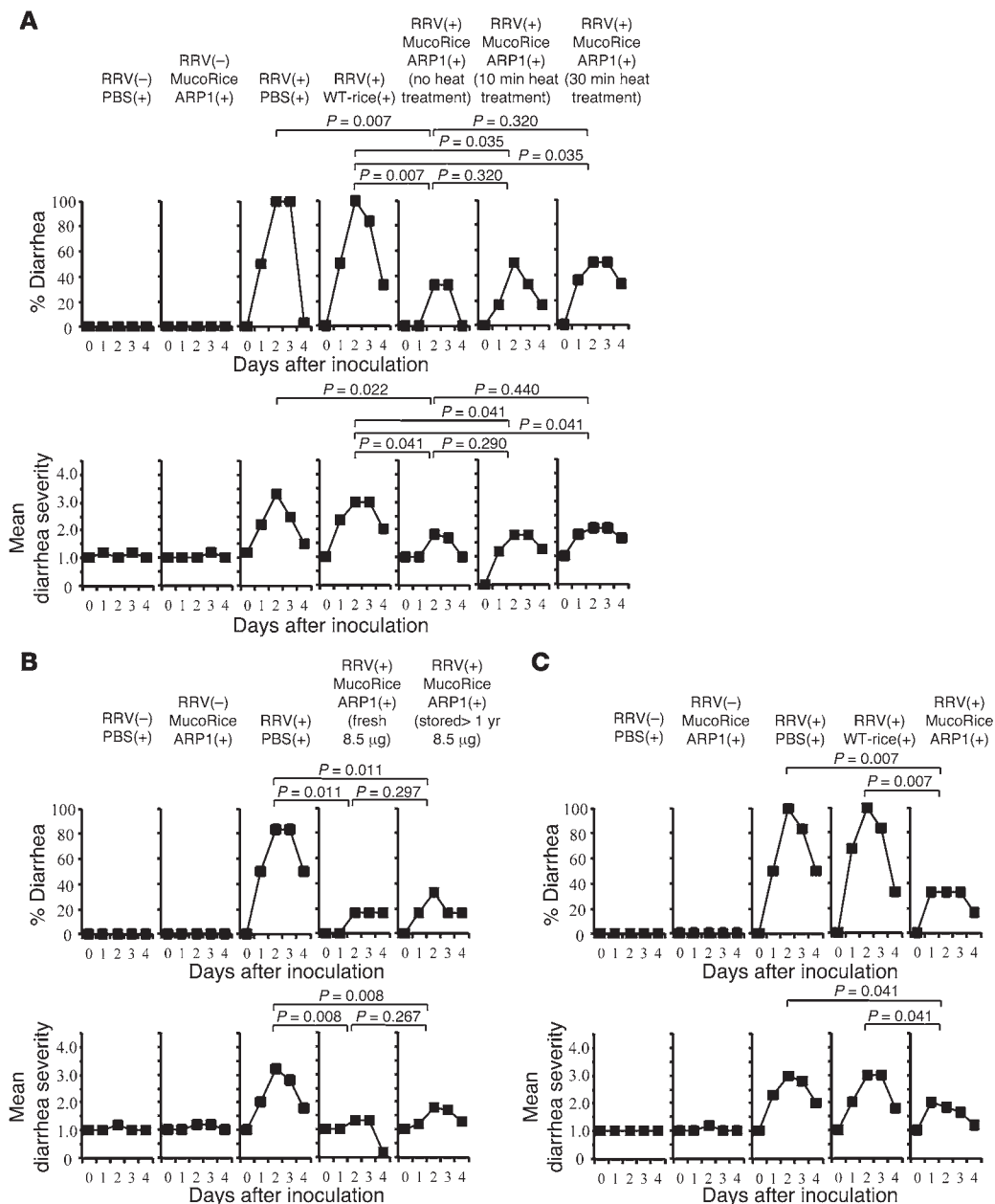


Figure 3

MucoRice-ARP1-mediated protection in a mouse pup model of RV infection. **(A)** Prophylactic administration of MucoRice-ARP1 containing rice water (8.5 µg of ARP1) given to 4-day-old neonatal mouse litters 9 hours prior to RRV inoculation and subsequently given twice daily for 4 consecutive days. Results are shown as percentage of mice with diarrhea and mean diarrhea severity. **(B)** Prophylactic administration of long-term stored MucoRice-ARP1 containing rice water given 9 hours prior to RRV inoculation and subsequently given twice daily for 4 consecutive days. **(C)** Therapeutic administration of MucoRice-ARP1 (8.5 µg of ARP1) given 9 hours after RRV inoculation and subsequently given twice daily for 4 consecutive days. Statistical significance among groups was calculated using the Kruskal-Wallis test followed by the Mann-Whitney *U* test.

Discussion

Although we have previously developed an oral rice-based vaccine expressing botulinum neurotoxin type A against botulism and CTB for cholera/enterotoxigenic *E. coli* (ETEC) (32, 33), expression of a cytokine or antibody such as human IL-10 or single-chain Fv (scFv) from an M cell-specific monoclonal antibody in rice resulted in insoluble proteins. Thus, we could not apply these proteins orally (data not shown). The present study markedly extends the potential of rice

plants into an antibody production system. Using the MucoRice technology, we successfully produced the variable domain of a RV-specific llama-heavy chain antibody and showed that oral administration of MucoRice-ARP1 afforded protection in mice. Unique features of this product, including water solubility, high expression level, heat stability, and long-term stability upon storage at room temperature, make MucoRice-ARP1 an attractive ready-to-use, oral, anti-RV product that can easily be distributed without the need for a cold chain.

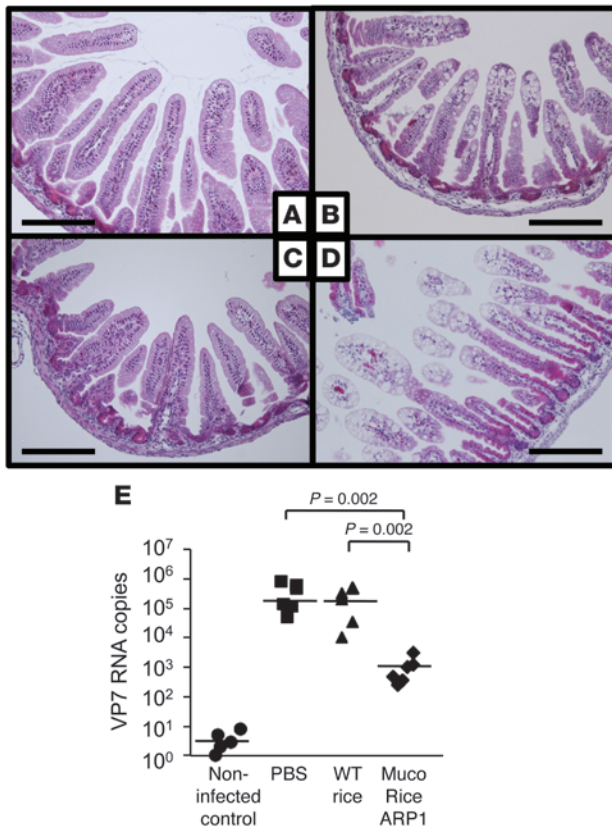


Figure 4

Histopathological examination and quantification of RV in the small intestine after RRV-induced diarrhea. (A–D) Histological sections of small intestine ileal mucosa from a noninfected mouse treated with PBS (A), RRV-infected mouse treated with PBS (B), RRV-infected mouse treated with MucoRice-ARP1 (C), and RRV-infected mouse treated with WT rice extract (D). In the RRV-infected mouse pups treated with PBS (B) or WT rice extract (D), large vacuoles are seen in the enterocytes lining the surface of the villous tips in the ileum. Histopathological lesions were absent in noninfected control mice (A) and pups treated with MucoRice-ARP1 (C). Sections were stained with H&E. Scale bars: 100 μ m. (E) Quantification of RV in the small intestine after MucoRice-ARP1 treatment. Results are given as VP7 RNA copies per weight (mg) of small intestine. Bars indicate the median RV load for each group of animals. Differences in virus load between the MucoRice-ARP1-treated group and the WT rice-fed group or PBS-treated (RRV alone) group were evaluated using the Kruskal-Wallis test followed by the Mann-Whitney *U* test.

The first important aspect of plant-based antibody system is that large amounts of antibodies can be produced at a low cost (39). Various plants have previously been used to produce antibodies and antibody fragments, including scFv, IgG, Fab, and VHH (40–43). The plant-based antibody (fragment) yields are reported as 28–136 mg kg⁻¹ (VHH) and 500 mg kg⁻¹ (fully assembled IgG) in tobacco leaves (40, 41) and 0.67% (scFv) and 3%–6% (Fab) of the total soluble protein in tobacco seeds (43) and *Arabidopsis thaliana* leaves (42), respectively. As suppression of internal storage protein production may enhance accumulation of foreign proteins (44), we introduced RNAi to the ARP1 production system and developed a T-DNA expression system by cointroduction of antisense genes specific for the 13-kDa prolamin and glutelin storage proteins to minimize their expression in order to allow expansion of space for ARP1 in the rice seeds (33). In comparison with those plant-based antibodies (scFv, IgG, Fab, and VHH) described previously (40–43), the MucoRice system achieved an extremely high yield of soluble antibodies (8.5 g soluble ARP1 kg⁻¹ of total weight or 11.9% of total protein) in rice seeds. Most important, MucoRice-ARP1 is highly water soluble (Figure 1C) due to the localization of the ARP1 antibody fragment in the PB-II and cytoplasm fractions, which are the soluble components of rice (Figure 1E).

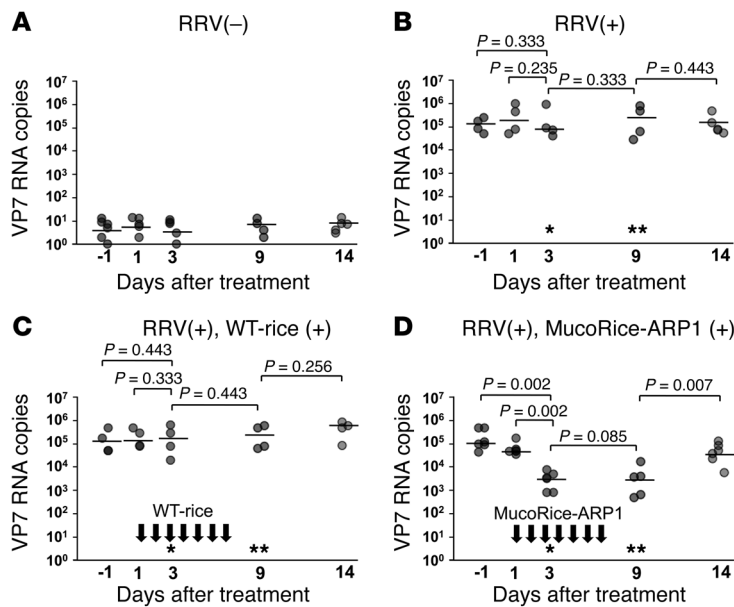
A second important aspect of the plant-based antibody system is that, in contrast with previous systems, there is no need for purification. As MucoRice-ARP1 originates from edible rice seeds, the obtained rice powder can be directly used as an ingredient for use in a broad range of nutraceuticals applied orally as a rice powder or rice water by simply dissolving the MucoRice-ARP1 rice powder in water (at room temperature), thus eliminating the costs for purification.

A third important aspect is that cold-chain transport and storage are unnecessary, as RV antibodies are primarily needed in developing

countries. Antibody-producing tobacco leaves need storage under –20 degrees, and antibody-producing tomatoes need a temperature below 4 degrees. In contrast, antibody-producing cereals such as rice and wheat as well as pea seeds can be stored at room temperature (39). In this study, we demonstrated that MucoRice-ARP1 was stable at room temperature for more than 1 year. Furthermore, in a boiling experiment, extracts from MucoRice-ARP1 demonstrated a higher heat resistance than the purified ARP1 from MucoRice-ARP1 and from yeast-based ARP1, suggesting that the presence of rice proteins in the sample contributes to an increased stability of ARP1. The high stability of MucoRice-ARP1 confers an economic advantage over products that require a cold-chain for distribution and storage. The cost effectiveness of MucoRice-ARP1, added to the fact that rice is one of the most important types of staple food, may contribute to making this product accepted and affordable and thus especially suitable for populations in developing countries where the burden of RV-induced diarrhea is high.

RV-induced diarrhea in humans is caused by RVs belonging to group A. A large number of serotypes have been isolated from infected individuals, although the most common serotypes worldwide are G1P[8], G2P[4], G4P[8], and G9P[8] (45). It has recently been shown that yeast produced ARP1 can neutralize a broad range of virus serotypes in vitro (M37 G1[P6], Wa G1[P8], Rv4 G1[P8], DS-1 G2[P4], P G3[P8], ST-3 G4[P4], Va70 G4[P8], 69M G8[P10], WI-61 G9[P8], F45 G9[P8]) (31). Moreover, it could also bind the recently emerged G8, G10, and G12 strains (31). In this study, we showed that ARP1 produced in rice (MucoRice-ARP1) neutralizes RV strains in a range similar to that of the yeast produced ARP1. Thus, it should be possible to use rice-based ARP1 treatment regardless of geographical differences in strain diversity.

The broad reactivity and neutralizing capacity of ARP1 indicates that it recognizes crossreactive epitopes. The outer layer proteins of RV, VP7 and VP4, both contain neutralizing epitopes, although these tend to be serotype specific (31). The middle layer protein, VP6, is the most abundant and immunodominant viral protein and contains the group and subgroup determining epitopes (31). Iturriza-Gómara et al. recently identified the epitope recognized by ARP1 and showed that it binds to polymeric VP6 (31), thus explaining its broad neutralization capacity. With regard to the role of anti-VP6 antibodies against RV-induced diarrhea, crossreactive anti-RV VP6 antibodies appear to be unable to neutralize infection by immune exclusion both in vitro and in vivo. However, some anti-VP6 secretory IgA bind to RV double

**Figure 5**

Inhibition of viral shedding after MucoRice-ARP1 treatment in SCID mice with chronic RRV infection. (A–D) MucoRice-ARP1 powder (200 mg) containing 1.7 mg of ARP1 was intragastrically administered to mice twice daily for 7 days. Fecal samples were collected on days –1, 1, 3, 9, and 14 ($n = 6$, each group). Control feces were collected from noninfected SCID mice. Positive control feces were collected from RRV-inoculated SCID adult mice treated with PBS. Bars indicate the median RV shedding level for each group of animals. Results are given as VP7 RNA copies per mg of stool. MucoRice-ARP1-treated mice showed a significantly lower amount of VP7 RNA copies on day 3 ($*P = 0.002$) and day 9 ($**P = 0.003$) compared with WT rice-treated or PBS-treated mice. Statistical significance among groups was calculated using the Kruskal-Wallis test followed by the Mann-Whitney U test.

and triple-layered particles, conferring protection by intracellular neutralization following transcytosis in mice and also in vitro using polarized Caco-2 cells (46–48). The mechanism by which these antibodies interfere with RV replication is not yet understood, and it is postulated that they may interfere with early replication events by inducing conformational changes in the VP6 layer, or alternatively, the anti-VP6 antibodies may interfere with the secretory pathways during late replication, preventing virus assembly and release (47). It is also known that not all anti-VP6 antibodies can neutralize RV replication intracellularly (49). Recently, the ARP1 llama-derived antibody that binds specifically to RV VP6 has been shown to bind to RV in ELISA and immune electron microscopy and to neutralize infection with a variety of RV genotypes in vitro using classical neutralization assays and in an in vivo mouse pup model, which suggests that these antibodies are likely to neutralize RV by immune exclusion (31, 50–53). Although the mechanism by which the llama antibodies neutralize infection is not yet understood, it is possible that the VP6-specific VHH could block a conformational change in the outer layer proteins of the RV particle, VP7 and/or VP4, preventing attachment and/or entry of the virus particle, as previously suggested (31, 50). This is supported by existing evidence that the phenotype of the outer layer VP4 can be altered by interactions with different VP7 proteins and of the marked impact of the protein-protein interactions among the constituent proteins of the RV inner, middle and outer layers (54). The neutralizing capacity associated with the recognition of

crossreactive epitopes within VP6 of these antibodies may also relate to their small size, as bivalent VHH antibodies showed a much reduced neutralizing activity compared with the monovalent VHH, suggesting that these may have no or limited access to the exposed VP6 of an infectious triple-layered RV particle (50).

A recent clinical trial in Bangladesh proved that yeast-derived ARP1 was effective in reducing the severity of the disease in RV-infected children with no adverse events considered to be related to ARP1 treatment (S.A. Sarker, M. Jäkel, S. Sultana, N.H. Alam, P.K. Bardhan, M.A. Salam, W. Theis, L. Hammarström, L.G.J. Frenken, unpublished observations), and, as there are no differences in the efficacy between the yeast-derived ARP1 and MucoRice-ARP1 in vitro, the latter has a potential to be equally effective in a clinical setting. With regard to the safety of the rice seed component, we have used a rice-based cholera vaccine in 5 cynomolgus macaques and did not observe any adverse events (55, 56). Rice seed itself is an edible plant; thus, taken together with the present results, we consider MucoRice-ARP1 to be safe and to be able to provide effective protection against RV-induced diarrhea. One remaining problem is the dose translation between mouse and human based on the body surface area normalization between animals and human adults (57, 58). The dose for neonatal (17 $\mu\text{g}/\text{d}$) or adult (SCID) mice (3.4 mg/d) translates as 0.7 mg of ARP1/kg/d (82 mg of MucoRice-ARP1 powder per kg/d) and 20.7 mg/kg/d of ARP1 (107 mg/d of MucoRice-ARP1 powder per mg/d), respectively, in 6- to 24-month-old children. In the recent Bangladesh study, in which 6- to 24-month-old children with RV diarrhea were treated, 5–10 mg of ARP1/kg 3 times per day was used, equivalent to a daily ARP1 dose of 15–30 mg/kg/d. Thus, the dose of rice-produced ARP1 we used for neonatal and SCID mice is lower, or at least equivalent, to the dose given to children in the Bangladesh study.

Therapeutic proteins produced using recombinant DNA technologies are generally complex, heterogeneous, and subject to a variety of enzymatic or chemical modifications during expression (59). Since efficacy, clearance, and immunogenicity can be highly dependent on the protein sequence and specific posttranslational modifications, it is important to characterize their structure by determining the sequence of the recombinant protein and amino acid modifications that may affect the safety and activity of therapeutic protein products using analytical tools such as mass spectrometry (60). However, to date there is no report showing the complete amino acid sequence of plant-derived therapeutics, including vaccines, antibodies, or enzymes. Using mass spectrometry, we elucidated the full amino acid sequence of rice-based ARP1, confirming that it does not contain any amino acid modification. The present study is thus the first, to our knowledge, to include this information for the safety and efficacy of the product.

In conclusion, we have developed a high-yield, purification-free, cold-chain-free, and heat stable, rice-based antibody fragment containing product for oral administration that provides protection against RV of different serotypes in vitro and reduction of virus shedding and disease in both immunocompetent and immunodeficient mice. MucoRice-ARP1 offers what we believe is a novel approach to the prevention and treatment of RV-induced diarrhea in both devel-



oped and developing countries and may provide an alternative to vaccination in individuals in whom current vaccines are contraindicated. It could also complement current vaccination programs by targeting different age groups and situations in which rapid protection or control of spread of infection is required. This technology can also be extended to the production of antibody fragments against other enteric pathogens such as norovirus, and may also be applicable to intestinal diseases beyond infections.

Methods

DNA constructs and transformation of rice plants. ARP1 was selected from a library of VHH fragments generated from llamas immunized with RRV (strain MMU18006, P5B[3], G3) (61), as previously described (29). Briefly, a phage display library was constructed from B lymphocyte cDNA encoding VHHs and selected by biopanning on RRV at a low pH (2.3). ARP1 was previously referred to as 2B10 (29) or VHH1 (30). The gene encoding ARP1 was synthesized with an optimized codon usage for plants and inserted into a binary T-DNA vector (pZH2B/3SSNos). This vector contains a cassette for overexpression of ARP1 and a combination cassette for RNAi suppression of production of the major rice endogenous storage proteins, prolamin (13 kDa) and glutelin (Figure 1A and ref. 35). The plasmid was transformed into a japonica variety of rice plants, Nippon-Bare, using a *Agrobacterium*-mediated method described previously (32).

Protein analyses. Total protein was extracted from transgenic rice plant seeds using a buffer containing 2% (wt/vol) SDS, 8 M urea, 5% (wt/vol) β -mercaptoethanol, 50 mM Tris-HCl (pH 6.8), and 20% (wt/vol) glycerol as previously described (62). The level of ARP1 was determined by Western blot densitometry analysis using purified yeast-derived ARP1 (BAC BV) as a standard. For detection of ARP1, a rabbit anti-ARP1 polyclonal antibody was prepared in our laboratory.

ARP1 was also extracted from MucoRice-ARP1 powder using PBS (rice water) or 8 M urea in PBS at room temperature. After centrifugation, the supernatants were analyzed by SDS-PAGE and Western blot. Purified ARP1 from MucoRice-ARP1 was produced from MucoRice-ARP1 containing rice water, using gel filtration on Sephadex G100 columns (GE Healthcare).

Mass spectrometry. Mass spectrometric analysis was performed as previously described (63). Samples were injected into a nanoflow LC system (Dina; KYA Technologies) and sprayed into a quadrupole time-of-flight tandem mass spectrometer (QSTAR Elite; AB SCIEX). The QSTAR analysis failed to detect 39 amino acids of the C-terminal peptide generated by trypsin because of the large mass (4256 Da) (Supplemental Table 1). To obviate this problem, a linear ion trap Orbitrap mass spectrometer (LTQ Orbitrap Velos; Thermo Fisher Scientific), which enables shotgun proteomics analysis with high resolution and high mass accuracy, was used. The data were analyzed using a Mascot Search Server.

Immune electron microscopy. The distribution of ARP1 in rice seeds was analyzed using immunoelectron microscopy as previously described, with some modification (32). Rice caryopses sections stained with a rabbit anti-ARP1 polyclonal antibody and gold particle-conjugated (18 nm) goat anti-rabbit IgG (Jackson) were examined using transmission electron microscopy (HITACHI). For localization of ARP1 in the seed, a frozen section was stained with rabbit anti-ARP1 polyclonal antibody and visualized using 3,3'-diaminobenzidine.

Binding to RRV by ELISA. ELISA plates were coated with HBC anti-RRV (38) as a capture antibody, followed by RRV (2×10^6 ffu ml⁻¹) and ARP1 (2-fold dilutions between 100 and 12.5 ng ml⁻¹). Biotinylated rabbit anti-VHH K492 antibody (BAC BV), followed by AP conjugated streptavidin (BD Pharmingen), was used for quantification of ARP1 bound to RRV. The assay was developed using para-nitrophenol phosphate (pNPP) (Sigma-Aldrich) as a substrate, and the optical density was read at 405 nm using a Varioskan

Flash (Thermo Electron Corporation). HBC was produced by vaccination of pregnant cows in a Swiss dairy farm with human strains of RV, i.e., Wa, RV3, RV5, and ST3, representing serotypes G1 to G4 (38).

In vitro heat stability test. To test the heat stability, samples with 100 ng ml⁻¹ of different ARP1 preparations (MucoRice-ARP1 containing rice water, ARP1 purified from MucoRice-ARP1, and ARP1 purified from yeast) and HBC containing 100 ng ml⁻¹ of total protein were boiled at 100°C for 10, 20, and 30 minutes. After cooling, 2-fold dilutions of each sample were tested in ELISA as described above for the ARP1-containing samples. For the HBC samples, a rabbit anti-RRV K230 antiserum (a gift from Lennart Svensson, University of Linköping, Linköping, Sweden) was used as a capture antibody for RRV. The functional anti-RRV IgG antibodies contained in the HBC samples were detected using AP-conjugated goat anti-bovine IgG (H+L) (Jackson ImmunoResearch Laboratories). The percentage of binding activity was calculated in relation to nonboiled samples in the same ELISA plate at a particular concentration before reaching binding saturation (25 ng ml⁻¹ ARP1 for ARP1-containing samples and 50 ng ml⁻¹ total protein for HBC samples).

In vitro neutralization assay. In vitro neutralization assays were carried out using MA104 cells and the human RV strains Wa G1P[8], ST-3 G4P[6], 69M G8P[10], F45 G9P[8] and Va70 G4P[8] as previously described (64). Briefly, 10^5 MA104 cells ml⁻¹ were seeded in 96-well plates. Forty-eight hours later, 2-fold dilutions of antibodies or rice protein preparations were incubated in duplicates with 200 ffu of RVs, and the mixture was subsequently used for infection of the seeded cells. Infected cells were detected by immunofluorescence staining using a monoclonal mouse anti-VP6 antibody (Austral Biologicals) and FITC-conjugated rabbit anti mouse IgG antibodies (Dako). Significant neutralization was defined by a reduction of the infected cells higher than 60% in relation to the control wells.

In vivo assays. Pregnant BALB/c mice were purchased from Japan SLC. Each dam was housed individually with her litter in cages in the same room under negative pressure in the animal facility on a 12-hour light/12-hour dark cycle. Food and deionized water were autoclaved and provided ad libitum.

To determine the level of protection against RRV infection conferred by MucoRice-ARP1 in immunocompetent mice (BALB/c), 4 day-old pups were infected orally using 2×10^7 ffu trypsin-activated RRV ($n = 6$ to 10 per group). Rice water derived from the supernatant of a mixture of MucoRice-ARP1 powder and PBS, containing a total of 8.5 μ g of ARP1, was intragastrically administered to the pups 9 hours prior to RRV inoculation and subsequently given twice daily for 4 consecutive days. In order to examine the heat stability and long-term stability at room temperature, MucoRice-ARP1 heat-treated at 94°C for 10 and 30 minutes or stored at room temperature over 1 year was intragastrically applied to the pups prior to RRV inoculation and subsequently given twice daily for 4 consecutive days. In order to examine the therapeutic effects of MucoRice-ARP1, MucoRice-ARP1 was intragastrically given to the pups 9 hours after RRV inoculation and subsequently given twice daily for 4 consecutive days. In the control groups, PBS or MucoRice-ARP1 was intragastrically administered twice daily for 4 consecutive days without RRV inoculation. The pups were examined daily for evidence of RV-induced diarrhea by gentle abdominal palpation. Diarrhea was recorded and scored blindly from 1 to 4 based on stool color, amount, and consistency as described previously, with a minor modification (65). Normal feces or absence of feces were given a score of 1. Exceptionally loose feces were given a score of 2. Loose yellow-green feces were given a score of 3. Watery feces were given a score of 4. A score of 2 or greater was considered diarrhea, as described previously (65). The percentage of mice with diarrhea for each group was calculated by dividing the number of diarrheic samples by the total number of mice scored for diarrhea each day. The diarrhea severity was determined by dividing the sum of all scores by the number of total number of mice scored for diarrhea each day. Finally, we calculated percentage of mice with diarrhea on a daily basis in each group based on accumulated data



after repeated experiments and compared the percentage of diarrhea and the mean diarrhea severity on day 2 after RRV inoculation among groups using the Kruskal-Wallis test followed by the Mann-Whitney test. Samples of small intestine were collected 3 days after infection for histopathological analysis and viral RNA quantification by real-time PCR against VP7 RNA.

Evaluation of histopathology. To evaluate histopathology, small intestinal samples were collected at 3 days after RRV inoculation ($n = 3$ for each group). Samples from duodenum, jejunum, and ileum were fixed in 4% paraformaldehyde for 12 hours. Subsequently, the samples were transferred to graded ethanol for dehydration, embedded in paraffin wax, and sectioned at 4 μm using a microtome. Sections were stained with H&E (65) and visualized under light microscope. Duodenal, jejunal, or ileal villi were examined for presence of enterocyte injury, inflammation, and vacuolization by a person blinded to the treatment given to the mice.

Quantification of viral RNA. Total RNA was isolated from small intestines of neonatal pups, using TRIzol reagent (Life Technologies) and treated with RNase-free DNase (QIAGEN) following the manufacturer's protocol. To evaluate viral shedding in the chronic RRV infection SCID mouse model, fecal specimens were prepared as 10% suspensions with PBS, and total RNA was isolated using TRIzol following the manufacturer's protocol. RV VP7 mRNA or viral genomic RNA was amplified at 58°C in the presence of 600 nmol l⁻¹ primers, 300 nmol l⁻¹ probe, and 5 mmol l⁻¹ Mn to generate a 121-bp-long amplicon. The sense primer (VP7 forward, 5'-CCAAGGAAAATGTAGCAGTAATC-3'; nt 791-815), the antisense primer (VP7 reverse, 5'-TGCCACCATTCTTTC-CAATTAA-3'; nt 891-912), and the probe (5'-6FAMTAACGCTGATCCAAC-CACAGCACCTAMRA-3'; nt 843-867) were designed on the basis of the VP7 gene sequence of RRV (GenBank AF295303). Reverse transcription reactions were carried out in a final volume of 20 μl using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. Each RT reaction sample was analyzed by the Light-Cycler 480 System II (Roche Applied Science) following the manufacturer's protocol. A standard curve was generated using a plasmid that contained a RRV VP7 gene, and the lowest level of detection of the PCR was 10 viral RNA copies. The presence of less than 10 copies of VP7 RNA per weight (mg) was defined as clearance of infection. The ratio of VP7 gene copy number to the weight of the stool sample (mg) or to the weight of the small intestine segment (mg) was compared among the groups.

In vivo assays in SCID mice. The efficacy of MucoRice-ARP1 against chronic RRV infection was determined in SCID mice. Pregnant C.B-17 SCID/SCID mice were purchased from Nihon Clea Inc. Four-day-old pups were infected orally with 2×10^7 ffu RRV. All of the mice developed diarrhea and chronic infection. The litters were weaned by removing each mouse from the dam at 21 days of age. After inoculation, all mice were confirmed to have low serum immunoglobulin levels by ELISA and examined for pathogen surveillance, but no specific pathogen, including murine norovirus, was found at 5 weeks of age. When mice were 6 weeks old (i.e., 6 weeks after RRV infection), 200 mg of MucoRice-ARP1 powder (containing 1.7 mg of ARP1) or nontransformed WT rice powder was intragastrically administered twice daily for 7 consecutive days. Mice were examined on days -1, 1, 3, 9, and 14 for diarrhea and viral shedding. Diarrhea scores, percentage of mice with diarrhea, and disease severity were measured as described above. Viral shedding was measured by VP7-specific real-time PCR using fecal samples. In order to examine the therapeutic

effects of MucoRice-ARP1 in SCID mouse pups, MucoRice-ARP1 was intragastrically given to the pups 9 hours after RRV inoculation and subsequently given twice daily for 4 consecutive days. Diarrhea scores, percentage of animals with diarrhea, and disease severity were measured as described above.

Statistics. Individual data for the percentage of mice with diarrhea, the disease severity scores, and differences in the intestinal virus load as assessed by real-time PCR were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney test. $P < 0.05$ was considered statistically significant.

Study approval. All mouse experiments were approved by the local ethics committee of the Institute of the Medical Science at the University of Tokyo.

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Address correspondence to: Yoshikazu Yuki, Department of Mucosal Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo, 108-8639, Japan. Phone: 81.3.5449.5274; Fax: 81.3.5449.5411; E-mail: yukiy@ims.u-tokyo.ac.jp.

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