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Protective Immune Response in BALB/c Mice Induced by the E_{72-296} Protein of Rubella Virus

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The goal of this study is to assess the protective efficiency of the E_{72-296} protein, a candidate for a safer rubella virus vaccine. BALB/c mice were immunized with purified E_{72-296} protein, RV JR23 strain or Attenuated RV Vaccine. Anti-RV antibodies were tested by indirect ELISA, and cell immune responses were examined by lymphocyte proliferation assay. NK cell activity and challenge tests were assayed to evaluate the safety of the E_{72-296} protein. In this study, the RV-specific ELISA antibodies induced by the E_{72-296} protein were obtained. Mice immunized with the E_{72-296} protein had effective protection against RV when challenged with RV JR23 ten days after triple immunization. Further analyses of cell-mediated immune responses showed that the E_{72-296} protein induced positive cell-mediated immunity (CMI) (stimulation index ≥2.0). In addition, it was noticed that E_{72-296} protein could not alter splenic NK cell cytotoxicity, which was reduced in mice immunized with the Attenuated RV Vaccine. This suggested that E_{72-296} protein was safer than latter. These results indicated that the E_{72-296} protein not only induced complete protection against RV challenge in mice, but is also a candidate for a safer vaccine.

Key words: Rubella virus; *Pichia pastoris*; E_{72-296} protein; NK cell activity; Protective immune response.

Rubella is a mild, self-limiting disease in humans [1-3], but it occasionally causes death and congenital rubella syndrome (CRS) of the fetuses in pregnant women [4]. Rubella virus (RV) belongs to the Togaviridae family of positive-strand RNA viruses and it contains two envelope glycoproteins, E1 and E2 (481 and 282 amino acids long, respectively). E1 glycoprotein, structurally homologous to viral class II fusing proteins and featuring three 2-sheet-rich domains [5], is a type I membrane protein inserted in the viral enveloping membrane [6]. Further studies of E1 have revealed that E1 carries the principal antigenic determinants, and is responsible for receptor recognition and low-pH-triggered membrane fusion upon internalization through receptor-mediated endocytosis [5].

In the past years, RA27/3 vaccine has been comprehensively used as an attenuated vaccine because of its high immunogenicity, durability to antibodies and less adverse reaction in children [7-8]. However, women who are in the first trimester of pregnancy cannot be vaccinated because of its uncertain safety[9].

Immune epitope studies were promoted for safer and more effective second-generation rubella vaccines such as subunit vaccines. Previous studies showed that E2 and C proteins

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alone are not sufficient to protect animals against RV infection and thus E1 has been examined in several studies [21]. Further studies have found that the presence of antibodies binding to amino acids 223-239 of *RV* E1 could be used as a measure for protection against viral diseases [10]. Moreover, various studies have concluded that the neutralizing anti-E1 antibodies instead of cell-mediated immunity contribute to the protection against RV [11-13]. For these reasons, it was deduced that a protein that contains major epitopes of E1 could elicit strong humoral immunity conferring protection against rubella. Then, various forms of E1, including purified recombinant E1[9,14] and E1-derived peptides[10,15], have been investigated for their immunogenicity. Although neutralizing anti-rubella virus antibodies are elicited following immunization of mice with those vaccines, the mechanisms underlying successful vaccination against E1 are poorly understood. Whether those vaccines can induce significant humoral immune responses in animal models against RV challenges, as well as the exact mechanism of protection, remain unclear. Therefore, the aim of this work was to assess the immunogenic properties and protective value of a recombinant pseudotype *Pichia pastoris* encoding the RV E_{72-296} protein against challenge of RV JR23 strain in a BALB/c mouse model.

MATERIALS AND METHODS

Strains, vectors, chemicals, media

Pichia pastoris host strain GS115 has previously been described [14]. pGAPZαA from Invitrogen was used for cloning and expression studies. All chemicals and media components were obtained from Sigma-Aldrich, primers were synthesized by Sangon Biotech Co., and enzymes were purchased from New England Biolabs.

Designation and synthesis of the E_{72-296} gene

The sequence of E_{72-296} from the RV JR23 strain was used to design a synthetic gene encoding 225 residues of region $E1_{72}$-$E1_{296}$ of the RV JR23 strain E1 glycoprotein. The synthetic E_{72-296} gene was produced by Sangon Biotech Co., and analyzed using SPSS 16.0.

Construction of plasmid pGAPZaA-E_{72-296}

The *RV* E_{72-296} codon-optimized cDNA sequence (completed by Sangon Biotech Co., Ltd) encoding region from $E1_{72}$ to $E1_{296}$ of *RV JR23* strain glycoprotein E1 was amplified by polymerase chain reaction (PCR) using a pair of specific primers containing 5'-CCGGAATTCGCAAGAATC TGGAACGGT-3' and 5'-TGCTCTAGATTATTA TTAATAAGGTCCAGCTCGAAT-3' (sites for EcoR I and Xba I are marked).

The PCR fragment with the E_{72-296} gene was purified with agarose gel and cloned into the pGAPZαA vector using the *EcoR* I and *Xba* I restriction sites. The positive clones were identified by colony PCR using the special primers. The resulting pGZPZαA-E_{72-296} clone was subjected to restriction enzyme digestion and sequence verification.

Transformation and Expression

Recombinant plasmid DNA for pGAPZαA-E_{72-296} and pGAPZαA were purified with agarose gel and linearized by the *Bln* I restriction enzyme. Competent *P. pastoris* GS115 cells were prepared according to the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). Linearized DNAs were transformed into yeast cells by electroporation according to the MicroPulser Electroporation protocol [22]. Apparatus operating instructions plated and cultured in YPD plates with Zeocin at 30 °C for two days to express the protein. The expression supernatant was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and Flow cytometry analysis.

Purification of the recombinant E_{72-296} protein

The culture supernatant was collected by centrifugation at 10,000 g for 10 minutes at 4 °C , and sterilized by filtration (Millex-GP Filter Unit, 0.22 µm, Millpore). The expressed fusion proteins were subsequently purified by cation-exchange chromatography using an Amersham column (COLUMN XK16/20). The E_{72-296} protein was then loaded on a SP Sepharose Fast Flow column (Äkta purifier Amersham Biosciences, Uppsala, Sweden) equilibrated in 20 mM HAc-NAc, pH 5.4, and eluted with a NaCl gradient in 20 mM HAc-NAc, pH 5.4. The fractions were subsequently analyzed on Coomassie stained 12% SDS-PAGE gels and the fractions containing the E_{72-296} glycoprotein were desalted by a ZebaTM Desalt Spin Column (No.89893, Pierce Biotechnology, USA) according to the instructions of the product. After being pooled and concentrated (Millpore-Amicon Ultra-15 Centrifugal Filter Units -3000NMWL), the

purified protein was assessed by Coomassie blue staining of 12% (v/v) SDS-PAGE, Western blotting, mass spectrometry (MALDI-TOF, Bruker Daltonics, Billerica, MA) and quantified using bicinchoninic acid assay (SinoBio).

Mice immunization

72 6-week-old female BALB/c mice (eight per group) were randomly divided into nine groups (as shown in Table 1). Two groups were intraperitoneally injected with 100 µg of E_{72-296} glycoprotein and 25 µl of alum adjuvant (Imject Alum, Thermo, Rockford, USA). Another two groups were intraperitoneally injected three times with 10^4 50% tissue culture infective doses ($TCID_{50}$) of the RV BRD 2 strain (Attenuated RV Vaccine) every two weeks (the infective doses were determined in previous studies). The third two groups were intraperitoneally injected with 10^4 $TCID_{50}$ of the RV JR23 strain every two weeks. Sera were collected 0, 14, 28, and 38 days after primary immunization to detect RV-specific ELISA antibodies. On day 38, sera were collected for neutralizing antibodies against RV, and the splenocytes of mice in groups 1, 2, 3 and 4 were isolated for lymphocyte proliferation response and lactate dehydrogenase release. Anti-RV IgG titers were determined in mouse serum using the CaptiaTM Rubella IgG kit and bound IgG was revealed by peroxidase-conjugated goat anti-mouse immunoglobulin G (Dako, CA, USA). All experimental protocols in this study were conducted in accordance with the policies established in the Guide to the Care and Use of Experimental animals.

Table1. Groups of Immunization

Groups	Immunization	Alum Adjuvant	Challenge
1	No		
2	RV JR23		
3	E72-296 vaccine	Yes	
4	BRD 2 strain (Attenuated RV vaccine)		
5	No		
6	No		RVJR23
7	RV JR23		RVJR23
8	E72-296 vaccine	Yes	RVJR23
9	BRD 2 strain (Attenuated RV vaccine)		RVJR23

RV infection and determination of apoptosis by Flow Cytometry (FCM)

Vero cells were plated at 20,000 cells/well in 6-flat bottom well plates. 24 h later, the cells were infected with the RV JR23 strain pre-incubated either in the presence or the absence of pooled serum. Pooled serum from each group (n=6-8 mice per group) of immunized or control mice was 0.22 µm-filtrated and treated at 56 °C for 30 min to inactivate complement. Sera were then 1:10 diluted (according the results of preliminary experiment and literature [9]). Sera in EP tubes were incubated at room temperature for 1 h with 100 $TCID_{50}$ of RV JR23 strain. 48 h later, apoptosis was assessed by using an annexin-V/PI kit following manufacturer's instructons (Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit, Invitrogen).

Lymphocyte proliferative response

Single-cell suspensions were prepared aseptically from mice spleens, and splenic lymphocyte proliferation was tested by MTT assays as previously described [16-18] and Concanavalin A (5µg/ml; Sigma) was used as a positive control.

Assay for natural killer (NK) cell activity

Single spleen cell suspensions (2×10^7 cells/ml) and target cells YAC-1 cells (4×10^5 cells/ml) were seeded into 96-well micro-plates (effect:target ration, 50:1). The cells were incubated in a, 5% CO_2 incubator at 37 °C for 4 h and LDH activity was measured as previously described [19-20].

RV JR23 challenge, viral testing and immunohistochemical analysis

Groups of BALB/c mice were immunized as previously described. Ten days after the last immunization, mice of groups 6, 7, 8 and 9 BALB/c mice were challenged with 10^4 $TCID_{50}$ of RV JR23.

Mice were sacrificed at three days post-infection, whole blood was collected for RT-PCR [22] and the spleen, liver, kidneys, heart and lungs of mice were aseptically removed, weighted and fixed for immunohistochemical analysis. The whole blood of protected and unprotected mice following RV challenge were collected and examined for the presence of viremia by reverse-transcriptase polymerase chain reaction (RT-PCR) as previously described [23-24]. For immunohistochemical staining procedures, anti-*Rubella virus* E1 antibodies (1:50, Abcam, UK) and goat-anti-mouse secondary antibodies (1:200, Dako, CA, USA) were used. The relative weight of the organs of each mouse was calculated as organ weight (mg)/body weight (g).

Statistics and analysis

Values are given as the mean±SD. Comparisons between two values were performed using ANOVA while statistical significance was assessed by conducting the Bonferroni test. A confidence level of $p<0.05$ was considered significant.

RESULTS

Efficient expression of E_{72-296} peptide in Pichia pastoris and E_{72-296} protein purification

The synthetic E_{72-296} gene (675 bp) with optimized-codon was sub-cloned and expressed in *Pichia pastoris*. The recombinant expression plasmid, designated as pGAPZαA-E_{72-296} was constructed and transformed into Pichia pastoris GS115 cells (Figure 1A and 1B). Cellular expression of the E_{72-296} protein was examined in GS115 cells by flow cytometry with anti-E1 monoclonal antiboies (Figure 1D), and 32.46% of GS115 cells showed positive in E_{72-296} expression. The data suggested that the E_{72-296} protein was efficiently expressed in Pichia pastoris GS115 cells.

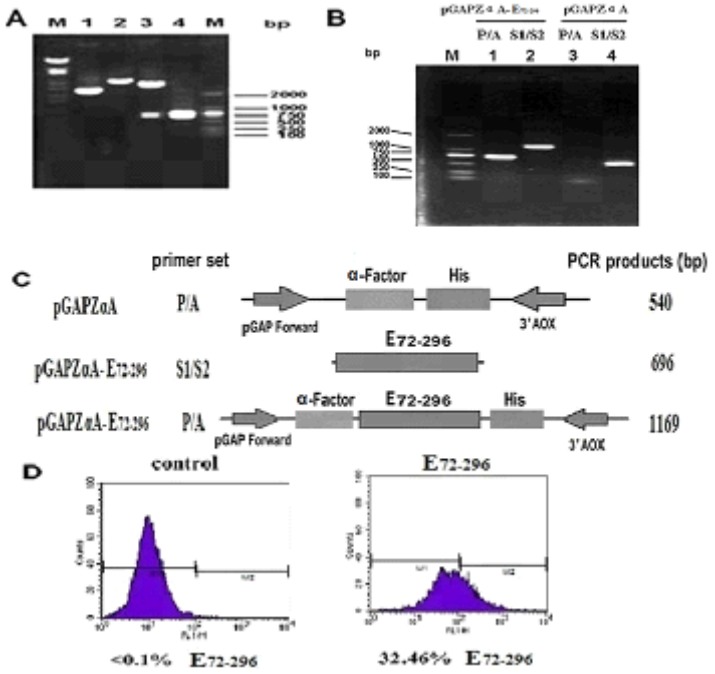

Fig. 1. (A) Identification of construct pGAPZαA-E_{72-296}. Lane M, DNA marker; lane 1, plasmid pGAPZαA-E_{72-296}; lane 2, pGAPZαA-E_{72-296} treated by EcoR I restriction enzyme digestion: 3.774kb; lane 3, pGAPZαA-E_{72-296} treated by EcoR I/Xba I restriction enzyme digestion: 3.0kb, 0.69kb; lane 4, PCR products of pGAPZαA-E_{72-296} : 0.69kb; lane M, DL2000 DNA marker. (B) and (C) Gel electrophoresis show pGAPZαA-E_{72-296} insertion (B) by PCR analysis and schematic representation (C) of PCR products and expected fragment sizes. lane M, DL2000 DNA marker; Lane 1, PCR analysis of pGAPZαA-E_{72-296} with primer set P/A; lane2, PCR analysis of pGAPZαA-E_{72-296} with primer set S1/S2; lane3, PCR analysis of pGAPZαA with primer set S1/S2; lane4, PCR analysis of pGAPZαA with primer set P/A.(D) Flow cytometry analysis of E_{72-296} demonstrates expression on Pichia pastoris yeast GS115 cells.

The E_{72-296} protein was obtained in relatively pure form after cation column purification using the SP Sephrose Fast Flow column and desalination using the ZebaTM Desalt Spin Column. The result of SDS-PAGE and western blot showed only one band (Figure 2A) and mass spectrometry analysis established that the exact molecular weight of the purified E_{72-296} glycoprotein was 21.06 kDa (Figure 2C). These analytical data indicated that the established purification procedure resulted in >90% of purity of the E_{72-296} glycoprotein.

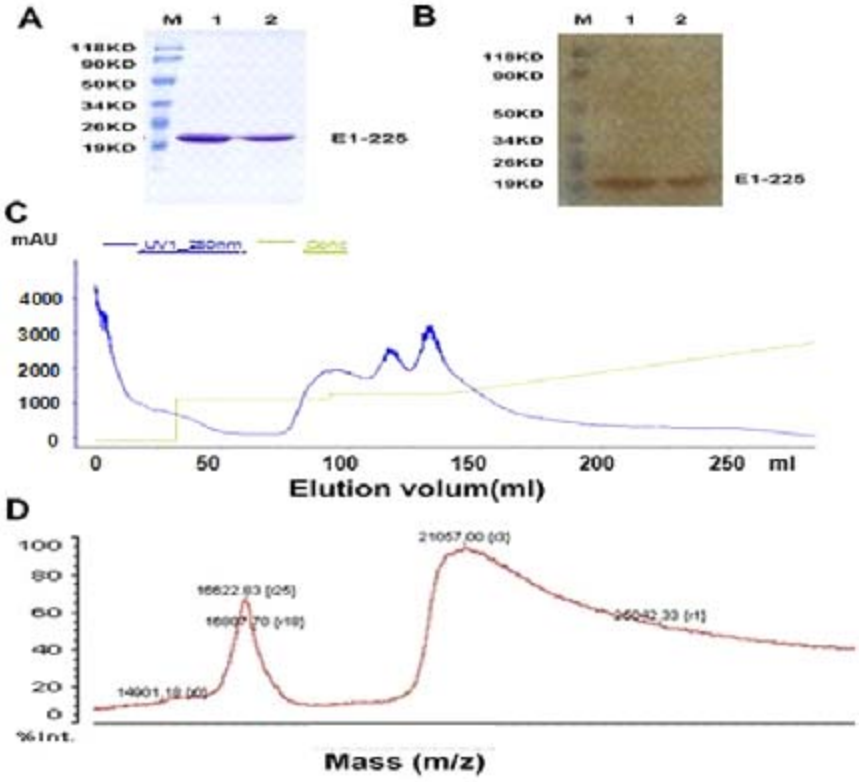

Fig. 2. Purification of rubella virus E_{72-296} protein. The E_{72-296} protein was purified by cation-exchange and desalted by Desalt Spin Columns. (A) and (B) Purified E_{72-296} protein was analyzed by Coomassie-stained SDS-PAGE (left) and Western blot (right). Lane M, molecular weight marker; lane 1-2, 0.22 M Nacl eluted fraction. (C) Chromatographic profile of the E_{72-296} protein purification. Approximately 250ml of supernatant was loaded on a 15 ml SP Sepharose Fast Flow column. After the column had been washed with three bed volumes of 20 mM pH 5.0 NaAc-HAc buffer (buffer A), the protein was eluted with 60 ml of buffer A containing 0.15 M Nacl, 45 ml of buffer A containing 0.17 M Nacl and a 180 ml linear gradient of 0.17-0.4 M Nacl in buffer A. The blue line indicates the elution profile of peptides monitored at 280nm. The yellow line indicates the content of Nacl in elution. There were three fractions, named fraction 1, fraction 2 and fraction 3 (from left to right in Fig.2 C). (D) Fraction 3 was analyzed by mass spectrometry. The profile shows the current intensity as a function of the mass of charge ratio.

Humoral responses induced by E_{72-296} protein immunization

Specific antibodies against RV appeared following injection of RV JR23, RV E_{72-296} and Attenuated RV Vaccine. Immunization with RV JR23, RV E_{72-296} and Attenuated RV Vaccine induced a brisk antibody response, with the antibody measurable in the ELISA that reacted well with the RV JR23 strain, RV E_{72-296} and Attenuated RV Vaccine (Figure 3A). Serum antibody levels in the RV JR23 group were significantly higher than those in other groups. However, there was no significant difference between *RV* E_{72-296} and Attenuated RV Vaccine treated groups in anti-RV antibody level (P=1.000) (Figure 3A). The results indicated that efficient humoral immunity was induced by the E_{72-296} protein.

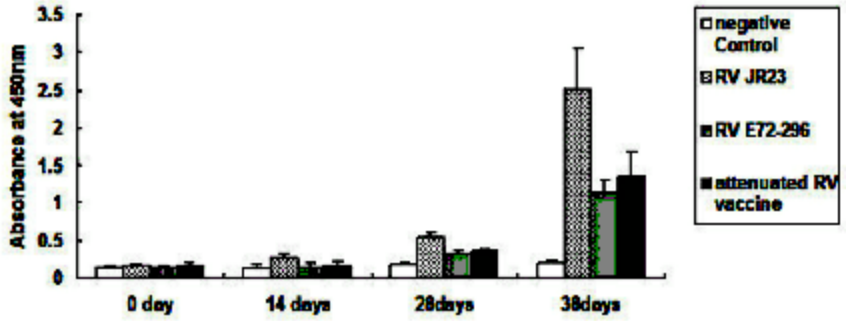

Fig. 3. Anti-RV IgG titer tested by ELISA. The assay was performed in triplicate, and the data are expressed as mean ±SE. Results were analyzed using ANOVA while statistical significance was assessed by conducting the Bonferroni test: *p<0.05; **p<0.01

Virus neutralization by antiserum in vitro

In these experiments, a dose of RV producing 33.65% late apoptotic of Vero cells was incubated in the presence or the absence of serum, and then added to Vero cell layers. By comparison, treatment of Vero cells with the apoptosis-inducing compound camptothecin produced 51.99% late apoptotic cells. As expected, pre-incubation of RV with RV immune mouse serum prevented apoptosis (Figure 4). RV samples pre-incubated with serum from an unimmunized mouse produced 0.02% late apoptotic cells. RV samples pre-incubated with sera from mice infected with RV JR23, immunized with the E72-296 protein and Attenuated RV Vaccine produced percentages of late apoptotic cells that were 12.63%, 14.28% and 15.16%, respectively.

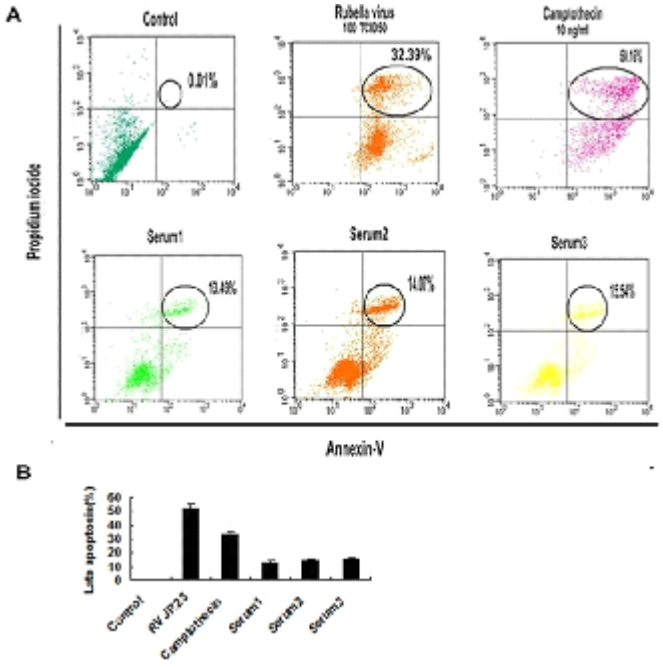

Fig. 4. Neutralization of rubella virus-induced apoptosis by mouse anti-$E_{72\text{-}296}$ protein antibodies. The sera were obtained from mice immunized with the RV JR23 strain (serum1), RV $E_{72\text{-}296}$ Vaccine (serum2) or Attenuated RV Vaccine (serum3) and they were the same as those used in Figure 4. The cells in the upper right quadrant indicate Annexin-positive/PI-positive, late apoptotic cells. Percentages of late stages of apoptotic cells in negative control group, rubella virus group, camptothecin group, serum 1 group, serum 2 group and serum 3 group were 0.02±0.0082, 51.9867±2.8854, 33.6467±1.3077, 12.63±1.827, 14.28±1.2621 and 15.16±0.7073, respectively (The assay was performed in triplicate, results were presented as means ± SD.)

Lymphocyte proliferation assay and splenic NK cell activity in immunized mice

Weak T cell responses were observed in mice immunized with the RV JR23 strain, RV E_{72-296} protein and Attenuated RV Vaccine (Figure 5A). T cell proliferations in mice inoculated with the RV JR23 strain, RV E_{72-296} protein and Attenuated RV Vaccine were significantly higher than those in the negative control group (P=0.003, P=0.001 and P=0.001). In contrast, no difference was observed in T cell proliferations among the groups of the RV JR23 strain, RV E_{72-296} protein and Attenuated RV Vaccine (P=1.000).

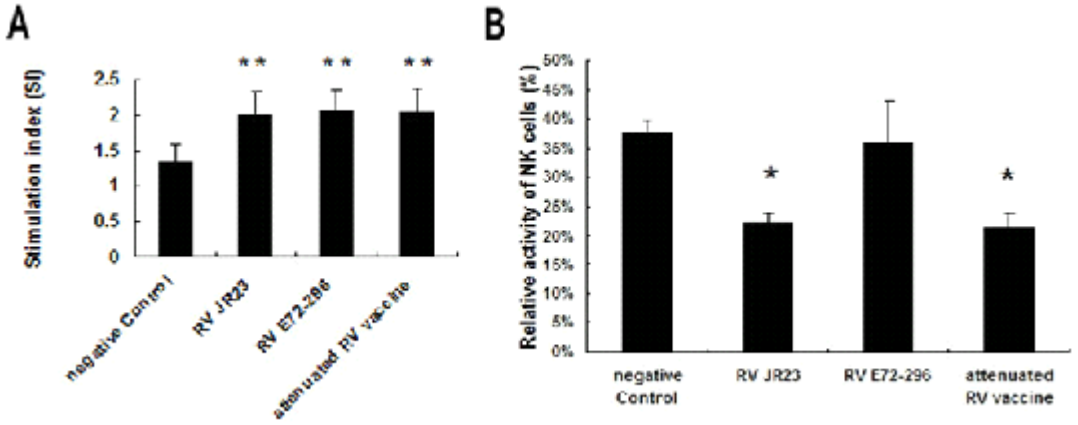

Fig. 5. Proliferative responses of splenocytes from immunized mice (A) and NK activity immunization with RV E_{72-296} vaccine and attenuated RV vaccine (B). The assay was performed in triplicate for every mice (n=6-8), and the data are expressed as mean ±SE. Results were analyzed using Student T test: *****p<0.05; ******p<0.01

The Attenuated RV Vaccine as well as the RV JR23 strain more significantly decreased NK cell activity in mice than those of the control mice (P=0.024,P=0.033), however, the RV E_{72-296} vaccine had almost no effect on NK cell activity as compared with the control (P=1.000). The results indicated that RV E_{72-296} vaccine had almost no effect on NK cytotoxicity as compared with the control, but Attenuated RV Vaccine or RV JR23 increased NK cell cytotoxicity. This indicates that the RV E_{72-296} vaccine was safer than the Attenuated RV Vaccine.

Challenge test in BALB/c mice

The effect of immunization with the RV E_{72-296} vaccine was assessed in a virus challenge experiment in BALB/c mice and the results of immunohistochemical analysis as well as RT-PCR are shown in Figure 6D. The whole blood from the mice of the RV JR23 group, RV E_{72-296} vaccine group and Attenuated RV Vaccine group was negative by RT-PCR while rubella virus was detected in unimmunized mice after being challenged with the RV JR23 strain (positive control group) (Figure 6C). Similar results were found in the immunohistochemical staining results of tissues. In unimmunized mice, RV antigens (RV E1 protein) were present in the spleen, liver, kidneys, heart and lungs at day 3 after being challenged. In the groups of the RV JR23 strain, RV E_{72-296} vaccine and Attenuated RV Vaccine, RV antigen staining was slight in these tissues at day 3 after being challenged (Figure 6A). The IOD per unit area for each group, measured by Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA), also presented the same results (Figure 6B). These results indicate that mice immunized with the RV E72-296 vaccine as well as the Attenuated RV Vaccine were protected against RV infection.

DISCUSSION

The RV E_{72-296} protein that contains residues 72-296 of the RV E1 protein was chosen based upon earlier research suggesting moderate to high antigenic responses in serological assays. In previous studies, four of five antigenic domains are located on E1 between residues 209-239 [16] and residues 245-285 [25]. Residues 273-285 of RV E1 have

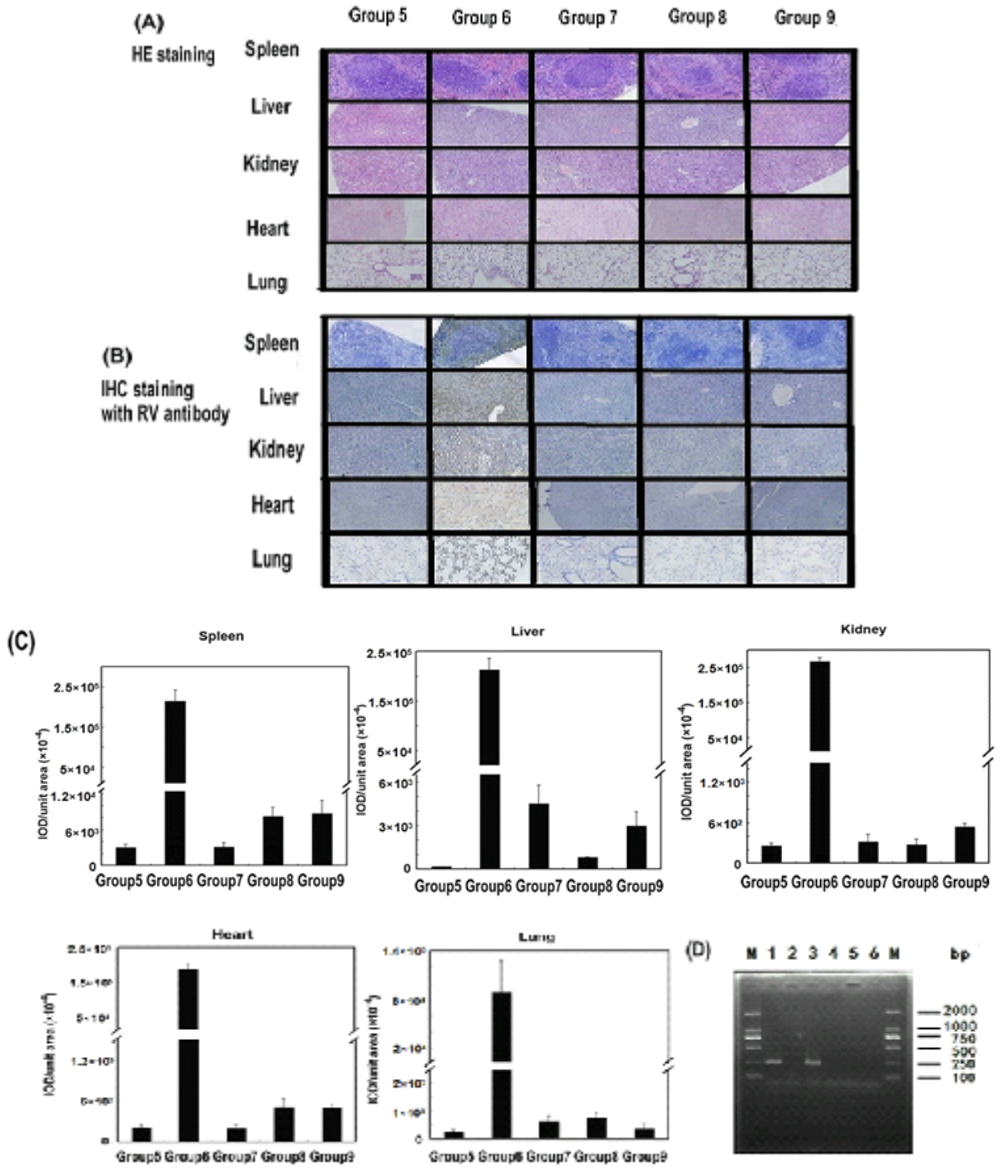

Fig. 6. Photomicrographs of spleen, liver, kidney, heart and lung obtained from various treatment groups (A or B). Data are representative of three independent experiments. Original magnification: × 200. (C) The intensity of immunohistochemistry. IOD, Integrated optical density. The intensity of immunohistochemistry was measured on samples from 3 photographs in each group. (D) RT-PCR analysis of whole blood for Rubella virus RNA. RT-PCR products were visualized on a 2.5% agarose/TAE gel. Positive and negative controls and molecular size markers are present in lane 1, 2 and M, respectively

been revealed by T-cell mapping studies to contain overlapping cytotoxic T-cells (CTL) and NT antibody epitopes [26]. Another study found that peptides E1(213-239), E1(234-252), E1(254-285) and E1(272-285) can increase lymphocyte proliferation [27]. They were recognized to be immunodominant antigenic sites and also known to contain antibody neutralization domains. E1(221-239) was shown to induce RV neutralizing antibodies 14 days after injection into mice in one study [28], and its presence was considered as a correlate for protection against viral disease. The epitope was completely buried at the centre of the E1e trimer [5], indicating that the neutralization mechanism was antibody-bound to

the site which would block E1 trimerization for entry [5]. Another reason was that the gene region of the RV E_{72-296} protein was located in the conservative gene region of E1 gene. It indicated that the E_{72-296} protein would be a more suitable candidate with the broadest application.

The E_{72-296} protein was expressed in the *Pichia pastoris* expression system, which offered economy, ease of manipulation, the ability to perform complex post-translation modifications, and high expression levels [29]. Proteins with a molecular weight larger than the E_{72-296} protein cannot be secreted from *Pichia pastoris* for some unknown reasons in preliminary experiments. Also, codon optimization contributed to the high expression level of the E_{72-296} protein by alleviating the translation inefficiency often caused by ribosomal pauses at rare codons interrupting translation elongation [30]. In this study, about 101mg/L of purified E_{72-296} was obtained by the *Pichia* pastoris expression system when the E_{72-296} codons were improved. The form of the secreted E_{72-296} protein seemed, by screening tools such as Western blot, to be a soluble monomer. It was surmised that the neutralizing epitope was exposed in the E_{72-296} monomer, and elicited neutralizing antibodies bound to the region of $E1_{223}$ to $E1_{239}$ would block E1 trimerization of the virus for entry. It was found that BALB/c mice immunized with E_{72-296} elicited an Ig G antibody response and the specific IgG titers observed demonstrated that no significant difference existed between the subunit vaccine and Attenuated RV Vaccine (BRD-2 strain), and the titers of both were significantly higher than that of the control group. To evaluate the neutralizing capacity of antibodies elicited following mouse immunization with the E_{72-296} protein, a new vitro method was used in this study. In this study, the method was based on the apoptosis observation of cells induced by RV and improved. The quantitative determination of apoptosis on FCM instead of an ELISA kit was used. Indeed, the results were more accurate and visualized than the absorbance values of ELISA. It was considered that the E1(221-239) epitope was clearly accessible on infectious virions [5], and the antisera of mice immunized with the E_{72-296} protein, which have reactive antibodies against the epitope, may react to it and block infection. Thus, little apoptosis occurred in cells when infected RV samples were pre-incubated with the antisera.

To determine the safety of RV E_{72-296}, several tests were performed. No behavioural abnormality were caused in the mice immunized with RV E_{72-296} by the examination, the body weight and the weights of the spleen, liver, kidneys, heart and lungs in BALB/c mice were not affected by RV E_{72-296}, no pathological changes were found in tissues determined by HE stain and NK cell toxicity tests were carried out. In this study, splenic NK cell activity of immunized mice was determined by measuring the release of cellular lactate dehydrogenase (LDH) on YAC-1 lymphoma cell lysis. It is known that NK cells represent a distinct lymphocyte subset with a central role in innate immunity, and serve important functions in influencing the nature of the adaptive immune response [31]. Moreover, NK cells can exert a beneficial effect in eliminating pathogen-infected cells and in controlling tumor development. In this study, low NK cell activity appeared to be associated with *RV*-infection. This decrease may be a consequence of either viral infection of NK cells or the secretion of soluble factors by infected cells that condition NK spontaneous cytotoxicity as previous studies showed [32]. No significant decrease of NK activity occurred in mice immunized with the RV E_{72-296} vacine but mice with the Attenuated RV Vaccine, indicating that the RV E_{72-296} vaccine was safer than Attenuated RV Vaccine because of little NK cell cytotoxicity.

The protective efficiency of RV E_{72-296} was determined by RT-PCR and immunohistochemical analysis after BALB/c mice were challenged. It was reported that RT-PCR assay for the detection of RV has been established [23], and the nucleic acid was extracted from oral fluid, whole blood, sera and PBMCs [21,23]. Previous studies have reported that the persistent time of RV antigen in PBMCs is 2-3 days [22] and the presence of replicating challenge virus in whole blood is at 8-72 h post inoculation [21] which were confirmed by preliminary experiments, thus in this study, the RV antigen in whole blood was detected 48-72 hours after challenge. The basal level of viral RNA present in the inoculum was not detected by RT-PCR at 8-72 h post inoculation using mice immunized with heat-inactivated RV in a prior study [21]. The negative RT-PCR results were obtained from the whole blood of RV-challenged mice that were pre-immunized

with RV, RV E_{72-296} or Attenuated RV Vaccine, while positive results were obtained from RV-challenged mice that were unimmunized. In addition, immunohistochemical analysis showed that the vaccinated mice were able to eliminate the infection and completely recover.

In conclusion, the results indicated that RV E_{72-296} was expressed in a secretory expression system that offered several advantages such as ease of manufacture, low cost and high yield. Above all, RV E_{72-296} was able to induce protective humoral immunity responses after intraperitoneal injection in mice, and the responses were similar to the responses obtained in mice immunized with the Attenuated RV Vaccine. Additionally, detection of NK cells activity in immunized mice suggested that RV E_{72-296} would be safer than attenuated RV strain (BRD-2) as a promising vaccine candidate. Furthermore, the possible protective effect of RV E_{72-296}, which may be exerted by eliminating the infection in less than three days, was demonstrated in the challenge model.

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