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ORIGINAL ARTICLE

Oral immunization with whole yeast producing viral capsid antigen provokes a stronger humoral immune response than purified viral capsid antigen

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Significance and impact of the study: Provoking sufficient antibody responses by oral immunization has been an enormous challenge because of the harsh conditions of the gastrointestinal (GI) tract. Immunization strategies using purified antigen to make oral vaccines are incapable of commercialization because excessive amount of antigen is required to provoke antibody responses. Therefore, resolving the problems concerning the cost and effectiveness of oral vaccines is a high priority. Our results suggest that recombinant yeast has great potential for inducing antigen-specific immune responses by oral immunization. We believe that oral immunization using recombinant yeast can be a breakthrough technology.

Keywords
capsid protein, nodavirus, *Saccharomyces cerevisiae*, vaccine.

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Abstract

Weak antibody responses to protein antigens after oral immunization remain a serious problem. Yeasts have a rigid cell wall and are inherently resistant to harsh conditions, suggesting that recombinant antigens made in yeast could have a greater chance of making contact with the immune cells of the gastrointestinal (GI) tract in intact form. We compared antibody responses to oral immunization with purified recombinant antigen, used in the conventional manner, and responses to whole recombinant yeast producing the antigen intracellularly. Recombinant capsid protein (CP) of red-spotted grouper necrosis virus (RGNNV) was used as model antigen and *Saccharomyces cerevisiae* as host. The purified CP was obtained from the *S. cerevisiae* producing the RGNNV CP. Whole recombinant yeast producing RGNNV CP provoked 9–27 times higher anti-RGNNV CP IgG titres than purified RGNNV CP. Moreover, sera from mice immunized with the recombinant yeast had neutralizing activity against RGNNV, while those from mice immunized with purified CP did not. These results show that whole recombinant yeast is a promising platform for antigen delivery by oral immunization.

Introduction

Delivery of protein antigens to immune cells via the oral route remains an enormous challenge because the oral delivery has been considered to be an ideal and easy way to deliver foreign antigens. However, oral immunization is notorious for resulting in experimental failure in the vaccine field. Most protein antigens are doomed to destruction in the harsh digestive conditions of the gastrointestinal (GI) tract (Murtaugh and Foss 2002). Moreover, antigens tend not to be immunogenic even when they overcome these extreme conditions and encounter mucosal immune cells, because the immune cells of the GI tract treat them as food or normal flora (Murtaugh and Foss 2002; Mestecky *et al.* 2007). Generally, immunization via the oral route requires 100–1000 times more antigen than immunization via the intramuscular or subcutaneous route (Thones *et al.* 2008), indicating that oral

immunization is not a commercial proposition. Therefore, it is essential to obtain strong antibody responses and cost-effective vaccination by developing novel vaccination strategies if the hurdles to oral immunization are to be overcome (Devriendt *et al.* 2012).

Saccharomyces cerevisiae (*S. cerevisiae*) is considered to be generally recognized as safe (GRAS) and is used as food and a feed ingredient (Food and Drug Administration 2001; Salnur *et al.* 2009; Finnis *et al.* 2010). *S. cerevisiae* cells range in size from 2 to 5 μm, a size that is suitable to be taken up by antigen-presenting cells (APCs) (Thomas 1988; Xiang *et al.* 2006). The best-known uptake mechanism for particulate antigen in gut-associated lymphoid tissue (GALT) is transportation through microfold (M) cells, presented in the follicle-associated epithelium, and this transportation allows subsequent uptake by APCs in the subepithelial dome region (Devriendt *et al.* 2012). Moreover, large amounts of recombinant yeast producing protein antigens can be easily obtained at low cost. Therefore, strategies using whole recombinant yeast to deliver protein antigens may have the potential to resolve these problems.

Nervous necrosis virus (NNV) infects more than 20 species of fish worldwide and results in massive mortality in hatchery-reared larvae and juveniles (Grotmol *et al.* 1997; Munday and Nakai 1997; Skliris *et al.* 2001). NNV is a nonenveloped icosahedral virus containing two positive-strand RNAs. A 3·1 kb RNA 1 encodes the viral replicase, while a 1·4 kb RNA 2 encodes the 37 kDa capsid protein (CP) (Schneemann and Marshall 1998; Tan *et al.* 2001). Recombinant CP of NNV is thought to be the most promising vaccine candidate for preventing NNV infection (Liu *et al.* 2006; Thiery *et al.* 2006). Most fish vaccines including a NNV vaccine have been developed as injection vaccines (Plant and LaPatra 2011). Injection vaccination is a reliable strategy for provoking protective immunity, but it has some obvious limitations; the processes of handling anesthetizing and injecting fish are problematic, and injection is also labour intensive and costly (Plant and LaPatra 2011).

Antigen delivery by oral route has been regarded to be the most ideal strategy. Currently, moreover, yeast components such as beta-glucans have been considered as supplements for feed for breeding fish (El-Boshy *et al.* 2010). It can provide valuable insights if orally administered *S. cerevisiae* producing NNV CP can provide protective immunity against NNV. For these reasons, we selected NNV CP as model antigen in the present study.

We investigated serum antibody responses to oral immunization with whole yeast producing a protein antigen intracellularly in mice. The immune responses were compared to those induced by purified antigen as used conventionally for oral immunization. *S. cerevisiae* were used as the host producing the CP of the red-spotted grouper NNV (RGNNV). Our evidence indicates that whole recombinant yeast has great potential as a system for provoking antigen-specific antibody responses.

Results and discussion

The production of CP by recombinant *S. cerevisiae* producing RGNNV CP (referred to as rS) was assessed by Western blot analysis with purified RGNNV CP as standard. As shown in Fig. 1, 48 μg of rS was found to produce 200 ng of CP. In other words, this result indicates that rS produces 4 μg of CP per mg of yeast culture. The experimental rS group received 10 mg of rS, equivalent to 40 μg of CP. Hence, the CP-rS (naked antigen) group was given 40 μg of purified RGNNV CP.

After the 3rd and 4th immunizations, serum anti-RGNNV CP IgG titres and neutralizing activities against RGNNV were determined. The median titre of the CP-rS group after the 3 immunization was 450 while that of the rS group was 4050 (Fig. 2a). The corresponding numbers after the 4th immunization were 450 and 12150, respectively (Fig. 2b). Boosting by repeated oral immunization with purified CP did not enhance the serum antibody response, whereas boosting with rS markedly increased serum anti-RGNNV CP IgG titre. The difference in anti-RGNNV CP IgG titre between the two mouse groups was

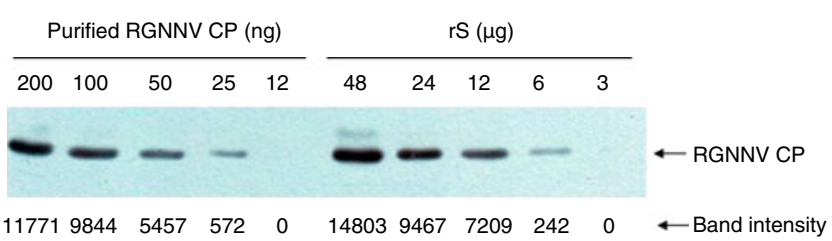

Figure 1 Western blot analysis of red-spotted grouper necrosis virus (RGNNV) capsid protein (CP) produced in rS. CP content of rS was determined by Western blot analysis with purified CP as standard. The amounts of purified CP and yeast cells loaded were based on Bradford protein assays and dry cell weight measurements, respectively. This figure demonstrates that 1 mg of rS contains 4·1 μg of RGNNV CP.

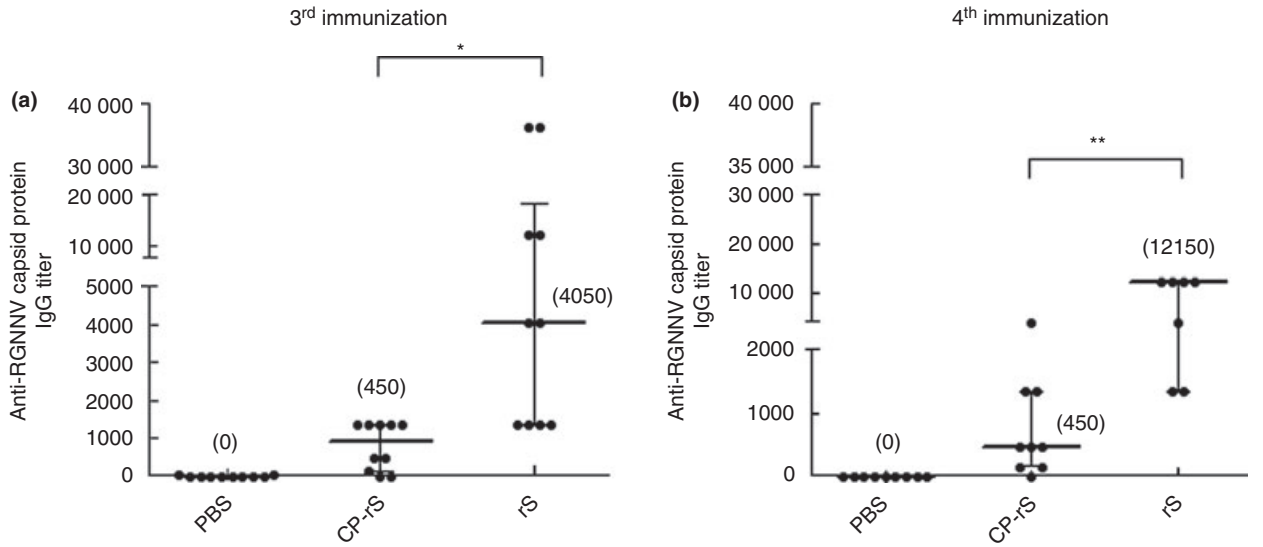

Figure 2 Serum anti-red-spotted grouper necrosis virus (RGNNV) capsid protein (CP) IgG titres following oral immunizations with purified CP and rS. Serum anti-RGNNV CP IgG titres were determined after the 3rd (a) and 4th immunizations (b). The central line represents the median, and the top (Q3) and bottom (Q1) lines, the 75th and 25th percentiles, respectively. 3rd immunization data: PBS, $n = 10$; CP-rS, $n = 10$; rS, $n = 10$. 4th immunization data: PBS, $n = 9$; CP-rS, $n = 9$; rS, $n = 7$. *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$.

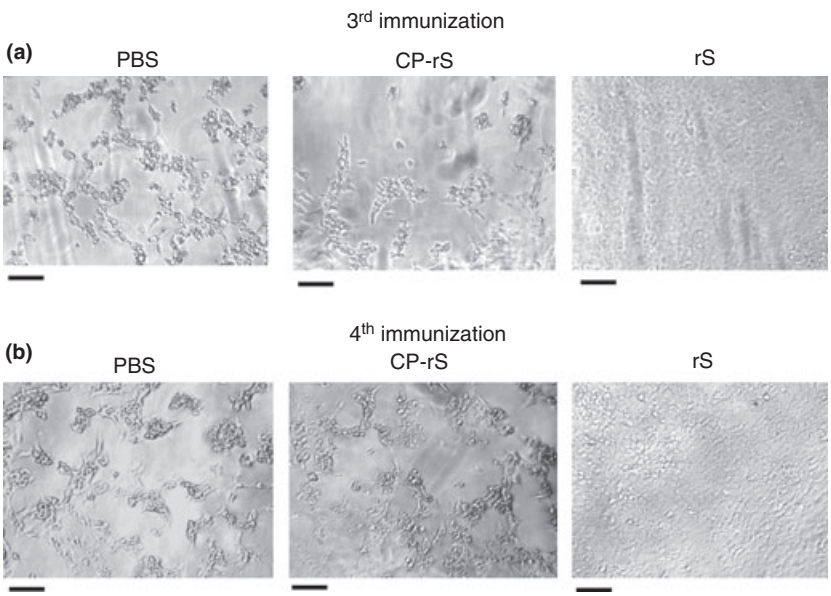

Figure 3 Microscopic analysis of cytopathic effect (CPE) in E-11 cells. The neutralization assay was performed as described in Materials and methods, and CPE was observed by microscope. a and b are the results obtained in the neutralization assay with mouse sera after the 3rd and 4th immunizations, respectively. Bars, 50 μm.

thus 9-fold after the 3rd immunization and 27-fold after the 4th immunization.

As shown in Fig. 3, sera from the mouse rS group significantly inhibited the cytopathic effect (CPE) of RGNNV in E-11 cells, while those from the CP-rS group did not. Similarly, sera from the rS group markedly reduced the amount of CP produced in response to the RGNNV as assayed by Western blot analysis, indicating that the sera had good neutralizing activity (Fig. 4). These results indicate that the antibody response induced by rS is superior to that induced by purified CP with respect to both amount and quality.

Several factors such as antigen integrity, antigen stability and type of adjuvant affect immune responses (Mach *et al.* 2006; Thones *et al.* 2008). Previously, it was suggested that yeasts may have adjuvant activity in oral immunization (Lee *et al.* 2013). Also, it has been shown that most yeast cells retain their morphology although

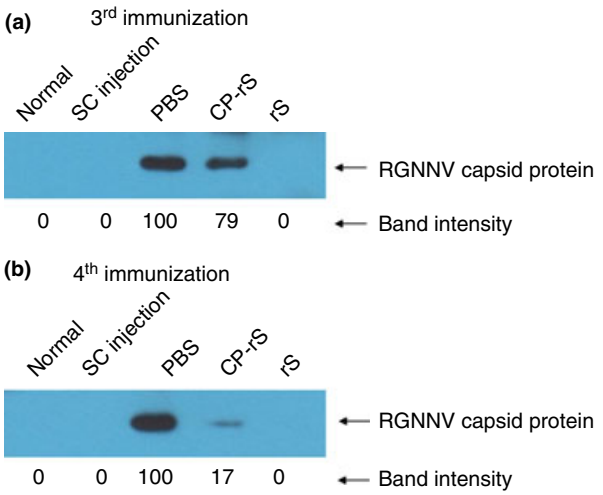

Figure 4 Western blot detection of capsid proteins (CPs) from E-11 cell cultures. Panels a and b are the results obtained in the neutralization assay with sera obtained from mice after the 3rd and 4th immunizations, respectively. The neutralization assay was conducted as described in Materials and methods. Normal refers to cell not infected with red-spotted grouper necrosis virus (RGNNV). E-11 cells were cultured with the mixtures of RGNNV (10^2 $TCID_{50}$) and mouse sera for 3 days, and the cells together with culture medium were harvested and analysed. SC injection is anti-RGNNV CP serum obtained from mice subcutaneously immunized with purified CP (Choi *et al.* 2013). The mice received subcutaneously three doses of 2 μg of purified RGNNV CP in combination with 2 μg of Freund's adjuvant, at 2-week intervals. PBS, CP-rS and rS are mouse sera from mice orally immunized with PBS, CP-rS and rS, respectively. The band intensity of PBS group was set at 100%.

exposed to an extremely low pH similar to that of the stomach (Graff *et al.* 2008).

The possibility of using yeast as a delivery system for protein antigens in oral immunization has been considered for a long time. In the present study, we evaluated only the systemic immune response following immunization with whole yeast producing RGNNV CP. We confirmed that the immunization strategy using CP encapsulated in yeast is more effective in inducing immunity to the CP in the rodent model than naked CP. The immune system of fish is very similar to that of mammals although there are some differences. Affinity maturation and memory responses are found in fish as in mammals (Sunyer 2013). Fish also have T-cell receptors and CD4, CD8, major histocompatibility (MHC) class I, MHC class II, CD28, CD40, CD80, CD86 antigens and produce cytokines for Th1 and Th2 responses (Sunyer 2013). The major immunoglobulin of fish blood is IgM, whereas that of mammals is IgG (Uribe *et al.* 2011). The IgM of fish is known to share some structural and functional characteristics with mammalian IgM (Rombout *et al.* 1993; Pucci *et al.* 2003). MHC molecules play a key role in the immune system by presenting peptides to T lymphocytes, and it has been suggested that the MHC class II genes of fish are similar to those of mammals and share a common origin with those of other vertebrates (Sultmann *et al.* 1993; Deakin *et al.* 2006). These properties of the fish immune system raise the possibility that passive immunization using neutralizing antibodies derived from mouse could provide protection against RGNNV infection and that oral immunization with rS could provoke the production of neutralizing antibodies in fish.

We found that rS, producing RGNNV CP, provokes an efficient antibody response to RGNNV CP, while it does not provoke an antibody response to the antigens derived from *S. cerevisiae* (Fig S1). This confirmed that *S. cerevisiae* itself is not immunogenic when it is administered orally. This result indicates that *S. cerevisiae* has great potential as a carrier to deliver foreign antigen in oral immunization.

The benefits of the recombinant yeast system for antigen delivery as veterinary and fish vaccines are as follows: First, additional manufacturing processes such as purification are not required. Second, the production of recombinant yeast is largely scalable: several thousand litres of recombinant yeasts can be obtained in one fermentation. Third, yeasts are suitable for use as feed ingredients.

In this study, we have shown for the first time that whole recombinant yeast producing RGNNV CP has potential as an oral vaccine. We believe that further development of this system will permit mass vaccination of fish at low cost.

Materials and methods

Preparation of yeasts producing RGNNV CP

A codon-optimized gene encoding the CP of RGNNV (opt-RGNNV-CP) was designed to enhance expression of the CP in yeast, and the opt-RGNNV-CP (Choi *et al.* 2013) was ligated into YEG-αMCS. *S. cerevisiae* Y2805 (*MATα pep::HIS3 prb1-Δ1.6R can1 his3-200 ura3-52*) was transformed with the YEG-αMCS-opt-RGNNV-CP. The resulting strain, referred to as rS, was cultured in YPDG broth containing 4% glucose, 4% galactose, 1% yeast extract and 2% peptone (all Duchefa Ltd, Haarlem, Netherlands) for 3 days at 30°C. Yeast cells were harvested, washed once with PBS, frozen at −70°C and completely dehydrated at −80°C for 3 days. Their dry weight was determined.

Purification of RGNNV CP

RGNNV CP was purified as described previously (Choi *et al.* 2013). rS was cultured for 48 h and disrupted with glass beads (Biospec Products, Bartlesville, OK, USA) by

vortexing. Cell debris was removed by centrifugation at 12000 ***g***, and the soluble fraction was dialysed against a Tris buffer (10 mmol l^{-1} Tris–Cl pH 7·2, 0·15 mol l^{-1} NaCl, 5% glycerol, 50 mmol l^{-1} L-glutamine, 50 mmol l^{-1} L-arginine + 0·05% Tween 80), followed by binding buffer for heparin chromatography (10 mmol l^{-1} Tris pH 7·6, 0·5 mol l^{-1} NaCl, 5% glycerol, 50 mmol l^{-1} L-glutamine, 50 mmol l^{-1} L-arginine, 0·1% β-mercaptoethanol + 0·05% Tween 80) for 3 h at 4°C. The preparation was loaded on to heparin resin (POROS50 HE, Applied Biosystems, Foster City, CA, USA) pre-equilibrated with binding buffer for heparin chromatography. The column was washed with five volumes of binding buffer, and CP bound to the resin was eluted by successive addition of buffers containing 0·65, 0·75, 0·85, 0·95 and 1·2 mol l^{-1} NaCl. The composition of the elution buffer was the same as that of binding buffer except for the addition of NaCl. The eluted CP was dialysed against storage buffer (10 mmol l^{-1} Tris pH 8·0, 0·5 mol l^{-1} NaCl, 5% glycerol, 50 mmol l^{-1} L-glutamine, 50 mmol l^{-1} L-arginine + 0·05% Tween 80) for 3 h at 4°C.

Western blot analysis of RGNNV CP

Western blotting was performed as described previously (Choi *et al.* 2013) with modification. Proteins were fractionated on a 12% polyacrylamide gel and transferred to a PVDF membrane (Millipore, USA). The PVDF membrane was blocked with 5% skim milk in Tris-buffered saline containing 0·1% Tween 20 (TBST). CP was detected using mouse anti-RGNNV CP serum (Choi *et al.* 2013), together with HRP-conjugated anti-mouse IgG antibody (Bethyl, Montgomery, TX, USA). The RGNNV CP was visualized on X-ray film using ECL Western blot substrate (Santa Cruz, Dallas, TX, USA). RGNNV CP band intensities were determined with NIH open-source software Image J (http://rsbweb.nih.gov/ij/).

Animals

All animal experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and with the Guidelines for Animal experiments of Chung-Ang University. Five-week-old female BALB/c (Orientbio Inc., Seongnam, South Korea) mice were acclimatized for 1 week prior to oral immunization.

Oral immunization of mice

Dosing amount and schedule for oral immunization were based on our previous study (Lee *et al.* 2013). Previously, we confirmed that the purified human papillomavirus (HPV) VLPs combined with freeze-dried *S. cerevisiae* provokes neutralizing antibodies efficiently when four times of oral immunizations were conducted at 2-week intervals. 2·5–10 mg of saponin per dose has been conventionally used to boost immune responses in oral immunization (Chavali *et al.* 1988; Pickering *et al.* 2006).

Mice were divided into three groups, each consisting of seven to ten mice. The CP-rS group received four doses of 40 μg of purified CP combined with 5 mg of saponin from Quillaja bark (total volume, 300 μl) by oral gavage at 2-week intervals. Purified CP protein was assayed with the Bradford protein assay. The rS group received four doses of 10 mg of rS combined with 5 mg of saponin (total volume, 300 μl) orally at 2-week intervals; the amount of rS was based on dry cell weight. Saponin from Quillaja bark was purchased from Sigma (St Louis, MO, USA). A 3-day regimen was used for immunization, such that the CP-rS and rS groups received 13·3 μg of purified CP per day and 3·3 mg of rS per day, respectively. The PBS group received orally 100 μl of PBS per day for 3 days. Ten days after the 3rd and 4th immunizations, mouse sera were obtained from tail veins.

Determination of serum anti-RGNNV CP IgG titre

Anti-RGNNV CP IgG titres were determined by indirect enzyme-linked immunosorbent assay (ELISA) (Choi *et al.* 2013). 96-well plates (Greiner Bio One, Frickenhausen, Germany) were coated overnight with 100 ng/well of purified RGNNV CP at 4°C and blocked with 5% skim milk in PBS containing 0·05% Tween 20 (PBST). The coated wells were incubated with serial dilutions of mouse sera for 1 h at 37°C, and mouse anti-RGNNV CP IgG bound to the RGNNV CP was detected with HRP-conjugated anti-mouse IgG antibody (Bethyl). Colour reactions were developed using *o*-phenylenediamine (Sigma). End-point titres were established at an optical density (OD) of 1·5 times the OD of the control serum.

Cell-based neutralization assay

Cell-based neutralization assays were conducted according to a previous protocol, with modifications (Shieh and Chi 2005). E-11 cells were seeded at a density of 1×10^4 cells per well in 96-well tissue culture plates and cultured for 48 h at 25°C. The cells were cultured in L-15 medium (Bioworld, St Louis Park, MN, USA) containing 5% FBS (Hyclone Thermo Scientific, Logan, UT, USA) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) and infected with RGNNV when they reached 80–90% confluence. Sera from the individual mice in each group were mixed and diluted 1 : 50 in culture medium, and 75 μl of each serum dilution was combined with 75 μl of RGNNV (10^2 $TCID_{50}$). The mixtures were

incubated for 30 min at room temperature and added to the seeded E-11 cells. The cells were cultured for 3 days at 25°C, and cytopathic effects (CPEs) were observed by microscope. The E-11 cells were harvested together with the culture medium, mixed with Laemmli sample buffer and loaded on to 12% polyacrylamide gels. The amounts of CP in the cultures were determined by Western blot analysis as described above.

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Conflict of interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparison of serum IgG levels of mice immunized orally with PBS, CP-rS and rS to the proteins derived from *S. cerevisiae* Y2805 and RGNNV VLP.