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Immunogenicity study of recombinant rubella virus capsid protein and generation of monoclonal antibodies

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This study was aimed at investigating immunogenic properties of yeast-expressed rubella virus capsid protein (RC) and generation of monoclonal antibodies with respect to their use in diagnostic assays. The immunogenicity of recombinant RC was studied using a long-term immunization scheme of BALB/c mice and evaluation of both humoral and cellular immune response. It was demonstrated that yeast-expressed RC posses high immunogenicity independently of the usage of adjuvants. RC induced high-titered IgG response in immunized mice. The IgG subclass distribution of RC-specific antibodies suggested an activation of both Th1 and Th2 T-helper cell subpopulations by the antigen. Enhanced secretion levels of cytokines IL-2 and IFN-γ were determined in antigen-treated spleen cell cultures of immunized mice, which directly demonstrated the activation of antigen-specific T cells. Four hybridomas producing monoclonal antibodies against RC were generated. The antibodies were of IgG1 subclass and specifically recognized RC both in ELISA and Western blotting. The obtained results might have implications for the development of novel immunoassays for the diagnosis of rubella virus infection.

Key words: rubella virus, capsid protein, immunogenicity, monoclonal antibodies

INTRODUCTION

Rubella virus (RV) is a member of the genus *Rubivirus* within the *Togoviride* family, which is a group of positive-strand RNA viruses with relatively simple virion structures and replication schemes. The virion consists of a lipoprotein envelope membrane and a nucleocapsid core [1]. RV is comprised of three structural proteins: two membrane glycoproteins, E1 and E2, and a capsid protein (C). The membrane glycoproteins are required for binding to host cells and membrane fusion during infection. The function of the capsid protein in the assembly pathway is to pack RV genomic RNA into nucleocapsids [2]. The RV is highly teratogenic and causes devastating malformations in human foetus during the first trimester of pregnancy. Usually, RV infection in children and adults is mild or asymptomatic [3]. The immunodiagnostics of RV infection is based on the detection of RV-specific IgG or IgM antibodies of direct virus detection. RV antigens currently used in serological assays are prepared from infected cell cultures [4]. Such antigens are relatively expensive and potentially infectious. Therefore, recombinant viral antigens provide a promising alternative for diagnostic assays. Also, monoclonal antibodies (mAbs) specific to viral antigens are important tools for various applications in immunodiagnostics [4–6]. Previously, we have employed recombinant viral nucleocapsid proteins and nucleocapsid-specific mAbs to develop highly specific and sensitive serological assays for the diagnosis of acute mumps and measles infections [5, 6].

Recently, we have successfully expressed recombinant RC in yeast *S. cerevisiae* [Petraityte, Sasnauskas, unpublished observations]. Before the use of the recombinant antigen in diagnostic assays, its antigenic and immunogenic properties should be characterized. In the current study, we investigated the immunogenicity of yeast-expressed RC, generated mAbs against RC and characterized their specificity.

MATERIALS AND METHODS

Antigens

Recombinant antigens – RC and hantavirus nucleocapsid protein (used as a negative control) – were expressed in yeast *S. cerevisiae* and purified using ultracentrifugation in CsCl gradient at the Institute of Biotechnology, Vilnius [Petraityte, Sasnauskas, unpublished observations].

Immunization of experimental mice

Three 6-week-old BALB/c mice (obtained from the Institute of Immunology of Vilnius University) were immunized subcutaneously with 50 μg of recombinant

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RC dissolved in phosphate-buffered saline (PBS). The mice were further injected two times every four weeks with 50 μg of RC in PBS. Serum samples after each immunization were collected from a tail vein and analyzed by an indirect enzyme-linked immunosorbent assay (ELISA).

Preparation and stimulation of spleen cell culture

Thirty-four days after the last immunization the spleen from the immunized mouse was removed. Erythrocytes were disrupted with 0.083% ammonium chloride. Spleen cells were washed with serum-free RPMI 1640 medium and then resuspended in RPMI 1640 growth medium containing 10% fetal calf serum. Single cell suspension was seeded into 96-well plates at a density of 0.5 mln cells/200 μl/well. To induce antigen-specific T-cell response, spleen cells were incubated with recombinant RC at concentrations of 5 μg/ml and 20 μg/ml, respectively. As a positive control, polyclonal T-cell stimulator Concanavalin A (Con A, Sigma) at a concentration of 20 μg/ml was used. After 48 h of incubation, the supernatants of spleen cell cultures were collected and stored at –20 ˚C until analysis.

Generation of monoclonal antibodies

The spleen cells from the immunized mouse with the highest RC-specific antibody titer (1:10000) were used to generate hybridomas. Hybridomas were generated essentially as described by Kohler and Milstein [7]. The mouse was boosted on day 35 after the 3rd immunization with 50 μg of recombinant RC in PBS. Four days later, spleen cells of the boosted mouse were fused with mouse myeloma Sp2/0 cells using polyethylene glycol (PEG) 1500 as a fusion agent (PEG/DMSO solution, HybriMax, Sigma). Hybrid cells were selected in growth medium supplemented with hypoxanthine, aminopterin and thymidine (50x HAT media supplement, Sigma). Positive clones were stabilized by limiting dilution cloning on a macrophage feeder layer. Hybridoma cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) containing 15% of fetal calf serum (FCS, Biochrom) and antibiotics. Heavy chain types of mAbs were determined by ELISA using the Monoclonal Antibody Isotyping Kit (Sigma). The specificity of mAbs was tested by ELISA and Western blotting as described below.

Enzyme-linked immunosorbent assay (ELISA)

The titers of RC-specific antibodies in mouse sera and the specificity of mAbs were tested by an indirect ELISA. Microtiter plates (MaxiSorp, Nunc) were coated with the antigen by adding 50 μl of the antigen solution (5 μg/ml) in the coating buffer (50 mM Na-carbonate, pH 9.5) and incubation overnight at +4 ˚C. The plates were blocked with 2% bovine serum albumin (BSA) in PBS and then incubated with serum samples or hybridoma supernatants. Following additional washing the plates were incubated with horse-radish peroxidase (HRP)-labeled anti-mouse IgG (Bio-Rad, 1:8000). After washing, the enzymatic reaction was developed with 3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) and stopped by adding 1 M H_2SO_4. The optical density (OD) was measured at 450 nm in a microtiter plate reader (Tecan).

For the determination of IgG titers in the sera of immunized mice, individual sera from mice were diluted in two-fold steps starting at 1:100 in PBS/0.01% Tween 20 and then incubated as described above. Antibody titers were defined as the reciprocal of the highest serum dilutions to yield OD_{450} values greather than three times the background.

Interleukin 2 (IL-2) and interferon γ (IFN-γ) concentrations in spleen cell cultures were determined by sandwich ELISA using Mouse IL-2 Opt-EIA Set and Mouse IFN-γ Opt-EIA Set according to the recommendations of the supplier (BD PharMingen).

Western blot analysis

Purified antigens and crude lysates of yeast cells were fractionated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% mini-gels and transferred to polyvinyldifluoride (PVDF) membranes (Millipore) under semidry conditions. Membranes were blocked with 1% gelatine in PBS, washed and then incubated with hybridoma supernatants. After washing, the membranes were incubated with HRP-labeled anti-mouse IgG (Bio-Rad, 1:5000). After several washing steps the enzymatic reaction was visualized using 4-chloro-1-naphthol substrate (Sigma). Prestained protein molecular weight (MW) markers were purchased from Fermentas.

RESULTS

Immunogenicity of recombinant rubella virus capsid protein

The ability of recombinant RC to induce humoral and cellular immune response in experimental mice was investigated. For this purpose, BALB/c mice were immunized three times without adjuvants. To evaluate B-cell response, the development of RC-specific IgG antibodies after each immunization was measured. After the first immunization, the titers of RC-specific IgG antibodies in the sera of immunized mice were approximately 1:1000. The antibody titers increased significantly (more than 10-fold) after repeated immunizations, indicating the ability of the antigen to induce true immune memory (Fig. 1). Sera of immunized mice taken 50 days after the final immunization continued to show high titers of RC-specific antibodies suggesting the induction of a long-term immunity (data not shown). To evaluate the ability of recombinant RC to activate T-helper cells, the IgG subclass distribution in the sera of immunized mice was measured. High levels of RC-specific IgG1 and IgG2a antibodies were produced with a non-significant predominance of the IgG2a subclass. After the third immunization, the titers of RC-specific IgG1 antibodies in the sera of immunized mice were 1.6×10^4, IgG2a antibodies – 1.8×10^4. IgG2b and IgG3 antibodies were developed at lower amounts: the titers were 2.5×10^3 and 1×10^3, respectively

(data not shown). This suggests a mixed Th1/Th2 response. In addition, the ability of recombinant RC to induce cellular immune response was assessed using spleen cell cultures. For this purpose, spleen cells of immunized mice were treated with different doses of recombinant RC (5μg/ml or 20 μg/ml, respectively) *in vitro* and the levels of cytokines IL-2 and IFN-γ were measured. After 48 h of incubation with recombinant RC, significant amounts of cytokines IFN-γ and IL-2 were determined in spleen cell cultures treated with recombinant RC (Fig. 2). Cytokine levels correlated with the antigen dose. In contrast, cytokine production was not detectable in RC-stimulated spleen cell cultures of naïve (non-immunized) BALB/c mice (data not shown). Taken together, these experiments have demonstrated that yeast-expressed recombinant RC is highly immunogenic and induces B- and T-cell responses in immunized BALB/c mice.

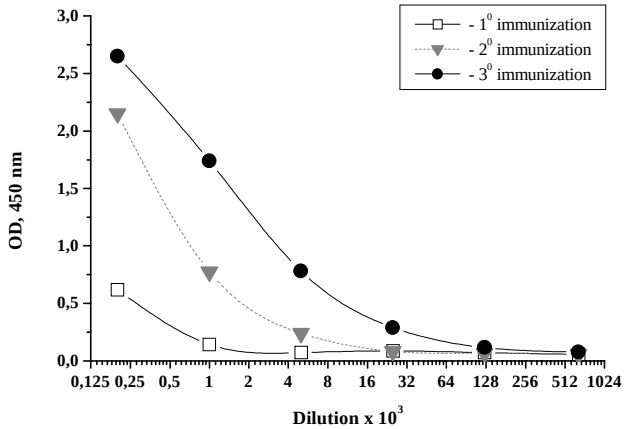

Fig. 1. Development of RC-specific IgG antibodies in BALB/c mice immunized 3 times with recombinant RC without adjuvants. Serum samples were collected from immunized mice after each immunization and tested by indirect ELISA. Optical density (OD) values *versus* serum dilutions are shown

Production and characterization of monoclonal antibodies

The spleen cells of immunized mice were used to generate hybridomas. Fusion of spleen cells with mouse myeloma cells resulted in four hybridoma clones producing mAbs against recombinant RC. The following clones were designed: 9B11, 10A1, 11B1, and 3E8. All mAbs were of IgG1 isotype and strongly reacted with recombinant authentic (non-His-tagged) and His-tagged RC both in ELISA and Western blotting (Fig. 3, lanes 3, 4). In

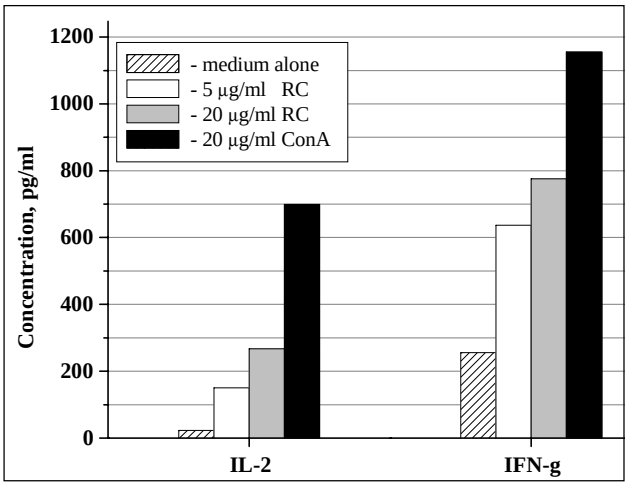

Fig. 2. Concentrations of cytokines IL-2 and IFN-γ (pg/ml) in spleen cell cultures of immunized BALB/c mice. Spleen cells were stimulated *in vitro* with different doses of RC: 5 μg/ml and 20 μg/ml, respectively. Positive control: 20 μg/ml Con A. Negative control: medium alone. Culture supernatants were collected after 48 hours of incubation.

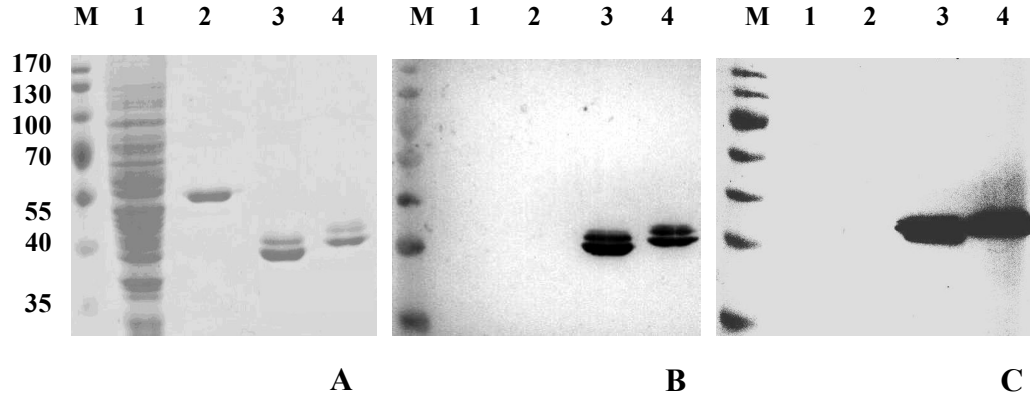

Fig. 3. Immunoreactivity of monoclonal antibodies with recombinant RC in Western blotting. SDS-PAAG electrophoresis of recombinant proteins (0.2 μg/lane) was performed in 12% gel. The proteins were transferred onto a PVDF membrane which was incubated with monoclonal antibody and then developed with anti-mouse IgG peroxidase conjugate (BioRad) and 4-chloro-1-naphthol substrate (Sigma)

(A) Coomassie blue-stained gel; (B) Immunoblot using mAb 10A1; (C) Immunoblot using mAb 3E8. Lane M – prestained protein molecular weight marker (Fermentas): 170, 130, 100, 70, 55, 40 and 35 kDa. Lane 1 – crude lysate of yeast *S.cerevisiae* cells (negative control); Lane 2 – recombinant yeast-expressed hantavirus nucleocapsid protein (negative control); Lane 3 – authentic (non-His-tagged) RC expressed in yeast *S. cerevisiae* and purified in CsCl gradient*;* Lane 4 - His-tagged RC expressed in yeast *S. cerevisiae* and purified in CsCl gradient.

contrast, no reactivity of mAbs with irrelevant antigens – yeast-expressed hantavirus nucleocapsid protein and yeast cell lysate proteins – was observed (Fig. 3, lanes 1, 2). This demonstrates the specificity of mAbs raised against recombinant yeast-expressed RC.

DISCUSSION

Recombinant viral proteins and virus-specific monoclonal antibodies (mAbs) are important tools for various applications in diagnostic assays [5, 6, 9]. Recombinant viral antigens are widely used to generate virus-specific mAbs. For mAb production and the development of diagnostic assays, recombinant viral antigens that induce an efficient immune response are required [10]. In the current study, we have demonstrated that yeast-expressed recombinant RC is highly immunogenic in mice. It induced an efficient immune response even in the absence of adjuvants. During immunizations, we have observed the development of RC-specific IgG antibodies and a significant increase of antibody titers after repeated immunizations. This indicates an activation of T-helper cells providing the necessary signals for Ig class switching and induction of memory cells. The subclass distribution of RC-specific IgG antibodies in immunized mice suggested an approximate balance of Th1 and Th2 cell activation during the immune response against RC. The production of the IgG1 antibodies indicates an important influence of IL-4 secreted by the Th2 subset, and the production of IgG2a antibodies indicates the role of IFN-γ secreted by the Th1 cell subset, in the regulation of Ig class switching. The results obtained with spleen cell cultures and direct measurement of antigen-induced IL-2 and IFN-γ secretion by spleen cells *in vitro* were consistent with the IgG subclass profile data. All these have data indicated that yeast-expressed recombinant RC induces an efficient immune response in mice. We have successfully used this antigen to generate hybridomas producing RC-specific mAbs. The specificity of mAbs was confirmed both by ELISA and Western blotting. In conclusion, the recombinant RC and the mAbs against RC might be further employed for the development of novel immunoassays for the serologic diagnosis of rubella virus infection.

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REKOMBINANTINIO RAUDONUKĖS VIRUSO KAPSIDĖS BALTYMO IMUNOGENIŠKUMO TYRIMAI IR MONOKLONINIŲ ANTIKŪNŲ SUKŪRIMAS

Santrauka

Šio darbo tikslas buvo ištirti raudonukės viruso kapsidės baltymo, ekspresuoto mielėse, imunogenines savybes ir sukurti monokloninius antikūnus, kuriuos vėliau būtų galima panaudoti diagnostikai. Rekombinantinio kapsidės baltymo imunogeniškumas buvo tiriamas atliekant ilgalaikę BALB/c linijos pelių imunizaciją ir matuojant humoralinį bei ląstelinį imuninį atsaką. Nustatyta, kad mielėse ekspresuotas kapsidės baltymas sukėlė stiprų imuninį atsaką, net ir nenaudojant adjuvantų. Imunizuotų pelių kraujo serume buvo rastas aukštas IgG klasės antikūnų titras. Ištyrus IgG poklasių pasiskirstymą paaiškėjo, kad antigenas sukėlė Th1 ir Th2 ląstelių subpopuliacijų aktyvaciją. Imunizuotų pelių blužnies ląstelių kultūrose, paveikus jas antigenu, buvo nustatyta aukšta citokinų IL-2 ir IFN-γ koncentracija. Tai rodo, kad buvo aktyvinamos antigenui specifinės T ląstelės. Buvo sukurtos 4 hibridomos, gaminančios monokloninius antikūnus prieš kapsidės baltymą. Antikūnai yra IgG1 poklasio ir specifiškai reaguoja su kapsidės baltymu tiriant imunofermentinės analizės bei imunoblotingo metodais. Gauti rezultatai galėtų būti panaudoti kuriant naujus raudonukės infekcijos diagnostikos metodus.