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Immunogenicity study of yeast-derived mumps nucleocapsid protein and production of monoclonal antibodies

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Objectives. This study was aimed to the expression of the nucleocapsid protein of mumps virus (mumps NP) in yeast cells, investigation of its antigenic properties and generation of monoclonal antibodies.

Materials and Methods. Mumps NP was expressed in recombinant yeast *S. cerevisiae* and isolated by ultracentrifugation. Self-assembly of mumps NP to virus-like particles was confirmed by electron microscopy. The immunogenicity of recombinant nucleocapsid-like particles was investigated using a long-term immunization scheme of different strains of mice and evaluation of both humoral and cellular immune response. Spleen cells of immunized mice were fused with mouse myeloma cells to generate hybridomas.

Results and Discussion. Yeast expression system was employed to produce recombinant mumps NP with respect to its use in mumps virus immunodiagnostics. Yeast-derived mumps NP was capable to self-assemble to virus-like particles that possessed high immunogenicity independently of the haplotype of immunized mice and the usage of adjuvants. The recombinant antigen was further used to generate hybridomas producing monoclonal antibodies specific to mumps NP. These antibodies recognized mumps virus-infected cells as shown by immunofluorescence assay. The obtained results are important for the development of highly sensitive and specific immunoassays and their application in epidemiological studies of mumps infection and monitoring immune responses to the vaccine.

Key words: mumps virus, nucleocapsid protein, immunogenicity, monoclonal antibodies

INTRODUCTION

The mumps virus is a member of the genus *Rubulavirus* in the family *Paramyxoviridae.* The genome is comprised of a single molecule of negative sense RNA of about 15kb and encodes seven proteins: the nucleocapsid protein (NP), large protein (L), phosphoprotein (P), matrix protein (M), small hydrophobic protein (SH) and two surface glycoproteins, haemaglutinin-neuraminidase (HN) and fusion protein (F). The function of the nucleocapsid protein appears to be packaging of the viral genome and formation of replication complexes along with the other proteins. Viral nucleocapsid proteins usually elicit a strong long-term humoral immune response in patients as well as in experimental animals. Therefore, immunoassays for the detection of antibodies specific to mumps NP have formed the basis for mumps diagnosis (1). Further studies on the structure, immunogenicity and diagnostic potential of mumps NP need high quantity of protein. Also, purified recombinant antigens are widely used to generate specific monoclonal antibodies using hybridoma technology. Antibodies to viral antigens are useful diagnostic tools. Here we report the high-level expression of mumps NP in recombinant yeast *Saccharomyces cervisiae*, the results of its immunogenicity analysis and the use of this recombinant antigen to generate monoclonal antibodies that recognize mumps-virus-infected cells.

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MATERIALS AND METHODS

Generation of recombinant mumps NP. The mumps virus NP gene was amplified by PCR from cDNA prepared from the wild-type mumps virus *Glouscester* strain isolated in the U.K. The mumps NP-encoding sequence was inserted into the yeast expression vec-

tor pFX7 under control of hybrid *GAL10-PYK1* promoter (2). The resulting plasmid pFX7-NP was used for transformation of the yeast *S. cerevisiae* strain AH22. After induction in growth medium supplemented with 3% galactose, yeast cells were harvested, resuspended in disruption buffer and homogenized in a blender using glass beads.

Purification and identification of NP. The homogenate was centrifuged, loaded onto a chilled 30% sucrose cushion and ultracentrifuged at 100000 g for 3 h. The pellet was resuspended in disruption buffer and ultracentrifuged at 100000 g for 48 h in CsCl gradient. Fractions containing NP were identified by SDS-PAG electrophoresis and imunoblotting. Formation of virus-like particles was confirmed by electron microscopy.

Immunization of animals. For the immunogenicity test, groups of inbred mice BALB/c (H-2^d), CBA/6 (H-2^k) and C57BL/2 (H-2^b) were given a single subcutaneous injection of 50 μg NP either dissolved in PBS (n = 5) or emulsified in complete Freund's adjuvant (n = 2). On day 22 after the first immunization, mice were boosted subcutaneously with 30 μg NP in PBS. For monoclonal antibody production, BALB/c mice were additionally boosted 3 days before fusion.

ELISA test. To evaluate specific antibody response in immunized mice, blood samples were collected from a tail vein before immunization and on days 14, 22, 35 and 60 after primary immunization. The ELISA test was performed in 96-well microtiter plates coated with recombinant mumps NP. Plates were developed using peroxidase-labeled secondary antibody against mouse IgG (Sigma) and TMB-one substrate (Fermentas).

Spleen cell proliferation test. Spleens of the immunized mice were removed on day 60 after primary immunization. Erythrocytes were depleted in 0.083% ammonium chloride. Spleen cells were resuspended in RPMI 1640 growth medium containing 10% fetal calf serum and seeded into 96-well plates, 1 mill. cells/100 μl/well. To induce antigen-specific proliferative response, spleen cells were incubated with recombinant mumps NP at a final concentration 25 μg/ml. To evaluate the proliferative capacity of spleen cells, cells were incubated with polyclonal stimulators: either LPS at a final concentration 10 μg/ml or Con A at 5 μg/ml. Cells were cultured for 72 h with or without stimulators and then stained with MTT (3). In a control experiment, spleen cells of mice immunized with an irrelevant antigen (recombinant polyomavirus nucleocapsid protein) were prepared and stimulated as described above.

Generation of monoclonal antibodies. Monoclonal antibodies to rNP were produced essentially as described by Kohler and Milstein (4). Briefly, spleen cells of the immunized BALB/c mice were fused with mouse myeloma NS0/1 cells using PEG 1500 as a fusion agent (PEG/DMSO solution, HybriMax, Sigma). Hybrid cells were selected in growth medium supplemented with hypoxantine, aminopterin and thymidine (50 × HAT media supplement, Sigma). Viable clones were selected by ELISA. Positive clones were stabilised by a limiting dilution assay. The isotypes of the monoclonal antibodies were determined by ELISA using a Monoclonal Antibody Isotyping Kit (Sigma, ISO-2). Hybridoma cells were maintained in complete Dulbecco's Modiefied Eagle's Medium (DMEM) containing 15% foetal calf serum (Biochrom) and antibiotics.

RESULTS

Characterization of yeast-derived recombinant mumps NP. Transformation of yeast *S. cerevisiae* with the recombinant plasmid resulted in a high-level expression

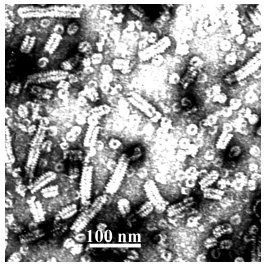

Fig. 1. Electron microphotograph of recombinant mumps NP expressed in *S. cerevisiae* and purified by CsCl gradient centrifugation

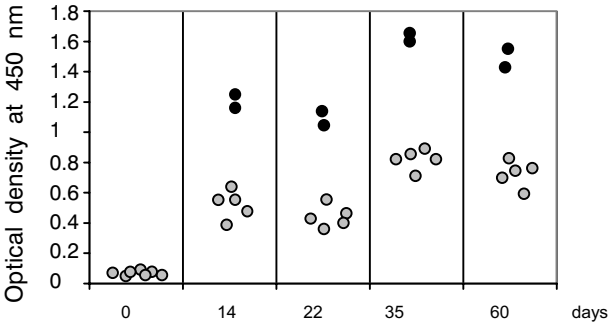

Fig. 2. Development of specific antibody response in mice BALB/c immunized with recombinant yeast-derived mumps NP

Mice were immunized subcutaneously with 50 μg mumps NP either in PBS or emulsified in complete Freund's adjuvant (FCA) and boosted on day 22nd with 30 μg of NP in PBS. Blood samples were collected before immunization and 14, 22, 35 and 60 days after primary immunization. The levels of NP-specific IgG antibodies were determined by indirect ELISA in blood samples diluted 1:400.

Black points – mice primed with NP/FCA (n = 2), grey points – mice primed with NP without adjuvant (n = 5)

of mumps NP. After purification on CsCl gradient the NP was the major protein present with only minor contaminants. Electron microscopy of the purified recombinant mumps NP revealed a characteristic herring-bone structure (Fig. 1).

Immunogenicity of recombinant mumps NP. The immunogenicity of recombinant mumps NP was investigated using a long-term immunization scheme of mice of different strains and evaluation of both humoral and cellular immune response. Mice were immunized twice with or without adjuvant stimulation. Specific antibody response to recombinant mumps NP was determined by ELISA in blood samples collected from the immunized mice at regular intervals. Cellular immune response was assessed by *in vitro* proliferation assay of spleen cells stimulated with recombinant mumps NP. A significant level of specific anti-NP antibodies in blood samples was observed after a single injection of recombinant mumps NP. Boost imunization on day 22 resulted in an increase of NP-specific IgG response (Fig. 2). This indicates that the affinity maturation of specific antibodies and switching of IgM to IgG occurred, although the usage of the adjuvant induced higher titers of NP-specific antibodies, immunization with NP without adjuvant also provided long-term humoral immune response and induction of antigen-specific T-cell help. When comparing different strains of mice for their capability to produce antibodies to NP, no significant differences were found. The immunogenicity of recombinant mumps NP was also demonstrated using spleen cell proliferation test. Stimulation *in vitro* of spleen cells from previously immunized mice induced a strong antigen-dependent proliferative response (Fig. 3A). Similar results were obtained with mice of different strains. Spleen cells of control mice immunized with an irrelevant antigen responded to polyclonal B- and T-cell stimulators (LPS and ConA, respectively), however, no detectable proliferative response to recombinant mumps NP was observed (Fig. 3B). These data suggest that recombinant mumps NP possess high immunogenicity independently of co-stimulation with the adjuvant and elicits a long-term immune response in experimental animals.

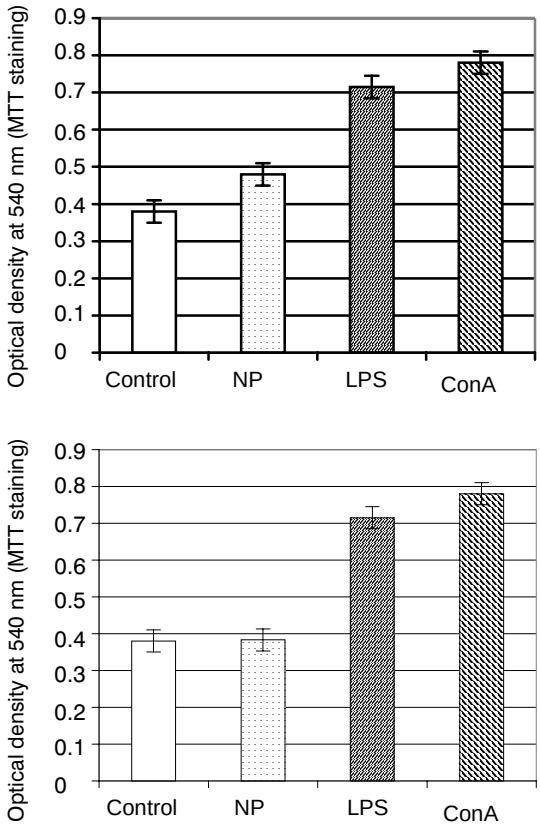

Fig. 3. Antigen-induced proliferative response of spleen cells from C57BL/6 mice immunized with recombinant mumps NP

Mice were immunized twice either with recombinant yeast-derived mumps NP (**A**) or an irrelevant antigen (**B**). Spleen was removed on day 60th after primary immunization. Spleen cells were incubated for 72 h in RPMI 1640 growth medium supplemented either with mumps NP or polyclonal stimulators and then stained with MTT.

Control – spleen cells cultured in growth medium without stimulators, *NP* – stimulation with mumps NP (25 μg/ml), *LPS* – stimulation with LPS (10 μg/ml), *ConA* – stimulation with ConA (5 μg/ml).

The results are expressed as the mean optical density measured at 540 nm in each set of 12 parallel wells.

Production and characterization of monoclonal antibodies. Fusion of spleen cells with mouse mye-

Fig. 4. Immunoreactivity of monoclonal antibodies with recombinant mumps NP in Western blotting

SDS-PAAG eletrophoresis of mumps NP (100 ng/lane) was performed in 12% gel. The proteins were transferred onto a PVDF membrane, which was incubated with monoclonal antibody 8H4 and then developed with anti-mouse IgG peroxidase conjugate (Sigma) and TMB-blotting substrate (Fermentas).

Lanes 1, 3 – prestained marker proteins (Fermentas), lane 2 – mumps NP.

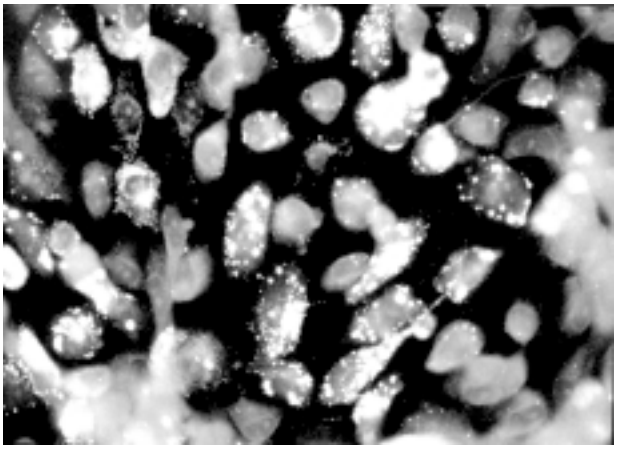

Fig. 5. Indirect immunofluorescence staining of mumps virus infected Vero cells using monoclonal antibody 5H7

loma cells resulted in 7 hybridoma clones producing monoclonal antibodies to mumps NP. Six antibodies were of IgG2a isotype and one was of IgG2b isotype. This indicates the ability of the recombinant antigen to stimulate preferencially Th1 cells. All of the monoclonal antibodies reacted with mumps NP in ELISA and Western blotting (Fig. 4). Also, they reacted with Vero cells infected with a mumps virus as shown by immunofluorescence analysis (Fig. 5).

DISCUSSION

The application of molecular technologies, such as the expression of viral proteins in yeast cells, has provided a powerful approach to designing new vaccines and diagnostics. Some mammalian virus proteins, especially HBV and HCV surface and core antigens (5), polyomavirus proteins (2) and others, are able to form virus-like particles in yeast providing highly immunogenic components for vaccine development and antigens for immunologic assays. We employed yeast expression systems to produce mumps NP with respect to its use in mumps virus immunodiagnostics. Recently, we have reported the high-level production of mumps NP in yeast *Pichia pastoris* (6). Here we report the expression of mumps NP in recombinant yeast *Saccharomyces cervisiae* and investigation of its antigenic properties. The mumps NP gene was inserted in the multicopy vector under control of the yeast *S. cerevisiae* GAL10-PYK1 promoter which resulted in a high-level expression. Recombinant mumps NP was effectively purified using two-step ultra-centrifugation and characterized by electron microscopy. Yeast-derived mumps NP was capable to self-assemble to virus-like particles that possessed a high antigenic potential in experimental mice. It elicited a long-term immune response, *e.g.*, development of IgG antibodies and stimulation of T-cells independently of the haplotype of immunized mice and the usage of adjuvants. Spleen cells of the immunized mice were further used to generate hybridomas producing monoclonal antibodies specific to mumps NP. Seven hybridoma cell lines producing monoclonal antibodies specific to mumps NP were obtained. All of these antibodies reacted with the antigen in ELISA and Western blotting. Also, they reacted with Vero cells infected with the mumps virus as shown by immunofluorescence analysis. Six antibodies were of IgG2a isotype and one was of IgG2b isotype. The isotype distribution suggests a significant influence of IFN-γ secreted by the Th1 cell subset in the regulation of Ig isotype switching during the immune response against recombinant mumps NP. The obtained results are important for the development of new immunodiagnostics of mumps infection and immune responses to vaccination by detection of nucleocapsid-directed antibodies in human serum.

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KIAULYTĖS VIRUSO NUKLEOKAPSIDĖS BALTYMO IMUNOGENIŠKUMO TYRIMAS IR MONOKLONINIŲ ANTIKŪNŲ SUKŪRIMAS

S a n t r a u k a

Šio tyrimo tikslai buvo kiaulytės viruso nukleokapsidės baltymo (NB) raiška mielių ląstelėse, šio baltymo antige-

ninių savybių tyrimas ir monokloninių antikūnų sukūrimas.

Tyrimo metodai. Kiaulytės viruso NB buvo gautas rekombinantiniame mielių *S. cerevisiae* kamiene ir išgrynintas ultracentrifuguojant. Elektroniniu mikroskopu siekta nustatyti, ar NB formuoja į virusus panašias daleles. Atlikus skirtingų linijų pelių ilgalaikę imunizaciją ir įvertinus humoralinį bei ląstelinį imuninį atsaką, buvo ištirtas rekombinantinių dalelių imunogeniškumas. Siekiant sukurti hibridomas, buvo sulietos imunizuotų pelių blužnies ląstelės su pelės mielomos ląstelėmis.

Rezultatai ir diskusija. Rekombinantinis NB buvo gautas mielėse, siekiant panaudoti jį kiaulytės viruso imunodiagnostikoje. Rekombinantinis NB formavo į virusus panašias daleles, sukėlusias stiprų imuninį atsaką skirtingų haplotipų pelėms, net ir nenaudojant adjuvanto. Imunizavus peles rekombinantiniu antigenu, buvo gautos hibridomos, gaminančios monokloninius antikūnus prieš NB. Kaip buvo nustatyta imunofluorescenciniu metodu, šie antikūnai atpažino kiaulytės virusu infekuotas ląsteles. Gauti rezultatai svarbūs kuriant naujus specifiškus ir jautrius imunodiagnostikos metodus, skirtus parotito infekcijos epidemiologiniams tyrimams bei imuninio atsako į vakciną įvertinimui.

Raktažodžiai: kiaulytės virusas, nukleokapsidės baltymas, immunogeniškumas, monokloniniai antikūnai