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Bursin-like peptide (BLP) enhances H9N2 influenza vaccine induced humoral and cell mediated immune responses

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ABSTRACT

Vaccination with H9N2 avian influenza whole-inactivated virus (WIV) has been shown to be ineffective at eliciting sufficient humoral and cellular immunity against H9N2 avian influenza virus. This study assessed the effects of a synthetic Bursin-like epitope peptide (BLP) as adjuvant for H9N2 WIV in mice. Titers HI and avian influenza virus neutralizing antibodies, subtypes of HA antibodies, T helper (Th) cytokine levels, cytotoxic T-lymphocyte activities and changes in spleen T-cell subsets and natural killer cells were determined. We found that BLP induced a balance between IgG1 and IgG2a secretion levels. WIV antigen alone induced mainly Th1 cytokines secretion, whereas BLP showed increased secretion of Th1 and Th2 cytokines, including interleukin (IL)-2, interferon-γ (IFN-γ) and IL-4, but not IL-10, and may be resembles a Th0 like response. BLP significantly promoted growth and expansion of natural killer cells and of $CD4^+$ and $CD8^+$ T-cell subsets in the spleen. Meanwhile, BLP induced a better cytotoxic T-lymphocyte response to H9N2 virus. Furthermore, virus challenge experiments confirmed that BLP contributed to inhibition replication of the virus from mouse lungs. Taken together, these findings suggest that BLP may be an effective adjuvant for H9N2 avian influenza vaccine.

1. Introduction

The H9N2 avian influenza virus (AIV) subtype was first detected in turkeys in 1966 and was associated with mild respiratory disease. Investigations of the H9N2 AIV subtype in domestic poultry populations in Hong Kong between 1975 and 1985 showed that the virus commonly spread in ducks but not in domestic chickens [1–5]. Since the early 1990s, however, the H9N2 AIV subtype has spread widely throughout Asia, in both wild birds and domestic chickens, resulting in serious economic loss in domestic poultry populations [6–8]. Moreover, this AIV subtype was shown to infect humans, inducing influenza [9], with the AIV H9N2 subtype first isolated from humans in 1999 [10].

Vaccination is still the best choice to provide protection against AIV infection [11]. Vaccination with inactivated pandemic influenza viruses have been shown to stimulate strong humoral immune responses, but could not elicit adequate cell mediated immune responses for effective protection against AIV infection [12]. Since adjuvants were shown to improve immune responses [13–15], combinations of pandemic influenza viruses with adjuvants, including aluminum salts, MF59, IC31® and chitosan, have been tested [16–19].

The tripeptide bursin (BS) is an endogenous B-cell differentiation factor [20]. The tetrapeptide D-LysAsnProTyr, which has a conformation similar to BS, has been shown to stimulate immune cell subsets, suggesting that BS may be a potential vaccine adjuvant.[21]. This study tested whether a synthetic peptide analogue of bursin, or bursin-like epitope peptide (BLP), could enhance immune responses in mice upon vaccination with H9N2 avian influenza whole-inactivated virus (WIV).

Abbreviations: AIV, avian influenza virus; BLP, bursin-like peptide; BS, bursin; CTL, cytotoxic T-lymphocyte; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; HA, hemagglutinin; HI, hemagglutination inhibition assay; HRP, horseradish peroxidase; IFN-γ, interferon-γ; IL-2, interleukin-2; LDH, released lactate dehydrogenase; MDCK, Madin–Darby canine kidney; NA, neuraminidase; NK cell, natural killer cell; PE, phycoerythrin; Th, T helper type; Th1, T helper type 1; Th2, T helper type 2; WIV, whole-inactivated virus.

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2. Materials and methods

2.1. Viruses and reagents

Avian influenza virus A/Duck/Jiangsu/NJ08/05 (H9N2) was kindly provided by Dr. Qi-Sheng Zheng. Virus titers were determined in Madin–Darby canine kidney (MDCK) cells, as described [22]. WIV was prepared by diluting the virus 1:4000 (v/v) in formalin [23,24]. Avian influenza virus A/chicken/Jiangsu/JS-1/2002 (H9N2) was isolated and maintained in our laboratory [25]. Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG was obtained from Boshide Corporation (Wuhan, China), and HRP conjugated goat anti-mouse IgG1 and anti-mouse IgG2a were obtained from Bethyl Corporation. Fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3 and phycoerythrin (PE)-labeled anti-mouse CD4, CD8 and pan-NK (CD49b) DX5 antibodies were obtained from Caltag Corporation. RPMI 1640 medium and FBS were obtained from Gibco Corporation (New York, USA), BLP (T-P-N-L-K-H-G) was synthesized by the solid phase method at Huachen Corporation (Xi'an, China) and had a purity >95%.

2.2. Immunization protocols

SPF female BALB/c mice aged 4–6 weeks were obtained from Nanjing Medical University. The mice were randomly divided into six groups of 30 each and intramuscularly immunized three times on days 0, 14 and 28 with mixtures of 100 μL WIV (A/Duck/Jiangsu/NJ08/05, 10^7 $TCID_{50}$/0.1 ml) and (i) 100 μL phosphate-buffered saline (PBS) or (ii) low (10 μg), medium (50 μg) or high (250 μg) dose BLP in 100 μL PBS. (iii) 100 μL oil-formulated inactivated AIV/H9N2 vaccine (Qian Yuan Hao Co., Ltd., Nanjing, China [10^7 TCID50/0.1 ml]) plus 100 μL PBS was used as a positive control and (iv) 100 μL PBS was a negative control. One week after final vaccination, 15 anesthetized mice in each group were challenged intranasally with 2.5×10^6 $TCID_{50}$ avian influenza virus A/chicken/Jiangsu//JS-1/2002 (H9N2) in 50 μl PBS. Five mice per group were humanely sacrificed and the viral titers in their lungs assessed by plaque formation assays using MDCK cells as described [26].

All animal experiments conformed to Nanjing Agriculture University Animal Care and Use Committee guidelines and were approved by the committee.

2.3. Detection of anti-hemagglutinin (HA) antibodies in serum

ELISA assays were performed as described [27]b Briefly, serum was collected on 7, 21 and 35 days after immunization. Aliquots were added to 96-well microtiter plates which had been coated overnight with 10 μg/ml recombinant influenza HA protein (expressed in *Escherichia coli* BL21) [28] and blocked. Aliquots of serum were added to the plates, which were then incubated overnight, washed, and incubated with HRP conjugates of goat anti-mouse IgG, IgG1 and IgG2a. Finally, TMB substrate was added and the reaction was stopped. The results were plotted as OD *versus* dilution (log scale). Titers at half maximal OD were determined by linear interpolation. Each serum sample was assayed in triplicate [29,30].

2.4. Hemagglutination inhibition (HI) assay

Serum HI antibody titers were determined using standard HI-microtiter assays, as described [31]. Briefly, serum was inactivated; duplicate samples were added to 96-well plates; and each sample was serially diluted twofold in PBS. Four HA units of avian influenza virus were added to each well, followed by the addition of chick erythrocyte suspensions. Hemagglutination was allowed to proceed for 1 h at room temperature.

2.5. Determination of AIV-neutralizing antibodies

Inactivated serum samples were incubated with 100 PFU of avian influenza virus A/chicken/Jiangsu//JS-1/2002 (H9N2), and the titers of AIV neutralizing antibody determined as described [26].

2.6. Spleen T-cell subtyping and natural killer cells

Spleen T-cells were subtyped and natural killer (NK) cell concentrations were determined in blood samples obtained seven days after the final immunization. Cell suspensions were prepared as described and incubated with FITC-labeled anti-mouse CD3 and PE-labeled anti-mouse CD4, CD8 and DX5 monoclonal antibodies, followed by flow cytometry as described [27].

2.7. Cytokine assays

Concentrations of interleukin (IL)-2, interferon (IFN)-γ, IL-4 and IL-10 were measured using ELISA cytokine kits (eBioscience, USA) according to the manufacturer's instructions.

2.8. Cytotoxic T-lymphocyte (CTL) assay

To determine whether BLP induces CTL responses, released lactate dehydrogenase (LDH) cytotoxic assays were performed using CytoTox 96® Non-Radioactive Cytotoxicity Assay kits (Promega, USA). Effector cells for cytotoxic assays were prepared as described [32–34]. Briefly, lymphocytes were isolated from spleens obtained 7 days after the last immunization and 10^7 cells were suspended in 5 ml complete RPMI 1640 medium. Syngeneic mouse spleen mononuclear cells were infected with 10^7 pfu avian influenza H9N2 virus strain JS-1 for 2 h. After three washes with complete RPMI 1640 medium, the cells were suspended in 5 ml complete RPMI 1640 medium, incubated for 3 h at 37 °C in 5% CO_2, and irradiated at 3000 rad. The vaccine-immunized spleen cell suspensions and the influenza-infected feeder spleen cell suspensions were mixed in a 25-cm^3 culture flask and incubated for 6 days at 37 °C in 5% CO_2. The cells were harvested and used as effector cells. NIH3T3 cells, kindly provided by Dr. Ping Jiang [35], infected with avian influenza virus A/chicken/Jiangsu//JS-1/2002 (H9N2) (moi 20 pfu/cell) for 24 h were used as target cells. Effector cells were washed three times, suspended in RPMI 1640 medium containing 10% FBS, and diluted in 96-well plates to yield effector-to-target cell ratios of 30:1 and 10:1. Two thousand target NIH3T3 infected cells were added to each well and the plates were incubated at 37 °C for 4 h. Each effector cell preparation was assayed in triplicate. Released LDH was measured according to the manufacturer's protocol, and the percent specific killing was calculated using the formula (experimental release − spontaneous release)/(total release − spontaneous release) [36].

2.9. Statistical analysis

Statistical analyses were performed using unpaired t-tests or one-way ANOVA F-statistics. Data are presented as the mean ± SD. Turkey multiple comparison tests were used to assess differences among the six experimental groups (five immunized and one control group). All statistical analyses were performed using GraphPad Prism 5 software, with differences were considered significant at $P < 0.05$ or $P < 0.01$.