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Maximal immune response and cross protection by influenza virus nucleoprotein derived from *E. coli* using an optimized formulation

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ABSTRACT

The highly conserved internal nucleoprotein (NP) is a promising antigen to develop a universal influenza A virus vaccine. In this study, mice were injected intramuscularly with *Escherichia coli*-derived NP protein alone or in combination with adjuvant alum ($Al(OH)_3$), CpG or both. The results showed that the NP protein formulated with adjuvant was effective in inducing a protective immune response. Additionally, the adjuvant efficacy of $Al(OH)_3$ was stronger than that of CpG. Optimal immune responses were observed in BALB/c mice immunized with a combination of NP protein plus $Al(OH)_3$ and CpG. These mice also showed maximal resistance following challenge with influenza A virus PR8 strain. Most importantly, 10 μg NP formulated with $Al(OH)_3$ and CpG induced higher protection than did 90 μg NP. These findings indicated that a combination of $Al(OH)_3$ and CpG may be an efficient adjuvant in the NP formulation.

Introduction

Seasonal influenza epidemics and the inevitable delay between viral identification and production of the specific vaccine have highlighted the urgent need for next-generation influenza vaccines that can preemptively induce broad immunity to different viral strains. Influenza (flu) is a serious hazard to human health, and vaccination is the most effective method for preventing flu. However, the conventional flu vaccine is produced in special-pathogen-free (SPF) chicken eggs, and it takes 4–6 months to obtain subtype-matched vaccine from the vaccine strain. Moreover, conventional flu vaccines induce protective effects depending on antibodies against highly variable hemagglutinin (HA) and neuraminidase (NA) (Gerdil, 2003). In most cases, it is difficult to prevent flu epidemics or pandemics caused by a new type of influenza A virus (Fedson, 2005; Palese, 2006). Therefore, universal flu vaccines based on conserved influenza A virus antigens are required to prevent flu outbreaks.

Nucleoprotein (NP) is a highly conserved internal antigen of the influenza A virus (Altmuller et al., 1989; Shu et al., 1993) and is the major target antigen for cytotoxic T lymphocyte (CTL) responses (Jameson et al., 1998, 1999; McMichael et al., 1983, 1986). At this time, several vaccines based on the NP antigen alone or in combination with other influenza A virus antigens have been developed including peptide vaccines (Adar et al., 2009; Atsmon et al., 2012; Gao et al., 2013; Jeon et al., 2002; Savard et al., 2012), DNA-based vaccines (Kheiri et al., 2012; Lalor et al., 2008; Luo et al., 2012; Price et al., 2009, 2010; Xu et al., 2011), virus vector-based vaccines (Price et al., 2009, 2010; Antrobus et al., 2012, 2014; Barefoot et al., 2009; Berthoud et al., 2011; Brewoo et al., 2013; Hessel et al., 2014; Kim et al., 2013; Lambe et al., 2013; Li et al., 2013; Lillie et al., 2012; Moraes et al., 2011; Mullarkey et al., 2013; Rohde et al., 2013; Sipo et al., 2011; Vitelli et al., 2013), recombinant attenuated Salmonella vaccines (RASVs) (Ashraf et al., 2011) or protein subunit vaccines (Luo et al., 2012; Haynes et al., 2012; MacLeod et al., 2013). The efficacy of these vaccines has been evaluated in animal models. Numerous studies have found that NP-based protein subunit vaccines can protect animals against homologous and heterologous influenza virus. As early as 1986, Wraith et al. (1987) purified NP of influenza A virus X31 (H3N2) and injected BALB/c mice with two 10-μg doses of NP s.c. at 4-week intervals or with one 50-μg dose i.p. They found that NP immunization resulted in significant protection (75%) of mice from a lethal challenge with PR8. However, the protective efficacy of NP protein-based vaccines requires improvement, possibly using an adjuvant. Next, Tamura et al. (1996) expressed rNP of PR8 in insect cells and found that intranasal immunization of mice with 5 μg rNP combined with the adjuvant cholera toxin B subunit (CTB)

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could accelerate viral clearance from the nasal site after a sublethal dose challenge of influenza virus. Moreover, rNP combined with CTB could protect 70–80% of mice against homologous challenge with PR8 (40LD50) and 40–70% of mice against heterologous challenge with B/Ibaraki/2/85 (B/Ibaraki) (40LD50). Later, Guo et al. (2010) used CTB in the formulation of rNP and found that intranasal immunization of mice with 10 μg rNP with CTB resulted in complete protection against the homologous influenza virus (10LD50), and immunization with 100 μg rNP with CTB provided good cross-protection against heterologous H5N1 and H9N2 avian influenza viruses (5LD50). MacLeod et al., (2013) found that NP delivered with adjuvant aluminum completely protected mice against homologous challenge with PR8. In contrast, immunization with NP delivered with alum and the detoxified LPS adjuvant monophosphoryl lipid A (MPL) provided some protection against the homologous viral strain but no protection against infection by influenza expressing a variant NP. Together, these data suggest that the NP protein subunit vaccine is immunogenic in mice and could provide protection against homologous and heterologous influenza viral challenge in the appropriate formulation.

Protein subunits are preferred in vaccine research because of their increased safety, single antigenic component, coverage of more antigen epitopes, and suitability for large-scale production. In our previous studies, the codon-optimized NP protein of the influenza virus A/Beijing/30/95 (H3N2) was expressed in *Escherichia coli* at high levels and administered at a high dose, and it was found to induce protection with high efficiency (Huang et al., 2012). In most cases, protein-based vaccines are poor immunogens and require the addition of adjuvants in the formulation to induce a protective and long-lasting immune response, even though they are advantageous over traditional vaccines considering safety and production cost. Insoluble aluminum salt is a universally used and safe adjuvant (Reed et al., 2009), and CpG is under development as an adjuvant for vaccines against cancer and infectious diseases (Cooper et al., 2004; Gupta and Cooper, 2008). In this study, aluminum and CpG were used to improve the immunogenicity of NP protein derived from *E. coli*. We investigated whether 10 μg NP in combination with adjuvant alum and CpG provides comparable cross protection as 90 μg NP without adjuvant in mice (Fig. 1).

Results

Adjuvant Al(OH)$_3$ and CpG significantly increased the humoral immune response induced by influenza A virus NP subunit vaccine

We explored whether adjuvant Al(OH)$_3$ and CpG increased the immune response elicited by NP protein (Fig. 2). We found that priming vaccination with NP alone or combined with adjuvant induced substantial anti-NP IgG (1.8×10^4–3.2×10^4), and the IgG titers were not significantly different from each other in the NP-immunized groups.

Boosting immunization performed on day 14 improved anti-NP IgG titers; an NP-specific IgG assay performed on day 28 (Fig. 2, middle) showed that 10 μg NP alone induced higher anti-NP IgG levels (geometric mean: 6.8×10^5). The addition of CpG (G4) induced significantly higher anti-NP IgG titers (geometric mean: 1.8×10^6) than did 10 μg NP alone (G3) (G4 > G3, $p<0.01$), and the addition of Al(OH)$_3$ (G5) induced similar anti-NP IgG titers (geometric mean: 2.8×10^6) as did G4 (G5 ≈ G4, $p>0.05$). Adding Al(OH)$_3$ plus CpG and 10 μg NP (G6) induced higher anti-NP IgG titers (geometric mean: 4.0×10^6) than did G4 alone (G6 > G4, $p<0.05$). However, the anti-NP IgG titers in G5 and G6 were similar ($p>0.05$). Immunization with 90 μg NP (G7) induced similar anti-NP IgG titers (geometric mean: 1.1×10^6) as did G3 and G4 (G7 ≈ G3 and G4, $p>0.05$), but lower titers compared with G5 and G6 (G7 < G5 and G6, $p<0.001$).

After the third immunization, anti-NP IgG titers did not improve in mice immunized with 10 μg NP protein alone (G3), NP protein formulated with CpG (G4) or NP protein formulated with Al(OH)$_3$ and CpG together (G6), compared with after the second immunization. However, anti-NP IgG titers in G5 ($p<0.05$) and G7 ($p<0.05$) improved significantly. As a result, after the third immunization, mice in G5, G6, and G7 showed comparable anti-NP IgG titers (G5 ≈ G6 ≈ G7, $p>0.05$), which were significantly higher than that in G3 (G5, G6 and G7 > G3, $p<0.001$). The anti-NP IgG titer in G4 was significantly higher than that in G3 (G4 > G3, $p<0.01$), comparable to that in G7 (G4 ≈ G7, $p>0.05$) and significantly lower than those in G5 and G6 (G4 < G5 and G6, $p<0.001$).

Antibody subtype analysis (Fig. 3) showed that CpG significantly improved anti-NP IgG2a titer (G4 > G3 and G7, $p<0.01$), but not anti-NP IgG1 titer ($p>0.05$), compared with immunization with NP alone (G3 and G7) (Fig. 3A and B). This decreased the anti-NP IgG1/IgG2a ratio (Fig. 3C), indicative of a potent Th1 response. Al(OH)$_3$ adjuvant significantly increased both anti-NP IgG1 (G5 > G4, $p<0.001$) and IgG2a (G5 > G4, $p<0.05$) titers compared with CpG (Fig. 3A, B), resulting in the highest IgG1/IgG2a ratio (Fig. 3C), which differed from the IgG1/IgG2a pattern in G4 (G5 > G4, $p<0.001$). In G6, lower anti-NP IgG1 (G6 < G5, $p<0.01$) and similar IgG2a (G6 ≈ G5, $p>0.05$) titers were induced, resulting in a lower IgG1/IgG2a ratio compared with group 5 (G6 < G5, $p<0.01$) (Fig. 3C). These results suggest that Al(OH)$_3$ improved the Th2 antibody response against NP protein, which may have been weakened by including CpG in G6. Slightly higher anti-NP IgG1 (G7 > G3, $p<0.05$) and similar IgG2a (G7 ≈ G3, $p>0.05$) levels were induced in G7 compared with G3, resulting in a similar IgG1/IgG2a ratio of these two groups (G7 ≈ G3, $p>0.05$) (Fig. 3).

Adjuvant CpG or Al(OH)$_3$ plus CpG improved the cellular immune response induced by influenza A virus NP subunit vaccine

To characterize the cellular immune responses elicited by NP protein in mice, IFN-γ-, IL-4-, and IL-10-secreting SMNCs after the third immunization were quantified using ELISPOT assays (Fig. 4).

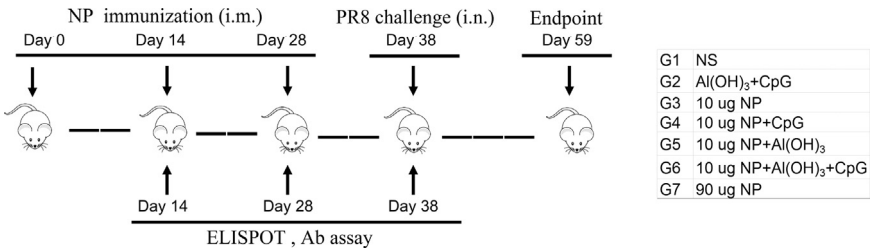

Fig. 1. Experimental schedule of the NP protein. The indicated mice were immunized intramuscularly with NP protein on days 0, 14 and 28. Blood was collected from the mice and analyzed using ELISA on days 14, 28 and 38, after which the mice were sacrificed, and SMNCs were separated and analyzed using the ELISPOT assay. The remaining mice were challenged on day 38 with 20 MLD50 of influenza A virus PR8 and monitored for 3 weeks until day 59. A summary of the mouse groups is provided in the right table.