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Improved Immune Response to Recombinant Influenza Nucleoprotein Formulated with ISCOMATRIX

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Current influenza vaccines elicit antibodies effective against homologous strains, but new strategies are urgently needed for protection against emerging epidemic or pandemic strains. Although influenza vaccine candidates based on the viral nucleoprotein (NP) or matrix protein do not elicit sterilizing immunity, they have the advantage of inducing immunity that may cover a larger number of viral strains. In this study, recombinant NP produced in *Escherichia coli* was purified and formulated in combination with the adjuvant ISCOMATRIX. This formulation increased a NP-specific immunity in mice, with a Th1 profile, and may constitute a promising low-cost influenza vaccine candidate, with ability to stimulate humoral and cellular immune responses.

Keywords: Influenza, recombinant nucleoprotein, ISCOMATRIX

Human influenza virus causes huge losses annually in terms of death and morbidity in the human population. This scenario is further aggravated when unusually virulent pandemic strains emerge, for which the population has no immunological coverage. The main health preventive measure against influenza is seasonal vaccination with vaccines formulated with recently circulating strains. All influenza vaccines currently in use are based on the induction of neutralizing antibodies, mainly against the viral hemagglutinin (HA), which elicit sterilizing immunity, preventing the virus replication after the initial contact [3]. The immunity generated by this type of vaccine is mainly type-specific and may have little or no cross-reactivity with the new strains that are constantly emerging in the field, given the high intrinsic genetic variability of this virus. Therefore, the vaccine strains must be changed every one or two years [http://www.cdc.gov/flu/about/qa/fluvaccine.htm].

New strategies of influenza vaccine production should maximize manufacturing technology and strain coverage. In recent years, the circulation of potentially new pandemic influenza strains gave a strong impetus to the development of new strategies for improving existing vaccines [4, 6, 11, 12, 17, 24, 29] or to develop new-generation vaccines. Strategies based on the use of recombinant viral vectors expressing the envelope protein hemagglutinin have been particularly attractive [8, 13, 22, 27, 28]. These strategies have also been successful when the same kind of vectors were used to induce immunity to conserved proteins of the virus, like the matrix protein and the nucleoprotein NP [1, 23, 38]. Genetic vaccination has also given promising results [14, 18, 21, 25, 33]. However, these approaches require sophisticated technology, and vaccines derived from these technologies would not be available soon in the market, mainly due to safety concerns and specific regulatory issues.

It has been known for a long time that the influenza A NP is a major target antigen for cross-reactive anti-influenza cytotoxic T lymphocytes (CTLs) [9, 35, 37]. Immunization with purified NP protected mice from lethal infection with related or unrelated virus [26, 31, 32, 36]. Recently, it has been reported that NP produced in *Escherichia coli* and formulated with appropriate adjuvants are able to confer a strong cellular immunity and protect against the challenge with homologous or divergent strains [7, 10, 30].

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In this report, we explored the antigenic potential of a recombinant influenza NP formulated with ISCOMATRIX (IMX), an adjuvant capable of inducing significant humoral and cellular immune responses in humans and animals [20]. Although there are several reports of experimental anti-influenza vaccines formulated with ISCOM or IMX, there are no reports on the use of these adjuvant systems combined with purified influenza rNP.

IMX contains saponins (immunostimulating component), cholesterol, and phospholipids and has been reported to integrate innate and adaptive immunity for CTL induction, to enhances cross-presentation of exogenous antigens through the MHC Class I pathway ($CD8^+$ T cells), and to prolong presentation in draining lymph nodes [34]. It also acts as an antigen delivery system, induces cytokines and chemokines, and is a potent activator of dendritic cells *in vivo* [20].

It is important to highlight that veterinary vaccines formulated with this adjuvant are already present in the market, and that it has also been extensively tested in humans [19].

Materials and Methods

Synthesis and Purification of rNP

The influenza strain A/PR/8/34 (H1N1) was provided by the World Health Organization and grown in the allantoic cavity of embrionated hen eggs, following standard procedures. The plasmid pET30a-NP was constructed by cloning the RT-PCR product derived from the full-length NP gene of A/PR/8/34 (H1N1) (GenBank Accession No. EF467822) into the pET30a plasmid vector (Novagen) using the following primers: forward 5'-CCCTCGCGACTGAGTGACATC-3' and reverse 5'-CCCTCGCGAATTTTTCATTAATTGTCG-3'. *Escherichia coli* BL21 (DE3) bacteria were transformed with pET30a-NP and grown to log phase, and protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside. The 6× His-tagged rNP was purified by a HisTrap HP 1 ml column (GE Healthcare) according to the manufacturer's instructions. Fractions eluting between 200 and 300 mM imidazol were pooled, diluted 20 times with 20 mM Tris-HCl (pH 7.0), and passed through a HiTrap SP HP column (GE Healthcare) equilibrated with 20 mM NaCl, 20 mM Tris-HCl (pH 7.0). The bound NP was extensively washed with fifty column volumes of 500 mM NaCl in 20 mM Tris HCl pH 7.0, in order to eliminate putative lipopolysaccharide (LPS) contamination, and was then eluted with 1 M NaCl in the same buffer.

Finally, fractions containing rNP were processed through a 5 ml HiTrap Desalting Column and subjected to sterilizing filtration. All buffers used were prepared with apyrogenic water and columns were sanitized, as indicated by the manufacturers.

The correct size and purity degree of the purified rNP were analyzed by SDS-PAGE in the presence of a reducing agent, stained with Coomassie Brilliant Blue R-250, and quantified by densitometry against bovine serum albumin standards, using the ImageJ software (US National Institutes of Health; http://rsb.info.nih.gov/ij/).

Immunological identity of rNP was determined by Western blot analysis, through sequential incubations with rabbit anti-influenza polyclonal antibody, donkey anti-rabbit antiserum conjugated to horseradish peroxidase, and 4-chloro-1-naphthol plus hydrogen peroxide as substrate.

Formulation of Experimental Antigens and Immunization Schedule

IMX adjuvant was kindly provided by Dr. Jose La Torre (CEVAN, Buenos Aires, Argentina). Eight-to-nine-week-old inbred female BALB/c mice were used in the immunization experiments. All experiments involving animals were approved by the Animal Care Committee of the Medicine School from the National University of Cuyo (IACUC No. 0020398/2011). The animals received a diet of commercial food pellets and water *ad libitum*.

Mice were subcutaneously immunized with 10 μg of rNP alone (control group) or with 10 μg of rNP formulated with 11 μg of IMX. Three weeks later, mice were boosted with the same formulations. On days 0 and 36 post prime inoculation, blood samples were collected to evaluate the presence of rNP specific antibodies in the serum. On day 36, mice were sacrificed by cervical dislocation and their spleen removed and processed to recover spleen cells in order to prepare splenocyte cultures for *in vitro* determination of cytokine production.

Analysis of Humoral Immune Responses

The humoral immune responses induced by the rNP antigen were evaluated by measuring total specific IgG, IgG1, and IgG2a levels by ELISA, in serum samples collected from the different groups of animals. Briefly, serum samples 10-fold serially diluted in PBS–3% BSA were incubated on microplates coated with 3 μg/ml of rNP. Bound antibody was detected with a goat anti-mouse IgG, IgG1, and IgG2a, conjugated with horseradish peroxidase (Thermo Scientific and BD Biosciences) after the addition of tetramethylbenzidine (TMB; Pierce-Endogen). Optical densities (ODs) were read at 450 nm in a microplate reader (Multiskan EX ,Thermo Scientific).

End-point ELISA titers were expressed as the reciprocal of the highest sample dilution that yielded an OD ≥ 2 times the mean value of preimmune control sera.

Splenocyte Cultures and Measurement of Induced Cytokines by ELISA

Spleens from immunized mice were aseptically removed and used to prepare single-cell suspension (4×10^5 cells) in RPMI-1640 culture medium supplemented with 4 mM L-glutamine, 24 mM $NaHCO_3$, 100 units/ml of penicillin, and 10% fetal calf serum. Spleen cells were cultured in flat-bottomed 96-well microtiter plates (Greiner Bio One), stimulated with 1 μg/well of rNP or culture medium for 48 h and tested for the presence of the cytokines by antigen-capture ELISA using OptEIA Set Mouse IFN-γ, IL-10, and IL-4 kits (BD Biosciences, San Diego, CA, USA). Negative controls were incubated in medium alone and positive controls with medium containing ConA (2.5 μg/ml). All assays were performed in triplicate. The concentration of cytokines in the culture supernatants was calculated by using a linear regression equation obtained from the absorbance values of the standards.

Statistical Analysis

Differences between groups were tested for significance by Student's unpaired t-test and Mann Whitney test using GraphPad Prism v.4.00 for Windows (GraphPad Software). P-values <0.05 were considered statistically significant. Data shown represent the mean values ± standard deviation of three independent experiments.

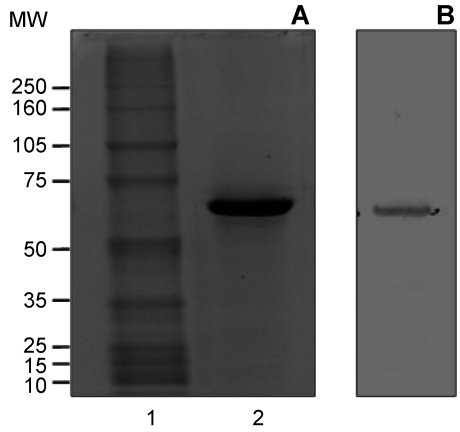

Fig. 1. Characterization of rNP.
(**A**) Purified rNP (2 μg) was fractionated by SDS-PAGE under reducing conditions and stained with Coomassie Brillant Blue. Lane 1, molecular weight markers; lane 2, 2 μg of purified rNP. (**B**) Western blot analysis. The proteins shown in panel A were transferred to a nitrocellulose membrane and rNP was detected using specific rabbit antibodies anti influenza virus.

RESULTS

Expression, Purification, and Characterization of rNP Protein

rNP was found in large quantities soluble in the cytoplasm of *E. coli* and could be purified by affinity columns, owing to the presence of a 6 × His tag at the 5' end of the gene. A second purification step was added using an ion-exchange column in order to minimize possible contamination with bacterial LPS. rNP bound strongly to the HiTrap SP Sepharose column, from which it was detached at high ionic strength. The rNP protein migrated as a single band with the expected apparent molecular mass of 60 kDa (Fig. 1A). No contaminants could be detected after Coomassie Brilliant Blue staining of the gel. Densitometric analysis of an overloaded gel (20 μg of rNP) indicated that the purity of this preparation was higher than 95%, (data not shown). The identity of the band was confirmed by Western blot analysis using polyclonal rabbit antibodies (Fig. 1B). In the conditions used, the yield of purified protein was 12 mg/l.

rNP-Specific Antibodies Generated After Experimental Immunization

BALB/c mice were injected by the subcutaneous route two times at 3-week intervals with 10 μg of rNP formulated with or without IMX, and NP-specific antibodies were determined in the animals sera by ELISA (Fig. 2A). Fifteen days after the second immunization, the rNP antigen induced seroconversion in all immunized mice. However, rNP formulated with IMX, significantly increased the mean titers of anti-NP IgG from 3,500 to 82,000 (Fig. 2A).

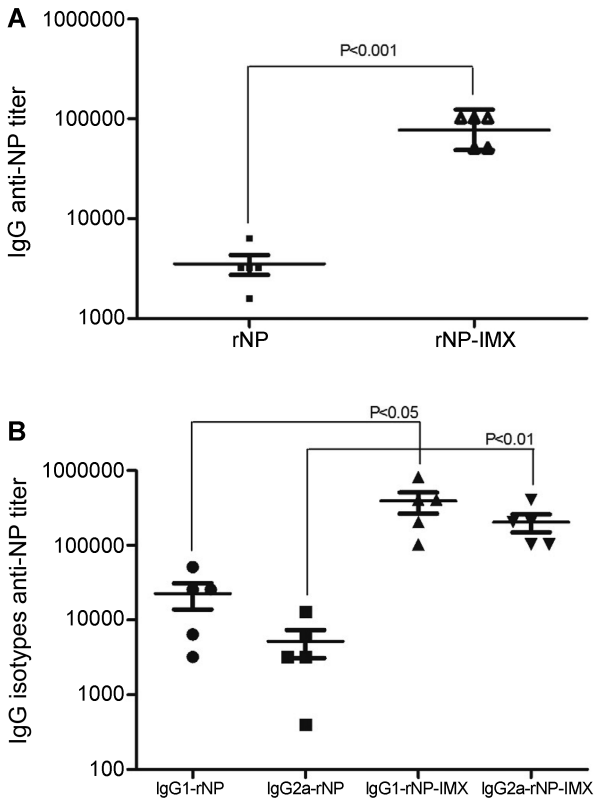

Fig. 2. Analysis of the immune responses.
(**A**) Total specific IgG titers of mice immunized with rNP or rNP-IMX. Filled squares or triangles indicate values for individual animals. Horizontal lines indicate mean values (± SEM). (**B**) Specific IgG isotypes, IgG1 and IgG2a, corresponding to the same groups shown in panel A. ELISA titers shown correspond to serum samples collected at day 36 post inoculation.

The experimental combination rNP-IMX resulted in increased titers of IgG1 (from 22,400 to 389,120) and IgG2a (from 5,200 to 204,800) (Fig. 2B). The ratio IgG2a/IgG1 was also significantly increased from 0.23 to 0.52 when the IMX was included. Since IgG2a and IgG1 have been used as indicators of the induction of Th1 and Th2 responses, respectively, the IgG2a/IgG1 ratio can help to define the T-cell phenotype induced by vaccination [2]. Thus, IgG2a/IgG1 ratios were used as indicators of Th1 or Th2 biased responses induced by immunization. The difference in magnitude of the total IgG titer compared with the anti-NP values of the subtypes IgG1 and IgG2a is likely due to use of different anti-mouse peroxidase conjugated.

Cytokines Elicited in Immunized Animals

The levels of IFN-γ, IL-10, and IL-4 induced by the different formulations of rNP were measured on *in vitro* cultured splenocytes, following stimulation with rNP. Immunization with rNP-IMX resulted in significant increased levels of IFN-γ production ($p<0.05$), compared

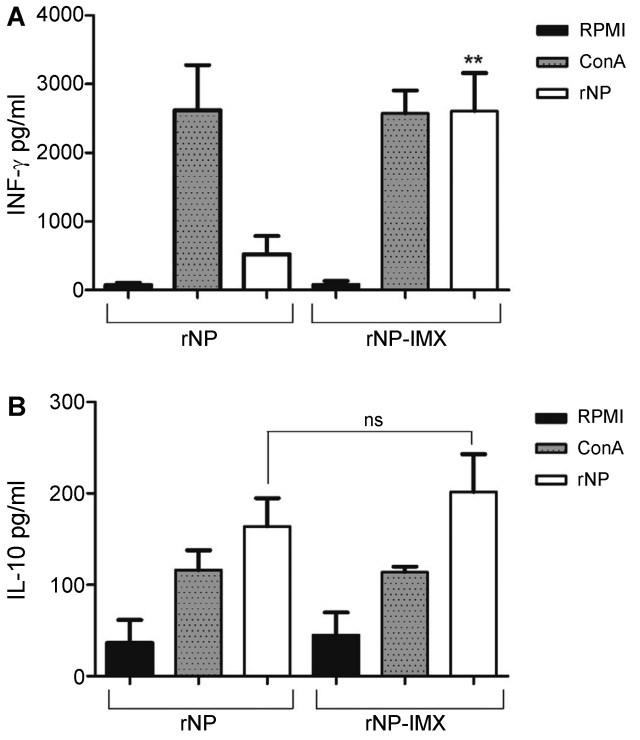

Fig. 3. Quantitative ELISA analysis of INF-γ (**A**) and IL-10 (**B**), secreted by splenocytes of mice immunized with rNP or rNP-IMX upon *in vitro* stimulation with rNP (1 μg/well).
Negative controls were incubated in medium alone and positive controls with medium containing Con A (2.5 μg/ml). The results are presented as the mean value ± SEM. The asterisks at the top of each bar indicate significant differences in comparison with control groups. **, $P < 0.01$; ns = not significant.

with that of the rNP control group (Fig. 3A), whereas no significant differences between both groups were found regarding IL-10 production (Fig. 3B). Barely detectable levels of IL-4 were present in the same supernatants (data not shown). Therefore, the rNP-IMX formulation triggered a higher response of IFN-γ, but a similar low response of IL-10, and IL-4. Taken together, these results suggest that immunization with rNP-IMX induces a specific Th1-type immune response in mice. These results are consistent with the IgG2a/IgG1 ratio induced in mice immunized with rNP-IMX in comparison with the mice vaccinated with unadjuvanted rNP.

Discussion

The results presented in this work confirm the findings of other research groups that the influenza nucleoprotein is produced in large quantities in a soluble form in the cytoplasm of recombinant *E. coli* bacteria, and can be rapidly purified by affinity columns [7, 30]. The analysis of selected immunological parameters, such as specific antibody levels, IgG isotypes, and cytokines (IFN-γ, IL-10, and IL-4), elicited after immunization of experimental animals, suggests that IMX possesses strong immunostimulatory properties when administered with purified influenza rNP, skewing the response toward a Th1 profile. Consistent with these results, the IgG2a/IgG1 ratio was higher in mice immunized with NP-IMX than in those vaccinated with unadjuvanted rNP.

Although the aim of this study was to develop a formulation of rNP capable of promoting a strong cellular response, the high titer of antibodies induced by rNP-IMX should also be taken into account. Very recently, LaMere *et al*. [16] have shown that systemic immunization with NP readily accelerated clearance of a 2009 pandemic H1N1 influenza virus isolate in an antibody-dependent manner. It was also demonstrated that anti-NP IgG specifically promoted influenza virus clearance in mice through a mechanism involving both FcRs and CD8(+) cells, and that anti-NP antibodies correlated with enhanced NP-specific CD8+ T-cell responses [15]. These works strongly suggest that the antibodies induced by immunization with rNP-IMX could also be an important attribute of a vaccine of this type.

The IMX system is an adjuvant and delivery system, and it has been shown that antigens formulated with IMX can transit from endosome to cytosol after incorporation to antigen presenting cells. This allows the processing of the antigen *via* the proteasome pathway for presentation through the MHC Class I pathway, thereby inducing a cytotoxic lymphocyte response, which is considered essential to eliminate the virus-infected cells.

New influenza vaccination strategies are urgently needed, due to uncertainties about future drifts and shifts in circulating viral strains. Previous reports have validated the concept of the feasibility and efficacy of a broad-spectrum T-cell-based influenza vaccine [1, 23, 38]. The trend of recent years regarding the development of new vaccines has underestimated the potential of vaccines based on viral subunits, probably because recombinant proteins are associated with the concept of antibody-producing vaccines. Although extensive data on the protective capacity of NP as a component of a genetic vaccine or expressed by viral vectors have been reported, it is not yet clear whether similar results could be obtained with rNP. In this sense, several laboratories have recently reported that immunization with adjuvanted rNP protects mice from challenge with virulent virus [7, 10, 30]. The data presented in this work confirm the concept that the combination of rNP with suitable adjuvant can be the basis of a broad-spectrum vaccine against influenza.

It should be mentioned that in the experiments reported in this work, the immunogens were formulated only with rNP protein for simplicity. However, the addition of other recombinant influenza proteins, such as Matrix 1 or Matrix

2 to the same formulation, might significantly improve the performance without increasing costs, since these proteins may also be produced in bacteria, under similar conditions [5, 30]. Additionally, it is important to note that the protein yields reported in this work have been obtained growing the bacteria at low cell density, which can be highly optimized using controlled fermenters.

At present, the number of countries with capacity to produce influenza vaccines is very limited. Indeed, the supply of virtually all developing countries is entirely dependent on external production. Simple technology, low costs of production, and broad coverage of the final product are ideal characteristics of an influenza vaccine of new generation.

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Author Disclosure Statement

The authors declare no competing financial interests in relation to the work described in this manuscript.

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