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A vaccination strategy to enhance mucosal and systemic antibody and T cell responses against influenza

Michael Vajdy [a,*], Barbara Baudner [b], Giuseppe Del Giudice [b], Derek O'Hagan [a]

[a] *Novartis Vaccines and Diagnostics, Inc., Emeryville, CA 94608, USA*
[b] *Novartis Vaccines, Inc., Siena, Italy*

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Abstract Influenza infections are a major cause of mortality and morbidity worldwide. Therefore, there is a need to establish vaccines and immunization protocols that can prevent influenza infections. Herein, we show that one intranasal (IN) followed by one intramuscular (IM) immunizations with a combination of cell culture produced hemagglutinin (HA) antigens derived from 3 different influenza strains induced significantly higher serum hemagglutination inhibition (HI) and serum IgG antibody titers as well as T cell responses, compared to 2 IM, 2 IN or 1 M followed by 1 IN immunizations. Moreover, while 2 IM immunizations did not induce any antibody responses in nasal secretions or cervical lymph nodes, which drain the nasal mucosa, IN immunizations alone or in combination with IM immunization induced mucosal and local responses. These data show that the IN followed by IM immunization strategy holds promise to significantly raise serum and local antibody and T cell responses against seasonal influenza strains, and possibly pandemic influenza strains, for which no pre-existing immunity exists.

Introduction

Infections with influenza virus cause considerable morbidity and mortality in the world [1]. Presence of serum HI activity against prevalent influenza viruses strongly correlates with protection from disease and an essential role of B cells in heterosubtypic cross-protection against lethal influenza A H5N1 virus infection has been reported [2]. Although in murine models, a controversial role of mucosal IgA has also been suggested [3], many murine and human studies support the importance of mucosal IgA responses in protection against influenza infection and disease [4]. In this regard, of particular importance have been the findings in both murine and human studies that mucosal IgA induced by intranasal immunization, as opposed to serum IgG induced by parenteral immunization, protected against multiple strains of influenza virus [2,5–14]. Therefore, recent efforts have focused on intranasal immunization strategies that induce both local IgA and systemic IgG responses [13–15].

Commercially available inactivated whole- and split-virus vaccines have been successful to prevent disease caused by influenza infection [16,17], and a licensed cold adapted live attenuated influenza vaccine is currently in use [13,15]. However, these vaccines suffer from limited efficacy in

* Corresponding author. 4560 Horton Street, M/S 4.3, Emeryville, CA 94608, USA. Fax: +1 510 923 2586.
E-mail address: michael.vajdy@novartis.com (M. Vajdy).

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generating long-lasting immunity, particularly in the elderly, and are not sufficiently cross-reactive to protect against antigenic variants [18–20]. Although these vaccines are known to induce serum immunoglobulin G (IgG) antibodies, they are poor stimulators of secretory IgA at respiratory mucosal sites and show sporadic CD8+ cytotoxic T lymphocyte (CTL) activation [1,21,22]. Efforts are currently under way to develop influenza vaccines that generate significant secretory IgA, as well as maintain high serum IgG titers, by exploiting mucosal immunization [18,23–25].

The role of intranasal vs. parenteral immunization and induction of local IgA as opposed to serum IgG in protection against replication in the nose, the lung or protection from disease is well established [26–29]. Therefore, IN immunizations alone or in combination with IM immunization may show superior local and systemic antibody responses. For optimal induction of immune responses through the IN route, effective and safe mucosal adjuvants are required. Mutants of the heat labile enterotoxin from enteropathogenic *Escherichia coli* have been shown to be safe in animal and human studies [30]. LTK63 is an effective mucosal adjuvant with no detectable toxic ADP-ribosyltransferase activity [30], and holds promise as a mucosal adjuvant.

We recently reported that mucosal followed by parenteral immunizations with *Helicobacter pylori*-derived protein antigens induced enhanced local and systemic responses compared to mucosal alone, parenteral alone, or parenteral followed by mucosal immunizations [31]. Moreover, data from a rhesus macaque study suggested that IN followed by IM immunizations significantly enhanced serum and vaginal antibody responses against HIV-env [32]. Therefore, in the current study we tested whether the combination of IN followed by IM immunization with cell culture derived HA from two strains of influenza A and a strain of influenza B virus induced enhanced local and systemic immune responses compared to IM followed by IN, IM alone or IN alone immunizations. Local responses were measured in cervical lymph nodes (CLN), which are widely accepted to drain the nasal mucosa [33–36], as well as in nasal washes.

Materials and methods

Animals and immunizations

Female BALB/c mice (6 or 8 per group) were immunized with canine kidney cell line (MDCK)-derived HA antigens from 3 different commercial influenza strains for the 2004/2005 season, i.e., New Caledonia (H1N1), Wyoming (H3N2), and Jiangsu (B) [37]. This HA preparation was of GMP grade and was recently used in a phase III clinical trial with adequate safety and immunogenicity [38]. The animals were immunized through 2 intramuscular (IM), 2 intranasal (IN), one IN followed by one IM, one IM followed by one IN, or one or two simultaneous IM/IN routes at a 28-day or a 14-day interval. The HA doses were 0.1 or 1 μg, while the LTK63 doses were 0.5 or 5 μg. For IN immunizations LTK63 was used, while for IM immunizations no adjuvants or delivery systems were used. Sera were collected from orbital sinus blood at 14 days after the first immunization and at 7 or 14 days after the final immunization. At 14 days or 7 days after the final immunization nasal washes were collected by holding the head in an upright position so that one nostril faced upward and applying 0.5 ml of PBS through the nostril pointing upward and collecting the wash from the other nostril. The nasal washes were snap frozen on dry ice and stored at −80 °C until use. At 7 days after the final immunization the mice were sacrificed and CLN and spleens were collected for detection of antigen-specific antibody-secreting cells and Tcell responses. The mice were purchased from Charles River Laboratories and maintained at the Novartis vivarium in Emeryville, which is an AAALAC (Association of Assessment and Accreditation of Laboratory Animal Care) accredited facility. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council.

Standard colorimetric and europium-based fluorescent ELISA

Titration of HA-specific immunoglobulin G (IgG) was performed on sera from individual mice collected at 2 weeks after the last immunization. Maxisorp 96-well flat-bottom plates (Nunc, Roskilde, Denmark) were coated overnight at 27–30 °C with 0.2 μg/well HA derived from H1N1, H2N3 or B influenza strains prepared in canine kidney cell lines in phosphate-buffered saline pH 7.4 (PBS). The coated wells were blocked for 1 h at room temperature with 300 μl of PBS pH 7.4, 0.1% BSA and 0.05% Tween-20 with 3% goat serum. The plates were washed with PBS pH 7.4, 0.1% BSA and 0.05% Tween-20, tapped and dried. Serum samples and serum standard were initially diluted 1:5000 with the dilution buffer (PBS pH 7.4, 1% BSA, 0.05%Tween-20), then transferred into coated-blocked plates in which the samples were serially diluted three-fold with the same buffer. Antigen-specific IgG was revealed with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). Antibody titers were expressed as the logarithm of the enzyme-linked immunosorbent assay titers that gave an optical density (OD) higher than the mean plus five times the standard deviation (SD) of the average OD obtained in the pre-immune sera. The titers were normalized with respect to the reference serum assayed in parallel.

Anti-HA IgG and IgA were measured in supernatants using a europium-based ELISA assay. Briefly, 96-well plates were coated with 1 μg/ml of HA from Jiangsu/B and blocked with 5% goat serum in PBS. The samples were then diluted in DELFIA assay buffer (PerkinElmer, Boston, MA) and added to the wells and incubated overnight at 4 °C. The samples were washed with DELFIA wash buffer and then goat anti-mouse IgG or IgA (Southern Biotechnology Associates, AL) was added to the plates and the plates were incubated at room temperature for 2 h. The plates were then washed and streptavidin–europium (PerkinElmer) was added to the plates at 1:1000 dilution in DELFIA assay buffer (PerkinElmer) and the plates were incubated at room temperature for 1 h under shaking. The plates were then washed and DELFIA Enhancement Solution (PerkinElmer) was added to the plates and incubated at room temperature for 5 min under shaking. The plates were read on a Wallac Victor 21420 Multi-label fluorescence reader at 616 nm. The data are presented as mean titers from 2 subgroups of 3 mice and 2 experiments plus standard deviation.