Effects of Different Adjuvants in the Context of Intramuscular and Intranasal Routes on Humoral and Cellular Immune Responses Induced by Detergent-Split A/H3N2 Influenza Vaccines in Mice

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Influenza A/H3N2 viruses have caused the most severe epidemics since 1968 despite current immunization programs with inactivated vaccines. We undertook a side-by-side preclinical evaluation of different adjuvants (Alum, AS03, and Protollin) and routes of administration (intramuscular [i.m.] and intranasal [i.n.]) for assessing their effect on the immunogenicity and cross-reactivity of inactivated split vaccines (A/H3N2/New York/55/2004). Humoral and T cell-mediated immune responses against the homologous virus and a heterologous drifted strain (A/H3N2/Wisconsin/67/2005) were measured in BALB/c mice at 2, 6, and 19 weeks postboost. The AS03- and Alum-adjuvanted i.m. vaccines induced at least an 8-fold increase over the nonadjuvanted vaccine in functional antibody titers against both the homotypic and heterotypic strains and low IgG2a and high IgG1 levels, suggesting a mixed Th1/Th2 response with a Th2 trend. The Protollin-adjuvanted i.n. vaccine induced the lowest IgG1/IgG2a ratio, which is indicative of a mixed Th1/Th2-type profile with a Th1 trend. This adjuvanted vaccine was the only vaccine to stimulate a mucosal IgA response. Whatever the timing after the boost, both hemagglutination inhibition (HAI) and micro-neutralization (MN) titers were higher with the AS03-adjuvanted i.m. vaccine than with the protollin-adjuvanted i.n. vaccine.

Our data indicate that the adjuvanted vaccines tested in this study can elicit stronger, more persistent, and broader immune responses against A/H3N2 strains than nonadjuvanted inactivated influenza vaccines.

Influenza A viruses are globally important human respiratory pathogens that cause seasonal epidemics and occasional pandemics, both of which can vary significantly in disease severity. During seasonal epidemics, circulating influenza viruses typically infect 10 to 20% of the total worldwide population, resulting in 3 million to 5 million cases of severe illness and 250,000 to 500,000 deaths every year (24). Influenza A/H3N2 viruses emerged in the human population in 1968, causing the so-called “Hong Kong” pandemic, and since then they have been responsible for the most severe epidemics. Influenza A/H1N1 viruses reemerged in 1977 (43) and have also been associated with yearly epidemics (4). Recently, the new swine-origin A/H1N1 virus was efficiently transmitted to humans, causing the first pandemic of the 21st century (13).

Vaccination is considered the most effective means to control influenza infections. However, due to the rapid antigenic evolution of the viral surface glycoprotein hemagglutinin (HA), trivalent vaccines have to be reformulated on a yearly basis with new representative strains. The efficacy of the detergent-split influenza virus vaccine is about 70% in children and young adults, and it is much lower in the elderly, in immunocompromised subjects, and in the case of antigenic mismatches between the vaccine and circulating strains (6). From 1997 to 2006, 5 antigenic drifts involving A/H3N2 viruses have been identified, and at least 4 of them resulted in a significant vaccine mismatch (45). In healthy individuals, the virus acts as a robust immunogen, eliciting neutralizing serum antibody that protects against reinfection with homologous strains. Both the humoral and cell-mediated arms of the adaptive immune response are involved in the resolution of active influenza infection, with neutralizing antibody titers correlating with in vivo protection (9).

A well-known mechanism of improving the immunogenicity of existing influenza vaccines involves the addition of adjuvants, and this has been particularly tested in the case of prepandemic A/H5N1 vaccines (5, 31). Conventional vaccine adjuvants were initially based mainly on aluminum salts. A new generation of more powerful adjuvants, based on oil-in-water formulations and/or specific agonists of Toll-like receptors (TLR), has been recently developed and evaluated. For instance, the AS03 adjuvant system is an oil-in-water emulsion containing DL-α-tocopherol and squalene (30). Such adjuvanted vaccines have been shown to confer some degree of heterotypic response and the potential for antigenic sparing in the context of A/H5N1 viruses (30). Another approach to improve immunogenicity of inactivated vaccines is to use alternate routes of administration. In this regard, the adjuvant...
Protollin, a TLR2/TLR4 agonist, has been optimized for mucosal delivery, which better mimics the natural route of infection and has the potential to elicit mucosal (IgA) immunity (26).

In this study, we performed a side-by-side preclinical evaluation of the immunogenicity of detergent-split A/H3N2/New York/55/2004 vaccines formulated with intramuscular (Alum and AS03) or intranasal (Protollin) adjuvants in mice. We also evaluated the potential of these vaccine formulations to induce a cross-reactive response to the heterologous drifted strain A/H3N2/Wisconsin/67/2005.

MATERIALS AND METHODS

Viruses, vaccines, and adjuvants. Reference wild-type A/H3N2/New York/55/2004 (or NY) and A/H3N2/Wisconsin/67/2005 (or WIS) virus strains (NIHSC, Hertfordshire) along with detergent-split influenza antigens (GSK Biologicals) from these two strains were used in this study. The Protollin adjuvant (26) consisted of proteosomes (outer membrane proteins derived from wild-type Neisseria meningitidis group B) noncovalently complexed with lipopolysaccharide (LPS) isolated from Shigella flexneri serotype 2a. The AS03 adjuvant (30) consisted of an oil-in-water emulsion-based adjuvant system containing 23.72 mg/ml α-tocopherol, 21.38 mg/ml squalene, and 9.72 mg/ml polysorbate-80 in phosphate-buffered saline (PBS). Alhydrogel, an aluminium hydroxide [2Al(OH)₃] gel suspension (Alum), was purchased from Brentag Biocsector. Detergent-split antigens were mixed with the adjuvants just prior to immunization.

Mouse immunizations. The immunogenicities of nonadjuvanted and adjuvanted detergent-split NY vaccines following intramuscular (i.m.) or intranasal (i.n.) administrations were assessed using groups of 6- to 8-week-old female BALB/c mice (Charles River). Animals were housed four per cage in HEPA-filtered cages, and food and water were available ad libitum. All animal procedures were approved by the Animal Protection Committee of the Centre Hospitalier Universitaire de Quebec in agreement with the Canadian Council on Animal Care. On day 0, mice were immunized i.m. with 50 μl containing 0.75 or 3.0 μg (based on HA content) of the NY antigen combined or not with 0.2% Alum (0.01 μg/50 μl) or AS03. Control groups received a saline solution or the adjuvants alone. In parallel, groups of mice were immunized i.n. with 30 μl of 0.75 μg of the NY antigen combined or not with 0.75 or 3 μg of Protollin based on LPS content (22) and also with 3 μg of the NY antigen combined with 3 μg of Protollin. Control groups received a saline solution or the Protollin adjuvant alone (3 μg/mouse). A second immunization was repeated on day 21 (boost) with the same formulations. Prior to boosting and 14 days postboost (p.b.), the animals were exsanguinated by cardiac puncture, and then serum samples were separated from clotted blood and stored at −20°C. Bronchoalveolar lavages (BAL) were also performed at that time, followed by 1.0-ml PBS injections with protease inhibitors. The same immunization protocols were repeated to assess long-term humoral and cellular immune responses with selected groups of mice: 3 μg of the NY antigen combined or not with Alum or AS03 (i.m.) and 0.75 or 3 μg of the NY antigen combined or not with 0.75 or 3 μg of Protollin (i.n.). On weeks 2, 6, and 19 p.b., 8 mice from each group were exsanguinated, and serum samples were separated and stored at −20°C. Splenectomies were removed aseptically, pooled, and used for in vitro restimulation studies and cytokine production.

Antibody assays. An array of assays was used to evaluate humoral responses. Antibody titers were first determined by enzyme-linked immunosorbent assay (ELISA) by using Nunc 96-well MaxiSorp plates coated overnight at 4°C with detergent-split NY antigen or detergent-split WIS antigen (0.025 μg/well). Serial dilutions of serum or BAL fluid in PBS supplemented with 0.1% (vol/vol) Tween 20 and 2% (wt/vol) dried milk powder were added to the plates. The presence of IgG1, IgG2a, and IgA was detected by the addition of horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies (AbD Serotec). After substrate addition (TMB; Fitzgerald Industries), samples were incubated for 30 min at 25°C, and the reaction was stopped by 0.2 M sulfuric acid. Plates were read at 450 nm. Antibody titers in the samples were calculated from a standard curve run on each plate (IgG1, mouse IgG1-UNLB, Cedarlane; IgG2a, mouse IgG2a-UNLB, Cedarlane; and IgA, purified mouse IgA, Bethesda Laboratories). Results were analyzed with the SoftMax Pro version 5 software, and values were expressed as nanograms of specific antibody per milliliter of serum or BAL fluid. Sera were also tested for the presence of specific functional antibodies by hemagglutination inhibition (HAI) and microneutralization (MN) according to WHO standard protocols (48) with some modifications (40). Due to the low volume of sera available from each mouse, samples from each group of mice were pooled for both assays and tested in duplicate, precluding statistical analyses.

Splenocyte cell culture and cytokine determination. Spleens from 8 mice per group were harvested on weeks 2, 6, and 19 p.b. into serum-free medium (RPMI 1640; Wisent) supplemented with 20 U/ml penicillin, 0.02 mg/ml streptomycin, and 2 mM l-glutamine (Sigma-Aldrich), and single-cell suspensions were prepared by homogenization. Cells were subsequently treated through a 70-μm cell strainer (Falcon; VWR International), incubated for 5 min at 37°C in 5 ml (per spleen) of 0.14 M NaCl (Sigma-Aldrich) and 17 mM Tris (MP Biomedicalals) at pH 7.2 to lyse red blood cells, and then plated on 96-well plates. For intracellular cytokine staining, 2 × 10⁶ splenocytes were stimulated for 2 h either with nonspecific stimulus phorbol myristate acetate (PMA), ionomycin (activation control), or specific stimulus, i.e., 1 μg of detergent-split influenza virus antigen of the homologous (NY) or the heterologous (WIS) strains. After 2 h of stimulation, 5 μg/ml of brefeldin A (eBioscience) was added, and the mixture was incubated overnight. Cells were harvested, and surface and intracellular cytokine staining were performed, as described previously (44). Briefly, cells were incubated with fluorescein isothiocyanate (FITC)– and PerCP/Cy5.5–labeled anti-CD4 and anti-CD8 antibodies (Becton Dickinson), respectively, for 30 min and then fixed with 1% paraformaldehyde and permeabilized. Rat anti-mouse interleukin 2 (IL-2), gamma interferon (IFN-γ), and IL-4 antibodies labeled with phycoerythrin (PE), allophycocyanin (APC), and PE-Cy7 fluorochromes (Becton Dickinson) and were concentrated at 0.25 μg/10⁶ cells and incubated for 30 min and then washed and analyzed by multiparameter flow cytometry (FACS Aria II). Data were acquired on the FACSDiva system (Becton Dickinson) and analyzed using the FCSExpress3 software. To analyze the CD4⁺ and CD8⁺ T cells, debris were gated out by forward-scatter and side-scatter characteristics. CD4⁺ and CD8⁺ T cells were also gated for various cytokines. The percentage of cells within the double-positive quadrants on dot plots was selected for cytokine data representation.

Statistical analysis. Serum IgG1, IgG2a, and IgA levels were compared using the paired t test. Lymphocyte and cytokine results (pooled data of three experiments) were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. All analyses were done using the GraphPad Prism (version 3.03) software.

RESULTS

Humoral response to a homotypic strain following immunization with nonadjuvanted and adjuvanted intramuscular vaccines. In this first study, mice were immunized (on days 0 and 21) intramuscularly (i.m.) with 0.75 or 3.0 μg of detergent-split NY antigen combined or not with Alum or AS03. The ability of these vaccines to elicit systemic humoral immunity was assessed by analyzing serum for specific antibodies (IgG1, IgG2a) by ELISA and for functional immunity by hemagglutination inhibition (HAI) and microneutralization (MN) assays at 2 weeks postboost (p.b.). In addition, we analyzed IgA antibodies at week 2 p.b. by ELISA in bronchoalveolar lavage (BAL) fluid to assess mucosal immunity. Mice immunized with 3 μg of the A/H3N2/New York/55/2004 (or NY) antigen combined with Alum or AS03 showed the highest levels of IgG1 and also high IgG1/IgG2a ratios. The presence of
both IgG1 and IgG2a isotypes was suggestive of a mixed Th1/Th2-type profile, with a trend for higher Th2 antibody response in these groups (Fig. 1). The highest ratios were seen with the groups immunized with 0.75 μg of the NY antigen combined with Alum (IgG1/IgG2a ratio of 133) and with 0.75 μg of the NY antigen combined with AS03 (IgG1/IgG2a ratio of 238). Nevertheless, the use of BALB/c mice that show a Th2 genetic background also directed this response toward a stronger Th2-type profile. In addition, reciprocal HAI titers (Fig. 2a) elicited by each of the i.m. adjuvanted formulations were 8-fold higher than those of the NY antigen alone (640 for 0.75 and 3 μg of the NY antigen combined with Alum or AS03 compared to 80 for 0.75 μg of the NY antigen alone and 160 for 3 μg of the NY antigen alone). For MN (Fig. 2b), reciprocal titers elicited by each of the adjuvanted formulations were 2- to 8-fold higher than those of the NY antigen alone (1,280 for 0.75 μg of the NY antigen plus Alum or AS03 compared to 160 for the NY antigen alone; 1,280 and 2,560 for 3 μg of the NY antigen combined with Alum and AS03, respectively, compared to 640 for the NY antigen alone). There was no detectable humoral response for the groups of mice immunized with the adjuvants alone (data not shown). Also, no mucosal immunity was elicited by nonadjuvanted and adjuvanted i.m. vaccines, since no IgA antibodies were detected in BAL samples at week 2 p.b. (data not shown).

Humoral response to a homotypic strain following immunization with nonadjuvanted and adjuvanted intranasal vaccines. Mice were immunized (on days 0 and 21) intranasally (i.n.) with 0.75 μg or 3 μg of detergent-split NY antigen combined or not with 0.75 or 3 μg of Protollin. Compared to mice that were immunized with the NY antigen alone, i.n. immunization with 0.75 or 3 μg of this antigen in the presence of either 0.75 or 3 μg of Protollin was associated with almost equally high levels of IgG1 and IgG2a and the lowest IgG1/IgG2a ratios at week 2 p.b. (between 5 to 7), which is indicative of a mixed Th1/Th2-type response with a trend for a predominant Th1 profile (Fig. 1), compared to those of AS03- or Alum-adjuvanted vaccines. The lowest ratios were seen with the groups immunized with the combination of 0.75 μg of the NY antigen and 0.75 μg of Protollin (IgG1/IgG2a ratio of 4.7) and of 0.75 μg of the NY antigen and 3 μg of Protollin (IgG1/IgG2a ratio of 5.6). In addition, adjuvanted formulations elicited at least 8-fold-higher HAI titers (Fig. 2a) than the NY antigen alone (320 and 640 for the combination 0.75 μg NY antigen and 0.75 and 3 μg of Protollin compared to 40 for the NY antigen alone; 320 for the combination of 3 μg NY antigen and 3 μg of Protollin compared to 40 for 3 μg of the NY antigen alone). For MN (Fig. 2b), reciprocal titers elicited by each of the adjuvanted formulations were 4- to 16-fold higher than those of the NY antigen alone (320 for both 0.75 and 3 μg of Protollin-NY antigen combinations compared to 80 for 0.75 μg of the NY antigen alone; 1,280 for 3 μg of Protollin combined with 3 μg of the NY antigen compared to 80 for 3 μg of the NY antigen alone). For the same antigen dose (3 μg), HAI and MN titers were 2-fold
higher with AS03-adjuvanted i.m. vaccines than with Protollin-adjuvanted i.n. vaccines. There was no evidence of humoral response for the groups of mice immunized with the adjuvants alone (data not shown).

Mucosal immunity (IgA production) in BAL fluids elicited by the NY antigen adjuvanted with 0.75 or 3 μg of Protollin was significantly increased compared to that of nonadjuvanted vaccines, for which no IgA was detected (Fig. 3). For the adjuvanted vaccine, there was a dose-dependent response, with the 3 μg of the NY antigen-Protollin combination eliciting the higher IgA response.

Humoral response to a heterotypic strain following immunization with nonadjuvanted and adjuvanted intramuscular and intranasal vaccines. We also tested the ability of serum samples from mice immunized with the detergent-split NY vaccine to recognize the A/H3N2/Wisconsin/67/2005 (or WIS) drifted strain. We found that the IgG1 responses with 3 μg of the NY antigen-Alum, -AS03, or -Protollin (3 μg) combinations were, respectively, 20%, 48%, and 27% of those obtained with the homologous strain (Fig. 1a), whereas the IgG2a responses were 22%, 69%, and 27%, respectively (Fig. 1b). Similarly, the adjuvants increased reciprocal HAI and MN titers for the heterotypic strain by at least 4-fold compared to those of the respective nonadjuvanted vaccine groups (Fig. 2a and b). The highest absolute heterotypic responses for the same antigen content (3 μg) were seen with AS03 by HAI and MN assays (reciprocal titers of 160 and 1,280, respectively, compared with 160 and 640 for Alum and 160 and 640 for Protollin at 3 μg).

The cross-reactive responses were also evident when assessing IgA antibodies (Fig. 3). The ratios of heterotypic/homotypic IgA responses were 131%, 69%, and 32% for 0.75 μg of the NY antigen combined with 0.75 μg of Protollin, 0.75 μg of the NY antigen combined with 3 μg of Protollin, and 3 μg of the NY antigen combined with 3 μg of Protollin, respectively.

Long-term humoral response to homotypic and heterotypic strains following immunization with nonadjuvanted and adjuvanted intramuscular and intranasal vaccines. We studied the long-term humoral responses at weeks 6 and 19 p.b. in a separate experiment with selected groups of mice (i.e., 3 μg of detergent-split NY antigen alone [i.m. or i.n.], 3 μg of detergent-split NY antigen combined with Alum or AS03 [i.m.], and 0.75 or 3 μg of
detergent-split NY antigen combined with 0.75 or 3 μg of Protollin [i.n.]. In those groups, we analyzed the reciprocal HAI and MN titers and found that they reached a peak at 6 weeks and then generally slightly decreased at 19 weeks, except for the AS03-adjuvanted group (by HAI and MN) and the Protollin-adjuvanted group (by MN only), for which there was no decrease at week 19 p.b. At week 6 p.b., there was at least a 16-fold increase in HAI and MN titers for all adjuvanted vaccines compared to the nonadjuvanted vaccine (Fig. 4a and b). Both absolute HAI and MN reciprocal titers were lower at week 6 p.b. with Protollin-adjuvanted vaccines (640 and 1,280, respectively) than with Alum (2,560 and 10,240)- or AS03 (2,560 and 5,120)-adjuvanted vaccines. Overall, the increases in reciprocal HAI and MN titers over time against the drifted WIS strain paralleled those of the homotypic NY strain (Fig. 4a and b).

T cell-mediated response to a homotypic strain following immunization with nonadjuvanted and adjuvanted intramuscular vaccines. Selected groups of mice were immunized twice with the different i.m. adjuvanted and nonadjuvanted vaccines (i.e., 3 μg of detergent-split NY antigen alone or combined with Alum or AS03). At weeks 2, 6, and 19 p.b., animals were sacrificed to evaluate the type of cellular immune responses elicited by the different vaccine formulations by measuring the intracellular cytokine production by CD4⁺ and CD8⁺ T cells in splenocytes stimulated with the homologous virus (NY) antigen using multiparameter flow cytometry. Specifically, the production of IL-2 and IFN-γ (representative Th1-type cytokines) as well as IL-4 (representative Th2 cytokine) by the same CD4⁺ and CD8⁺ T cell population was evaluated. The percentage of IL-2-producing CD4⁺ T cells by NY antigen-stimulated splenocytes of mice immunized i.m. with the Alum-adjuvanted vaccine was 2-fold higher (P < 0.05) (0.33% ± 0.057/0.16% ± 0.03, 0.55% ± 0.1/0.25% ± 0.05, and 0.54% ± 0.1/0.27% ± 0.08 at weeks 2, 6, and 19 p.b., respectively) than that of the NY antigen alone (Fig. 5a). The percentage of IFN-γ-producing CD4⁺ T cells also increased up to 1.8-fold on average (0.23% ± 0.05/0.13% ± 0.055 at week 2 p.b., 1.25% ± 0.1/0.65% ± 0.1 at week 6 p.b., and 1.1% ± 0.06/0.65% ± 0.1 at week 19 p.b.) compared to that of the NY antigen group (Fig. 5a). The percentage of IL-4-producing CD4⁺ T cells was also significantly (P < 0.05) increased (2.1-fold on average) in the Alum-adjuvanted group (0.62% ± 0.1/0.24% ± 0.05, 0.76% ± 0.2/0.40% ± 0.06, and 0.62% ± 0.05/0.36% ± 0.05 for weeks 2, 6, and 19 p.b., respectively) compared to that of the nonadjuvanted group (Fig. 5a). Finally, immunization with the AS03-adjuvanted group induced a higher (but not statistically significant) production of IL-2- and IFN-γ-producing CD4⁺ T cells than the NY antigen group alone (Fig. 5a).

Next, we measured IL-2, IFN-γ, and IL-4 cytokine production by CD8⁺ T cell NY antigen-stimulated splenocytes isolated from immunized mice. Compared to the NY antigen-only group, immunization with the Alum-adjuvanted vaccine elicited a 2-fold significant (P < 0.05) increase in IL-2-producing CD8⁺ T cells and a 1.9-fold significant (P < 0.01) increase in IFN-γ-producing CD8⁺ T cells at week 6 p.b. (Fig. 5b). The IFN-γ response remained high after week 19 p.b. in the Alum-adjuvanted vaccine group (1.8% ± 0.05/1.0% ± 0.03). Furthermore, IFN-γ-producing CD8⁺ T cells were also significantly higher (P < 0.01) in the Alum-adjuvanted group than in the AS03-adjuvanted group at weeks 6 and 19 p.b. IL-4 production by CD8⁺ T cells in both the Alum-adjuvanted and nonadjuvanted vaccine groups was close to that of the saline group (only 0.25% of the CD8⁺ T cells). Immunization with the AS03-adjuvanted vaccine elicited a slight increase in the percentage of IL-2- and IFN-γ-producing CD8⁺ T cells, but this increase was not statistically significant compared to that of the nonadjuvanted vaccine group (Fig. 5b).

T cell-mediated response to a homotypic strain following immunization with nonadjuvanted and adjuvanted intranasal vaccines. We next evaluated the production of IL-2, IFN-γ, and IL-4 by CD4⁺ and CD8⁺ T cells in groups of mice immunized i.n.
Induction of cytokine-producing CD4+ (a) and CD8+ (b) T cells in splenocytes of 8 mice/group/time point immunized intramuscularly with 3 μg of A/New York/55/2004 detergent-split vaccines with or without adjuvants (Alum or AS03) following homologous (NY) antigen stimulation. Representative FACS analysis data of the week 6 postboost group illustrating the percentage of T cells gated out by anti-CD4 and anti-CD8 antibodies that coproduce IL-2, IFN-γ, and IL-4 cytokines as determined by intracellular staining. Double-positive (CD4/CD8- and cytokine-positive) cells were taken for graphical representation of cytokine production at weeks 2, 6, and 19 postboost. The results (pooled data of splenocytes from various animal groups stimulated with homologous A/New York/55/2004 detergent-split virus in triplicate) were analyzed for their homogeneity of variance by one-way ANOVA followed by a Tukey-Kramer posttest using GraphPad Prism (version 3.03) software. Significant differences between the various groups are indicated (*, P < 0.05; **, P < 0.01; and ****, P < 0.001).
with the different vaccine combinations, including 3 μg of detergent-split NY antigen alone or 0.75 μg of detergent-split NY antigen combined with 0.75 μg of Protollin or 3 μg of detergent-split NY antigen combined with 3 μg of Protollin by using multiparameter fluorescence-activated cell sorting (FACS) analysis. Percentages of IL-2-producing CD4+ T cells by splenocytes stimulated with the homologous virus (NY) antigen were 2-fold higher (P < 0.01) in mice immunized with the 0.75 μg Protollin-adjuvanted vaccine compared to the NY antigen-only group at weeks 6 (0.6% ± 0.1/0.3% ± 0.1) and 19 (0.53% ± 0.05/0.26% ± 0.05) p.b. (Fig. 6a). Percentages of IL-2–producing CD4+ T cells in mice immunized with the 0.75 μg Protollin-adjuvanted vaccine were also significantly higher (P < 0.05) than the 3 μg Protollin-adjuvanted group at weeks 2 and 19 p.b. Similarly, the 0.75 μg Protollin-adjuvanted group elicited a 2.2-fold increase (P < 0.05) in IFN-γ–producing CD4+ T cells compared to that of the NY antigen group at weeks 6 (0.52% ± 0.2/0.23% ± 0.05) and 19 (0.5% ± 0.1/0.23% ± 0.05) p.b. A 2.13-fold increase (P < 0.05) was also observed in IL-4–producing CD4+ T cells in mice immunized with the 0.75 μg Protollin-adjuvanted vaccine compared to the nonadjuvanted vaccine at weeks 6 (0.58% ± 0.05/0.26% ± 0.1) and 19 (0.51% ± 0.07/0.25% ± 0.1) p.b. Interestingly, immunization with higher antigen (3 μg) and Protollin (3 μg) doses elicited a slight decrease in IL-2 and IL-4 production by CD4+ T cells compared to that of the lower-dose (0.75 μg) adjuvanted vaccine (Fig. 6a). However, the percentages of IFN-γ–producing CD4+ T cells remained similar (0.5% and 0.52%) in groups immunized with either the high- or low-dose adjuvanted vaccine formulations.

Production of IL-2, IFN-γ, and IL-4 cytokines by CD8+ T cells was also assessed in the same groups of immunized mice. Only the combination of detergent-split NY antigen (0.75 μg) and Protollin (0.75 μg) elicited a significant (P < 0.05) increase (~2-fold) in the percentage of IL-2–producing CD8+ T cells over the group immunized with the NY antigen at both weeks 6 (0.44% ± 0.05/0.24% ± 0.06) and 19 (0.5% ± 0.1/0.24% ± 0.06) p.b. (Fig. 6b). The percentage of IFN-γ–producing CD8+ T cells was also significantly (P < 0.05) higher (1.7-fold) in the group of mice immunized with the 0.75 μg Protollin-adjuvanted vaccine than in the NY antigen-only group at all time points p.b. tested (0.53% ± 0.05/0.30% ± 0.05, 0.65% ± 0.05/0.37% ± 0.1, and 0.6% ± 0.1/0.37% ± 0.1 at weeks 2, 6, and 19 p.b., respectively). The percentage of IFN-γ–producing CD8+ T cells was also significantly (P < 0.05) higher in the group of mice immunized with the 0.75 μg Protollin-adjuvanted vaccine than in the 3 μg Protollin-adjuvanted group at week 6 p.b. However, no significant differences in IL-2 and IFN-γ production were observed between the group immunized with a higher dose of antigen and Protollin and the nonadjuvanted group (Fig. 6b). The IL-4 response was generally low for all the vaccine formulations tested, with ~0.25% of the CD8+ T cells producing this cytokine over the 19-week p.b. period.

T cell-mediated response to a heterotypic strain following immunization with nonadjuvanted and adjuvanted i.m. and i.n. vaccines. To assess the ability of the different vaccine combinations to induce cross-reactivity against a drifted strain, we measured IL-2, IFN-γ, and IL-4 cytokine production by CD4+ and CD8+ T cells in splenocytes of both i.m. and i.n. immunized groups of mice upon stimulation with a heterologous virus (WIS) antigen. The numbers of IL-2– and IL-4–producing CD4+ and CD8+ T cells were not significantly different between the saline, nonadjuvanted, and i.m. or i.n. adjuvanted groups (data not shown). There was also no significant difference in IFN-γ–producing CD8+ T cells for the heterotypic strain between all i.m. adjuvanted groups, the nonadjuvanted group, and the saline group (data not shown). However, the percentages of IFN-γ–producing CD8+ T cells were significantly (P < 0.05) higher in the two Protollin groups at weeks 6 and 19 p.b. than in the saline group (Fig. 7). No significant difference was seen, however, between the nonadjuvanted and i.n. adjuvanted groups.

DISCUSSION

The most utilized influenza vaccine today is still the nonadjuvanted detergent-split virus formulation. Despite good immunogenicity and protection in healthy adults, the efficacy of this vaccine is less than optimal in the elderly and in immunocompromised hosts. Furthermore, the protection conferred by nonadjuvanted detergent-split vaccines is short-lived and seriously compromised in the case of antigenic drifts (6, 45). We have thus investigated the kinetics and the magnitude of humoral and cellular immune responses as well as the cross-immunogenicity of detergent-split A/H3N2 vaccines combined with selected adjuvants (Alum, AS03, and Protollin) and administered by the i.m. and i.n. routes in BALB/c mice. Generally, we observed that both i.m. and i.n. adjuvanted vaccines induced higher humoral and cellular immune responses than nonadjuvanted vaccines. We showed that the i.n. Protollin-adjuvanted vaccine elicited a vigorous mucosal response (IgA production) and also induced the lowest IgG1/IgG2a ratio in favor of a mixed Th1/Th2-type response with a trend for a Th1 profile. On the other hand, i.m. vaccine combinations with Alum or AS03 induced a mixed Th1/Th2-type response but with a trend for a predominant Th2-type profile associated with higher reciprocal HAI and MN titers than the i.n. Protollin-adjuvanted vaccine against both the homotypic and the drifted WIS strains even at week 19 p.b. However, neither the route of immunization nor the different adjuvants used were able to elicit a satisfactory cellular response against the heterovariant A/Wisconsin/67/2005 strain (2).

The Protollin adjuvant was reported to exhibit strong immunogenic properties for both bacterial polysaccharide and viral glycoprotein antigens (8, 17, 25, 35). Ligation of TLR during the early stages of infection by pathogen-associated motifs provides critical costimulatory signals for the initiation of adaptive immune responses. Individual TLRs have distinct pathogen-associated motif specificities, with TLR4 recognizing LPS (3) and TLR2 binding bacterial lipopeptides (1). In that regard, Protollin (proteosomes-LPS) can activate antigen-presenting cells via both TLR2 and TLR4, inducing a more polarized Th1 response (8). More importantly, proteosomes have been shown to be potent intranasal adjuvants inducing proinflammatory cytokines (IFN-γ and IL-5) in mouse splenocytes (25) and having a favorable safety profile in preclinical studies as well as in humans (17, 29, 38, 46). An i.n. trivalent detergent-split vaccine adjuvanted with proteosomes not only demonstrated a good safety profile in a large phase II clinical trial, but it also was effective against a drifted A/H3N2 strain (28). The intranasal mucosa needs a strong adjuvant to overcome immune tolerance. Herein, we show that Protollin is necessary to trigger the appropriate IgA and helper T cell response. There were significantly more IL-2–producing CD4+ T cells and IFN-γ–producing CD8+ T cells after immunization with the low-dose (i.e., 0.75 μg NY antigen-Protollin combination) than with the...
high-dose (i.e., 3 μg NY antigen-Protollin combination) adjuvanted vaccine. The low-dose adjuvanted vaccine elicited a 2-fold increase on average in Th1 and Th2 type-producing CD4+ T cells and in IFN-γ- and IL-2-producing CD8+ T cells compared to the NY split antigen alone. Interestingly, this response was maintained up to week 19 p.b. It is possible that higher doses of antigen induce other Th2-related immunosuppressive cytokines (i.e., IL-10 or others), which may in turn inhibit the production of Th1-related cytokines (41), suggesting a feedback control shown only by a high dose of the flu antigen. However, it is not clear from...
these mouse studies whether the 2-fold increase in cytokine levels observed with the Protollin-adjuvanted vaccines is biologically significant. Further studies ideally in the ferret model are required to assess the importance of this difference in eliciting protection against influenza challenge.

We also examined the effect of Alum as an adjuvant in combination with the detergent-split virus vaccine administered by the i.m. route. Alum has been used for a long time as a vaccine adjuvant (18, 34, 36), and it preferentially enhances Th2-related responses, particularly in BALB/c mice (7, 10). Recently, the role of Alum as an adjuvant has been revisited for boosting the immunogenicity of prepandemic vaccines (23). It was demonstrated that Alum activates the innate inflammatory pathway (inflammasome) in the presence of LPS priming, resulting in the production of some proinflammatory cytokines (e.g., IL-1β and IL-18) after activation of caspase 1 (15, 16, 20, 32). We found that Alum coadministered with the NY antigen induced not only a trend for the highest Th2 type antigen-specific antibody responses but also a mixed CD4+ T cell response of Th1 and Th2 types in BALB/c mice. Furthermore, the Alum-adjuvanted vaccine elicited higher levels of IL-2 and, most importantly, IFN-γ production by CD8+ T cells than the split antigen alone, and this response remained high up to week 19 p.b. It is well established that virus-specific CD8+ T cells play an essential role in limiting viral replication by inducing, to some degree, a cytotoxic lymphocyte response, although cytotoxicity of IFN-γ-producing CD8+ T cells was not evaluated here. It would be interesting to further characterize the subsets of effector and memory CD4+ and CD8+ T cells in immunized animals to gain additional knowledge.

Finally, we also assessed the ability of different vaccine formulations to induce cross-reactivity against an A/H3N2 (WIS) drifted strain by evaluating the antibody and cytokine responses. While adjuvanted vaccines clearly increased the production of cross-reactive antibodies compared to that of the nonadjuvanted vaccines, we did not observe any significant differences in T cell-mediated responses (in particular IFN-γ production) in stimulated splenocytes. Whether such humoral response could confer cross-protection against a drifted virus requires additional experiments using other animal models, such as ferrets. Limitations of our study include the lack of statistical evaluations of HAI and MN titers between vaccine groups due to a low volume of blood and pooling of the replicates as well as the absence of challenge studies. However, our data unequivocally pointed toward more important increases in HAI and MN titers among the adjuvanted vaccine groups, which have been shown to correlate with clinical protection.

In summary, this study demonstrates that adjuvanted detergent-split antigen vaccines elicited generally higher humoral and cellular responses than the nonadjuvanted vaccines in mice. Each adjuvant had its own merits, with AS03 inducing the most persistently homologous Th2 type response, Protollin eliciting the most balanced IgG1/IgG2a ratio and a mucosal (IgA) response, and Alum inducing higher levels of Th2 antibody response and a mixed Th1/Th2 type cellular immune responses. These results, therefore, suggest that the detergent-split A/H3N2 vaccine combined with selected i.m. and i.n. adjuvants could be more protective than the actual nonadjuvanted detergent-split vaccines in humans, particularly in vulnerable and elderly populations, as well as in the advent of an antigenic drift. Further evaluations using both immunization routes in combination (e.g., i.n. priming followed by i.m. boosting) may lead to additional observations that could have practical applications.
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REFERENCES
1. Aliprantis AO, et al. 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. Science 285:736–739.
2. Baldo V, Baldwin T, Floreani A, Fragapani E, Trivello R. 2007. Response of influenza vaccines against heterovariant influenza virus strains in adults with chronic diseases. J. Clin. Immunol. 27:542–547.
3. Beutler B. 2000. Tlr4: central component of the sole mammalian LPS sensor. Curr. Opin. Immunol. 12:20–26.
4. Bramer TL, et al. 2002. Surveillance for influenza—United States, 1997–98, 1998–99, and 1999–2000 seasons. MMWR Surveill. Summ. 51:1–10.
5. Bresson JL, et al. 2006. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/194/2004 (H5N1) vaccine: phase I randomised trial. Lancet 367:1657–1664.
6. Bridges CB, et al. 2000. Effectiveness and cost-benefit of influenza vaccination of healthy working adults: a randomized controlled trial. JAMA 284:1655–1663.
7. Bungener L, et al. 2008. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine 26:2350–2359.
8. Chabot S, et al. 2005. A novel intranasal Protilitin-based measles vaccine induces mucosal and systemic neutralizing antibody responses and cell-mediated immunity in mice. Vaccine 23:1374–1383.
9. Couch RB, Kassel JA. 1983. Immunity to influenza in man. Annu. Rev. Microbiol. 37:329–349.
10. Cox JC, Coulter AR. 1997. Adjuvants—A classification and review of their modes of action. Vaccine 15:248–256.
11. Cox RJ, et al. 2006. The humoral immune response and protective efficacy of vaccination with inactivated split and whole influenza virus vaccines in BALB/c mice. Vaccine 24:6585–6587.
12. Crapper DR, Krishnan SS, Dalton AJ. 1973. Brain aluminium distribution in Alzheimer’s disease and experimental neurofibrillary degeneration. Science 180:511–513.
13. Dawood FS, et al. 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N. Engl. J. Med. 360:2605–2615.
14. Doherty PC, Turner SJ, Webbry RG, Thomas PG. 2006. Influenza and the challenge for immunology. Nat. Immunol. 7:449–455.
15. Eisenbarth SC, Colegio OR, O’Connor W, Sutterwala FS, Flavell RA. 2005. Crucial role for the Nalp3 inflammasome in the immunostimulation of healthy working adults: a randomized controlled trial. JAMA 294:143–151.
16. Eisenbarth SC, Flavell RA. 2009. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet 370:580–589.
17. El H, Willingham SB, Ting JP, Re F. 2008. Cutting edge: inflammasome activation by alun and alun’s adjuvant effect are mediated by NLRP3. J. Immunol. 181:17–21.
18. Lindblad EB. 2004. Aluminium adjuvants—in retrospect and prospect. Vaccine 22:3638–3668.
19. Lindblad EB. 2004. Aluminium compounds for use in vaccines. Immunol. Rev. 197:497–505.
20. Mallett CP, et al. 1995. Intranasal or intragastric immunization with proteosome-Shigella lipopolysaccharide vaccines protects against lethal pneumonia in a murine model of Shigella infection. Infect. Immun. 63:2382–2386.
21. Mannhalter JW, Neychev HO, Zlabinger GJ, Ahmad R, Elbl MM. 1995. Modulation of the human immune response by the non-toxic and non-proteogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation. Clin. Exp. Immunol. 116:141–153.
22. O’Hagan DT, Akin F, Podda A. 2007. The humoral immune response and protective effi- cacy of vaccination with inactivated split and whole influenza virus vaccines in BALB/c mice. Vaccine 21:3296–3298.
23. Osterballe O. 1982. Side effects during immunotherapy with purified group pollen extracts. Allergy 37:553–562.
24. Papenburg J, et al. 2011. Evaluation of serological diagnostic methods for the 2009 pandemic influenza A (H1N1) virus. Clin. Vaccine Immunol. 18:520–522.
25. Redmond WL, Marinck BC, Sherman LA. 2005. Distinct requirements for deletion versus anergy during CD8 T cell peripheral tolerance in vivo. J. Immunol. 174:2046–2053.
26. Roman F, et al. 2010. Immunogenicity and safety in adults of one dose of influenza A H1N1v 2009 vaccine formulated with and without AS03A-adjuvant: preliminary report of an observer-blind, randomised trial. Vaccine 28:1740–1745.
27. Scholtissek C, von Hoehningen V, Rott R. 1978. Genetic relatedness between the new 1977 epidemic strains (H1N1) of influenza and human influenza strains isolated between 1947 and 1957 (H1N1). Virology 93:27–44.
28. Seeds RE, Gordon S, Miller JL. 2009. Characterisation of myeloid receptor expression and interferon alpha/beta production in murine plasmacytoid dendritic cells by flow cytometry. J. Immunol. Methods 350:106–117.
29. Skowronski DM, et al. 2007. Estimating vaccine effectiveness against laboratory-confirmed influenza using a sentinel physician network: results from the 2005-2006 season of dual A and B vaccine mismatch in Canada. Vaccine 25:2842–2851.
30. Treanor J, et al. 2006. Intranasal administration of a proteosome-influenza vaccine is well-tolerated and induces serum and nasal secretion influenza antibodies in healthy human subjects. Vaccine 24:254–262.
31. Vogelbruch M, et al. 2000. Aluminium-induced granulomas after inaccurate intradermal hyposensitization injections of aluminium-adsorbed depot preparations. Allergy 55:883–887.
32. WHO. 2002. WHO manual on animal influenza diagnosis and surveillance. World Health Organization, Geneva, Switzerland. http://www.who.int/vaccine_research/diseases/influenza/WHO_manual_on_animal-diagnosis_and_surveillance_2002_3.pdf.