Published in final edited form as:

Biochem Biophys Res Commun. 2015 November 27; 467(4): 766–770. doi:10.1016/j.bbrc.2015.10.067.

LACTOFERRIN ACTS AS AN ADJUVANT DURING INFLUENZA VACCINATION OF NEONATAL MICE

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Abstract

Health policy precludes neonatal vaccination against influenza. Hence, morbidity and mortality are high under 6 months of age. Lactoferrin may activate diminished numbers of dysfunctional dendritic cells and reverse neonatal vaccine failures. Aluminum hydroxide/ALUM recruits neutrophils that secrete lactoferrin at deposition sites of antigen. We theorized lactoferrin + influenza antigen initiates an equivalent antibody response compared to ALUM. Three-day-old mice received subcutaneously 30 micrograms of H1N1 hemagglutinin + 200 µg of bovine lactoferrin versus hemagglutinin + ALUM. Controls received hemagglutinin, lactoferrin, or ALUM. After 21 days, sera measured anti-H1N1 (ELISA) and neutralizing antibody (plaque assays). ELISA detected equal antibody production with lactoferrin + hemagglutinin compared to hemagglutinin + ALUM; both sera also neutralized H1N1 virus at a 1:20 dilution ($p < .01$). Controls had no anti-H1N1 antibody. Neonates given lactoferrin had no anaphylaxis when challenged four weeks later. Lactoferrin is a safe and effective adjuvant for inducing antibody against influenza in neonates.

Keywords

adjuvant; lactoferrin; influenza; hemagglutinin; immunization; newborn

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Introduction

Immunization of children prevented many deaths during the 20th century; however, newborn and young infants require early, safe and effective immunizations to save lives in this century (1). Pneumonia caused by influenza, pneumococci, and pertussis remain a significant worldwide cause of morbidity and mortality among infants in the early life (2). In 2015, guidelines to prevent influenza in young infants recommended giving inactivated influenza vaccine at 6 months of age (3). This leaves babies unprotected against influenza between 0 to 6 months. The most vulnerable infants are those born prematurely or having low birth weight since these babies are often undernourished (4). Two options for prevention of infantile influenza are proposed. First, immunization of pregnant women would be protective via transplacental antibody (5). The second approach is ‘cocooning’ or vaccination of all caregivers of infants less than one year of age (6). To date, regulatory, financial and societal issues have rendered these strategies unsuccessful. Hence, the third approach is development of a safe and effective influenza vaccine for newborn infants.

Adjuvants facilitate the processing of microbial antigens so vaccination has heightened success. In 1932, vaccines added aluminum hydroxide gel (ALUM) to enhance protection against diphtheria (7). Today, ALUM remains the major adjuvant used in US vaccines. A developmental deficit in both mice and humans includes decreased secretion of interleukin-12 (IL-12) by neonatal dendritic cells that reverse apoptosis of Th1 cells (8, 9). The Th2 bias of the neonate and IL-4 secretion by these lymphocytes are responsible for apoptosis of Th1 cells and represent a leftover component of immunosuppression during fetal-maternal engraftment *in utero* (10). Use of ALUM as an adjuvant in neonates is problematic because aluminum hydrogel inhibits IL-12 secretion that is required for the induction of Th1 cellular responses (11). ALUM and MF59 are common adjuvants in influenza vaccines. Fig. 1 shows adjuvants elicit migration of neutrophils and monocytes to the site of adjuvant and antigen injection (12). Once on site, neutrophils release lactoferrin (LF) from their secondary granules. We theorize that injecting LF rather than a traditional adjuvant eliminates the neutrophil recruitment step and LF immediately hastens DC recruitment, maturation and activation (13, 14). In this research, we tested if the breast milk protein, LF, is the natural adjuvant sought by scientists and caregivers for better vaccination of babies during the first month of life.

Materials and methods

Mice

We studied Balb/c mice of both genders between three to 28 days of age following breeding and birth. We purchased the Balb/c mice from Jackson Laboratory (Bar Harbor, ME). Thereafter, we maintained breeding colonies in the vivarium of the medical school. The investigators strictly followed the protocol approved by the Animal Care and Use Committee of the University of Missouri – Columbia.

Reagents

Professor Adolfo Garcia-Sastre (Icahn School of Medicine at Mount Sinai) provided Influenza A/Puerto Rico/8/34 (H1N1) virus. We maintained stock virus at -80°C until grown for our enzyme-linked immunoassays (ELISA) and plaque assays. We purchased Madin-Darby canine kidney (MDCK) cells from ATTC (ATCC[®] CCL-34[™]). We stored MDCK cells at -80°C until re-cultivated for the plaque assays. Immune Technology Corporation (AA 1-529) was the source for recombinant H1N1 hemagglutinin (His tag). We purchased all ELISA materials from BD Biosciences (San Jose, CA). We purchased Alhydrogel[®] adjuvant 2% from INVIVOGEN (San Diego, CA). We suspended bLF in endotoxin- and preservative-free sterile saline (Vitality Medical, Cottonwood Heights, UT). The Food Science and Technology Institute, Morinaga Milk Industry Company, Ltd., Zama City, Japan provided a gift of 1 gm of purified, freeze-dried bovine lactoferrin powder. Biochemical reagents came from Scientific Fisher or Sigma-Aldrich.

Experimental Protocol

Fig. 2 shows the workflow for vaccination. We suspended the dose of H1N1 hemagglutinin (30 μg) in either 100 μg of bovine lactoferrin in sterile, endotoxin- free saline or a comparable volume of Alhydrogel[®]. We followed immunization with three assessments: a) serum anti-H1N1 antibody measurements, b) plaque assays determining H1N1 neutralizing antibody, and c) determinations of bLF safety as an adjuvant. At 24 d of age, sera was prepared from whole blood obtained by cardiac puncture (27 G needle scalp vein set) under isoflurane anesthesia (Allivet[™]) and followed by euthanasia using isoflurane followed by cervical dislocation; sera was stored at -80°C until studied.

Immunoassay for Anti-H1N1 Antibody

Influenza A virus (strain A/Puerto Rico/8/1934 H1N1) in carbonate-bicarbonate buffer at 10^6 plaque-forming units (pfu)/mL were bound to 96 well plates (Costar) overnight at 4°C . The plates were washed x3 and blocked for 1 h at 25°C . Serum dilutions: 1:10, 1:50, 1:100, & 1:200 were prepared and incubated with bound virus for 2 h at 25°C . After washing, we incubated the bound sera with goat anti-mouse IgG (1:5000 dilution, Southern Biotech) for 1 hour at 25°C . After incubation and washing x 3, we added colorimetric reagent (BD Bioscience) and incubated the plates in the dark for 30 min at 25°C . Thereafter, we added Stop reagent (BD Bioscience) and read absorbance at 450 nm (BioTek Epoch plate reader).

Plaque Assay for H1N1 Neutralizing Antibody

MDCK cells were incubated in six-well plates until confluency was $>90\%$. Serial dilutions (1:10, 1:20, 1:40, 1:80, & 1:160) were made of heat-inactivated (56°C for 1 hr) immune or control sera. We added one hundred plaque-forming units of A/PR/8/1934 virus to each serum dilution and incubated for 75 minutes. We then overlaid the monolayer with the dilute sera containing H1N1 virus and incubated for 1 hour at 37°C with intermittent mixing. Thereafter, we covered the monolayer with 2X minimal essential media (MEM) containing antibiotics, 0.3% bovine serum albumin, 1 $\mu\text{g}/\text{mL}$ TPCK trypsin to which we added agarose (final concentration 1%). First, we incubated the covered infected plates for 3 days at 37°C , second, we subsequently fixed with 20% neutral buffered Formalin[™] overnight, and third,

we stained with 10% crystal violet for 1 hour. After washing, we determined plaques visually and recorded the number/well (Figure 3, Panel B).

Safety of Bovine Lactoferrin as an Adjuvant

We monitored infant mice daily after vaccination. Namely, we watched weight gain or loss, motor activity, hair and other physical characteristics. We separately gave four 3-day-old mice a single dose SQ dose of 100 µg of bLF, while six other 3-day-old mice received 100 µg of bLF SQ and this dose was repeated SQ seven days later. We studied two separate groups of these mice. One group quantified anti-bLF antibody, while the second group ascertained whether prior bLF administration could have caused later anaphylaxis. After 21 days, the first group had blood drawn, as described previously, for preparation of sera to measure anti-bLF antibodies using an immunoassay. Alternatively, at 28-days of age, the second group of pups received 2 g/kg of bLF intraperitoneally. We observed these pups for 2 hours for signs or symptoms of anaphylaxis, including sneezing, coughing, respiratory distress, movement disorders, shaking/tremors, and/or fur changes (15).

Statistical Methods

Sample size calculations recommended a minimum of nine mice per treatment group. The student t test analysed differences between bLF + HA- and ALUM + HA-treated neonatal mice. Descriptive statistics included means ± standard deviation. We used a statistical software program for calculations (16). A *p* value <0.05 was considered significant.

Results

Fig. 3 (Panel A) shows that HA injection alone produced minimal anti-H1N1 antibody, however, when the adjuvants bLF or ALUM were added to the HA antigen, equivalent levels of anti-H1N1 antibody were produced between 3- and 24-days of life. Sera from neonatal mice given only bLF or ALUM without HA also had no detectable anti-H1N1 antibody. Fig. 3 (Panel B) shows wells ❶ through ❹ and the effects of adding 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and no plaque-forming units (pfu) of H1N1 A/PR/8/1934 virus. In the plaque assay, we added 100 pfu + 1:10 to 1:160 dilutions of heat-inactivated immune or non-immune sera to individual wells. Using a 1:20 serum dilution of sera from HA + bLF-immunized pups, the plaque assay had 16 ± 3 plaques (mean ± standard deviation, *n* = 14 animals and 3 litters), while a 1:20 serum dilution of sera from HA + ALUM-treated pups had 15 ± 2 plaques (*n* = 16 animals and 3 litters). This difference in neutralizing capacity was not significant between the two groups (*p* = 0.52). By comparison, control wells with 100 pfu of H1N1 virus added to MDCK cells had 66 ± 3 plaques which was four-fold higher than wells that contained the 1:20 dilution of immune sera + H1N1 virus (*p* < 0.01). We observed no neutralizing antibody in sera recovered from pups given HA only, bLF only and ALUM only.

Monitoring of neonatal mice during the 21 days after immunization identified no manifestations of illness. Table 1 shows bovine LF given SQ to 3-day-old mice in a single dose or a single dose + a booster SQ injection had no Ig (anti-IgG or anti-IgE) antibody against this protein. Moreover, no mice had signs or symptoms of anaphylaxis when

challenged with a large (2 g/kg) intraperitoneal dose of the bovine lactoferrin four weeks after the same regimens.

Discussion

This research study tests the hypothesis that lactoferrin could replace ALUM as an adjuvant when immunizing neonatal mice against H1N1 influenza virus. The assumption was ALUM causes tissue injury that induces chemotaxis of neutrophils to the injection site as a “clean-up” mechanism (7). Upon arrival, we propose neutrophils release lactoferrin from their secondary granules as a microbicide and a regulator of the immune responses (17, 18). Although we have yet to quantify cellular and immune effectors at the nuchal injection site, we conclude bovine lactoferrin facilitated production of IgG against the H1N1 virus and this antibody had protective characteristics. Bovine LF was at least as effective as aluminum hydrogel as an adjuvant. We conclude the lactoferrin-related mechanism of action is recruitment and activation of dendritic cells on the 3rd day of life (13, 14) well before immature dendritic cells undergo and attain sufficiency by 6 days of life in Balb/c mice (8, 19).

Since recombinant H1N1 hemagglutinin is costly, we did not perform dose response experiments to ascertain the optimal dose of H1N1 hemagglutinin. Rather we extrapolated doses of H1N1 hemagglutinin and ALUM used to immunize Balb/c mice used as models for H1N1 pulmonary infection (20, 21). In the preceding study, adult Balb/c mice produced anti-H1N1 antibody after immunization with H1N1 HA, but our neonatal mice do not. Neonatal and mature Balb/c mice have enhanced antibody production if ALUM is included as an adjuvant with influenza hemagglutinin (20).

We extrapolated the dose of bovine LF for use as an adjuvant from studies of murine macrophages intended to enhance bacillus Calmette-Guerin vaccination (22). Still, we acknowledge we did not seek the optimal dose of bovine LF because of cost-related concerns. However, our findings strongly suggest bovine LF enhanced the response to H1N1 hemagglutinin in neonatal mice (Fig. 3, panel A).

Lactoferrin is the major whey protein of mammalian milk (23) and is a natural, safe and effective immunomodulator. We did not observe any detrimental effects after administering bLF to neonatal Balb/c mice. Vaccine experts are prospectively monitoring adverse effects of vaccination, especially influenza (24). Recently, investigators have attributed adverse events after vaccination to pre-existing neurologic disease (25). Nevertheless, aluminum is among the environmental factors, along with genetic factors, considered responsible for unfavorable outcomes after vaccination (26). If a natural adjuvant, namely lactoferrin, could replace many adjuvants in current vaccines and further reduce the risks of immunization, parents would be more willing to vaccinate their infant or child.

Lastly, our rationale for studying a neonatal Balb/c mouse is researchers have used this animal to study pneumonia caused by H1N1 influenza (21) and to define immunologic events (27). Having found bLF and H1N1 hemagglutinin is effective in producing neutralizing antibody and is safe, we can now proceed with *in vivo* studies of intranasal

infection in 2-week-old mice and observe whether immunization on the 3rd day of life alleviates influenza-related pneumonia. The model lends itself to studying viral burden in the lung and the associated immune response as we have previously described in life-threatening group B streptococcal pneumonia (28).

By performing future studies, we can take steps forward to make neonatal vaccinations common, safe, and further reduce the burden of infectious disease in the 1st year of life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research funding included the MU Research Board grant (MPS, BH), NIH/NIAID grant AI091797 (BH), and the Leda J. Sears Trust (MPS, HZ). The investigators appreciate bovine lactoferrin generously provided by Hiroyuki Wakabayashi, Ph.D. and Koji Yamauchi, Ph.D., at Morinaga Milk Industry Company, Ltd. Adolfo Garcia-Sastre, Ph.D., kindly provided Influenza A/Puerto Rico/8/34 (H1N1) virus.

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Fig. 1. Inflammation induced by ALUM injection with a vaccine antigen. ALUM induces tissue injury that recruits blood neutrophils and monocytes. Neutrophils secrete lactoferrin from their secondary granules that enhance maturation and activation of monocytes and dendritic cells. We eliminated the ALUM step by simply injecting bovine lactoferrin (bLF) + influenza hemagglutinin (HA).

Fig. 2. Workflow of the immunization of 3-day-old mice against H1N1 influenza and subsequent detection of anti-H1N1 IgG antibody, viral neutralizing antibody, and safety related to bovine lactoferrin, ALUM and H1N1 hemagglutinin exposures. The testing involved testing sera from mice given bLF + HA SQ (n = 14 pups from three litters), sera from pups receiving ALUM + HA SQ (n = 16 pups from three litters), and pups immunized with HA alone SQ and no adjuvants (n = six pups from two litters).

Table 1

Safety of Bovine lactoferrin Administration to Neonatal Mice

Outcome	3-day-old Mice: One Nuchal Dose Bovine LF (100 µg/kg)	3-day-old Mice: One Nuchal Dose Bovine LF (100 µg/kg) + Booster Dose 7 Days Later
Presence of Murine anti-Ig Against Bovine Lactoferrin	0/4	0/6
Presence of Anaphylaxis after 2 g/kg body weight given i.p.	0/4	0/6

Abbreviations: LF = lactoferrin; Ig = IgG, IgM, IgA & IgE; i.p. = intraperitoneal injection