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Virus aggregating peptide enhances the cell-mediated response to influenza virus vaccine

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ABSTRACT

Given the poor immunogenicity of current H5N1 influenza vaccines, additives and adjuvants remain a viable solution for increasing efficacy. Here, we demonstrate that a 20-amino acid peptide (EB) possessing influenza antiviral activity also enhances the immune response to H5N1 vaccination in mice. The addition of EB to formalin-inactivated whole-virus vaccine induced virion aggregation and these aggregates were readily engulfed by phagocytic cells *in vitro*. *In vivo*, mice vaccinated with a suboptimal dose of inactivated vaccine containing EB peptide had reduced morbidity, improved viral clearance, and faster recovery than mice receiving vaccine alone. This phenomenon was not accompanied by an increase in virus-specific antibodies. Instead, cell-mediated immunity was enhanced as demonstrated by increased interferon-γ production from splenocytes. This data demonstrates that the EB peptide may a useful adjuvant for boosting the efficacy of poorly immunogenic influenza vaccines.

1. Introduction

The 2009 H1N1 influenza virus pandemic clearly demonstrated the importance of influenza vaccination in reducing infections rates and controlling virus dissemination in large populations [1,2]. Despite the fact that influenza vaccination programs have existed for over 40 years [3], several limitations exist in both the production of vaccine stocks, and the immunological response to influenza virus. First, influenza viruses undergo constant mutation as they circulate in human and animal populations, allowing them to successfully evade host immune responses to previously encountered virus variants [4,5]. Second, influenza vaccines are most commonly produced in embryonated chicken eggs; a process which can be severely affected by contamination of eggs, poor replication of seed viruses, and egg supply shortages [6–9]. Vaccine seed viruses chosen for production and distribution may be mismatched to the circulating strain, providing inadequate protection [10]. Additionally, influenza vaccines are often less effective in high risk groups such as the very young, elderly or immunocompromised [11]. Finally, vaccines against certain influenza subtypes, such as H5N1 viruses, are poorly immunogenic [12–14]. These drawbacks highlight the need to continually evaluate and improve upon our existing vaccines.

Highly pathogenic avian H5N1 viruses have been at the forefront of the influenza vaccine discussion since they crossed the avian to human species barrier in 1997. Over the past decade, H5N1 viruses have become endemic to poultry populations in many parts of the world, broadened their host range, and are associated with a 60% mortality rate in humans [15–20]. Formulation of an effective H5N1 virus vaccine is a crucial component in control strategies aimed to limit spread and severity of potential pandemic viruses. However, several challenges exist when producing H5N1 vaccines, including their continual evolution and poor immunogenicity [12–14,21–24]. One method to improve vaccine efficacy is through inclusion of vaccine additives or adjuvants. Adjuvants boost vaccine efficacy by inducing a stronger protective immune response and/or lowering the dose of antigen required to induce a response (dose sparing). Adjuvants exert their effect through a variety of mechanism including concentration and retention of antigen at the injection site, and/or promoting uptake and subsequent effector functions of phagocytic cells such as macrophages and dendritic cells [22,25–29].

We previously demonstrated that a 20-amino acid peptide derived from fibroblast growth factor-4, designated "EB", displayed antiviral activity against multiple subtypes of influenza

Abbreviations: EB, entry blocker peptide adjuvant; hpi, hours post-infection; TCID50, 50% tissue culture infectious dose; PR/8, influenza virus A/PuertoRico/8/34 H1N1; VN/1203, influenza virus A/Vietnam/1203/04 H5N1; HA, hemagglutinin protein/assay; HAU, hemagglutinating units; HI, hemagglutination inhibition; dpi, days post-infection.

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viruses [30]. The EB peptide inhibited virus binding to host cells *in vitro*, and prevented influenza virus-induced morbidity and mortality in mice. Here, we demonstrate that the EB peptide aggregates influenza virions while keeping them structurally intact. We hypothesized that the EB-induced aggregation would enhance uptake by phagocytic cells and boost downstream immune responses to influenza virus. These studies support that hypothesis and show that EB increases the protective immune response against poorly immunogenic H5N1 virus vaccines. Surprisingly, EB did not increase humoral immunity to H5N1 vaccines, but instead enhanced cell-mediated responses to formalin-inactivated whole virus.

2. Materials and methods

2.1. Ethics statement

All procedures involving animals were approved by the University of Wisconsin-Madison and St. Jude Children's Research Hospital IACUC committees and were in compliance with the Guide for the Care and Use of Laboratory Animals.

2.2. Viruses and cells

A/Puerto Rico/8/34 (PR/8, H1N1) and A/Vietnam/1203/04 (VN/1203, H5N1) viruses were propagated in 10-day-of-age embryonated chicken eggs (Sunnyside Farms, Beaver Dam, WI) at 37 °C. Allantoic fluid was harvested, centrifuged for clarification, and stored at −70 °C. VN/1203 virus for animal challenge was propagated in Madin Darby canine kidney (MDCK, ATCC, Manassas, VA) cells. Supernatants were harvested 72 h post infection (hpi), centrifuged for clarification, and stored at −70 °C. Viral titers were determined either by hemagglutination (HA) assay as described previously [30] and reported as hemagglutination units (HAU) or by fifty percent tissue culture infectious dose ($TCID_{50}$) analysis in MDCK cells and calculated by the method of Reed and Muench [31]. MDCK cells were cultured in Modified Eagle's Medium (MEM, CellGro, Herndon, VA) supplemented with 4.5 g of glucose per liter, 2 mM L-glutamine, and 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA) at 37 °C, 5.5% CO_2. RAW 264.7 cells (ATCC, Manassus, VA) were cultured in RPMI 1640 medium (CellGro) supplemented with 4.5 g of glucose per liter, 1 mM L-glutamine, 1 mM sodium pyruvate, and 10% heat inactivated FBS.

2.3. Laboratory facilities

All H5N1 virus experiments were conducted in a Select Agent-approved bio-safety level-3 enhanced laboratory. Investigators were required to wear appropriate respirator equipment (RACAL, Health and Safety Inc., Frederick, MD). Mice were housed in HEPA filtered negative pressure cages (M.I.C.E. racks, Animal Care Systems, Littleton, CO).

2.4. Virus Inactivation and purification

Allantoic fluid containing influenza virus was treated with 0.1% in formalin (v/v) at 4 °C for 5 days. To verify inactivation, MDCK cells and 10-day-of-age embryonated chicken eggs were inoculated with a neat dilution of formalin-treated virus and viability was assessed after 72 hpi. All viruses, both inactivated VN/1203 and PR/8 and live PR/8 virus were purified by overlaying allantoic fluid onto a 30–60% discontinuous sucrose gradient. The samples were centrifuged for 90 min at 26,000 RPM in an SW-28 rotor. The virus layer was extracted from the 30–60% interface and pelleted by another round of centrifugation for 60 min. Virus pellets were resuspended in phosphate buffered saline (PBS), refluxed through an 18 g needle to break up aggregates, and titered by HA assay.

2.5. Density ultracentrifugation

Purified PR/8 virus (512 HAU) was treated with PBS alone (mock) or EB peptide (30 μM) for 1 h at 37 °C and layered on continuous 20–60% sucrose gradients. Samples were subjected to ultracentrifugation 90,000 × *g* for 60 min. Fractions (500 μl) were collected from the bottom of the tube and 7 μl of each was dotted to nitrocellulose, blocked with 3% milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS), and probed with goat anti-H5 hemagglutinin (1:1000 in TTBS) for 1 h at room temp, followed by donkey anti-goat IgG (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were detected by enhanced chemiluminescence (Pierce, Rockford, IL). Results were quantitated by densitometry (Image J, NIH, Bethesda, MD) and reported as relative pixel intensity (RPI). The density of each sucrose fraction was determined by measuring refractive index in a Bausch and Lomb 334610 Refractometer (Rochester, NY). The attachment activity from each fraction was determined by HA assay and is represented as HAU/50 μl.

2.6. Electron microscopy

Purified PR/8 (512 HAU) or inactivated VN/1203 viruses (1 μg total protein) were treated with PBS alone (mock) or EB peptide (30 μM) for 1 h at 37 °C. Samples were adsorbed to poly-L-lysine coated grids, stained with 2% phosphotungstic acid (pH 7) and air dried as described [32]. Grids were examined in a JEOL JEM-1200EX electron microscope. Results are indicative of 3 independent experiments.

2.7. Virus labeling and flow cytometry

Purified PR/8 virus was labeled with fluorescein isothiocyanate (FITC-PR/8) using the EZ-Label FITC protein labeling (Pierce, Rockford, IL) according to manufacturer's instructions. Infectivity of labeled virus was confirmed by $TCID_{50}$ analysis and was re-titered by HA assay for use in flow cytometry experiments. The FITC-PR/8 (512 HAU) virus was treated with increasing concentrations of EB peptide for 1 h at 37 °C and then added to RAW 264.7 macrophages (2×10^5) in 1 ml of RPMI 1640 containing 1% heat inactivated FBS. Samples were incubated with gentle rocking for 1 h at 37 °C, followed by 2 washes with PBS. Cells were fixed with 1% paraformaldehyde and virus-cell association measured on a LSR-II Benchtop flow cytometer (BD Biosciences, Franklin Labs, NJ). Single cell populations of mock infected cells were gated using forward and side scatter properties. At least five-thousand events from these gates were recorded from each experimental group in duplicate or triplicate. Results are indicative of 2 independent experiments.

2.8. Vaccine formulation

The vaccine was composed of 1 μg of formalin inactivated and sucrose purified VN/1203 or PR/8 virus diluted into sterile PBS. Adjuvant groups were supplemented with 0.5 mg/ml aluminum ammonium sulfate or 200 μM EB peptide. All groups were incubated for 1 h at 37 °C prior to administration.

2.9. Animals, vaccination and challenge

Blood was drawn from the tail vein from 4 to 6 week old female BALB/C mice (n = 5–6/group) followed by hind-limb intramuscular injection with 50 μl of vaccine preparation. All mice were prebled (Day 0) and vaccinated, followed by a boost of vaccine 15 days after the initial injection. Mice were bled again at day 28