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Identification of HLA class II H5N1 hemagglutinin epitopes following subvirion influenza A (H5N1) vaccination

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ABSTRACT

Prophylactic immunization against influenza infection requires CD4+ T-helper cell activity for optimal humoral and cellular immunity. Currently there is one FDA approved H5N1 subvirion vaccine available, although stockpiles of this vaccine are insufficient for broad population coverage and the vaccine has only demonstrated modest immunogenicity. Specific activation of CD4+ T-helper cells using class II H5N1 HA peptide vaccines may be a useful component in immunization strategy and design. Identification of HLA class II HA epitopes was undertaken in this report by obtaining PBMCs from volunteers previously immunized with an H5N1 inactivated subvirion vaccine, followed by direct *ex vivo* stimulation of CD4+ T cells against different sources of potential HA class II epitopes. In the 1st round of analysis, 35 donors were tested via IFN-γ ELISPOT using pools of overlapping HA peptides derived from the H5N1 A/Thailand/4(SP-528)/2004 virus, recombinant H5N1 (rHA) and inactivated H5N1 subvirion vaccine. In addition, a series of algorithm-predicted epitopes coupled with the Ii-Key moiety of the MHC class II-associated invariant chain for enhanced MHC class II charging were also included. Specific responses were observed for all 20 peptide pools, with 6–26% of vaccinated individuals responding to any given pool (donor response frequency) and a magnitude of response ranging from 3- to >10-fold above background levels. Responses were similarly observed with the majority of algorithm-predicted epitopes, with a donor response frequency of up to 29% and a magnitude of response ranging from 3–10-fold (11/24 peptides) to >10-fold above background (7/24 peptides). PBMCs from vaccine recipients that had detectable responses to H5N1 rHA following 1st round analysis were used in a 2nd round of testing to confirm the identity of specific peptides based on the results of the 1st screening. Sixteen individual HA peptides identified from the library elicited CD4+ T cell responses between 3- and >10-fold above background, with two peptides being recognized in 21% of recipients tested. Eight of the putative MHC class II epitopes recognized were found in regions showing partial to significant sequence homology with New Caledonia H1N1 influenza HA, while eight were unique to H5N1 HA. This is the first study to identify H5N1 HA epitope-specific T cells in vaccine recipients and offers hope for the design of a synthetic peptide vaccine to prime CD4+ T-helper cells. Such a vaccine could be used to provide at least some minimal level of H5N1 protection on its own and/or prime for a subsequent dose of a more traditional but supply-limited vaccine.

1. Introduction

Vaccination against influenza H5N1 will likely be the only effective means of limiting morbidity and mortality in the event of a world-wide pandemic. Over the last several years, cases of direct avian-to-human transmission have been reported mainly in southern China and Southeast Asia [1,2]; however the more feared human-to-human transmission of virus has been limited to a few probable cases [3]. Should the virus re-assort its genetic material, allowing for direct human-to-human transmission, the potential exists for a world-wide pandemic. Traditional egg-based vaccines such as the tri-valent seasonal influenza vaccine, although highly effective against seasonal influenza subtypes, may not elicit sufficient cross-protection against H5N1 influenza. Early attempts to propagate H5N1 virus in embryonated chicken eggs for vaccine production were met with disappointment as the viral pathogenicity hindered high titer propagation resulting in relatively few vaccine doses. This limitation has been addressed by propagating subvirion vaccines, which includes strain-specific H5N1 HA and neuraminidase proteins combined with the internal viral proteins from the non-pathogenic A/PR/8/1934 (H1N1) strain. This approach, in addition to replacing the polybasic cleavage site

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between HA1 and HA2 has resulted in higher viral titers (increased vaccine supply) with minimal virulence in chicken eggs. Despite these improvements in manufacturing capability, clinical testing of these vaccines has induced only weak to modest immunogenicity [4–7], although a more recent clinical trial demonstrated that two 3.8 μg doses of a split-virion adjuvanted vaccine induced 77% seroconversion [8]. Stockpiling of such vaccines is of questionable utility due to the potential loss of potency over time and the emergence of mutant strains through antigenic drift, rendering such vaccines less effective.

Influenza infection has been most thoroughly investigated in murine model systems. Studies have shown that a lack of B cells in mice can lead to increased mortality following viral challenge [9,10], implicating the importance of having strong anti-viral humoral immunity, although the induction of CD8+ effector responses also contributes to viral clearance and recovery [11]. It has been demonstrated that activation of both arms of the immune system yields the most effective anti-viral response, and in most instances, relies heavily on the aid of CD4+ T cells. Activated CD4+ T cells provides indirect "help" for B cells and CD8+ T cells, as well as providing essential support for the induction of memory B and T cells [12,13]. Additional effector functions have been described for CD4+ T cells in the direct control of viral infections [14,15], including influenza-specific cytolytic activity [16,17]. Studies have also demonstrated that while CD4-depleted mice can clear the highly lethal PR8 murine influenza virus [18], the combination of CD4+, CD8+ and B cells greatly increases viral clearance and survival in mice [19,20], suggesting that a multi-pronged response is most efficient for protection. The contribution of each cell type in protecting humans against H5N1 infection is currently unknown and may depend in large part on the pathogenicity and overall virulence of the circulating strain. Taken together, H5N1 vaccines designed to induce multiple arms of the immune system and generate broad immunity will likely be most effective against an H5N1 outbreak.

The utilization of the Ii-Key technology to augment and drive stronger CD4+ T-helper cell activity may be advantageous to the development of an H5N1 vaccine. Specifically, modification of MHC class II epitopes with a fragment of the MHC class II-associated invariant chain termed Ii-Key has been shown to facilitate extracellular epitope loading of class II molecules, thereby bypassing the need for intracellular antigen processing. It has been suggested that extracellular peptide loading may activate naive T cells more quickly [21]. Such Ii-Key peptides have also been shown to enhance antigen-specific T cell responses *in vitro* [22] as well as T and B cell responses *in vivo* relative to non-Ii-Key-modified vaccine peptides [23–26]. Clinical testing of an Ii-Key class II Her2/neu peptide vaccine has shown it to induce a robust and specific immunological response in breast cancer patients [27].

In preliminary studies, mice primed with algorithm-predicted H5N1 HA MHC class II epitopes linked to Ii-Key demonstrated improved immunological response to a clinically tested rHA H5N1 subunit vaccine (unpublished observations). Specifically, priming with predicted class II H5N1 HA/Ii-Key epitopes derived from highly conserved regions of H5N1 HA increased the T-helper cell and antibody responses to a rHA boost. Unrelated studies also have demonstrated the utility of antigen-specific CD4+ priming prior to boosting with a recombinant vaccine, resulting in a more robust immunological response [28]. Therefore, it seems reasonable to pursue the use of Ii-Key-modified vaccine peptides as part of an overall H5N1 vaccine strategy with the goal of extending the limited supplies of more traditional H5N1 vaccines under development by using Ii-Key vaccines as a pre-emptive vaccine. As a "stand-alone" vaccine Ii-Key-modified H5N1 HA epitope(s) from conserved regions of H5N1 HA may provide some degree of protection against multiple H5N1 strains that may emerge in a pandemic.

Towards the development of such a vaccine, we have acquired PBMCs from subjects of an H5N1 subvirion vaccine trial to assess and identify specific CD4+ T cell epitope responses. Both algorithm-predicted class II HA peptides modified with Ii-Key as well as a library of overlapping peptides (peptide pool array) covering the entire H5N1 HA sequence were used as a source of potential MHC class II epitopes. The current study is the first to characterize CD4+ responses to an H5N1 subvirion vaccine and identify potential MHC class II epitopes suitable for H5N1 vaccine development.

2. Materials and methods

2.1. PBMC samples

The original double-blinded clinical trial involved 451 healthy adults who received two intramuscular doses of either 90, 45, 15 or 7.5 μg of an H5N1 subvirion influenza A vaccine (rgA/Vietnam/1203/2004), followed by safety, tolerability and hemagglutination inhibition analysis [4]. Six months following the second immunization, 337 study participants were given a third immunization, as a follow-up to the original study [5]. Of these participants, 35 study subjects (age 23–78) were recruited back to the University of Rochester site 20–29 months following study completion for collection of blood for PBMC isolation. PBMC samples were subsequently shipped to Antigen Express using a liquid nitrogen dry shipper and stored in liquid nitrogen until analysis.

2.2. Synthetic peptides, recombinant HA protein and H5N1 subvirion vaccine

For the identification of immunodominant class II HA epitopes, an influenza peptide array was utilized. This array, provided by BEI Resources (Manassas, VA), included 94 overlapping peptides (16–17 mers, overlapping by 11–12 amino acids) covering the entire A/Thailand/4(SP-528)/2004 HA protein and is >99% homologous to the HA of the Vietnam/1203/2004 strain used in the trial. Initial screening (1st round) of PBMCs to identify class II epitopes was performed by IFN-γ ELISPOT using a matrix-based approach. Briefly, the 94-peptide H5N1 HA array was divided amongst 20 different peptide pools, with 10 peptides represented in each pool (2 mg/ml), with the exception of Pools 5–10, which had 9 peptides each and Pool 20, which only included 4 peptides. Using a matrix-based strategy to more rapidly and efficiently identify potential new class II epitopes, similar to that described by Kaufmann et al. [29], each peptide was included in two different pools, such that a positive response in two different pools would permit identification of the individual peptide of interest. Individual peptides were subsequently retested in a 2nd round ELISPOT analysis to confirm reactivity.

In addition to screening a library of overlapping peptides, T cell responses were tested against twenty-four predicted Class II H5N1 epitopes. The SYFPEITHI algorithm (www.syfpeithi.de) was used in a manner to maximize the likelihood of identifying promiscuous, yet highly conserved HA epitopes from the H5N1 HA A/Duck/Anyang/AVL-1/2001 amino acid sequence (GenBank, accession #AF468837). Epitope peptides were selected based upon their cumulative binding affinity to six of the most common HLA-DRβ1 alleles (DRβ1*0101, DRβ1*0301, DRβ1*0401, DRβ1*0701, DRβ1*1101, and DRβ1*1501). It was later observed that all but one donor tested in these studies had at least one of these alleles (data not shown). Peptides were synthesized (NeoMPS, San Diego, CA) to include the Ii-Key motif (LRMK) for enhanced interaction with the class II molecule, which was covalently linked to the N-terminus of each epitope via a linker sequence (5-aminopentannoic acid, *ava*). Peptides were dissolved in 20% DMSO and frozen at −80 °C until use.