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Broad cross-protective anti-hemagglutination responses elicited by influenza microconsensus DNA vaccine

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ABSTRACT

Despite the routine development and distribution of seasonal influenza vaccines, influenza remains an important pathogen contributing to significant human morbidity as well as mortality each year. The seasonal variability of influenza creates a significant issue for vaccine development of seasonal strains that can afford protection from infection or disease based on serotype matching. It is appreciated that the globular head of the HA antigen contained in the vaccines generates antibodies that result in HAI activity that are a major correlates of the protection against a particular strain. Due to seasonal genetic changes in the HA protein, however, new vaccine strains are needed to be developed continually to match the new HA antigen of that seasons virus. A distinct advantage in seasonal vaccine development would be if a small group of antigens could be developed that could span many seasons without needed to be replaced due to this genetic drift. Here we report on a synthetic microconsensus approach that relies on a small collection of 4 synthetic H1HA DNA antigens which together induce broad protective HAI immunity spanning decades of H1 influenza viruses in mice, guinea pigs and non-human primates. The protective HAI titers induced by microconsensus immunogens are fully functional *in vivo* as immunized ferrets were completely protected from A/Mexico/InDRE4487/2009 virus infection and morbidity associated with lethal challenge. These results are encouraging that a limited easy-to-formulate collection of invariant antigens can be developed which can span seasonal vaccine changes allowing for continued immune protection.

1. Introduction

Despite an intensive vaccine program, influenza infections remain a major health problem, due to the viruses' ability to change its envelope glycoprotein hemagglutinin (HA) through shift and drift, permitting influenza to escape protection induced by current vaccines or natural immunity. Each year the components of seasonal Flu vaccines are selected by the World Health Organization (WHO) based on reporting from 112 distinct National Influenza Centers (NICs) regarding which H1N1, H3N2 and Influenza B strains are currently circulating and which strains are the most likely to cause significant human suffering in the coming season. While new seasonal vaccines are built on the premise of the induction of protective antibodies, the immunity generated by traditional seasonal Flu vaccines offer little to no cross-protection against strains of Influenza that are not seasonally selected by the WHO [1,2]. Thus, if strains of influenza other than those selected by the WHO emerge and circulate, immunity driven by the vaccine may be only partially protective or even completely ineffective [3]. In 2007, for example, seasonal influenza vaccine coverage was estimated at only 30% due to mismatches between the expected emerging strains and the strains that ended up actively circulating [4]. According to the early estimates of 2014–2015 seasonal influenza vaccine effectiveness from CDC, overall vaccine effectiveness (VE) was only 23% during the period of Nov.10th, 2014–Jan.2nd 2015 [5]. This interim low VE estimate was also due to the fact that more than two-thirds of circulating H3N2 viruses are different from the 2014–15 H3N2 vaccine component. Moreover, seasonal vaccines provide coverage for only those selected viruses which are thought to likely to circulate during a given flu season, with current approaches not able to anticipate or provide protection against pandemic influenza viruses. The 2009 Novel H1N1 Pandemic highlights the lack of cross-reactivity

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of seasonal vaccines towards non-targeted strains, including pandemic strains. It was determined early in the most recent Influenza Pandemic that immunization of the seasonal vaccine did not confer protection against Novel H1N1 infection [6,7]. The lack of cross-reactivity against Influenza strains not selected by the WHO or of emergent pandemic strains has led to increased efforts focused on the creation of a more "Universal" influenza vaccine approach capable of neutralizing multiple variants of influenza virus, including those not specifically matched to the vaccine strain.

The basis for the efficacy of influenza vaccines is primarily the generation of neutralizing antibodies, in particular the induction of antibodies that prevent hemagglutination [8,9]. It is believed that hemagglutination inhibition (HAI) antibody titers correlate with protection against influenza. While higher HAI titers ($\geq$110) may be used to predict the conventional 50% clinical protection rate in children, in general, an HAI titer of $\geq$40 is associated with a 50% or more reduction in influenza infection risk in adults [8,10–12]. Historically, the induction of antibodies capable of this type of cross-neutralization through vaccination has been difficult to achieve due largely to the fact that most antibodies that prevent hemagglutination bind to the HA1 "head" region of the hemagglutinin (HA) protein, an area which is subject to high variability thus resulting in limited type specific immunity [13,14]. In contrast, the HA2 "stem" region of hemagglutinin is more highly conserved, and has been proposed as a target for more universal vaccine development [15–18]. While this approach shows promise, the general inability of anti-stem antibodies to display anti-HA activity, and the higher titers needed for them to be effective has generated challenges for their clinical implementation. Importantly, the protection offered from these vaccines in diverse challenges in ferrets appears to be amelioration of disease symptoms with less ability to prevent infection than anti-HA antibodies, thus creating complexity for evaluation in clinical trials. The prospect of developing an influenza vaccine that can protect more fully against diverse viral isolates via the generation of antibodies that prevent hemagglutination remains highly appealing.

In this regard, synthetic DNA vaccines have emerged as an attractive immunization approach against important infectious diseases targets. From the point of view of universal influenza synthetic DNA vaccine design, sequence identities between the vaccine candidate and the infecting virus should be an important consideration. Utilization of a focused consensus DNA immunogen derived from the most common amino acid in every position in a limited alignment minimizes the degree of sequence dissimilarity between a vaccine and infecting viruses, thus potentially maximizing vaccine-induced immune responses. Recently, we have applied many strategies, including codon/RNA optimization, the addition of highly efficient immunoglobulin leader sequences [19–21], use of 'centralized' immunogens [22,23], new formulations [24] combined with highly efficient DNA delivery methods such as CELLECTRA® based *in vivo* electroporation (EP) [25,26] using synthetic DNA vaccine design. Through these combined changes, we have dramatically improved magnitude and breadth of immune responses induced by these synthetically developed DNA vaccines in small animals, macaques [26,27], and most recently and importantly, in humans [28,29].

In this report, we designed four distinct plasmid-encoding influenza H1 HA micro-consensus antigens; each containing a limited consensus sequence that was generated based on analysis of a subset of the broader collection of primary sequences of H1 HA antigens deposited in the NCBI database. We evaluated the immunogenicity of each individual micro-consensus plasmid delivered by EP in mice. Then we combined these plasmids as a single vaccine formulation and evaluated its immunogenicity in guinea pigs and rhesus macaques. We observed that this influenza DNA vaccine containing four plasmid-encoding micro-consensus H1 HA immunogens was capable of inducing protective levels of HAI titers to diverse isolates of H1N1 Influenza. Challenge of ferrets with a lethal dose of unmatched A/Mexico/InDRE4108/2009 virus confirmed 100% protection from influenza-driven mortality. The data support further study of this synthetic micro-consensus approach in combination with EP delivery as a potential platform against various influenza infections.

2. Materials and methods

2.1. Phylogenetic analysis of influenza H1 HA protein sequences

206 primary H1 HA protein sequences of seasonal and pandemic virus isolates were retrieved from Influenza Research Database (IRD, https://www.fludb.org/brc/home.spg?decorator=influenza). The alignment applied in the phylogenetic study was performed using Clustal X (version 2.0) and a phylogenetic tree was constructed based on Neighbor-joining evaluation of the alignment.

2.2. Design and construction of influenza H1-1, H1-2, H1-3 and H1-4 DNA vaccines

After constructing the H1 HA phylogenetic tree, four micro-consensus-based HA antigens covering four major clusters were developed. As indicted in the phylogenetic tree (Fig. 1A), influenza sequences were grouped into four major clusters. Primary influenza H1 HA sequences in each cluster (23, 33, 79 and 58 sequences, respectively), were therefore used to develop four micro-consensus antigens covering these four major clusters. All sequences were aligned using MegAlign (DNASTAR, Madison, WI) and four micro-consensus full-length HA sequences (H1-1, H1-2, H1-3 and H1-4) were developed. Instead of targeting specific domain (s) of HA protein, these microconsensus immunogens represent four different full-length consensus HA proteins and each includes all domains of HA protein. Sequences for H1-1 through H1-4 were used to construct comparative models using standard homology modeling protocols in Discovery Studio 4.5 (Biovia, San Diego, CA). Energy-minimized models were aligned and superimposed to assess overall structural similarity and for epitope comparison purposes.

The micro-consensus sequences were codon/RNA optimized, and the synthesized *H1-1, H1-2, H1-3 and H1-4* genes were cloned into the expression vector pGX0001 under the control of the cytomegalovirus immediate-early promoter, respectively. The constructs were named as pH1-1, pH1-2, pH1-3 and pH1-4, respectively.

2.3. Expression of pH1-1, pH1-2, pH1-3 and pH1-4

Rhabdomyosarcoma (RD) cells were cultured in 6-well plates and transfected with 3 μg of pGX0001 (empty vector), pH1-1, pH1-2, pH1-3 or pH1-4 using FuGENE6 Transfection Reagent (Roche Applied Science, 11815091001), respectively. Forty-eight hours after transfection, cells were washed three times with PBS and lysed in 100 μl Modified RIPA cell lysis buffer. The total protein lysates (50 μg) were fractioned on a SDS-PAGE gel, transferred to a PVDF membrane (Amersham). The Western blot analysis were performed with anti-HA (A/New Caledonia/20/99) (eEnzyme, MIA-0011), anti-HA (A/Brisbane/59/2007) monoclonal antibodies (Sino Biological Inc., 11052-MM06), anti-HA (A/California/06/2009) (eEnzyme, MIA-0014) and visualized with horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, NA931V) using an ECL™ Western blot analysis system (GE Healthcare, RPN2132).

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