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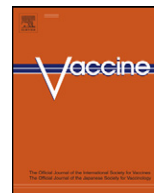

Meeting report

FDA/NIH/WHO public workshop on immune correlates of protection against influenza A viruses in support of pandemic vaccine development, Bethesda, Maryland, US, December 10–11, 2007

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ABSTRACT

The goals of the workshop were to identify gaps in our knowledge and abilities to address the unique challenges encountered in the development of vaccines intended to protect against pandemic influenza and to facilitate implementation of a global research agenda to improve efficacy assessment of pandemic influenza vaccines. This workshop included discussions on: (i) current knowledge regarding immune correlates of protection against seasonal influenza; (ii) human immune responses to avian influenza infection and vaccines for novel influenza viruses; (iii) limitations of currently available assays to evaluate vaccine immunogenicity; and (iv) potential insights from animal models for correlates of protection against avian influenza.

1. Introduction

Highly pathogenic (HP) avian influenza (AI) A viruses of the H5N1 subtype are currently causing wide-spread infections in domestic birds throughout Southeast Asia, with gradual spread into Central Asia, Europe, and Africa. As of 28 May 2008, WHO reports 383 H5N1 human cases and 241 deaths of human infection have occurred (>60% case fatality rate). It is feared that adaptation of the HPAI viruses that allows human-to-human transmission will result in a global pandemic. Therefore, the development of an effective vaccine against avian influenza A (H5N1) has been declared of high priority by Public Health authorities around the world.

The first H5N1 vaccine for human use was generated from the human isolate of influenza A/Vietnam/1203/2004 (H5N1) with the use of a plasmid rescue system to introduce attenuating modifications (deletion of the multibasic amino acid sequence in HA that is associated with virulence in poultry) and reasonable growth properties in eggs (internal genes are from A/PR/8/34). A H5N1 inactivated vaccine manufactured with this viral seed (Sanofi Pasteur) was approved by the FDA in 2007 for the US stock piles for emergency use only. Evaluation and licensure of pandemic influenza vaccines rely on immunological endpoints borrowed from seasonal influenza vaccines. Currently, the principal immunological endpoint used in seasonal vaccine studies is the hemagglutination-inhibition (HI) assay. Using this endpoint, it has been noted that the immune response to one A/VN/1203/2004 H5N1 vaccine is significantly lower than to seasonal human influenza vaccines, with HI titers of ≥1:40 observed in only 54% of vaccinees receiving two doses of 90 μg HA [1]. The need to develop pandemic vaccines that are effective and can be manufactured in amounts sufficient for global distribution has stimulated the influenza research community to develop novel approaches to H5N1 vaccines [2]. This in turn has raised questions concerning evaluation of influenza vaccine immunogenicity and effectiveness. Examples of questions that need to be addressed are: (i) is it appropriate to extrapolate what we know from seasonal influenza vaccination to pandemic influenza vaccines? (ii) what is the impact of vaccinating populations that lack pre-existing immunity? (iii) what end-points correlate with protection against highly pathogenic influenza viruses? (iv) how should immune responses against new generation vaccine candidates including live attenuated viruses (LAIV), plasmid DNA vaccines, virus like particles (VLP), and viral vectors be evaluated? (v) can we establish the protective levels associated with existing or new immunological endpoints and accurately quantify the responses following vaccination? The public workshop to address these issues was co-sponsored and co-organized by scientists from CBER/FDA, NIAID/NIH, and WHO. The meeting was attended by 200 participants, from 15 countries, with other participants joining by webcast (http://www.fda.gov/cber/pandemic/panflu121007.htm). All the influenza vaccine manufacturers, including those involved in the development of pandemic influenza vaccines were represented at this meeting.

The conclusions in this Meeting Report have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy

2. Correlates of protection against seasonal influenza (session I)

Session I included presentations on humoral and cellular immune responses against seasonal influenza infections and vaccines, including inactivated vaccines (TIV) and live attenuated cold adapted vaccines (LAIV). Lessons learned from murine studies and human studies were discussed. The session included presentations by Robert Couch (Chair, Baylor College of Medicine), Brian Murphy (NIAID, NIH), Tom Jefferson (Cochrane Collaboration), Jack Bennink (NIAID, NIH), and Harry Greenberg (Stanford University).

A half-century of experience with influenza vaccines shows that homotypic immunity following infection or vaccination is powerful

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and long-lived. However, when tested against drifted strains of the same type, immunity varies with the extent of antigenic variation (antigenic drift). Heterosubtypic immunity (HSI) (i.e., protection against influenza strains that have a different hemagglutinin (HA) subtype) is weak in humans. Protection against infection or severe clinical symptoms correlates with serum antibodies against the HA of the infecting virus. The anti-HA antibodies may block viral infection, reduce viral replication in the upper respiratory tract (URT), and prevent dissemination to the lung. Secretory IgA is likely the most relevant antibody in the URT, but serum IgG is protective in the lungs. Unfortunately, measurement of antibodies in mucosal secretions is difficult (identified as a key research priority). Therefore, studies on correlates of immunity have focused on serum antibodies. A serum antibody hemagglutination inhibition (HI) titer of 40 is accepted as the level of serum HI antibody associated with >50% reduction of the risk of contracting an influenza infection or influenza disease [3]. However, it should be kept in mind that other immune parameters also contribute to protection so that HI titer alone may not guarantee immunity or predict susceptibility. In addition to anti-HA antibodies, the protective potential of other antibodies was discussed. These may include antibodies against neuraminidase (NA), which are known to reduce the severity of infection. Anti-M2 antibodies were reported to reduce the severity of infection in mice. However, titers of anti-M2e antibodies in humans are low and proof of their contribution in resolving infections is lacking.

T cell responses against internal (more conserved) viral proteins contribute to heterosubtypic protection in mice. There is limited information about the specificity and characteristics of influenza-specific T cell responses in humans. Recent studies demonstrate an increase in the percentage of influenza-specific IFN-γ-producing CD8+ T cells in children following immunization with live attenuated influenza vaccine (LAIV) but not trivalent inactivated vaccine (TIV), supporting the idea that replicating virus activates cell mediated immune responses.

In addition to measuring serum antibodies, it is helpful to quantify influenza-specific IgA and IgG antibody secreting cells (ASC) present in the circulation following vaccination. Both antibody responses and the number of ASC were more significantly increased in children post-vaccination (compared with adults), probably reflecting the lower levels of pre-vaccination immunity. Interestingly, TIV but not LAIV significantly increase the percent of influenza-specific memory B cells measured 30 days after vaccination. In addition to the number of ASC, influenza-specific CD4+ T cell responses were predictive of antibody responses.

In summary, it was concluded that no single end-point can be used as a surrogate of protection; protection reflects the sum of various immune responses, including antibody and cell-mediated responses. The above correlates have largely been derived from challenge studies in humans, carried out under carefully controlled conditions. There are only a small number (4) of randomized controlled trials that meet the criteria set forth by the Cochrane collaboration and provide data to test the assumption that vaccines that induce an anti-HA antibody response that meet regulatory requirements provide protection at a population level. However, an analysis of these studies concluded that the correlates currently in place are valid. It was emphasized that clinical studies of influenza vaccine efficacy should include end-points that reflect a broad range of immune responses. These studies should be designed carefully to avoid risk of bias and the trials' outcome should be reported accurately in publications, so that meaningful conclusions can be reached about immunological endpoints that predict protection or clinical benefit.

3. Immune responses to avian influenza infections and vaccines for novel influenza viruses in humans (session II)

Session II included presentations on the status of avian influenza infections in humans, a description of immune responses in poultry workers and description of clinical trials to evaluate inactivated and live attenuated avian influenza vaccine candidates. The session included presentations by Jacqueline Katz (Chair; CDC), Nancy Cox (CDC), Maria Zambon (Health Protection Agency, UK), David Cho (NIAID, NIH), Ruth Karron (Johns Hopkins University), Laszlo Palkonyay (WHO), and Frederick Hayden (WHO).

Nancy Cox presented an update on avian H5N1 influenza viruses in birds and human exposure. An extensive genetic and antigenic heterogeneity of H5N1 infections in poultry birds has been reported, with 9 of 10 different genetic clades isolated in the past 3 years. The H5N1 viruses isolated from humans reflect the heterogeneity in birds [4]. Reverse genetics techniques have been used to generate attenuated 6:2 PR8/H5N1 (HA, NA) reassortant viruses representing clades (1, 2, and 3) and subclades (2.1; 2.2; 2.3). These can be used for vaccine development and *in vitro* assays to measure vaccine immunogenicity or immune responses in exposed individuals.

Serologic responses in poultry workers exposed during the 1997 H5N1 outbreak, showed neutralizing titers that ranged between 1:10 and 1:40, while titers of $\geq$1:80 were reported in convalescent patients and individuals with more intense exposure. The general findings in several studies of serologic responses in human following exposure to avian viruses are that the incidence of seroconversion is low and antibody responses after mild/asymptomatic infections are short-lived.

The occurrence of human infections with H9N2 viruses prompted examination of responses to the two antigenically and genetically distinct lineages of H9N2 viruses called G1 and G9 in poultry workers in Hong Kong. While only 2% of the subjects were seropositive for antibodies to G1-like viruses, approximately 36% were seropositive to G9-like viruses. Reactivity with H9N2 viruses likely reflected the presence of age-related pre-existing antibodies to H2N2 viruses that are cross-reactive with H9N2 viruses. The fine specificity of this cross-reactivity was not established. However, prescreening volunteers for avian influenza studies is often desirable.

Clinical trials of both inactivated and live attenuated avian influenza vaccines have been conducted in the USA, Europe, Asia, and Australia. Inactivated H5N1 virus vaccines have been administered as whole virus or split virus preparations, and in some instances have been formulated with adjuvants. All vaccines are reasonably well tolerated but require high doses when no adjuvant is included. Microneutralization results suggest that adjuvanted vaccines induce antibody responses with broader reactivity to heterologous H5 strains.

Clinical trials of cold adapted live attenuated avian influenza vaccines have shown that replication capacity of these attenuated strains in humans is variable (H7N3 > H9N2 $\gg$ H5N1), with seroconversion rates reflecting level of replication in the URT.

The WHO recently established the South East Asia Influenza Clinical Research Initiative that will provide a network of sites and resources in areas with a high incidence of HP avian influenza infections. The aim of this group is to support clinical protocol-based studies to improve the understanding of pathogenesis, immunology, diagnostics, and therapies for human H5N1 virus infections. The initiative aims to develop a central inventory of resources that can be shared between groups. This initiative could support vaccine trials and studies to establish correlates of "protective immunity".

4. Assays to evaluate vaccine immunogenicity (session III)

This session included discussion of the assays that are currently available for evaluation of vaccine immunogenicity and their limitations. Novel *in vitro* assays were also described. This session included presentations by John Wood (NIBSC, UK), Guus Rimmelzwaan (Erasmus Medical Center, Netherlands), Gary Nabel (VRC, NIH), Maryna Eichelberger (CBER, FDA), Hana Golding (CBER, FDA), Walter Gerhard (Wistar Institute, Philadelphia), and Janet McElhaney (University of British Columbia, Canada).

The most commonly used *in vitro* assays to measure immunogenicity of type A influenza vaccines are HI, virus neutralization (VN) and single radial hemolysis (SRH) assays. Each method was outlined and their limitations for evaluation of avian influenza vaccines presented. The HI assay, which is used for evaluation of seasonal influenza antibodies, is based on agglutination of chicken or turkey RBC. This assay is not sensitive for measuring anti-H5 or anti-H7 antibodies due to differences in their receptor binding specificities. Use of horse RBC in the HI test provides an increased sensitivity for avian influenza HI. However, this assay needs standardization. The VN assay utilizes MDCK cells which are sensitive to infection with diverse types and strains of influenza, including avian influenza viruses of all clades. Comparison of the Horse HI and VN assays for antibodies against clade 1 and clade 2 avian influenza viruses showed an apparent good correlation when a positive microneutralization titer was defined as $\geq$1:80, although future studies and assay validation are needed. The SRH assay is currently used in a small number of labs and evidence for it's applicability for measuring anti-H5 antibodies is lacking. The WHO has initiated a collaborative study to evaluate H5N1 serological techniques and to establish an international standard for H5N1 antibody. These studies will be conducted at 17 participating laboratories that will share blinded sera samples, vaccine stocks, reference sera and SOPs.

Assays that provide an alternative to the traditional assays or evaluate different aspects of the immune response were then presented. These assays include a pseudovirion reporter gene based assay for measurements of anti-H5N1 antibodies that neutralize H5N1 virus entry, neuraminidase enzyme inhibition (NI) assays, identification of antibody epitopes using whole genome phage display libraries that can be used to map the binding sites of monoclonal antibodies and polyclonal sera; M2e-specific assays, and assays to enumerate influenza-specific T cell responses. Many of these assays could be used to supplement the traditional serological assays for more complete assessment of influenza immunity.

5. Correlates of protection against avian influenza: potential insights from humans and animal studies (session IV)

This session focused on the evaluation of vaccine immunogenicity and efficacy in animal models, evidence for heterosubtypic immunity, previous challenge studies in humans, and the potential use of surrogate measures of efficacy. The session included presentations by Kanta Subbarao (Chair, NIAID, NIH), Jacqueline Katz (CDC, Atlanta), Suzanne Epstein (CBER, FDA), John Treanor (University of Rochester, NY), and Jerry Weir (CBER, FDA).

Kanta Subbarao provided a comprehensive description of the mouse, ferret and monkey models available to measure vaccine immunogenicity and efficacy. Many avian influenza viruses, including HP H5N1 viruses replicate efficiently in mice and cause disease without prior adaptation, but not all avian influenza strains are lethal in mice. Intranasal infection under anesthesia results in viral pneumonia, with clinical signs that include ruffled fur, hunching, labored breathing, hypothermia, weight loss, and mortality that can be used to evaluate vaccine efficacy after virus challenge. However, protection from lethality is not a sensitive indicator of vaccine efficacy and should be accompanied by studies evaluating viral replication, histopathological changes and immunohistochemical studies. The vast array of reagents available to analyze immune responses in mice allows detailed studies of immune responses in this model. In addition, mice provide a good model for passive transfer of antibodies in order to establish a correlate of protective immunity. For example, several human monoclonal antibodies against H5N1 (A/Vietnam/1203/2004-like viruses) provided protection against both homologous (the parent wild type virus) and heterologous (different clades) strains of highly pathogenic H5N1 viruses.

The ferret provides a more natural animal model since they are susceptible to both human and avian influenza viruses. Although reagents are limited in this model, clinical signs of disease and viral replication provide multiple parameters that can be used to assess vaccine-induced protection. The effect of vaccine is primarily to reduce pulmonary viral replication and enhance clearance. Therefore, protection against homologous and heterologous challenge strains is best assessed by determining reduction of virus replication. Other animal models including hamsters, guinea pigs, cotton rats, cats, and non-human primates have been used to study influenza pathogenesis and immunity. However, each of these animal models has weaknesses that limit broad use.

Animal models provide good evidence for heterosubtypic immunity, with immunization with H3N2 viruses providing protection against lethal infection with influenza viruses of a different subtype. Several immune effector mechanisms could contribute to this protection, including CD8+ T cell responses to conserved antigens and antibody responses to conserved epitopes such as the extracellular M2 peptide. Epidemiologic data from the 1957 H2N2 pandemic support the idea that heterosubtypic immunity may play an important role in humans as a first line of defense against emerging influenza strains with pandemic potential.

How can pandemic vaccine efficacy be evaluated? Past studies involving vaccination against seasonal influenza have compared virus replication and signs of illness in vaccinated and non-vaccinated individuals following natural infection, or after challenge with wild-type or attenuated virus strains in adults and children, respectively [3]. These studies have limitations due to expense of testing virus challenge preparations for safety, the absence of information on viral replication and pathogenesis in the lower respiratory tract, and the lack of virulence of recent viruses in adults even when individuals are seronegative. Human challenge studies to establish correlates of protection against avian influenza are unlikely to be feasible.

The serological criteria currently used for approval of pandemic vaccines in the US are based on seasonal influenza vaccines, with a seroconversion rate (i.e. percent of subjects with a minimum 4-fold rise in HI titer) for persons younger than 65 of >40% and seroprotection rate (i.e. percent of subjects with HI titer >1:40) for this same age group of $\geq$70%. These criteria may not be valid for pandemic influenza vaccines. Furthermore, these serological endpoints may not capture the major protective mechanisms against new vaccine platforms such as LAIV, DNA vaccines, VLP, and viral vector delivered influenza antigens. There is certainly a need to establish the protective levels associated with newly defined immunological endpoints and to accurately quantify these responses following vaccination.

6. General recommendations of workshop participants

The meeting concluded with a panel discussion that addressed many of the issues that had been raised during the sessions and discussed approaches to expedite the evaluation of pandemic influenza vaccines (panelists: Robert Couch, Jacqueline Katz, Maria Zambon, Kanta Subbarao, Robert Belshe, Ed Kilbourne, Wendy Keitel, and Peter Wright).

No formal recommendations were issued. However the main conclusions of the workshop are summarized below:

- Surrogate markers of vaccine efficacy: There is a need to correlate protection with both quantitative and qualitative measures of immunological endpoints. This can include established serological assays (HI as the gold-standard, virus neutralization titers), additional immune parameters (e.g. NA inhibition titers, cell-mediated responses) as well as novel assays to evaluate quality of the antibody and/or cellular response.
- It may be premature to extrapolate what we know from seasonal influenza vaccination to pandemic influenza vaccines. Particularly the use of a pre-determined serological endpoint (such as 1:40 HI titer) to predict vaccine efficacy, although until a better understanding is obtained, this remains a useful benchmark. In general, the higher the antibody response, the broader the immune response is likely to be, and thus induction of a high level of anti-H5 HA antibodies by a vaccine candidate would be desirable. To assess the breadth of response to H5 clades, it was suggested investigators focus on viruses from clades that are circulating in humans, and that data from 3 to 4 clades was likely to be sufficient; it was noted however that such data should be updated as new clades emerge in humans. This means sponsors should be encouraged to set aside sufficient sera from clinical trials to enable additional future studies to be conducted.
- In order to facilitate the standardization of key laboratory assays to evaluate vaccine responsiveness and cross-reactive neutralization of avian A viruses, there is an immediate need for production of reference standard reagents including low pathogenicity avian viral stocks, immune sera (from animals and humans) and sharing of common laboratory protocols.
- It is important to develop tools to measure mucosal immune responses.
- Animal challenge models are useful for large proof-of-concept studies and for comparison of vaccine formulations and novel adjuvants. Vaccines undergoing clinical evaluation may benefit from parallel studies in ferrets using the same formulations and dose range. Challenge with homologous and heterologous avian influenza strains (i.e. the same or different clades) could provide important information on the breadth of protection.

In conclusion, a more programmatic approach to pandemic vaccine trials, with use of standardized assays and reference reagents should facilitate comparison of clinical trial outcomes and expedite vaccine development.

7. WHO closed-session working groups

Following the workshop, WHO convened two closed-session working groups. Working group 1 was tasked with identifying (a) the improvements and standardization of non-HI assays that will be needed to enable evaluation of new influenza vaccines, and role WHO should play in establishing these and (b) the activities that need to be undertaken to improve the use of human challenge studies for evaluation of new vaccines. Working group 2 was tasked with making recommendations on correlates to be used for evaluating efficacy of pandemic influenza vaccines and whether any changes are needed to current WHO standards.

Working group 1 identified the following priority activities and actions:

(a) The ability to perform adult human challenge studies was considered a high research priority and the establishment of a Working Group was suggested to drive the development of and assist in implementation of such studies. Other clinical studies were recommended as tools to identify correlates of immune protection included LAIV challenge studies in children using shedding of the vaccine virus as the read-out, as well as natural history studies.

(b) A wider range of immunological parameters should be followed in human and animal challenge studies including an assessment of humoral, cellular, and mucosal immunity. The establishment of a second Working Group was suggested to promote and coordinate development of improved methodology for assays, particularly standardizeable means of measuring mucosal immunity in the clinic, and also to promote the development of standardized neuraminidase inhibition assays.

(c) It was recognized that there is a need for improved bridging of immune readouts between human and animal model studies with emphasis on:
- identifying parameters in animal models that mimic human responses to vaccination and infection;
- where possible performing all the immune evaluations both in humans and animal trials;
- promoting the development of better ferret reagents.

It was recommended that a Working Group coordinate development of tools for animal models and studies that bridge to clinical trials.

(d) For influenza vaccine quality control purposes there is a need to evaluate NA quantity and conformational integrity in vaccines.

Working group 2 concluded that current WHO guidance [5], which provides three criteria for assessment of the efficacy of candidate vaccines by testing anti-HA antibodies in clinical trial recipients, and also recommends testing a subset of sera for neutralizing antibodies, is scientifically valid. The Working Group also recommended support for studies that evaluate the cross-protection offered by H5 vaccines in animal models and the cross-immunity induced by such vaccines using sera from clinical trials in neutralization tests against different H5 clades currently infecting humans. Such studies should be used as an opportunity to advance the standardization of such methods, and to generate data for the public domain.

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