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REVIEW

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The use of cell-mediated immunity for the evaluation of influenza vaccines: an upcoming necessity

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ABSTRACT

Influenza vaccines are a fundamental tool for preventing the disease and reducing its consequences, particularly in specific high-risk groups. In order to be licensed, influenza vaccines have to meet strict criteria established by European Medicines Agency. Although the licensure of influenza vaccines started 65 years ago, Hemagglutination Inhibition and Single Radial Hemolysis are the only serological assays that can ascertain correlates of protection. However, they present evident limitations. The present review focuses on the evaluation of cell-mediated immunity (CMI), which plays an important role in the host immune response in protecting against virus-related illness and in the establishment of long-term immunological memory. Although correlates of protection are not currently available for CMI, it would be advisable to investigate this kind of immunological response for the evaluation of next-generation vaccines.

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Introduction

Influenza vaccines constitute the only available means of preventing influenza and its complications. Although influenza is a vaccine-preventable disease, it still constitutes a major health problem, accounting for about 3 to 5 million cases of severe illness and responsible for 290,000 up to 650,000 respiratory deaths per year.[1] Young children, pregnant women, immunocompromised subjects, subjects of any age with specific chronic medical conditions and the elderly have a higher risk for influenza-related co-morbidities; these may be life-threatening, requiring hospitalization, and even lead to death. In healthy children younger than 24 months of age, the risk of hospitalization is comparable to that of high-risk groups, or even higher. Specifically, children aged < 24 months run a significantly higher risk of being hospitalized than older children; in addition the youngest children have the greatest risk of hospitalization as a consequence of flu. Influenza-associated deaths in children often occur soon after symptom onset, mostly within 1 week. Wong et al.[2] found that the period between symptom onset and death was even shorter in previously healthy children than in children with high-risk medical conditions. Although no explanation for this observation is currently available, it has been hypothesized that abnormal immune regulation could underlie severe infection in certain previously healthy children.[3]

Flu complications range from moderate (ear and sinus infections) to serious. The latter include pneumonia, myocarditis, encephalitis, myositis, rhabdomyolysis, multi-organ failure (such as respiratory and kidney failure) and sepsis. Flu also can make chronic health problems worse.[4]

The elderly show reduced vaccine effectiveness as a result of immunosenescence. It is traditionally accepted that aging leads to a gradual decline of both innate and adaptive immune responses, thereby reducing the response towards infections and vaccines; today, however, immunosenescence is seen more as a remodeling of the immune system, causing an altered regulation of the various compartments. Indeed, while certain activities show a -deterioration,[5] others are up-regulated[6] or remain unchanged.[7] In addition to age, other factors influence the effectiveness of influenza vaccines: the antigen match between the circulating influenza strains and those strains contained in the vaccine itself, the vaccinee's immunocompetence, and the antibody levels induced by previous infections or vaccinations.[8,9]

Criteria for influenza vaccine licensing

The evaluation of vaccine immunogenicity constitutes a critical aspect of vaccine marketing. In order to evaluate the host immune response to vaccines that provides protection, correlates of protection are used. Although the words "correlates" and "surrogates" are often used synonymously, their meanings are different. As specified by Plotkin,[10] "an immune function that is responsible for and statistically interrelated with protection is a correlate, while an immune response that is simply an easy measurement but not functional in protection is a surrogate". In the case of influenza vaccines, correlates of protection for influenza are usually represented by serum antibody titers, which are mainly measured by means of the Hemagglutination Inhibition (HI) assay.[11] Indeed, antibodies can protect against influenza, as demonstrated by the fact that their parental or intranasal administration reduces infection rates in animal models[12,13] and IgG trans-placental passage provides neonatal protection.[14,15] Furthermore, in the human influenza challenge, treatment with an anti-M2e monoclonal antibody has proved effective and safe.[16]

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Several serological assays are commonly used to evaluate vaccine effectiveness; these include usually Single Radial Haemolysis (SRH), HI test and Virus Microneutralization (MN).

However, although the licensure of influenza vaccines began 65 years ago, HI and SRH are the only serological assays for the evaluation of humoral effectiveness that have correlates of protection established by the European Medicines Agency (EMA) which have to be met in order to obtain vaccine licensure. For this reason, they are considered the gold standard. Every year, vaccine manufacturers have to conduct clinical trials for the annual update of influenza vaccine composition. Specifically, pre- and post-vaccination serum samples are collected (approximately 21 days after the first blood draw) from 2 groups of at least 50 individuals aged 18–60 years and >60 years. Immunogenicity is assessed by means of three criteria identified by the Committee for Medicinal Products for Human Use (CHMP).

The proportion of vaccines that achieve an HI titer of 40 or SRH > 25 mm2 should be >70% in

18–60 year-olds and >60% in the over-60s. The seroconversion rate (SCR) (at least a 4-fold increase in titer) should be >40% in 18–60-year-olds and >30% in the over 60s. A mean geometric increase (ratio of pre- to post-vaccination) of >2.5 is required in 18–60-year-olds and >2 in over-60s. In the US, the same criteria are used by Food and Drug Administration (FDA), but the lower boundary of the 95% confidence interval (CI) has to be higher than or equal to that of the SCR and geometric mean titer (GMT) criteria.[17]

At least one of the 3 criteria must be met by seasonal influenza vaccines, and all 3 criteria by pandemic influenza vaccines in order to be licensed.

Since different classes of antibodies are identified by the three serological assays, different degrees of correlation among them have been observed.[18]

The SRH and HI tests recognize antibodies which bind the influenza virus and fix complement[19,20] and viral HA, respectively, preventing the agglutination of erythrocytes caused by the influenza virus (Figure 1).

The SRH assay is based on the measurement of the hemolysis areas,[20,21] which correlates with influenza antibody concentration, since hemolysis is the outcome of antigen-antibody binding. The assay is specifically suitable for use in large-scale clinical trials, owing to its rapidity, reproducibility and reliability.[22,23] The SRH assay has a higher sensitivity for influenza B strains.[24,25]

The HI assay is the gold-standard assay of antibody titers against HA, and is based on erythrocyte agglutination due to the ability of antibodies that specifically recognize HA to inhibit the binding of viral surface protein HA to sialic acid sites on the surface of red blood cells.[21] Avian (chicken or turkey) or mammalian (horse or guinea pig) erythrocytes are usually chosen for the assay. HI titers are quantified as the reciprocal of the highest serum dilution (titer) (1/dilution factor) that inhibits hemagglutination by binding with the virus.[26]

HI also presents limitations, including low sensitivity for influenza B and avian viruses, unsuitability for LAIV evaluation, high inter-laboratory variability due to many factors and the absence of standardized protocols.[25] Regarding seasonal influenza A strains, SRH and HI show similar sensitivity.[24] A ≥ 25 mm^2 zone is defined as a correlate of protection for SRH.[27] Concerning HI, an HI antibody titer ≥40 and a minimum 4-fold increase in antibody titer post vaccination are historically considered an immunological correlate of protection against infections caused by influenza viruses, and is associated with a 50% reduction in the risk of developing influenza. These data are based on a challenge study performed by the group of Hobson on

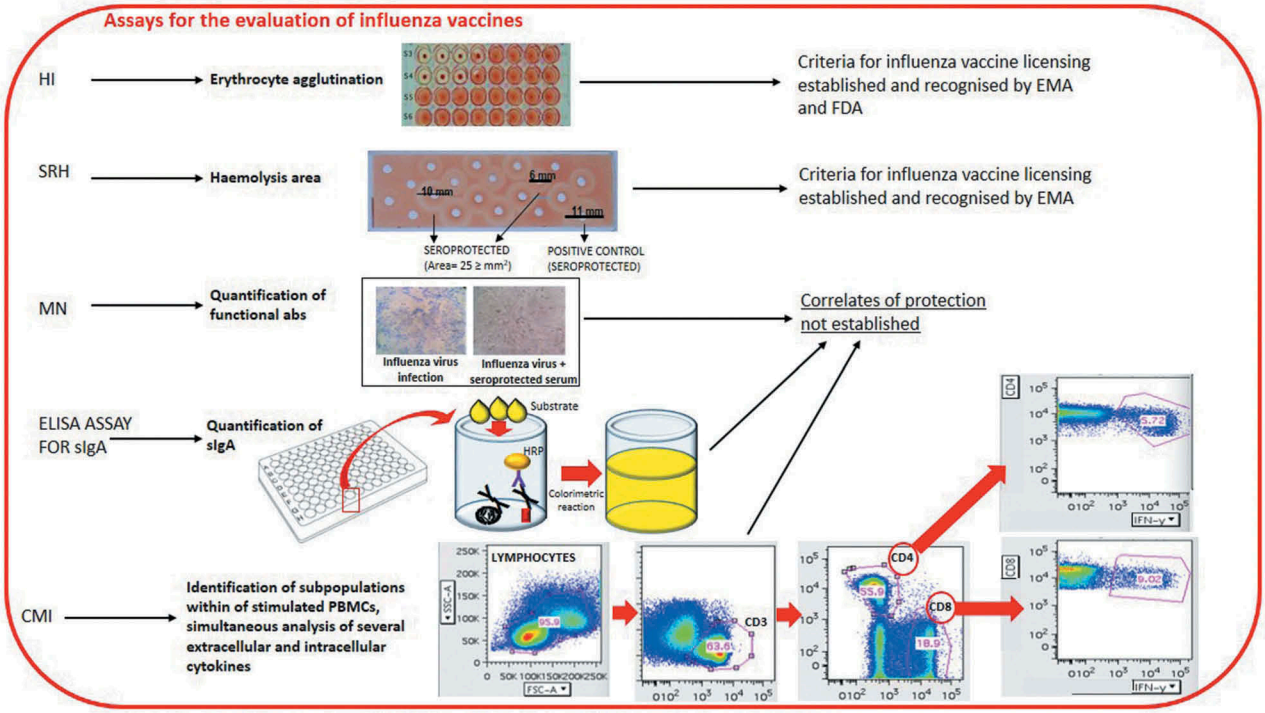

Figure 1. Some of the assays currently used for the evaluation of influenza vaccines

adults[27] and epidemiologic studies conducted mainly on young adults. [*rev. in*[17,23,28]]. However, the biological role in establishing protection of titers higher than 1:40 remains to be elucidated. Indeed, in an analysis of 12 studies conducted on healthy adults, de Jong et al.[29] found that a mean titer of 28 was protective in 50% of the subjects. However, they reported protective titers ranging from 15 to 65, owing to differences in the studies. By contrast, a subsequent meta-analysis by Coudeville found that 17 was a median 50% protective titer.[30] In addition, application of the bayesian random-effects model developed by Coudeville to the data from Hobson's study revealed that a titer of 29 provided a 50% level of protection. Regarding the correlation between HI titers and the establishment of clinical protection against influenza, the investigation revealed an increase in protection up to an HI titer of 150. Reaching HI titers higher than 150 did not provide a further significant protective ability; however, it is possible that HI titer > 160 must be reached in order to be protective both in children less than 6 years of age and in the elderly, since these two groups have an elevated risk of suffering influenza-related complications.[31] Concerning children, Black and colleagues found that 50% protection corresponded to an HAI titer of 110,[31] whereas few data are available on subjects aged over 65 years, to whom the protective titer of 40 is applied, as in younger adults.[32]

In addition, over the last decade, an increasing proportion of circulating human influenza A(H3N2) viruses have exhibited lower levels of hemagglutination as a consequence of binding to HA.[33]

The MN assay identifies functional antibodies that recognize the globular head of hemagglutinin (HA); these antibodies are able to halt receptor binding and internalization during membrane fusion, thus constituting the principal immune mediators toward influenza viruses.[34] Hence, the assay provides a true measure of the antibodies able to neutralize the ability of the virus to enter mammalian cells and replicate (Figure 1).[35] In the MN assay, sensitive cells are inoculated with a mixture of viruses and serum (i.e. the serum samples tested) and the read-out is performed by microscopically observing cytopathic effects (CPEs). The neutralization titer (i.e. antibody titer of tested previously serum samples serially diluted two-fold) is defined as the serum dilution by means of which 50% of the wells are protected against a virus-induced CPE. CPE is evaluated by checking the 96-well plate under an optical microscope for the presence of local lesions in the cell monolayer, in terms of hole(s) in the cell monolayer, surrounded by destroyed cells, or complete destruction of the cell monolayer in the well.[11] However, the assay is labor-intensive and displays poor reproducibility among laboratories.[36,37] Currently, no threshold for protection against influenza has yet been established for the MN test.[23]

All the above-mentioned assays measure antibody titers in the peripheral blood, but they do not evaluate the establishment of local mucosal immunity.

Evaluating local mucosal immunity is particularly important with regard to the efficacy of live attenuated influenza vaccines (LAIVs). Although these vaccines entered the market in 2013, we still do not have specific knowledge of the immunological mechanisms they induce, nor do they have correlates of protection.[38] A study conducted by Gorse[39] on older, chronically ill adults found that LAIVs, but not trivalent inactivated vaccines (TIVs), were able to induce heterosubtypic immunity in terms of both humoral and cellular immune responses. Comparison of the immune response in children and adults immunized with LAIVs or TIVs, revealed that both vaccines were effective; however, significant differences emerged in B-cell and antibody responses elicited by LAIVs or TIVs in the two groups.[40,41]

Specifically, inactivated influenza vaccine (IIV) proved more effective in adults, whereas LAIVs provided higher protection in children,[42] although the immune mechanisms underlying these differences have not yet been clarified.[41] With particular regard to influenza virus-specific HI, only a small increase in serum HI responses was observed in adults immunized with LAIV, and these responses were considerably lower than those induced by IIV. Both LAIV and IIV similarly induced only transient T-cell responses to replication-competent whole virus in adults. In contrast, stronger influenza virus-specific secretory IgA (sIgA) responses were induced by LAIV than by IIV.[41] A previous investigation conducted by the same group[40] reported that LAIVs showed a greater ability to induce several T-cell responses ($CD4^+$, $CD8^+$, and γδ T cells) in young children, indicating that they play an important role in providing heterosubtypic immunity.

Measuring sIgA and identifying a subpopulation of resident memory T-cells[43] could constitute valuable alternative tools for assessing the effectiveness of LAIV (Figure 1). In addition, a recently published meta-analysis performed by the group of Wen[44] allowed the identification of differentially expressed genes responsible for distinct immune responses following LAIV and TIV vaccinations. Specifically, whereas LAIV mainly promoted the upregulation of genes associated with the innate immune system, TIV up-regulated genes correlated with both the innate and the humoral immune responses. The importance of these data lies in the fact that they provide more information about the activating pathways underlying the different immune responses to LAIV and TIV immunization – knowledge which may enable us to enhance the efficacy of vaccinations in children, adults and the elderly.[44] Wang and colleagues[45] first investigated the correlates of protection by calculating Spearman's rank correlation coefficient (r) for antibody levels for SRH, HI and MN against H3N2 influenza in children and adolescents; in these two age-groups, few data on the transferability of the two thresholds established for HI and SRH are available.[31] They reported significant correlations among HI, MN and SRH. Specifically, correlation of 0.50 ($P < .01$), 0.53 ($P < .01$) and 0.82 ($P < .01$) were observed between HI and MN, between HI and SRH, and between MN and SRH. MN was the most sensitive of the three serological assays investigated for the evaluation of antibody response against influenza H3N2.[45] This result could be directly linked to the principle underlying the three tests. While SRH and HI assays are based on complement fixation and HA binding, respectively, MN recognizes specifically functional antibodies involved in virus neutralization. Hence, MN can detect a higher proportion of protective antibodies than SRH and HI.[25] Specifically, HI has been seen to have lower sensitivity than SRH and MN, as demonstrated by the fact that 34% and

16% of the subjects, respectively, showed HI and SRH titers below the detection limit, in comparison with 7% on the MN assay. Previous investigations had also reported a higher sensitivity of MN than HI.[11,19,20] Moreover, MN is reckoned to be more sensitive than HI in detecting protective antibodies towards some pandemic influenza strains.[46,47] With regard to the MN assay, a recent study conducted by Tsang[48] reported a correlation between antibody titers ≥ 40 and the establishment of 49% protection against H3N2 influenza virus within households.

Another limitation that should be considered is that the currently available correlates of protection for influenza vaccines regard healthy subjects and not high risk groups, such as older adults, young children and subjects affected by certain medical conditions;[46] in these groups, HI displays lower effectiveness in predicting protection. Indeed, the efficacy of an influenza vaccine is not always correlated with the extent of the humoral immune response.[49] Evaluation of the efficacy of novel influenza vaccines is heavily based on serological assays; however, both seroconversion and seroprotection rates, as well as antibody titers, when used as the only predictors of vaccine efficacy, present limitations, as has increasingly been recognized.[50-54] Immune senescence is responsible for the age-related decline of immune responses, since the elderly present alterations in the immune function and inflammatory response; this results in more serious outcomes of viral and bacterial infections as well as lower vaccine responses.[55] Specifically, elderly subjects who are affected by febrile influenza illness may not be able to mount an antibody response (with reductions in antibody titers and lower antibody avidity) although they test PCR+ for influenza virus.[56] For this reason, an optimal correlation between antibody titer and strain-specific vaccine efficacy cannot be obtained by using the traditional measures of immune response to influenza vaccines. Hence, in the elderly, the cell-mediated immune response can help to establish clinical protection against the increased risk for complications of influenza infections.[57,58] In addition, in the elderly cytotoxic T-lymphocyte (CTL) response and granzyme B synthesis have shown a stronger correlation with protection than that provided by antibodies.[59,60]

Another limitation of standard serology methods is that new vaccines may not contain HA in their formulations, but other viral proteins, which means that their protective actions cannot be determined; some example are DNA- or RNA-based vaccines with sequences encoding nucleoprotein (NP) and M proteins.[61]

In young adults, correlates of protection for T-lymphocytes have been identified,[62,63] but these have not been transferred to older adults. Moreover, attempts to transfer the thresholds indicating clinical protection against influenza infection to older adults, with a view to developing novel influenza vaccines, could be unsuccessful, since these thresholds may need to be associated with antibody responses. Supporting the need for new correlates of protection, a recent study by Neidich[64] on influenza-vaccinated adults suffering from obesity revealed for the first time that, although obese subjects displayed similar seroconversion and seroprotection rates to healthy-weight subjects, they were twice as likely to develop influenza or influenza-like illness (ILI). Not only did an HI titer ≥ 40 not represent a serological correlate of protection in obese adults, but also MN titers could not be applied to this group at high risk of influenza and ILI, in accordance with studies performed on obese mice.[65]

The use of flow cytometry

Cell-mediated immunity (CMI) plays an important role in host immune response in protecting against virus-related illnesses, including influenza, and in the establishment of long-term immunological memory.[66]

Today, clinical trials aimed at evaluating vaccine immunogenicity and, in particular, at increasing our knowledge of the mechanisms underlying the immune response are making greater use of techniques involving the simultaneous and accurate measurement of subpopulations of stimulated peripheral blood mononuclear cells (PBMCs) and several extracellular and intracellular cytokines, chemokines and cytotoxic activity, by means of flow cytometry (Figure1) or Enzyme-linked ImmunoSPOT (ELISPOT) assays.[67]

Identifying significant changes in the phenotype, differentiation and activity of T-lymphocytes induced by vaccine administration could provide reliable correlates of protection.[68]

Although our knowledge of memory T-cell responses has increased, we still know little about the duration of these responses and their involvement in various pathologies. Protection against influenza involves both B and T lymphocytes. Specifically, CD8 T-cell memory and antigen-selective B cells require CD4 T-cells. Although conserved influenza peptides/antigens have been seen to induce the formation of both CD8 and CD4 T-lymphocytes, their generation does not reach a sufficiently elevated level to maintain immunological protection for years.[69] Long-term heterotypic protection against several influenza viruses have been induced by memory T-lymphocytes,[70-72] as demonstrated by the fact that seasonal influenza viruses induced CD4 T[70,73] and cytotoxic T-lymphocytes[71] that are able to recognize the pandemic H1N1 2009 (pdmH1N1) virus.

Intracellular cytokines released by an entire population can be identified through the use of intracellular cytokine staining (ICS). However, the extracellular release of cytokines by the Golgi is prevented by the use of an inhibitor of protein transport, such as monensin or Brefeldin A in the last 4 or 16 hours of cell culture.[74,75] PBMCs or diluted whole blood can then be stimulated overnight by using different stimuli, such as Staphylococcus enterotoxin B (SEB), recombinant ESAT-6 protein or anti-CD28 and anti-CD49d co-stimulatory antibodies. The end of the incubation is followed by fixing, permeabilization and staining with fluorescent-labeled anti-cytokine antibodies. The PBMCs are then analyzed by means of a flow cytometer.[76,77] The profile of secreted cytokines enables T-lymphocyte sub-populations to be distinguished.

ICS can be conducted both on isolated PBMCs, either fresh or cryopreserved in freezing medium (before at −80°C and then in liquid nitrogen)[78] and on whole blood; in the former case, however, a lower inter-laboratory coefficient of variation has been observed.[79]

Evaluation of t and B cell responses

Natural influenza virus infection stimulates $CD4^+$ and $CD8^+$ T cells, which act in synergy to provide protection in the case

of vaccine mismatch or pandemic outbreak. By contrast, only the currently available LAIVs, and not IIVs, have been seen to efficiently elicit T-cell responses, especially in children.[40,80,81] The establishment of T-cell-mediated immunity induced by next-generation vaccines could overcome limitations linked to both specific subtype protection and antigenic mismatch. The different T-cell sub-populations can be identified through the use of specific antibody combinations that recognize cytokines secreted by a specific T-cell subset, such as Interferon (IFN)-γ by $CD4^+$ T helper (Th)1 T-cells[82] and cytotoxic T (Tc)1 $CD8^+$ T cells,[83] Interleukin (IL)-17A by Th17 $CD4^+$ T-cells,[84] or IL-4, IL-5, IL-9, IL-10 and IL-13 synthetized by $CD4^+$ Th_2 cells.[84]

Although the cell-mediated immune response upon influenza vaccination is increasingly being investigated, and the observation that granzyme B production correlates with protection and increased CTL response to influenza vaccination in the elderly (*vide supra*), no correlates of protection regarding either the phenotype or the magnitude of the T-cell response following vaccination have yet been established. Evaluation of the cytotoxic potential of $CD8^+$ T-lymphocytes is a further method of evaluating immune response; this involves measuring the degranulation and granule contents of specific T-cell subsets. Degranulation is typically measured in terms of CD107a expression on the cell surface. In normal conditions, CD107a is expressed in internal granular membranes, whereas during degranulation its transient expression can be identified on the cell surface.[85] T-cell responses can be evaluated by analyzing several cytokines, cell surface markers and other functional markers, such as perforin, CD107a, and CD154, with up to 10-color resolution[86] and CD40 ligand expression with regard to $CD4^+$T cell response.

The profile of ab-producing B lymphocytes has been investigated in infected or immunized subjects by monitoring the surface markers CD19, CD20, CD27, CD38, and CD138. Acute plasmablasts constitute the cell population which usually appears in the blood after infection during the phase of immune response. These cells are $CD19^{low}CD20^{-}CD27^{high}CD38^{high}CD138^{+/-}$cell populations, which differ from steady-state plasmablasts.[87] Their number has been seen to peak on day 6 or 7 in the case of booster responses, and somewhat later (≥day 10) in the case of new responses.[11,88-91]

In order to identify novel correlates of protection, Nakaya and colleagues investigated early features of the innate and adaptive immune responses that could predict the HI titer 4 weeks after vaccination in 56 healthy young adults immunized with TIV or LAIV during the annual influenza seasons in 2007, 2008 and 2009.[92] The study highlighted the presence of a large number of genes showing a different expression; most of these participated in the response involving type I IFN and had a high expression in antibody secreting cells (ASCs), the latter probably due to rapid plasmablast proliferation 7 days after vaccination,[89] in the PBMCs of LAIV and TIV vaccinees, respectively.[92]

Gijzen et al.[49] utilized granzyme B as a marker of T cell-mediated cytotoxicity and the production of Th1 and Th2 cytokines, such as IFN-γ, TNF-α, IL-2, IL-10, IL-4, IL-13, GM-CSF, to determine the cellular immune response with a view to establishing correlates of protection. Their study demonstrated that both granzyme B and cytokine assays could be used to evaluate cellular immunity and thus be examined as correlates of protection.

The group of Jürchott[93] evaluated the baseline protective immune response to the A(H1N1)pdm2009 influenza strain following seasonal vaccination of 17 young (<31 years old) and 20 older (≥50 years) subjects who were seronegative against this strain by analyzing 36 sub-populations of lymphocytes. They also correlated this response with the serological immune response to the A(H1N1)pdm2009 strain after seasonal influenza vaccination. The seasonal vaccine for the season 2011–2012 (and 2013–2014 season) contained A (H1N1)pdm09/California/7/2009, together with A(H3N2)/Perth/16/2009 and B/Brisbane/60/2008 (or A(H3N2)/Texas/50/2012 and B/Massachusetts/2/2012) as vaccine strains.

The A(H3N2) and the B strains circulated before 2009 in humans and accumulated slight modifications by means of antigenic drift over the time,[94] whereas the California strain was a new virus of the subtype A(H1N1). The study revealed that the serological response to A/California/7/2009 depended on age and number of strains for which the donors were seronegative at the baseline. More specifically, a trend toward a higher risk of no response and no seroprotection was observed in elderly donors. In addition, the analysis of several cell counts of immune sub-populations allowed these authors to identify the axis of $CD4^+$ T cells, $CD4^+$ naïve T-cells and $CD4^+$ recent thymic emigrant T-cells as good candidates for response predictors. Specifically, they reported that the baseline $CD4^+$ T-cell count, and especially that of naive $CD4^+$ T-cells, constituted the best correlates for the evaluation of a successful immune response to A(H1N1)pdm09, but not to the A(H3N2) Perth or the influenza B Brisbane strains. Indeed, no marked deviations in $CD4^+$ T-cells and their subsets were noted in Brisbane and Perth seronegative donors regarding the response to these strains, while the cell counts of $CD8^+$ T-cells and $CD19^+$ B cells in Brisbane seronegative donors, and of monocytes and dendritic cells in Perth seronegative donors, differed considerably between the protected and non-protected groups.[67] The significant differences between non-responders and responders concerning the immune cell sub-populations could be due to the fact that the H3N2 and the influenza B strains – or similar strains – were circulating in humans before 2009.

In agreement with Jürchott's results, Nayak's group also reported that $CD4^+$ T-cell expansion was predictive of neutralizing antibody responses to a monovalent 2009 A(H1N1) pdm09 vaccine.[95] By contrast Tebas[96] found that the A (H1N1)pdm09 vaccine was poorly immunogenic in well-controlled HIV-infected patients as a consequence of their low $CD4^+$ T-cell counts. The further subdivision of naïve $CD4^+$ T-lymphocytes into $CD31^+$ recent thymic emigrants (RTE) and $CD31^-$ non-RTE fractions was not correlated with improved prediction, though both sub-populations were predictive of protection. Conversely, no association between baseline influenza selective $CD4^+$ $CD40L^+$ T-cells and protection against the A(H1N1)/California/7/2009 strain was observed.

Recently, Tsang and colleagues revealed that the analyses of human immune changes highlights the presence of baseline predictors of post-vaccination immune responses.[97]

A recent investigation conducted by Mbawuike[98] evaluated cell-mediated immune responses upon re-vaccination of 177 subjects by using an inactivated influenza A/H5N1 (A/H5N1/Vietnam/1203/2004 and A/H5N1/Indonesia/05/05) vaccine; they also considered the effects exerted by the vaccine dose (15- or 90-mcg), adjuvant and the age of the subjects immunized.

Concerning LAIV vaccines, neither the quantization of mucosal or serum antibodies, nor that of chemokines or cytokines provide information regarding protection, and no association between protection and the administration of either IIV or LAIV vaccines was observed when the commonly used methods for the evaluation of immunity were implemented.[99]

Conclusions

The formulation of an influenza vaccine that provides broad protection even in high risk groups, and the optimization of the vaccines currently available, require more thorough knowledge of the immune response of the host following influenza vaccination. A further need is to establish new correlates of protection for influenza vaccines, particularly for the evaluation of next-generation vaccines, since correlates of protection can vary according to the vaccine type, vaccine formulation, and the age and medical status of vaccinees. For the last 70 years, serological assays have been the only tests used to assess influenza vaccine efficacy, and the HI assay is a well-standardized and widely used test. However, the standardization of a T-cell assay may constitute a valuable approach, not least with a view to the development of more immunogenic, effective and cross-reactive novel vaccines. Traditionally, influenza vaccines have been aimed at eliciting antibodies involved in virus neutralization. However, those which recognize the HA-head region, even though potently neutralizing, can usually target only related viruses that do not present marked antigenic diversity. By contrast, although antibodies that target the conserved stem region have less neutralizing activity *in vitro*, they are endowed with cross-reactivity.[100,101] Even though HI plays a paramount role as a correlate of protection for conventional influenza vaccines, in addition to CMI, assays based on other vaccination-induced antibodies, and which recognize epitopes different from HA, could constitute valid alternatives. Indeed, the human immune response is complex, involving both humoral and cellular responses, and various correlates of protection may conceivably exist.

Novel assays able to measure the Fc-mediated functions of anti-influenza antibodies have been developed, since it has been demonstrated that, in addition to neutralization, Abs can mediate further functions by using their Fc region. Specifically, they are important for anti-influenza immunity *in vivo*, playing a role in complement-dependent cytotoxicity (CDC),[102-106] antibody-dependent phagocytosis (ADP),[107,108] and antibody-dependent cellular cytotoxicity (ADCC).[109,110] These antibodies represent a connection between the innate and adaptive immune responses. It has been proved that antibody Fc-receptor interaction is not only able to enhance the efficacy of widely neutralizing antibodies,[111] but is also necessary for broadly neutralizing anti-influenza Abs to guarantee protection *in vivo*[112] and that these antibodies are correlated with protection against experimental influenza challenge for several candidate universal vaccines.[113] Indeed, in the absence of elevated HI titers towards circulating strains in the elderly, older adults usually present ADCC antibodies.[114] The majority of the currently used cell-based assays of the Fcγ function of antibodies are based on Natural Killer (NK) cells, and quantify activation marker expression, cytokine and lytic protein release or the killing ability of NK cells through flow cytometry or ELISpot techniques.[115-117] These methods include NK viral inhibition assays, rapid-fluorimetric ADCC assay (RFADCC), granzyme delivery assays, lactate dehydrogenase release assay, and NK cell activation assays which assess IFN-γ and/or CD107a. However, these techniques also present limitations, owing to the long execution, complexity and difficulty of reproduction and standardization across laboratories; in addition, the results of the assays may be biased by the possible presence of polymorphisms in the Fc-receptor of effector cells collected from human donors.[118]

This review focuses on the main aspects of T- and B-cell responses following influenza vaccination, as evaluated by means of flow cytometry. Hence, efforts should be made to identify other immunological parameters, such as T – cell-mediated immune response, as correlates of protection, especially in view of the fact that the scenario of influenza vaccine is evolving rapidly and novel influenza vaccines will probably be developed in the foreseeable future.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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