Correlates of Protection

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What is a correlate of protection?

Correlate of protection
- An immune response that is responsible for and statistically correlated with protection
  - All individuals protected or population average e.g. 50%?

Surrogate correlate of protection
- An immune response that substitutes for the true immunological correlate of protection, which may be known or not easily measured
  - *i.e. non mechanistic correlate of protection, which does not cause protection but nevertheless predicts protection through its (partial) correlation with another immune response that mechanistically protects*.
- Differ – vaccine type and formulation, age, health status,
Objective of protection of an influenza vaccine?

**Definitions.** ILI = influenza-like illness. URI = upper respiratory tract illness. Hosp = hospitalization.

Need for well controlled clinical trials and laboratory confirmed cases (virus culture or PCR) correlate: individual protected at time of sampling however pre exposure can be later.

Influenza vaccines

Adjuvants- enhance and broaden immune response
e.g. MF59, AS03, AF02, ISCOM ALUM
Annual Vaccination

• Trivalent vaccine (quadrivalent 2 B strain?)

• Inactivated vaccines standardised by HA content
  • (generally 15μg HA per strain)

• High risk individuals recommended for vaccination
  • Cardiac disease
  • Respiratory disease
  • Metabolic disease
  • >65 years old
  • children (2 doses in younger children),
  • pregnant women etc.)
Regulatory criteria: Annual update

EU (CPMP/BWP/214/96)
2 clinical trial of tolerability and efficacy of vaccines

At least 50 subjects
18-60 years old
over 60 years old

Pre vaccination and post vaccination blood samples (approximately 3 weeks)
All sera shall be analysed for HA antibodies by HI or SRH

Regulatory criteria: immunogenicity studies

<table>
<thead>
<tr>
<th>Parameter</th>
<th>18-60 years old</th>
<th>&gt;60 years old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroprotection Rate (SPR) (HI titre ≥40 or SRH &gt; 25mm²)</td>
<td>&gt;70%</td>
<td>&gt;60%</td>
</tr>
<tr>
<td>Seroconversion Rate (SCR) (4 fold increase)</td>
<td>&gt;40%</td>
<td>&gt;30%</td>
</tr>
<tr>
<td>Mean geometric increase (Ratio pre to post vaccination)</td>
<td>&gt;2.5</td>
<td>&gt;2</td>
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**Seasonal vaccines**
EMEA (CHMP) : must meet at least one of the three parameters
FDA : GMT and seroconversion : lower bound of 95% CI ≥ criterion

**Pandemic vaccines**
EMEA (CHMP) : all three parameters must be met
FDA: lower bound of 95% CI ≥ criterion for all parameters
Immune response induced by influenza vaccination

Protection is multifaceted but an assay only measures one parameter

Haemagglutinin (HA) major viral antigen

Antibodies to HA directed against
- Receptor binding site - inhibiting viral entry
- Stalk - block fusion of viral and host membranes
- Glycosylation sites may interfere with antibody binding

HI assay
(haemagglutination inhibition assay)

- Influenza HA binds to sialic acid receptors on surface of erythrocytes – agglutination

- HI assay measures the ability of serum antibodies to inhibit haemagglutination

No agglutination 50% Agglutination 100%
**HI assay**

- Sera pre treated with RDE overnight at 37° C
- Inactivation at 56° C for 45 min
- Serial two-fold dilutions (start 1:10) in V-well
- 8 HAU of virus to sera for 1 h
- 0.7% turkey red blood cells in PBS for 30 min

**HI as a “correlate” established in efficacy trials**

HI titre of 18-36 associated with 50% protection

Hobson et al, 1972
Relationship between antibody titres and the influenza infection in adults

HI titres associated protection in adults

50% protection
- HI titre 42 for H1N1
- HI titre 44 for H3N2 viruses
Metaanalysis: Probability of protection by HI titre

international standard for antibody to pdmH1N1 reduced variability

Wood et al. Vaccine. 2012:210-7
HI assay

Technically simple & easy to automate but large variation between laboratories (8-128 fold)

Insensitive for detection of influenza B and avian (H5 and especially H7) antibody responses (horse cells)

need better understanding HI as correlate of protection for pandemic vaccines

Standardisation of laboratory protocols and introduction of international standards to reduce inter laboratory variation

Single radial haemolysis

• Agarose gels containing influenza adsorbed to sheep RBCs & guinea pig complement. Sera added to wells and antibody to influenza diffuses in gel causing zones of complement mediated lysis
• Size of haemolysis zone proportional to amount of influenza HA antibody (IgG)
• SRH zone area 25mm² indicative protection
SRH zone areas associated 50% protection in adults

- 50% protection was associated with SRH zone areas of 20.0-25.0 mm²
- H1N1 (HI 42) and SRH zone area 21mm².

Single radial haemolysis

Sensitivity compared to HI
- Comparable for seasonal A viruses and greater for B viruses & 1997 Clade 0 H5 viruses (equivalent sensitivity to VN - Stephenson et al, 2003)
- Better reproducibility between labs- seasonal strains (Wood et al, 1994): HI 32 fold variation, SRH 3.8 fold
- Critical factors: type of erythrocyte & complement, serum controls
Is an HI titre of 40 a suitable correlate of protection in all age groups?

Protective HI titre defined in adults

But what about infants and children?

Young children may not have been previously primed by infection thus no influenza specific humoral and cellular response

Relationship between antibody, infection and clinical attack rates in children

![Graph showing the relationship between antibody, infection, and clinical attack rates in children.](image)

(Davies and Grilli, Epidemiol Inf 1989)
Antibody titres needed to provide protection in children after 2 doses of inactivated vaccine

<table>
<thead>
<tr>
<th>Probability of protection</th>
<th>H3N2 antibody titre</th>
</tr>
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<tbody>
<tr>
<td>22%</td>
<td>40</td>
</tr>
<tr>
<td>50%</td>
<td>110</td>
</tr>
<tr>
<td>60%</td>
<td>151</td>
</tr>
<tr>
<td>70%</td>
<td>215</td>
</tr>
<tr>
<td>80%</td>
<td>330</td>
</tr>
<tr>
<td>90%</td>
<td>629</td>
</tr>
</tbody>
</table>

Hi titre 110 predicted the conventional 50% clinical protection rate
Hi titre of 330 predicted 80% protective level1

Virus Neutralisation assay

- Detects antibodies which neutralise infection (HA) and release of virus (NA)
- 2 main protocols: incubation time & method of detection (1-2 days NP (automation) or ≥3 days virus in supernatant)
- ≥ sensitivity to HI & SRH for seasonal (De Jong, 2003) & H5N1 viruses (live virus)
- Correlates of immunity unknown, but VN titre of 1:20-80 used to indicate a seropositive for H5N1
Virus Neutralisation assay

• Poor reproducibility between labs- different protocols (31-724 fold) (Stephenson et al, 2007)
• Validation & standardisation - amount of virus & growth kinetics; differences in serum treatment and dilution, time allowed for virus neutralisation (longer periods of time can also detect NA antibodies)
• Epitope mapping required-identify targets

Challenges for Serology

Validated, standardized assays to reduce laboratory variation
(Protocols, reagents and international antibody standard)
• Need for HI titres to be associated with laboratory confirmed influenza (culture and PCR)
• Need for definition of HI titres associated with protection for pandemic strains
Influenza infection stimulates multiple arms of the immune system:
- Systemic antibody to HA and NA, and multiple internal proteins
- Mucosal antibody
- Multiple cellular responses
- Long-lived protection against reinfection

**Live attenuated influenza vaccine (LAIV)**
- 1 dose of vaccine sufficient to elicit an immune response
- Induction of innate antiviral state shortly after vaccination
- Serum & mucosal antibody responses
- T cell responses
- Broader cross-protection against antigenic drifted variants than inactivated virus vaccines in naïve populations
High levels of efficacy observed despite low HI seroresponse rates to LAIV

<table>
<thead>
<tr>
<th>Study</th>
<th>A/H1N1 responses</th>
<th>A/H3N2 responses</th>
<th>B strain responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belshe, year 1</td>
<td>Did not circulate</td>
<td>46</td>
<td>68</td>
</tr>
<tr>
<td>Belshe, year 2</td>
<td>Did not circulate</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td>Tam, year 1</td>
<td>60</td>
<td>60</td>
<td>26</td>
</tr>
<tr>
<td>Tam, year 2</td>
<td>58</td>
<td>61</td>
<td>79</td>
</tr>
<tr>
<td>Vesikari, year 1</td>
<td>50</td>
<td>60</td>
<td>59</td>
</tr>
<tr>
<td>Vesikari, year 2</td>
<td>56</td>
<td>60</td>
<td>59</td>
</tr>
<tr>
<td>Bracco, year 1</td>
<td>Did not circulate</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Bracco, year 2</td>
<td>28</td>
<td>14</td>
<td>14</td>
</tr>
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Seroresponse, %
Efficacy, matched strains, %

Problem: no correlate of protection for LAIV

<table>
<thead>
<tr>
<th>Assay</th>
<th>Adults</th>
<th>Children</th>
<th>Efficacy study</th>
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<tbody>
<tr>
<td>Serum HI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal IgA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-G ELISPOT</td>
<td></td>
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<tr>
<td>Serum IgG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Neutralising Ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA antibody</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliva IgA</td>
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NA

Presence quantified in inactivated vaccines
Number of assays now available allowing re-evaluation of NA antibody response (mismatched HA strains)
Subjects clinical illness significantly lower HI and NA antibodies
NA antibodies 10-30 fold higher in subjects with no illness that those with serologically proven infection and illness (Ogra et al. JID 1977)

Serum Anti-Neuraminidase Antibody associated with Resistance to Influenza in Man

- Volunteers no HA antibody and varying NA specific antibody infected with wt influenza A/Hong Kong/1968 (H3N2)
- Clinical response related to the level of serum anti-NA antibody.
- Fever present -low levels of serum NA antibody prior to challenge
- No infection-significantly higher NA antibody
- Afebrile illness intermediate level of anti-NA antibody.
- Duration of virus excretion and maximal level of virus shed inversely related to anti-neuraminidase antibody level
- Conclusion: NA associated with resistance to clinical expression of influenza A virus in man

Enzyme Linked Immunosorbent Assay (ELISA)

Qualitative or quantitative measurement of antibody
- Enzyme linked to detector antibody soluble chromogenic measured in a spectrophotometer
- Measurement of nasal wash IgA, Serum antibody classes & subclasses
- Used for measuring antibody to other antigens than HA

(a) Indirect ELISA

Potential role of mucosal immunity

- Protection of the upper respiratory tract
- Role in otitis media
- Prevent viral shedding reduce transmission
- Intracellular neutralization of viruses
- No standardised collection method or assays

- TIV induces a rapid strong systemic but short lived local antibody response
TIV induces systemic and tonsillar but not nasal antibody secreting cell (ASC) response

0 7 14 21
days after vaccination

0 7 14 21
days after vaccination

blood ASC
nasal mucosa
ASC

high basal number of influenza specific ASC in the nasal tissue
no change after influenza vaccination

tonsils important activating, effector & memory site
for upper aerodigestive tract

M2e

- M2e tetrmeric membrane protein responsible acidification interior virion
- M2e highly conserved all influenza A subtypes
- M2e expressed high levels infected cells
- Poorly immunogenic, no/little immune response after infection
- CDC 50% people infected pdm2009 M2e antibody
- M2e vaccines coupled to immunogenic carrier (HBsAg, flagellin)
anti-M2e antibody binds M2e expressed on infected cells

Infected cell killed by NK cells complement mediated lysis or phagocytosised

Immune response induced by influenza vaccination

T cells critical to adaptive immune response often cross reactive with conserved epitopes

Induction of suitable priming to induce memory B and T cells

Isolation of lymphocytes

Standardisation of timing of blood samples, preparation (and freezing) of lymphocytes
Intra and inter laboratory variation
Quantification of T cell responses:

**Traditional methods:** Measures response of bulk population
- CD4 proliferation (stimulation specific antigen)
- $^3$H-Thymidine incorporation into DNA
- CD8 cytotoxic activity
  - Release of $^{51}$Cr from labeled target cell killed by effector

**Modern Methods:** Measure single cell
Production of cytokines after *in vitro* stimulation with antigen
- Intracellular staining
- ELISPOT
- Multiple analytes in one sample
- Staining with MHC/peptide complexes (tetramers) HLA typing

Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans

Correlation between CD4+ and total symptom scores in the H1N1 study
relationship between T cell responses to NP and M and less severe illness was associated with preexisting CD4+ T cells not CD8+ cells

Wilkinson et al. *nature medicine* 2012
Frequency of total preexisting CD4+ T cells strongly correlated inversely with illness duration

![Graph showing correlation between CD4+ T cells and illness duration](image)

Majority of subjects with ≥ 100 SFC/10^6 PBMC protected against influenza after LAIV

![Graph showing probability of infection vs. Interferon-γ ELISPOT](image)

Overall vaccine efficacy 62%

<10% of subjects in study had ≥ 100 SFC/10^6 PBMC
CD4+ T-cell response after Matrix M adjuvanted H5N1 vaccine

Days post vaccination

0.0 0.2 0.4 0.6 0.8 1.0
Total influenza specific CD4+ T-cells (%)

30µg- 1.5µg+ 7.5µg+ 30µg+

* = p<0.01 # = p<0.001

3-4 fold increase in CD4+ T cells associated MN antibody

Galli et al. PNAS 2009:106:3677
Cytotoxic T lymphocytes (CTL) CD8+ CTL recognition and elimination of virus-infected cells

CTLs are cross-reactive and recognize epitopes shared by influenza A viruses

CD8+ cells as a correlate

- Magnitude and repertoire of influenza-specific T cells for each person determined by
  - unique history of influenza infection(s) and vaccination
  - immunological status
  - HLA phenotype

- Unique immunological fingerprint for each person complicates the establishment of CD8+ T cell immunity as a correlate of protection.

- Require standardised assays for the quantification of influenza virus-specific T cell responses
**CD8+ T cells in influenza viral clearance in man**

63 volunteers intranasally inoculated with live attenuated A/Munich/1/79 virus.

- Clinical observations and virus shedding measured 7 days

- **All subjects with a demonstrable CD8+ T cell response cleared the virus.**

- Individuals lacking specific antibody, high levels of CD8+ T cells correlate with reduced viral shedding following experimental infection.

- CD8+ T cells showed cross-reactivity to virus subtypes.

Virus-specific cytotoxicity inversely correlated with the extent of virus shedding in the absence of specific antibodies


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**Heterosubtypic CTL immunity in man**

<table>
<thead>
<tr>
<th>Primising</th>
<th>Cross reactivity</th>
<th>CTL cross reactivity</th>
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</thead>
<tbody>
<tr>
<td>H3N2</td>
<td>H1N1</td>
<td>NP, M1, or PB2</td>
</tr>
<tr>
<td>Seasonal influenza</td>
<td>H5N1</td>
<td>Healthy subjects not exposed to H5N1 virus</td>
</tr>
<tr>
<td>pdmH1N1</td>
<td>1918 H1N1</td>
<td>T cells specific NP418 epitope</td>
</tr>
<tr>
<td>Seasonal Influenza</td>
<td>pdmH1N1</td>
<td>Healthy humans prior to pdm H1N1</td>
</tr>
</tbody>
</table>
CD8+ T cells are important in recovery from virulent influenza infections

- Role of CD8+ T cells determined mainly from adoptive transfer or depletion in animal models e.g.,

- No CD8+ T cells (transgenic mice) delayed viral clearance and increased mortality after infection with a virulent influenza virus strain (A/PR/8/34)

- Compared with wild type, transgenic mice had significantly delayed pulmonary (lung) viral clearance (Bender et al. JEM, 1992, 175; 1143-1145)

- Mice that received (I.V) influenza virus-specific CD8+ T cell clones (T9/13) had a three $\log_{10}$ reduction in lung virus titres and by days 6-8, the lung epithelial pathology returned to normal. (Mackenzie et al. Immunology 1989 67 375-381)

LAIV or infection, but not TIV, prime mice for robust recall of pulmonary CD8+ CTL responses to pdm-LAIV

Animal models: Immunogenicity and protective efficacy studies

Mice
- Proof of concept & dose range studies
- Seasonal strains require adaption
- Immunological reagents

Ferrets
- Protective efficacy studies
- Clinical disease reflects human disease
- Lack of immunological reagents

Highly pathogenic virus

Intranasal challenge high does virus (~10^5 ID_{50}) or a lethal dose
Evaluation of homologous or heterologous challenge
Clinical signs and symptoms, Viral replication

**Upper respiratory tract**
- Nasal washes
  - (no sacrifice no. of time points)

**Lower respiratory tract**
- Systemic spread requires euthanasia

**Contrasts**

- **Controls**
  - Vaccinated

Days post challenge

Viral titre (log_{10} EID_{50}/ml)

- Eye swabs
- Lung
- Spleen
- Brain
Antibody is not always associated with protection for pandemic strains:
2 doses of H7N1 aluminium adjuvanted vaccine protected

Adjuvants
- Enhance humoral and cell-mediated responses
- Broader and longer lasting immune response
- Allow dose sparing
Novel adjuvant systems

MF59 (squalene emulsion)
AS03 (squalene/tocopherol emulsion)
AF03 (squalene emulsion)
Squalene (oil in water emulsions)
Polyoxoidonium (poly-electrolyte)

Virosomal vaccine (virus like particle-liposomes)

Toll like receptor (TLR) agonists
- rHA Glucopyranosyl Lipid A (GLA-SE) (TLR4 agonist)
- Covaccine (TLR 4 agonist)
- Flagellin HA fusion protein (TLR 5 agonist)
- M2e NP ImmunoStimulatory Sequence (ISS), C295 (TLR-9 agonist)
- IC31 (dI:dC – TLR9 agonist)

Immunostimulating complex (ISCOMATRIX, Matrix M)

Challenges

- New generation vaccines not only dependent upon induction of HA specific antibodies
- Need for detailed evaluation of humoral and cellular immune response including kinetics of response
- Evaluation in all age groups (including pediatric and elderly populations)
- Multifaceted immune response- correlates may vary with vaccine formulation
- How will new generation vaccines be licensed,?

LAIV does not require annual trial in Europe
Challenges for correlates of protection

- Multiple correlates of protection
- Need for well defined correlates of protection (H5, H7 and pediatric population)
- Definition of suitable priming in cellular assays
- Validated, standardized assays to reduce laboratory variation (Protocols, reagents and international antibody standard)
- Head to head comparison of vaccines
- Development of novel standardized assays for novel vaccines
- Further evaluation of correlates of protection necessary