Application of Reverse Genetics to Influenza Vaccine Development

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Proteins and RNA’s of Influenza A Virus

- **NP** (Nucleoprotein)
- **PA** (Matrix Protein)
- **PB1, PB2, PA** (Replication)
- **NS2 (NEP)** (RNA transport, replication)
- **M2** (Ion channel, Penetration)
- **Lipid Bilayer**
- **HA (Hemagglutinin)** (Attachment, Penetration)
- **NA (Neuraminidase)** (Release)
- **M1** (Matrix Protein, Assembly)
Licensed Vaccines for Influenza

Principle: Induction of a protective immune response against the hemagglutinin gene.

Trivalent vaccines containing A/H1N1, A/H3N2 and B strains

- **Conventional inactivated vaccine**: Reassortant viruses containing HA and NA from wt influenza A viruses and internal genes from A/PR/8/34 virus + wt influenza B virus.

- **Live attenuated cold-adapted (ca) vaccine**: Reassortant viruses containing HA and NA from wt influenza A and B viruses and internal genes from master ca strains A/Ann Arbor/6/60 or B/Ann Arbor/1/66, respectively.
Antigenic Drift

- Gradual alteration of the influenza surface proteins (mainly HA) within a subtype resulting in the inability of antibody to previous strains to neutralize new viruses.
- Antigenic drift results from point mutations in the HA and NA genes.
- The composition of the influenza vaccine has to be updated annually as a consequence of antigenic drift.
Antibody Binding Sites of HA

Site A
Site B
Site D
Site E
Site C

Receptor Binding
Loop
Fusion Peptide

External
Membrane
Internal

Generation of Reassortant Influenza Viruses

Vaccine donor virus with phenotype of attenuation or high growth in eggs

Circulating wild type virus

Reassortant vaccine virus with phenotype of attenuation or high growth in eggs
Human Influenza Vaccines Generated by Plasmid-based Reverse Genetics

2 plasmids encoding genes from circulating wt virus

6 plasmids encoding genes from a vaccine donor strain

Vaccine grade cells

Hoffmann et al 2002, Vaccine and PNAS
Biosafety Measures Used for Generation of Reassortant Influenza Vaccines

The reassortant virus is generated at the biosafety level recommended for work with the wild-type virus (BSL-2 for human influenza viruses).
Influenza A viruses in humans in the last century

H1N1 → H2N2 → H3N2

Spanish Influenza → Asian Influenza → Hong Kong Influenza

1918 → 1957 → 1968 → 1977

Influenza A reservoir

15 HA subtypes
9 NA subtypes
## Recent Outbreaks of Avian Influenza in Poultry

<table>
<thead>
<tr>
<th>Year</th>
<th>Subtype</th>
<th>High pathogenicity?</th>
<th>Location</th>
<th>Birds killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1983</td>
<td>H5N2</td>
<td>yes</td>
<td>Pennsylvania</td>
<td>17 million</td>
</tr>
<tr>
<td>1995</td>
<td>H5N2</td>
<td>yes</td>
<td>Mexico</td>
<td>?</td>
</tr>
<tr>
<td>1997</td>
<td>H5N1</td>
<td>yes</td>
<td>Hong Kong</td>
<td>1.6 million</td>
</tr>
<tr>
<td>1999-2000</td>
<td>H7N1</td>
<td>no</td>
<td>Italy</td>
<td>13 million</td>
</tr>
<tr>
<td>2002</td>
<td>H7N2</td>
<td>no</td>
<td>Virginia</td>
<td>4.7 million</td>
</tr>
<tr>
<td>2003</td>
<td>H7N7</td>
<td>yes</td>
<td>Netherlands</td>
<td>30 million</td>
</tr>
<tr>
<td>2004</td>
<td>H5N1</td>
<td>yes</td>
<td>Asia</td>
<td>&gt;100 million</td>
</tr>
<tr>
<td>2004</td>
<td>H5N2</td>
<td>yes</td>
<td>Texas</td>
<td>?</td>
</tr>
<tr>
<td>2004</td>
<td>H7N3</td>
<td>yes</td>
<td>Canada</td>
<td>?</td>
</tr>
</tbody>
</table>
Avian Influenza Viruses Infecting Humans

- **H5**
  - Hong Kong 1997: 18 cases, 6 deaths
  - Hong Kong 2003: 3 cases, 2 deaths
  - Vietnam, Thailand 2004: 39 cases, 28 deaths
- **H7**
  - Case reports
  - Netherlands 2003: 79 cases of conjunctivitis, 13 ILI, 1 death, 3 person-person transmissions
- **H9**
  - Hong Kong and Southern China 1999: 7 cases
  - Seroprevalence in poultry workers 1999
  - Hong Kong 2003: 2 cases
Potential Social and Economic Impact of an Influenza Pandemic

- Mathematical model* estimates for first year of a pandemic in absence of effective interventions:
  - 89,000 - 207,000 deaths in the U.S.
  - 314,000 - 734,000 hospitalizations
  - 18 - 42 million outpatient visits
  - additional 20 - 47 million illnesses
  - economic impact: $71- $166 billion

Pandemic Influenza Vaccines Generated by Plasmid-based Reverse Genetics

2 plasmids from avian influenza virus

\[ \text{HA} \quad \text{NA} \]

\[ \text{PA} \quad \text{PB1} \quad \text{PB2} \quad \text{NP} \]

\[ \text{M} \quad \text{NS} \]

6 plasmids from vaccine donor strain

Vaccine grade cells

Hoffmann et al 2000
Strategy for Production of Live Attenuated Influenza Virus Vaccine (Genetic Reassortment)

Attenuated Donor Virus

Attenuated Reassortant Vaccine Virus

Virulent Wild Type Virus
General Approach to the Evaluation of Candidate Vaccine Viruses

• Genotype and sequence the HA and NA genes
• Compare the antigenicity of the vaccine reassortant with the parent virus
• Pathogenicity in chickens (USDA)
• Pathogenicity and level of replication in mice or other suitable animal model
• Efficacy of protection from challenge with wild-type virus in mouse or other suitable animal model
ts and ca Phenotypes of the H9N2-AA ca Reassortant Virus

Chen et al. Vaccine 21: 4430-36; 2003
The AA ca Genes Attenuate the H9N2 Reassortant Virus in Mice

Mice were infected with $10^5$ EID$_{50}$ in 50 µl intranasally. Organs were harvested on days 2, 3 and 4 post-infection.

Chen et al. Vaccine 21: 4430-36; 2003
A Live Attenuated H9N2-G9/AA ca Vaccine Protects Mice against Replication of Homologous and Heterologous H9N2 Challenge Viruses

Mice were challenged with wt H9N2 viruses 4 weeks after intranasal infection with $10^5$ TCID$_{50}$ of vaccine virus or controls

Chen et al Vaccine 21: 4430-36; 2003
## Shedding of IV and IN Administered H9N2-AA ca Virus in Chickens

<table>
<thead>
<tr>
<th>Route</th>
<th>Virus</th>
<th>Virus isolation from swabs</th>
<th>Antibody detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oropharynx</td>
<td>Cloaca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shedding  Titer</td>
<td>Shedding  Titer</td>
</tr>
<tr>
<td>IV</td>
<td>H9N2 wt</td>
<td>8/8  4.3</td>
<td>8/8  4.2</td>
</tr>
<tr>
<td></td>
<td>AA ca</td>
<td>0/8  &lt;0.9</td>
<td>0/8  &lt;0.9</td>
</tr>
<tr>
<td></td>
<td>H9N2/AA ca</td>
<td>0/24 &lt;0.9</td>
<td>0/8  &lt;0.9</td>
</tr>
<tr>
<td>IN</td>
<td>H9N2 wt</td>
<td>7/8  4.7</td>
<td>7/8  1.5</td>
</tr>
<tr>
<td></td>
<td>AA ca</td>
<td>0/8  &lt;0.9</td>
<td>0/8  &lt;0.9</td>
</tr>
<tr>
<td></td>
<td>H9N2/AA ca</td>
<td>0/8  &lt;0.9</td>
<td>0/8  &lt;0.9</td>
</tr>
</tbody>
</table>

Chen et al. Vaccine 21: 4430-36; 2003
The Influenza H5 Hemagglutinin Gene

HA1

Avirulent

Highly pathogenic

1997 HK human isolates

2003/4 HK human isolates

HA2

....RETR*GLF

....RKKR*GLF

...REERRRKKRR*GLF

...REERRRKKRR*GLF

The presence of multiple basic amino acids adjacent to the HA cleavage site increases the tissue range of the virus in birds.
Modifications Engineered into the HA Gene of HK/491/1997

HK/491/1997 wildtype HA:

CCT CAA AGA GAG AGA AGA AGA AAA AAG AGA ↓ GGA TTA TTT
Pro Glu Arg Glu Arg Arg Arg Lys Lys Arg Gly Leu Phe

Modified HA:

CCT CAA AGA GAG ACT CGA ↓ GGA TTA TTT
Pro Glu Arg Glu Thr Arg Gly Leu Phe

↓ Site of cleavage of into HA1 and HA2 domains.

Subbarao et al Virology 305: 192-200; 2003
Strategy for the Generation of a Candidate Reassortant H5 Vaccine

Modify the cleavage site of the H5 HA gene

Influenza Vaccine
Donor Strain
A/AA/6/60 ca OR A/PR/8/34

Li et al JID 1999; 179: 1132-38; Subbarao et al Virology 2003
In Vitro Characterization of the H5N1/PR8 virus

**Genetic Analysis:**
- HA and NA genes sequenced
- HA gene lacks multibasic amino acid cleavage site motif
- Remaining genes characterized by RFLP and partial sequence analysis

**Growth characteristics:**
- Failure to plaque in MDBK cells in the absence of trypsin

Subbarao et al Virology 305: 192-200; 2003
## Intravenously Administered H5N1/PR8 Virus Is Not Highly Pathogenic for Chickens

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mortality</th>
<th>Oropharyngeal swabs</th>
<th>Cloacal swabs</th>
<th>Sero conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shedding /total</td>
<td>Mean Titer</td>
<td>Shedding /total</td>
</tr>
<tr>
<td>H5N1 wt</td>
<td>8/8</td>
<td>8/8</td>
<td>6.1</td>
<td>8/8</td>
</tr>
<tr>
<td>PR8</td>
<td>0/8</td>
<td>1/8</td>
<td>0.94</td>
<td>6/8</td>
</tr>
<tr>
<td>H5N1/PR8</td>
<td>0/8</td>
<td>0/8</td>
<td>&lt;0.9</td>
<td>0/8</td>
</tr>
</tbody>
</table>

Subbarao et al Virology 305: 192-200; 2003
Intranasally Administered H5N1/PR8 Virus Replicates in Lungs But Is Not Lethal for Mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean virus titer in lungs ($\log_{10} \text{EID}_{50}$)</th>
<th>$\text{LD}<em>{50}$ ($\log</em>{10} \text{EID}_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 6</td>
</tr>
<tr>
<td>H5N1 wt</td>
<td>5.9</td>
<td>6.4</td>
</tr>
<tr>
<td>PR8</td>
<td>6.0</td>
<td>6.2</td>
</tr>
<tr>
<td>H5N1/PR8</td>
<td>5.7</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Subbarao et al Virology 305: 192-200; 2003
# Immunogenicity and Protective Efficacy of Formalin Inactivated H5N1/PR8 Vaccine

## Table

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>HAI titer vs</th>
<th>Protection against challenge</th>
<th>Percent survival foll wt challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homologous</td>
<td>Heterologous</td>
<td>Lung virus titer</td>
</tr>
<tr>
<td>FI H5N1 wt</td>
<td>80</td>
<td>40</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td>FI H5N1/PR8</td>
<td>120</td>
<td>80</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td>Live H5N1 wt</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
<td>10</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Subbarao et al Virology 305: 192-200; 2003
Biosafety Measures Used for Generation of Pandemic Influenza Vaccines

- The reassortant virus is generated at the biosafety level recommended for work with the wild-type virus.
- Risk assessment data are generated regarding *in vitro* and *in vivo* properties of the recombinant (modified?) candidate vaccine virus.
- Based on risk assessment data, the biosafety level required for manufacture of the vaccine virus may be reduced.