Characterization and immunogenicity in mice of recombinant influenza haemagglutinins produced in *Leishmania tarentolae* expression system

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Influenza, the vaccine and the haemagglutinin (HA)
Influenza virus

- Influenza virus belongs to the *Orthomyxoviridae* family and is the causative agent of a highly contagious respiratory illness that affects humans and causes public health and economic problems.
- The envelope of influenza virus derives from host cell membrane and contains the HA and NA spike proteins.
- The HA molecule is the major viral antigenic determinant of the influenza particle, and the selection applied by the host immune system constantly selects for drift variants that can no longer be neutralized by circulating Ab. This is the reason why the influenza vaccine has to be reformulated, or at least partly reformulated, almost every year.
Current influenza vaccine

- The current vaccine is a trivalent or tetravalent vaccine containing two types A influenza strains (H1N1 and H3N2) and a strain from one or both influenza B lineages.
- Most influenza vaccines are produced in embryonated hens’ eggs and high yields are obtained.
- However, current egg-based production processes are labor-intensive requiring millions of embryonated eggs every year.

➢ New technology: influenza vaccine based on recombinant HA protein produced using the *L. tarentolae* expression system
Influenza HA protein

The HA molecule is:
- the major viral antigenic determinant of the influenza particle.
- the receptor binding protein of influenza.
- a fusion type I membrane glycoprotein containing 6 intra-chain disulfide bonds and 7 N-linked potential glycosylation sites depending on the HA type and A subtype.

The HA homotrimer is synthesized as a single polypeptide (HA0) of around 550 amino acids cleaved by host cell protease into HA1 and HA2.

The HA protein agglutinates red blood cells.

Skehel JJ et al, Vaccine, 2002
The *Leishmania tarentolae* expression system
Leishmania: presentation of the system

- *L. tarentolae* is an unicellular protozoan isolated from a lizard. Jena Bioscience commercializes kits for the cloning and expression of genes of interest into *L. tarentolae*.
“Pros” of the system

- *L. tarentolae* is a BSL1 organism.
- It is an eukaryotic host as easy to handle as *E. coli*: no specific labware, no cell biology equipment are required.
- It can be cultivated at 26 °C in a standard culture medium (BHI medium supplemented with hemin).
- It has a short doubling time of 8 hours and can be grown to a density of > $10^8$ cells/ml.
- It consists of a fully eukaryotic protein expression machinery with post-translational modifications, including eukaryote glycosylation, phosphorylation and disulfide bond formation.
- The gene of interest is cloned into shuttle vectors allowing, first, the cloning in *E. coli* and then, the expression in *L. tarentolae*. 
Multiple possibilities of the system

- The constitutive or inducible, intracellular or secretory expression of the target proteins is possible depending on the vector used.
- Numerous cytosolic, membrane-localized and extracellular proteins have been expressed with the LEXSY system at Jena Bioscience and in their customer’s laboratories.
- Only stable expression was feasible for constant protein production, but Jena now commercializes a new vector that allows episomal (non-integrative)/multicopy expression of the protein of interest.
Cloning and expression of the HA genes of influenza

- **HA protein cloned:**
  - without the TM/Cyt tail of the protein but with its own signal sequence and an His Tag in the 3’ end of the gene.
  - with optimization of codon usage for *L. tarentolae*.
  - in the pLEXSY-I-neo-2 vector
- **Integration into the « odc » locus of the chromosome containing the gene coding for the T7 polymerase.**
- **Induction of the HA gene expression by tetracycline.**

**HA complete:** approximately 550 aa, 62 kDa. Around 57kDa without the TM/Cyt tail
Results
Cloning and expression of 8 different rHA in the *L. tarentolae* expression system

- **Cloning of the different HA genes in the *L. tarentolae* integrative system, and expression in the supernatant:**
  - A (H1N1) pandemic 2009: A/Texas/04/09 (H1N1) and A/California/07/09 (H1N1)
  - A/PR/8/34 (H1N1)
  - A/Brisbane/59/07 (H1N1)
  - A/Uruguay/716/07 (H3N2)
  - A/Perth/16/09 (H3N2) **no secretion**
  - A/Vietnam/1194/04 (H5N1) rg14
  - B/Brisbane/60/08
Production of influenza rHA in the *L. tarentolae* expression system using Biostat Qplus12 (Sartorius)

- Recombinant parasite expressing rHA
- Preculture in shaker, in the dark
  - Volume: 100-200ml
  - Duration: 2-3 days

- Bio-fermenter
  - Volume: 700ml
  - Temperature: 26°C
  - Duration: 2-3 days

- Bio-generator
  - Volume: 400ml

- Harvest of culture aliquots every day to document:
  - OD600nm
  - Metabolites (Gln, Glu, Gluc, Lac, NH₄⁺)
  - HA expression on SDS-page gel and Western-blot
  - Cell mobility (microscopy)

- Regulation of parameters:
  - pO2
  - pH
  - Temperature

A yield of 1.5-5mg/L of rHA was obtained after purification for the A/California strain
Glycosylation of the rHA in the *L. tarentolae* expression system

**HA from A/Vietnam/1194/04 (H5N1) rg14**

Reducing condition, non purified rHA, anti-H5N1 serum. Glycosylated (G) De-glycosylated (DG)

*L. tarentolae* appropriately glycosylates the rHA of influenza
DLS analysis of the purified A/California rHA protein produced in the *L. tarentolae* expression system.

The rHA produced *in L. tarentolae* is mainly monomeric, the one produced in insect cells by Protein Sciences* is mainly polymeric.
The rHAs produced in *L. tarentolae* are immunogenic after two injections in the presence of an oil-in-water emulsion adjuvant (AF03)
The HA of 8 different influenza strains were cloned and expressed in the integrative/inducible *L. tarentolae* expression system.

A yield of 1.5-5mg/L of rHA could be obtained using the Biostat Qplus12 biofermenters (Sartorius) after purification for the A/California strain.

*L. tarentolae* appears to appropriately glycosylate the influenza rHA.

Monomeric form of HA is predominant.

Purified rHAs were immunogenic in Balb/c mice at 10µg with adjuvant (2 injections).
Perspectives

● **Increasing the yield:**
  - The use of the non-integrative and multi-copy *L. tarentolae* expression system: 10-fold increase of the yields.
  - The medium composition could be improved.

● **Increasing the immunogenicity**
  - C-terminal trimerization tags such as T4 foldon could be used to oligomerize the protein and increase the immunogenicity.
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