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

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ABSTRACT

The membrane displayed antigen haemagglutinin (HA) from several influenza strains were expressed in the *Leishmania tarentolae* system. This non-conventional expression system based on a parasite of lizards, can be readily propagated to high cell density (>10^8 cells/mL) in a simple incubator at 26 °C. The genes encoding HA proteins were cloned from six influenza strains, among these being a 2009 A/H1N1 pandemic strain from swine origin, namely A/California/07/09(H1N1). Soluble HA proteins were secreted into the cell culture medium and were easily and successfully purified *via* a His-Tag domain fused to the proteins. The overall process could be conducted in less than 3 months and resulted in a yield of approximately 1.5–5 mg of HA per liter of biofermenter culture after purification. The recombinant HA proteins expressed by *L. tarentolae* were characterized by dynamic light scattering and were observed to be mostly monomeric. The *L. tarentolae* recombinant HA proteins were immunogenic in mice at a dose of 10 μg when administered twice with an oil-in-water emulsion-based adjuvant. These results suggest that the *L. tarentolae* expression system may be an alternative to the current egg-based vaccine production.

1. Introduction

Influenza A and B viruses are responsible for annual Flu epidemics and occasionally give rise to pandemic outbreaks caused by the transfer of influenza A viruses or virus gene segments from animal reservoirs to humans.

Vaccination is the most cost-effective public health measure to prevent disease and mortality caused by influenza virus infection [1,2]. The haemagglutinin (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 antibodies. This is the reason why the influenza vaccine has to be reformulated, or at least partly reformulated, most years.

The current vaccine is a trivalent or tetravalent vaccine containing two types of A influenza strains (H1N1 and H3N2) and a strain from one or both influenza B lineages [3]. Most influenza vaccines are produced in embryonated hens' eggs and high yields are currently obtained. In fact, the egg-based influenza vaccine production process has been optimized through the use of "high growth" reassortants for A strains, and recently for B strains, which maximizes virus replication in eggs. However, current egg-based production processes are labor-intensive requiring millions of embryonated eggs every year.

Several new approaches are being applied toward the development of influenza vaccines that do not require the use of egg substrate. These include virus propagation in mammalian cell culture [4], the production of recombinant virus-like particles (VLPs) based on the HA and matrix (M) proteins with or without neuraminidase (NA) [5], and the development of live vector vaccines based on adenovirus [6]. Another promising approach is the

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expression of the recombinant HA protein, using prokaryotic or eukaryotic cell systems.

HA is a receptor-binding and a fusion Type I membrane glycoprotein containing six intra-chain disulfide bonds and approximately seven N-linked potential glycosylation sites depending on the HA type (A or B) and A influenza subtype [7,8]. The structure of the HA ectodomain has been determined by X-ray crystallography. The HA homotrimer is synthesized as a single polypeptide (HA0) of around 550 amino acids that is subsequently cleaved by a host cell protease to form the HA1 and HA2 domains [9]. The HA1 globular head domain contains the principal neutralizing epitopes, however the HA2 stem region also contains neutralizing epitopes that span the inter-subunit interfaces of the intact HA trimer [10,11].

Several laboratories have proposed to produce an influenza vaccine based on recombinant HA protein. Among them, Protein Sciences is the most advanced with a licensed influenza vaccine based on a full length trimeric HA extracted from the membranes of recombinant baculovirus-infected insect cells [12]. The vaccine contains 45 μg of HA per strain from each circulating influenza A strain and from a single B influenza strain.

Leishmania tarentolae is a unicellular eukaryotic protozoan parasite of lizards that does not infect humans, established as a novel host for recombinant protein production. Interesting features of proteins produced in *L. tarentolae* are their animal-like N-glycosylation pattern and the recombinant protein yields that could reach milligrams per liter of culture. *L. tarentolae* has also the advantage of a high specific growth rate and cultivation to high cell density. Several proteins have already been produced in such a system, for instance erythropoietin, proprotein convertase 4, human laminin-332, a tissue type plasminogen activator and an active acetyl serotonin methyl transferase [13–15].

In this proof-of-concept study the production of various HA influenza proteins in *L. tarentolae* is described. The integrative/inducible expression system commercially available from Jena (Jena Bioscience, Jena, Germany), namely the pLexsy-I-neo2 vector and the *L. tarentolae* T7-TR strain were used to produce six recombinant HA proteins coming from six different influenza strains. The production of secreted recombinant HA proteins was particularly easy and the purified products obtained were immunogenic in mice.

2. Materials and methods

2.1. Cloning of the HA protein encoding genes into the pLexsy-I-neo2 vector

In order to express recombinant HA proteins into the *L. tarentolae* system, the pLexsy-I-neo2 vector was chosen. This vector allows the integration of the gene of interest into the chromosomal *ornithine decarboxylase* (*odc*) locus of the *L. tarentolae* T7-TR recipient strain that constitutively expresses bacteriophage T7 RNA polymerase and TET repressor under the control of host RNA polymerase I. Induction of the expression of the protein of interest is carried out *via* the T7 promoter inducible by tetracycline addition as per the manufacturer's instructions (user's guide EGE-1400, Jena Bioscience, Jena, Germany).

The HA genes from A/PR/8/34(H1N1), A/California/07/09(H1N1), A/Brisbane/59/07(H1N1), A/Uruguay/716/07(H3N2) and B/Brisbane/60/08 that were cloned into the pLexsy-I-neo2 vector contained the natural signal sequence of each HA gene strain; however the transmembrane region and cytoplasmic tail were deleted and replaced by a His-6x Tag.

The signal sequence of the vector (coming from *Leishmania mexicana*) was maintained only for the A/Vietnam/1194/04(H5N1) strain. The corresponding genes coding the HA sequences of A/California/07/09(H1N1) and B/Brisbane/60/08 were optimized for codon usage by *L. tarentolae* before cloning. Confirmation of genomic integration of the expression cassette containing HA sequences was performed by diagnostic PCR as recommended by the manufacturer.

2.2. L. tarentolae cell growth condition in shake flasks/biofermenters and induction of HA expression

For shake flask cultures, recombinant parasites were cultivated in 100 mL BHI medium supplemented with hemin and antibiotics at 26 °C, and agitated at 100 rpm in the dark. In order to induce the production of the recombinant HA protein, the T7 promoter driven transcription was induced by addition of 10 μg/mL of tetracycline into the supplemented medium at inoculation of the parasites.

For fermentation, 1 L Biostat Qplus 12 fermenters (Sartorius AG, Aubagne, France), were used. Briefly, 700 mL of supplemented BHI medium were inoculated with 1/10 of a recombinant parasite starter culture in exponential growth (0.4 DO_{600}) and cultivated in the dark at 26 °C, 100 rpm, 40% pO_2, pH 7.4 ± 0.1. Culture parameters were registered using the MFCS/WIN software (Sartorius AG). Induction was performed as for shake flask cultures.

Shake flask and fermenter cultures were harvested 72 h after induction and centrifuged 30 min at 5000 × *g*. Supernatants were filtered on 0.22 μm filter and analyzed for the production of the recombinant HA by Western-blot.

2.3. Characterization

2.3.1. SDS page and Western-blot

About 40 μL of supernatants were analyzed for the presence of the expected proteins *via* a reducing NuPAGE Novex 4–12% Bis–Tris gel (Life technologies, Carlsbad, USA). Proteins were then transferred to nitrocellulose membranes (BioRad Laboratories, Hercules, USA), and non-specific binding sites on the membrane were blocked in PBS (Eurobio, Montpellier, France) containing 0.1% Tween 20 and 5% non-fat dry milk (DIFCO-BD, Sparks, USA). When commercially available, specific anti-HA polyclonal sera were used as primary antibody and an anti-species IgG HRP conjugated as secondary antibody. Development was conducted using an OPTI-4CN™ substrate kit (BioRad Laboratories). Otherwise, the recombinant HA proteins were detected with an anti-His Tag® Antibody HRP Conjugate Kit (Novagen, Darmstadt, Germany) 2000-fold diluted.

2.3.2. Dynamic light scattering (DLS)

Dynamic light scattering (DLS) was used to determine the hydrodynamic radius distribution of samples through measurement of the decay rates of scattered light and calculation of translational diffusion coefficients. The hydrodynamic radii (R_H) were obtained from the diffusion coefficients (D) *via* the Stokes–Einstein equation:

$$R_H = \frac{k_B T}{6\pi\eta D}$$

where η is the viscosity of the solvent, T is the absolute temperature, and k_B the Boltzmann constant.

Dynamic light scattering experiments were performed at 25 °C, using a Viscotek 802 DLS instrument (Viscotek Europe Ltd., Basingstoke, UK) using a 50 mW diode laser with wavelength 827.9 nm. Typically, 10 scans of 10 s were obtained for each sample. Data were analyzed using Omnisize 3.0 software (Viscotek Europe Ltd.).

2.4. Purification of the recombinant HA

Histidine-tagged recombinant HA proteins were purified by Immobilized Metal Ion Affinity Chromatography (IMAC). Briefly, cell culture medium containing the expressed HA protein was concentrated 5-fold using a Vivacell 100/10 K concentration system at a cut-off of 10 K (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The concentrated cell culture medium was then diluted in Tris–HCl 50 mM pH 8, NaCl 0.5 M, imidazole 10 mM buffer and loaded onto a His-Trap nickel-actived affinity column (HISTRAP FF, GE Healthcare, Life Sciences, Velizy-Villacoublay, France). Elution of recombinant HA was performed at pH 8 in imidazole buffer 500 mM, Tris–HCl 50 mM, NaCl 0.5 M. Finally, the purified recombinant HA protein was dialyzed in PBS buffer (Eurobio).

2.5. Formulation

The various recombinant HA proteins were formulated with or without an oil-in-water emulsion-based adjuvant (AF03 Sanofi Pasteur). Adjuvanted formulation was performed by mixing the antigen with the adjuvant 1:1 (volume to volume) just prior to injection.

2.6. Immunization of mice

Groups of five to ten 8-week-old female BALB/c mice received two intramuscular (i.m.) injections 4 weeks apart of 10 μg of purified recombinant HA protein in the presence or absence of AF03 adjuvant. Influenza-naïve control mice received injections of PBS following the same immunization schedule. Blood samples were collected under anesthesia just before the booster injection (day 28) and 3 weeks after the second injection (day 50). Antibody responses were measured using a standard Hemagglutination Inhibition (HI) assay described below.

2.7. Serology

The presence of HI antibodies against the various influenza strains was assessed using chicken red blood cells (cRBCs) for the H1N1 strain and horse red blood cells (hRBCs) for the H5N1 strain. Assays were performed on individual Receptor Destroying Enzyme (RDE) treated serum samples collected at each time-point and titers were expressed as the reciprocal of the highest dilution showing no hemagglutination. A value of 5 corresponding to half of the initial dilution (1/10) was arbitrarily given to all sera determined negative.

The ELISA dosage of specific antibodies in the serum samples (IgG) was performed according to the following protocol: in brief, 96-well microplates were coated overnight at +4 °C with 100 ng/well (100 μl) of the corresponding recombinant HA produced in baculovirus (Protein Sciences). Plates were then blocked and serial two-fold dilutions of the sera were added to the wells and incubated for 90 min at 37 °C. After washings, an anti-mouse IgG peroxydase conjugate (Jackson, Suffolk, England) was added and the plates incubated for another 90 min at 37 °C. After extensive washing, the substrate TMB (Zymed, Invitrogen, Cergy Pontoise, France) was applied. The optical density (OD) was measured at 450–650 nm with an automatic plate reader (Molecular Devices, St Grégoire, France). The blanks (mean value) were subtracted from the data. The antibody titers were calculated using the CodUnit software (Dipole, Vaugneray, France), for the OD value range of 0.2–3.0, from the established curve of the anti-strain specific reference serum placed on each ELISA plate. The threshold of antibody detection was 20 (1.3 $\log_{10}$) European Unit (EU). All final titers were expressed in $\log_{10}$. For each titer <1.3 $\log_{10}$, an arbitrary titer of 1.0 $\log_{10}$ was applied.

3. Results

3.1. Expression of recombinant HA from L. tarentolae

The entire HA encoding genes of different influenza strains were cloned and expressed without the transmembrane region and cytoplasmic tail in order to favor the secretion of the protein into the cell culture medium. The signal sequence of the protein was conserved except for the A/H5N1 strain and a His-6x Tag was added in C terminal. Six different HA genes were cloned into the LEXY integrative vector and expressed (A/California/07/09(H1N1), A/Brisbane/59/07 (H1N1), A/PR/8/34(H1N1), A/Uruguay/716/07(H3N2), A/Vietnam/1194/04(H5N1), and B/Brisbane/60/08). Initially, production was performed in 100 mL shake flask culture. As an example, a Western-blot of the His-Tag purified recombinant HA from A/Vietnam/1194/04(H5N1) strain is shown in Fig. 1. As shown in this figure, a major band at 57 kDa corresponding to the expected size of the HA molecule without the transmembrane region and cytoplasmic tail was obtained. Deglycosylation using PNGase F gave rise to a 6 kDa loss product, suggesting that the HA was appropriately glycosylated.

3.2. Recombinant HA production in biofermenters

In order to assess if the *L. tarentolae* system can be scaled up and the HA yield optimized, 700 mL biofermenters were used to grow the parasite and to produce the HA protein from the A/California/07/09(H1N1) strain. For that purpose, recombinant pre-cultures were prepared in shake flasks and used to seed the biofermenters. The production of HA protein was maximum72 h after induction with tetracycline. A yield of 1–3 mg/700 mL (approximately 1.5–5 mg/L) was consistently obtained after His-Tag column purification as measured by Bradford quantification. It should be noted that 72 h post induction the parasite culture was in exponential growth reaching around 2×10^8 cells/mL.

3.3. Dynamic light scattering analysis

It was expected that the recombinant HA would be produced as a monomeric form in *L. tarentolae* supernatant since no transmembrane and cytoplasmic tail were present to trimerize the protein. In order to verify this hypothesis, DLS was performed on

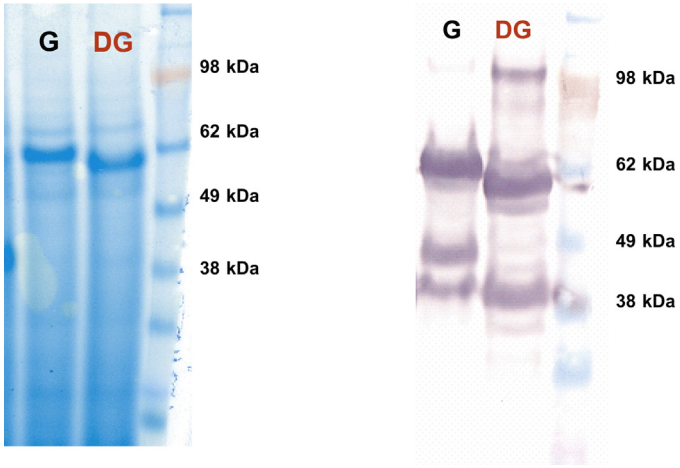

Fig. 1. Purified A/Vietnam/1194/04(H5N1) HA protein. 4–12% Bis–Tris NuPage gel on the left (reducing conditions) and Western-blot on the right using a specific anti-H5N1 rabbit polyclonal serum 2000-fold diluted (eEnzyme® LLC, Gaithersburg, USA) and a goat anti-rabbit IgG (H+L) HRP conjugated (1000-fold diluted, Zymed®, San Francisco, USA). G: glycosylated, DG: deglycosylated.

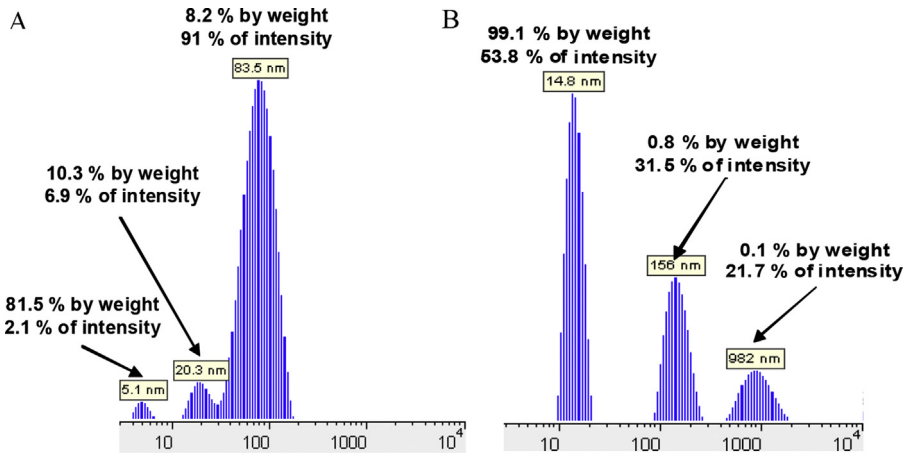

Fig. 2. DLS analysis of the purified A/California/07/09(H1N1) rHA proteins produced in the supernatant of *L. tarentolae* (A), or produced by Protein Sciences using the baculovirus expression system (B).

the A/California/07/09(H1N1) HA protein purified from *L. tarentolae* supernatant fluid after fermentation and on the baculovirus produced HA from Protein Sciences (Interchim, Montluçon, France) as a control. Fig. 2 shows the hydrodynamic radius (R_H) distribution of A/California/07/09(H1N1) HA produced in *L. tarentolae*. Although the aggregate peaks (at R_H 83.5 nm) represent a large fraction of the scattering intensity, the largest fractions by weight were estimated to be more than 81% corresponding to monomeric HA (R_H of 5.1 nm).

In comparison, DLS analysis was performed on A/California/07/09(H1N1) HA protein commercially available from Protein Sciences. The recombinant HA produced using the baculovirus expression system purified from the insect cell membrane showed a major peak (more than 99% by weight) at a R_H of 14.8 nm, that likely corresponded to oligomeric HA and the absence of monomeric HA. Aggregates were also visible presenting higher R_H of 156 and 982 nm, but corresponding to minor populations (0.8% and 0.1% by weight).

3.4. Serum antibody responses elicited by different recombinant HA proteins

To assess the immunogenicity of the recombinant HA proteins produced in the *L. tarentolae* system, mice were immunized twice, at 4 weeks interval with 10 μg of the purified recombinant HA from different influenza strains administered in the presence or absence AF03 adjuvant. The antibody responses elicited by the recombinant HA proteins were measured in individual sera collected from each animal post-1 (day 28), or 2 (day 50) immunizations by HI assay using RBCs. Fig. 3 shows that the A/PR/8/34(H1N1) recombinant HA product was not immunogenic in the absence of the AF03 adjuvant. Following two vaccinations in the presence of AF03 adjuvant

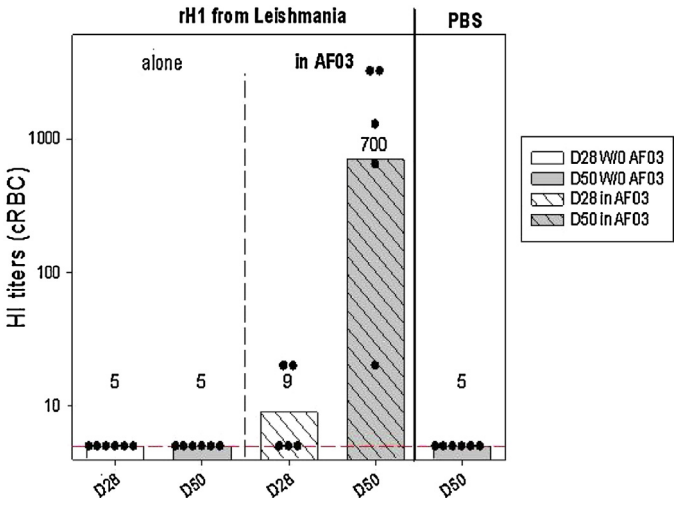

Fig. 3. Antibody response measured by HI (HI titer/50 μl determined using cRBC: chicken Red Blood Cells). Mice, 8-week-old, were injected i.m. two times 4 weeks apart and bled 3 weeks after the last injection of rHA (purified HA from A/PR/8/34(H1N1)) with or without (W/O) AF03; one control group received two injections of PBS.

a strong antibody response was measured with a mean HI titer of 700. A/Vietnam/1194/04(H5N1) and A/California/07/09(H1N1) recombinant HA proteins were also tested for their immunogenicity in mice by HI assay and ELISA using recombinant HA for coating. The results obtained after two immunizations are summarized in Table 1. All three recombinant HA proteins induced functional HI antibodies after two injections in the presence of AF03

Table 1
Hemagglutination inhibition (HI) and ELISA IgG response ($\log_{10}$ EU) response in mice after two immunizations.

Immunogen/influenza strain	Formulation	HI titer	ELISA IgG titer
rH5 A/Vietnam/1194/04(H5N1)	W/O adjuvant	6 (5–10)	3.896 (3.067–4.209)
	AFO3	60 (5–3200)	5.525 (5.303–5.769)
rH1 A/PR/8/64(H1N1)	W/O adjuvant	5	Not done
	AFO3	700 (20–3200)	Not done
rH1 A/California/07/09(H1N1)	W/O adjuvant	53 (5–160)	4.631 (4.343–5.028)
	AFO3	3378 (1280–5120)	6.437 (6.009–6.805)

Mice, 8-week-old, were injected i.m. twice 4 weeks apart and bled 3 weeks after the last injection of rHA with or without (W/O) AF03. Mean titers and ranges of individual values are presented in brackets. The experiment was done once with each recombinant HA except for A/California rHA W/O adjuvant for which a titer of 46 was obtained in a separate experiment.

adjuvant, recombinant H5 protein being the least immunogenic (HI titers of 60 and 3378 for the H5 and H1 California respectively). ELISA titers obtained for A/Vietnam/1194/04(H5N1) and A/California/07/09(H1N1) recombinant HA proteins were inline with HI titers.

4. Discussion

Six influenza recombinant HA proteins were successfully cloned and expressed into the integrative/inducible *L. tarentolae* expression system. The transmembrane region and cytoplasmic tail of each HA protein were removed from the constructs to avoid the production of cell membrane anchored recombinant HA. Under these conditions it was expected that the recombinant HA proteins would be produced in the supernatant of the parasite and consequently would be easily purified. His-Tag was also added to allow an easy purification step, although in the final product the sequence would have to be removed.

The expression system from Jena allowing to perform both constructs, recombinant HA proteins were expressed with their native signal sequence in the pLEXY vector, except for the HA of the A/H5N1 strain for which the signal sequence from *L. mexicana* that is contained in the vector was used. We did not compare the same protein expressed with the native A/H5N1 HA signal sequence. However, for other recombinant HA proteins produced subsequently, the production of HA proteins was impaired when the *L. mexicana* sequence was used. Therefore, we decided to use the native sequence signal of each HA gene. We also observed that without optimization of the codon usage for *L. tarentolae*, some HA proteins could not be correctly produced or could be produced but with a very low yield. In view of these results, all the newly considered HA sequences were systematically codon optimized. Literature reports of the production of recombinant HA proteins show that optimized gene sequences allow better expression in the host without truncated protein production and also aid in maintaining proper protein folding and function [16–19].

We observed that the recombinant HA proteins were glycosylated as shown by the PNGase F profile obtained for the A/H5N1 strain. Glycosylation is known to be important for immunogenicity and appropriate folding of the HA protein [7,20]. Oligosaccharides at certain positions on the HA can mask antigenic sites. Yeast have been reported to hyperglycosylate foreign proteins and, therefore, are not the expression systems of choice for the HA proteins. Conversely, *E. coli* does not glycosylate recombinant proteins, which could results in unstable/unfolded recombinant HA protein expression.

Production of recombinant HA proteins was performed first in shake flasks and then in biofermenters. Although *L. tarentolae* is a eukaryotic cell, it grew better in biofermenters that are usually used for prokaryotic cells, than in biogenerators generally used for eukaryotic cells. A yield of 1.5–5 mg of recombinant HA per liter of initial culture was reproducibly obtained after purification of fermenter cultures. Some assays were recently performed using the newly non-integrative and multi-copy *L. tarentolae* expression system [21] showing a 10-fold increase of the yields that could be obtained in biofermenters for the A/Califonia/20/09(H1N1) strain (data not shown).

In view of the growth parameters established in the present study for the production of several influenza recombinant HA proteins in *L. tarentolae*, we anticipate that the total process from cloning of the HA gene to the recombinant HA protein expression and purification could be conducted in less than three months, a time frame fully compatible with an influenza annual production campaign.

DLS analysis showed that the A/California/20/09(H1N1) recombinant HA produced in *L. tarentolae* was mostly monomeric compared to the oligomeric recombinant HA produced in the baculovirus system by Protein Sciences. These results were expected since the HA proteins expressed by the *L. tarentolae* system were produced without the transmembrane region and the cytoplasmic tail. It is well known that these domains could be very important for the correct association of the monomeric HA [22,23].

In the present study, the three HA proteins tested were found to be immunogenic as measured by HI assay and ELISA for two of them, when administered twice, at a dose of 10 μg, in the presence of AF03, a squalene-based emulsion adjuvant. AF03 is present in the A/Vietnam/1194/2004 NIBRG-14(H5N1) adjuvanted pandemic influenza vaccine, Humenza™ although never marketed, it was injected into hundreds of human volunteers during its clinical development [24]. At low immunization dose (0.3 μg of vaccine), the AF03-adjuvanted pandemic influenza A (H1N1) 2009 vaccine elicited higher HI antibody titers than the unadjuvanted vaccine in both naïve and trivalent inactivated split-virion vaccine-primed animals [25]. AF03-adjuvanted H5N1 inactivated split-virion vaccine was also demonstrated to elicit antibody responses to protective levels in humans at doses as low as 1.9 μg [26,24]. Similar observations were made with other squalene emulsion adjuvants such as MF59 from Novartis Vaccines & Diagnostics (Cambridge, MA) and AS03 from GSK Biologicals (Rixensart, Belgium) which are both present in marketed influenza vaccines [27]. Although different in their surfactant composition and manufacturing process, all these squalene emulsion adjuvants have been reported to recruit monocytes at the site of administration and to induce their maturation into potent antigen presenting cells [27,28].

Concerning the recombinant HA proteins produced in the *L. tarentolae* system, an increase in ELISA titers was observed when the recombinant HA proteins were formulated with AF03 compared to the unadjuvanted proteins. Notice that a Th2 response (IgG1 > IgG2a) is usually observed after immunization with influenza recombinant HA proteins with and without AF03 (data not shown).

A mean HI titer of 700 was reached with the recombinant HA derived from the A/PR8/34(H1N1) strain. Of the five mice injected with this recombinant HA in the presence of adjuvant, four showed a high HI titer equivalent or above 640 while the remaining mouse showed a low response of 20. It is possible that this mouse did not receive the full intended volume of vaccine during the injection. In addition, mean HI titers of 60 and 3378 were reached with 10 μg of the respective recombinant HA proteins prepared from A/Vietnam/1203/04(H5N1) and A/California/07/09(H1N1)). Ten micrograms is the average dose that we use to study the immunogenicity of a recombinant HA, whatever the expression system, in the presence of an adjuvant or not. In our model, i.e. 8-week-old female BALB/c mice immunized twice by the intramuscular route 4 weeks apart, 10 μg is an adequate dose to observe sustained HI titers regardless of the strain from which the HA is derived. Reports in the literature have shown that 5 or 10 μg of recombinant HA is commonly used in the presence of adjuvant [29–32]. A HI titer of 40 has been adopted as a serologic correlate of protection for the evaluation of human influenza vaccines [33]. While a standard of protection has not been established in mice, protective antibody responses in human are generally observed when HI titers are above 40 [34,35]. As this study was essentially for proof of concept, protection was not performed in a first approach. However, for the development of effective vaccines against epidemic and potentially pandemic influenza viruses safety and efficacy can be evaluated in mice, ferrets and/or macaques [36]. For example, Chang et al. studied the protective efficacy of recombinant HA in mice. They first showed a poor HI (<50)

response induced in mice after two IM immunizations, given 2 weeks apart, with 5 μg of unadjuvanted recombinant HA from A/NC/20/99(H1N1) strain; this response was enhanced (mean HI titers of 160) when the recombinant HA was administered in the presence of 20 μg of JVRS-100 adjuvant. In a second step, they showed that a single and two dose-vaccination regimen with H1N1 HA protein and JVRS-100 adjuvant were protective following challenge in mice [29]. In another study, protective efficacy of the HA recombinant protein from A/California/07/09(H1N1) strain produced in *E. coli* was demonstrated in ferrets. Vaccination of ferrets with the recombinant HA gave rise to a mean HI titer of 256 after two immunizations in the presence of Titermax adjuvant. A dose response was observed with a mean HI titer reaching 2048 when 30 μg of adjuvanted recombinant HA was administrated. Interestingly the HA1 globular head that presented the most multimeric forms provided better protection in ferret compared to the monomeric HA ectodomain [37]. Other studies did not show any protective data but only HI titers in the presence of various adjuvants: recombinant HA from an A/H1N1 influenza strain produced in the *Pichia pastoris* yeast was administrated to BALB/c mice with Freund's complete adjuvant. An average HI titer of 32 was observed in mice groups immunized twice with 10 or 50 μg of HA two weeks after the boost. Of note, no dose-dependent response was observed [30]. The recombinant HA from an A/H5N1 strain also produced in the *Pichia* system was found to be immunogenic in mice at a dose of 10 μg when formulated with 2% aluminum hydroxide gel. In that study HI titers reached a maximum of 280 after 3 injections [31].

In summary, the results obtained in this study concerning HI titer are in agreement with the literature describing the immunogenicity of different recombinant HA proteins, often administered in the presence of adjuvant. However, Protein Sciences recombinant HA is immunogenic without adjuvant [12,38]. This could be due to the fact that they expressed and purified the full length influenza HA from membranes of recombinant baculovirus-infected insect cells resulting in a trimeric protein more closely related to the native HA although the purification step is expected to be more complex with a membrane associated HA. Expressing only the ectodomain of HA as in the present study to facilitate purification resulted in a monomeric HA inducing functional antibodies only in the presence of an adjuvant. C-terminal trimerization tags such as T4 foldon could be used to oligomerize the protein. However the oligomerization of different HA proteins with such peptides has been reported to be inconsistent and this approach may also be more challenging from a regulatory approval perspective due to the introduction of exogenous peptide sequences [39].

There are substantial differences in HI titers among the various HA subtype strains expressed in the *L. tarentolae* expression system, from a titer of 60 for the A/Vietnam/1194/04(H5N1) to 3378 for the A/California/07/09(H1N1) in the presence of the AF03 adjuvant, and from a titer of 5 for the A/PR/8/34(H1N1) to 53 for the A/California/07/09(H1N1) without adjuvant. It is well known that the relative immunogenicity of various strains of each HA subtype is different, as reported previously [40]. In our hands, the A/California/07/09(H1N1) was particularly immunogenic, while the A/H5N1 strains have been shown to be poorly immunogenic when injected as an inactivated split-virion vaccine in mice. Another hypothesis to explain the differences in immunogenicity could be the content of monomeric *versus* polymeric forms of the various recombinant HA produced in the *L. tarentolae* expression system depending on the HA subtype.

In conclusion we have demonstrated for the first time that recombinant HA proteins from various influenza strains can be readily cloned and expressed using the *L. tarentolae* expression system. The monomeric HA proteins produced using this system were shown to be glycosylated and immunogenic in mice.

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