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Original Communication

Expression of recombinant Influenza A H1N1 neuraminidase in *Rachiplusia nu* larvae

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ABSTRACT

Two recombinant baculoviruses carrying the full-length or transmembrane-deleted neuraminidase (NA) genes of influenza were constructed with the aim to select the best strategy to express this viral antigenic protein in *Rachiplusia nu* larvae. These variants of NA were efficiently expressed with the expected molecular weight (~60 kDa) in the *Sf*9 cell line. The transmembrane-deleted (Tm-less) NA variant was mainly detected in the supernatants and showed poor enzymatic activity due to the destabilization of the tetramer. The full-length NA variant integrity was confirmed by the presence of enzymatic activity and the yield in the supernatants was 6.57 ± 0.51 mg/l of culture. Although the full-length variant was not completely secreted, it happens to be the best option for NA expression. *R. nu* larvae produced 1.20 ± 0.20 mg of full-length recombinant NA/g larva. Thus, to produce 1 mg of NA, 153 ml of suspension culture with 1 x 10^6 cells/ml or only six larvae are needed. The full-length NA expressed in larvae was captured by a Concanavalin A affinity matrix, thus indicating the presence of glycosylated residues.

KEYWORDS: neuraminidase, influenza A H1N1, expression, baculovirus, *Rachiplusia nu*

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INTRODUCTION

The last influenza A (H1N1) outbreak in 2009 demonstrated how rapidly a new strain of influenza virus can emerge and spread around the world. Current influenza vaccine production methods may not be sufficient to respond rapidly enough in the case of a pandemic [1]. Production of the surface antigens hemagglutinin (HA) and neuraminidase (NA) glycoproteins using recombinant DNA technology provides an alternative approach to the current egg-based manufacturing process.

Different eukaryotic expression systems, including yeast [2, 3], plants [4, 5] and the baculovirus-insect cell system [6-10], have been explored to produce recombinant influenza antigens.

The baculovirus-insect cell expression vector system (BEVS) allows obtaining viral antigenic proteins in a short time, and might be particularly suitable for influenza, where both an annual adjustment of the seasonal vaccine and a rapid response to pandemic influenza are required. Recently, a recombinant trivalent HA vaccine (FluBlock) that demonstrated safety, immunogenicity and efficacy after several human clinical trials has been developed using insect cells [11, 12]. However, the main disadvantage of recombinant protein production in insect cell culture at an industrial scale is the high cost because specialized facilities and bioreactors are needed. A low-cost alternative is the production of recombinant proteins using live insect larvae as

"biofactories" [13]. In comparison with *Sf*9 cell line cultures, other advantages of live insect larvae include: increased recombinant protein yields, easy scale-up and higher efficiency of certain types of post-translational modifications. This approach has been more widely adopted in Asian countries where the expression vectors are based on *Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV), which infects the silkworm *B. mori* [14]. In Europe and the United States, in contrast, the industrial interest is focused mainly in the larvae of the cabbage looper moth, *Trichoplusia ni*, as host for recombinant protein production by *Autographa californica* multiple nuclear polyhedrosis virus (*Ac*MNPV). This baculovirus also infects *Spodoptera frugiperda* and *Rachiplusia nu* larvae, which are plagues of many important crops in Latin America.

NA is the second main glycoprotein of influenza A viruses. NA (MW ~240 kDa) is a homotetrameric protein composed of four identical monomers of a theoretical MW of ~60 kDa. Each NA tetramer has a mushroom-like morphology in which the individual monomers are linked as a pair of dimers by disulfide bonds. NA is a type II membrane protein anchored in the membrane by an unprocessed N-terminal lipophilic sequence [15]. The largest part of the total structure extends above the membrane, forming a distal box-like "head" domain located on top of an elongated "stalk" region. The antigenic and enzymatic sites are localized in the head domain. NA acts in a decisive manner during the last stages of the influenza replication cycle by hydrolyzing sialic acids from host glycoproteins that are recognized by the viral HA. As a result, newly assembled viruses are prevented from binding to the infected cell surface and from aggregating with each other through HA-sialic acid interactions. This allows the release of the virus from the infected cell and facilitates viral spread from one cell to another [15].

Antibodies directed against NA are usually not neutralizing, but block its receptor-destroying activity [16]. They do not prevent infection, but can reduce viral replication below a pathogenic threshold so that infection can occur without disease. The cleavage from the binding site at the host cell is as essential as the binding of the influenza virus. Immunizations with NA preparations have been proposed as vaccines that allow infection but prevent viral spread and reduce the severity and duration of the disease. Several studies suggest that the inclusion of NA in current vaccines is required to induce a broader and more protective immunity [17-19]. Moreover, a NA subunit vaccine "FluNhance" (Protein Science Co., USA) is currently in clinical trial in phase II challenge [20]. Several reports have described the expression of influenza virus antigens in insect larvae [20-25]. Particularly, in *R. nu* larvae, recombinant protein production has not yet been explored in depth. Previously, we have demonstrated the efficacy of this host to achieve high-level expression of recombinant horseradish peroxidase secreted in hemolymph [26-29].

In this work, we studied two genetic constructions, the full-length and transmembrane-deleted (Tm-less) variants of NA derived from the A/Argentina/ HNRG13/2009 (H1N1) influenza virus, with the aim to achieve high-level expression of secreted NA in hemolymph of *R. nu* larvae.

MATERIALS AND METHODS

Materials

Analytical reagents

The molecular weight standards for Western blot PageRuler™ Prestained (Thermo Scientific, Rockford, IL, USA) and BenchMark™ (Invitrogen, Gaithersburg, MD, USA) protein ladders were used. The nitrocellulose membranes were from GE Healthcare (Piscataway, NJ, USA). The Quick Start™ Bradford reagent for total protein determination was obtained from BioRad (Hercules, CA, USA). Cellfectin™ reagent was from Invitrogen. The neuraminidase substrate MUNANA (2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic), the methyl-alpha-mannopyranoside and the Concanavalin A (ConA) matrix were from Sigma-Aldrich (Saint Louis, MO, USA). The goat anti-rabbit rhodamine-conjugated secondary antibody was obtained from Millipore (Bedford, MA, USA). The recombinant influenza A virus H1N1 NA was from RYD Systems (Minneapolis, MN, USA). The rabbit anti-NA (A/California/06/2009/H1N1) polyclonal antibody was purchased from Immune Technology Corp. (New York, NY, USA).

Molecular biology

The full-length *na* sequence corresponding to the novel H1N1 influenza A strain prevalent in Argentina

in 2009 (A/Argentina/HNRG13/2009(H1N1)) were obtained from the National Center for Biotechnology Information (NCBI) data bank. The cDNA containing two restriction sites sequences for EcoRI and PstI was synthesized by GenScript (Piscataway, NJ, USA) and provided cloned in the pUC57 plasmid. The transfer vector pAcGP67-B was from BD Biosciences Pharmingen (San Diego, CA, USA). The EcoRI, PstI restriction endonucleases, T4 DNA ligase, Pfu polymerase, and the Lambda DNA EcoRI/HindIII marker were provided by Promega (Madison, WI, USA). The AxyPrepMiniprep Kit was from Axygen Biosciences (Union City, CA, USA).

Virus, cell line and larvae

The Baculogold Bright AcMNPV DNA was purchased from BD Biosciences, Pharmingen. The insect cell line IPBL-Sf9 from *S. frugiperda* (*Sf*9) was purchased from the Asociación Banco Argentino de Células (ABAC, Pergamino, Buenos Aires, Argentina). Sf900 II insect culture media and the antibiotic-antimycotic solution were from Invitrogen (Carlsbad, CA, USA). The fetal calf serum was from Nutrientes Naturales S.A. (Buenos Aires, Argentina). Eggs of *R. nu* larvae were from Agidea (Buenos Aires, Argentina).

Methods

Recombinant baculovirus construction

The pUC57 containing the full-length NA gene was amplified in *Escherichia coli* DH5α under ampicillin selection, purified, and then digested with EcoRI and PstI restriction endonucleases to obtain the *na* (1407 bp) fragment. Afterwards, the *na* digested fragment was cloned in the pAcGP67 transfer vector downstream the baculovirus polyhedrin promoter and the GP67 insect signal peptide sequence (full-length construction).

On the other hand, the following primers were designed to remove the cytoplasmic and transmembrane domain of the *na* gene: NA forward (5´-3´): GCGAATTCGGCACTCAATTCAACTTGGG (underlined sequence corresponds to EcoRI site) and NA reverse (5´-3´): CGGCTGCAGTGACAATTA CTTGTC (underlined sequence corresponds to PstI site). After deletion of the Tm domain by PCR, the *na* gene was also cloned into the pAcGP67 transfer vector as described (Tm-less construction). Both plasmids were purified, quantified and sequenced.

A monolayer of $1x10^6$ *Sf*9 cells was co-transfected with each recombinant transfer vector (1 µg) and 250 ng Baculogold Bright DNA using Cellfectin™ reagent in independent assays. A negative control plate was used for each co-transfection assay. After 4 days of incubation at 27 °C, the cell culture medium was collected and centrifuged at 3,000 x *g* for 10 min. Since the green fluorescent protein (GFP) reporter is encoded in the baculovirus genome, co-transfection and homologous recombination efficiency was determined by monitoring GFP expression under fluorescence microscopy. An aliquot of 2 x 10^6 cells was seeded in T-25 flasks and infected with 100 µl of the 4th day co-transfection supernatant. After incubation for 4 days, 100 µl supernatant of the first amplification step was used to initiate a second amplification round. Further amplifications were performed by infecting the cells at a low multiplicity of infection (MOI) of 0.01 and harvesting the supernatants at 3-5 days post-infection (p.i.).

Baculovirus titrations

Both recombinant baculoviruses were used as viral stocks and titrated by the end-point dilution assay method as described by Reed and Muench [30]. The titers were converted to plaque forming units (pfu)/ml once the TCID50 value was obtained by using the formula: pfu/ml = TCID50/ml x 0.69, assuming a Poisson distribution where TCID50 would be equivalent to 0.69 infectious units [31].

***Sf*9 suspension cultures**

Suspension cultures were grown in sterile Erlenmeyer flasks under continuous shaking at 100 rpm in *Sf*900 medium supplemented with 1% (v/v) fetal calf serum and 1% antibiotic-antimycotic solution under a controlled environment at 27 °C and then sub-cultured before they reached a density of 6 x 10^6 cells/ml. The suspension volume did not exceed 10% of the total volume of the Erlenmeyer flask. All the assays were carried out using *Sf*9 cells from suspension cultures in log-phase with 95-99% viability.

Expression kinetics of full-length and Tm-less NA in *Sf*9 cell line cultures

In the next step, 10 ml of suspension culture (2 x 10^6 cells/ml) was infected with each recombinant baculovirus named rAcGPNA and rAcGPNATm-less at a MOI of 2 in independent assays. Triplicates

were done in all cases. A negative control (infection with baculovirus without the foreign gene) was included as well. Expression kinetics experiments were performed by collecting 1 ml of suspension culture at 1, 2, 3, 4 and 5 days p.i. and centrifuging at 10,000 x *g* for 5 min to separate the cell pellet from the supernatant. Both fractions were frozen at -20 °C until they were analyzed.

Insect larvae rearing and infection

R. nu larvae were fed with a high-wheat germ diet and reared in twelve-well plates at 23-25 °C in a 70% humidified chamber, with a 16:8 light:dark photoperiod.

For all the experiments, fifth-instar larvae (20 days of age) were sedated by incubation on ice for 5 min and then injected with 50 µl of the recombinant baculovirus stock near the third prolegs. To characterize and quantify the recombinant proteins produced, four groups of 20 fifth-instar *R. nu* larvae (200 mg average weight) were injected with four doses (5×10^2, 5×10^3, 5×10^4 and 5×10^5 pfu) of each recombinant baculovirus in independent assays. The larvae were collected between 72 and 96 h p.i., weighed and frozen immediately at -20 °C until they were processed for recombinant NA analysis.

Preparation of protein extracts

The cell pellet (2×10^6 cells) was washed once with PBS and then lysed in 100 µl of radioimmunoprecipitation assay (RIPA) buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice. The whole cell lysate was obtained from the resulting supernatant after centrifugation at 10,000 x *g* for 10 min.

Frozen insect larvae were homogenized in extraction buffer (RIPA, 1 mM PMSF and 1 mM dithiothreitol) and incubated for 30 min. One ml of extraction buffer was used for 0.2 g larvae. The resulting suspension was centrifuged at 10,000 x *g* for 30 min at 4 °C, and the pellet was discarded. The supernatant was filtered through Whatman paper to remove the lipidic fraction remaining at the top. This crude extract contained the total soluble proteins.

Determination of total protein concentration

The Quick start™ Bradford microassay protocol was used to determine the total protein concentration [32].

Western blot analysis

Whole cell lysates, supernatants and crude or adsorbed NA fraction on ConA-agarose were resolved by SDS-PAGE (10-15% gels) and transferred onto nitrocellulose membranes. Samples were heated for 5 min at 100 °C with sample buffer [125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 0.01% (w/v) bromophenol blue, 10% (v/v) 2-mercaptoethanol]. For protein size determination, PageRuler™ Prestained protein ladder was used. Membranes were incubated overnight at 4 °C in blocking solution (0.05% PBS-Tween – 3% skim milk). After washing, rabbit anti-NA (A/California/06/2009/H1N1) polyclonal antibody (1:1000 in 0.05% PBS-Tween – 1% skim milk) was added. Anti-rabbit IgG-horseradish peroxidase-labeled conjugate (1:1000 in 0.05% PBS-Tween – 1% skim milk) was used as the secondary antibody. Protein bands were detected using the enhanced chemiluminescence Western blotting detection system followed by autoradiography.

Immunofluorescence

*Sf*9 cells were grown on glass slides in six-well plates and infected with r*Ac*GPNA or mock at a MOI of 2. At 48 h p.i., the medium was removed, and the cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min. Non-fixed cells were washed in PBS and the fixed cells were permeabilized with Triton-X100 for 30 min. After washing and blocking, the samples were incubated with rabbit anti-NA antibody for 1 h at room temperature. The antiserum was removed and the slide washed three times with 0.05% PBS-Tween. The cells were incubated with goat anti-rabbit rhodamine-conjugated secondary antibody for 1 h at room temperature. After three washing steps, the cells were examined for fluorescence under a confocal microscope.

NA enzymatic activity

NA enzymatic activity was determined by measuring the fluorescence of 4-methylumbelliferone released by the hydrolysis of 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid substrate. Cell lysates and supernatants harvested at different times were diluted in assay buffer (50 mM Tris, 5 mM $CaCl_2$, 200 mM NaCl, pH 7.5) and assayed for activity after 30 min incubation at 37 °C using 200 µM of substrate. The fluorescence was read (as relative

fluorescence units, RFU) at excitation and emission wavelengths of 365 and 415 nm, respectively, in a multi-well microplate reader (Flex Station 3). A calibration curve was constructed using a commercial recombinant influenza A virus H1N1 NA to quantify the recombinant NA.

NA adsorption from *R. nu* larvae extract on ConA-agarose

The extraction buffer used for *R. nu* larvae homogenization was changed to ConA adsorption buffer (20 mM Tris-ClH buffer, pH 7.4, containing 500 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) by using PD10 columns, and a ConA matrix (1 ml) was equilibrated with the same buffer. The extract was loaded onto the column and the pass-through fraction collected for further analysis. The matrix was washed with ConA adsorption buffer until the absorbance at 280 nm reached baseline. Different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 M) of methyl-alpha-mannopyranoside in ConA buffer were used for elution. Three fractions of 1 ml each were obtained from the different eluents. Results were analyzed by Western blot or SDS-PAGE followed by Coomassie blue staining.

Statistical analysis

Main effects and interactions in the NA activity assays were assessed by ANOVA. Differences between sampling days were additionally explored by calculating confidence intervals (0.05 family-wise error rate) for all possible differences between days using the Bonferroni method as a mean separation procedure. The analysis was made using the S-PLUS 2000 software Professional Edition for Windows, Release 3.

RESULTS AND DISCUSSION

Two constructs were obtained for NA expression: a full-length variant and a mutant lacking the transmembrane and intracellular domains to enhance the secretion of NA to the culture supernatant. In the latter, the deletion was achieved by PCR. Figure 1 shows both NA constructions named p*Ac*GPNA and p*Ac*GPNATm-less after being cloned in the p*Ac*GP67 transfer vector.

pAcGPNA and pAcGPNATm-less were co-transfected with the linearized *Ac*MNPV DNA in independent experiments and two recombinant baculoviruses were obtained (rAcGPNA and rAcGPNATm-less). Homologous recombination between the plasmid and the viral DNA rescued the lethal deletion and the GFP expression was monitored under a fluorescence microscope.

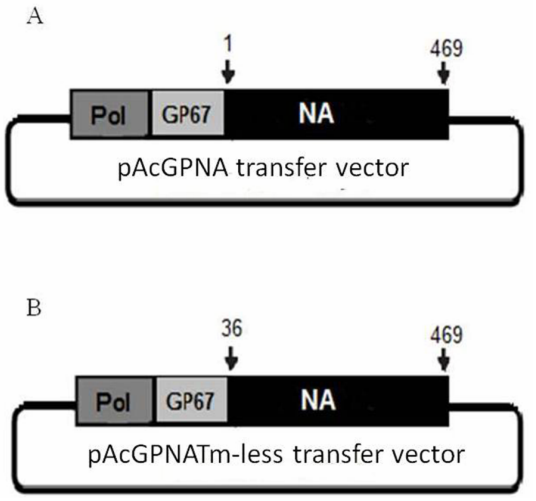

Figure 1. Transfer vector schemes.
(A) Transfer vector schemes containing the full-length neuraminidase (NA) sequence and **(B)** the mutant sequence lacking the transmembrane and intracellular domains (Tm-less NA). Both genes were cloned downstream the polyhedrin promoter and the GP67 peptide signal.

Cloning the influenza antigen genes into a baculovirus vector can be accomplished within a few weeks and this recombinant expression system provides a eukaryotic environment that generally leads to the proper folding and post-translational modifications required for biological activity. It has been reported for *Sf*9 cells that intracellular recombinant proteins are expressed in higher yields than secreted recombinant proteins [33]. However, the subsequent purification steps from the cell culture medium are easier than those of the cell homogenate. The choice of the localization of the protein (intracellular or secreted) is a compromise between the expression level obtained and the complexity of the downstream purification process.

We used the GP67 signal peptide to enable efficient translocation of the proteins into the secretory pathway and thus increase the amount of soluble protein in the extracellular medium. First, we characterized the NA expression achieved by both strategies in *Sf*9 cell line cultures.

Expression of NA in *Sf*9 cells

Enzymatic activity assay was used to demonstrate the integrity of recombinant NA and to determine its expression level. The full-length NA activity was significantly higher in cell lysates than in supernatants ($p < 0.001$), reaching a maximum on day 2 p.i. (Figure 2, ~2 x 10^6 RFU/ml). Within the supernatant fraction, maximum activity was detected on day 4 p.i. (Figure 2, ~1 x 10^6 RFU/ml). About 40% of total NA activity (cell lysate plus supernatant) was measured in the supernatant on day 4 p.i. whereas the other 60% remained intracellular. The specific activity in the supernatant at day 3 and 4 was similar and the viability was 30% lower at day 4 than that at day 3. The integrity of the full-length NA variant was confirmed. The molecular weight and localization of both NA variants were analyzed by Western blot. In cell pellets, the full-length variant showed the monomeric, glycosylated ~60 kDa form in addition to a high molecular weight band corresponding to a dimeric form of the protein (~120 kDa) and a band of approximately 50 kDa corresponding to a non-glycosylated form. The course of the infection (days 1 to 5 p.i.) showed a similar pattern. As an example, the cell pellet on day 2 p.i. is shown in Figure 3A. As the lower band did not increase along the infection time, it seems that it was not related with the poor secretion of NA. On the other hand, in the supernatants, two bands of ~120 kDa and ~60 kDa appeared on day 4 p.i. (Figure 3A). These results indicate that day 3 is the best choice in term of productivity to recover the full-length variant from supernatants.

To assess the punctual localization, immuno-fluorescence analysis was performed using anti-NA antibodies (red, rhodamine) and GFP detection (green). NA was localized in the plasma membrane of the infected cells while GFP was distributed in the cytoplasm (Figure 3B). No red immuno-fluorescence signal was seen in controls. These results indicate that NA was anchored or exposed in the insect cell membrane, probably in a way similar to their native localization in the influenza virus envelope.

In contrast, the Tm-less mutant showed very poor intracellular expression. The activity of the NA Tm-less variant was expectedly low in cell lysates ($p < 0.001$) but gradually increased in the supernatants during the five days p.i. (Figure 2). The maximum specific activity recorded for this mutant was on day 4 p.i. (186,200 RFU/mg), when almost 80% of NA was present in the supernatant. Although most of the Tm-less NA was efficiently secreted, its activity

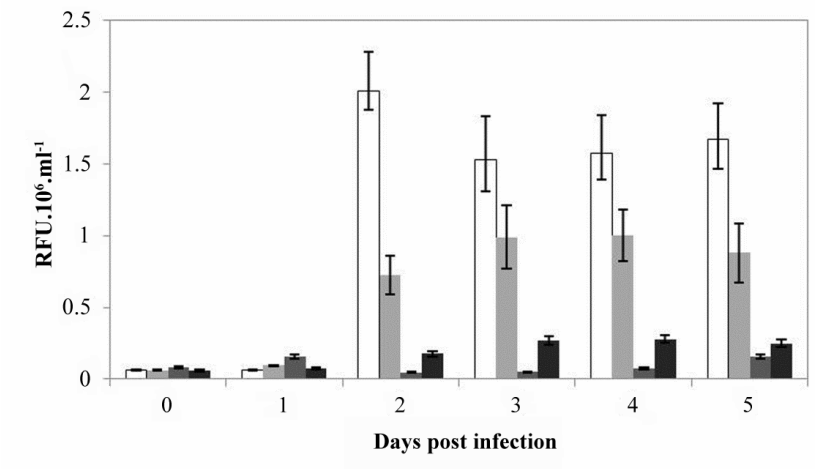

Figure 2. Time course of the enzymatic activity of full-length NA and Tm-less NA in cell lysates (CL) and supernatants (S) of infected *Sf*9 suspension cultures (N = 3, mean ± SEM).

NA activity was expressed as relative fluorescence units (RFU)/ml of suspension at the indicated days post infection (0-5 days)**.** □ Full-length NA in CL, ■ Full-length NA in S, ■Tm-less NA mutant in CL, ■ Tm-less NA mutant in S. The plot indicates mean ± SD from N = 3 independent experiments. Analysis by ANOVA test, **P < 0.001.**

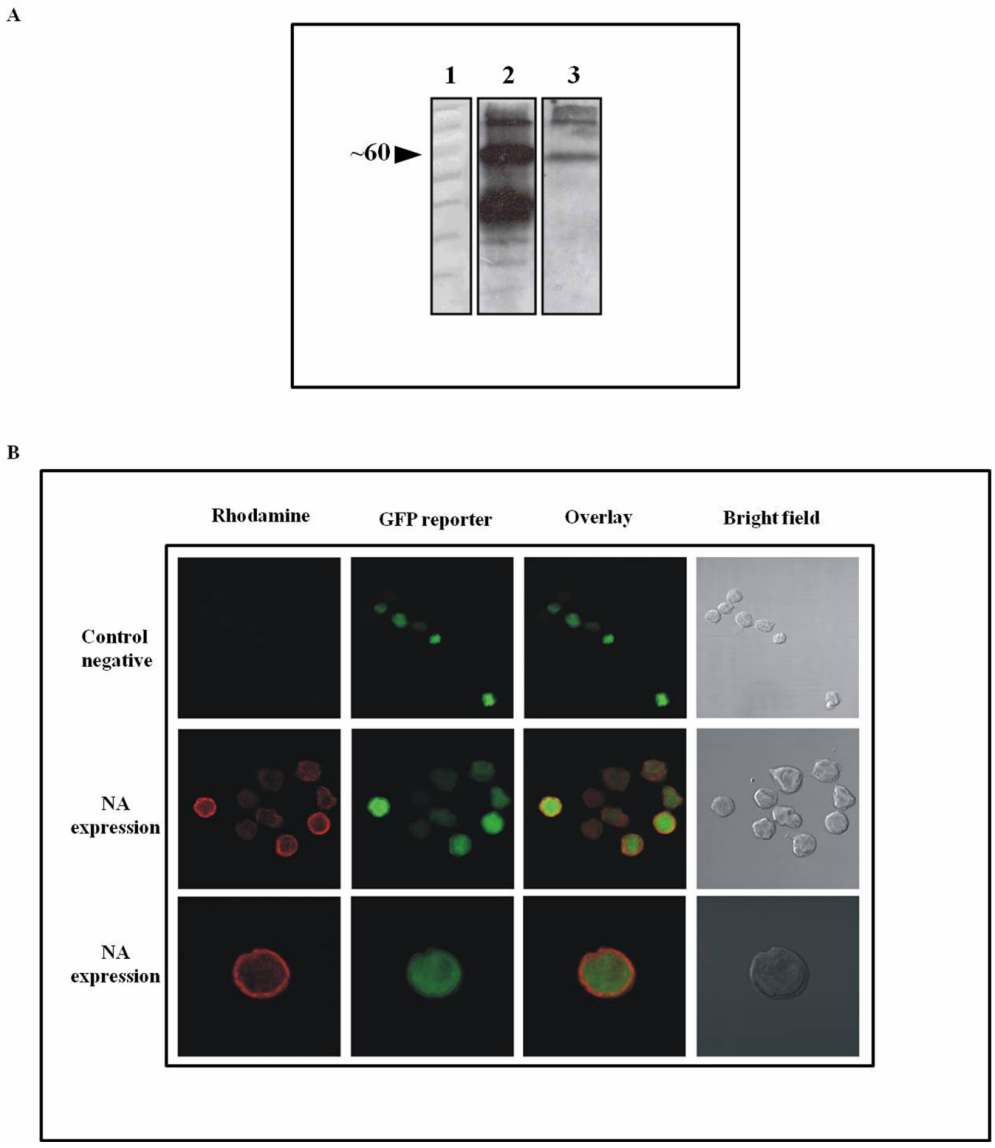

Figure 3. (A) Expression of full-length NA in cell lysates (CL) and supernatants (S). Anti-NA Western blot analysis shows the expression in the CL fraction on day 2 post infection (lane 2) and in the S fraction from day 4 post infection (lane 3). Lane 1, molecular weight marker. **(B) Immunolocalization of the expressed recombinant full-length NA.** First row: Negative control (cells infected with a baculovirus without the NA gene), second and third rows: NA expression on day 2 post infection of *Sf*9 cell line infected with r*Ac*GPNA. Red: rhodamine. Green: GFP reporter also expressed with r*Ac*GPNA.

was about four-fold lower than that of the full-length NA (Figure 2).

The ~60 kDa monomeric form appeared in the culture medium and its expression increased along time as judged by the Western blot of the cell lysate samples (data not shown). In addition, from day 3 to 5 p.i., an additional low molecular weight band appeared. Taking into account that the cell viability remained intact at day 3, this additional band indicated that some kind of degradation might occur under the assayed conditions. The secreted form of NA was present predominantly as monomers of ~60 kDa, even in the absence of reducing agents (data not shown).

The expression kinetics revealed that the Tm deletion significantly enhanced the secretion of NA in the supernatants. Also, in spite of the presence of the Tm domain in the full-length variants, our results suggest that a part of them is efficiently secreted into the culture medium directed by the potent GP67 signal peptide. Moreover, our results indicate that the decrease in biological activity of the Tm-less NA variant may be related to the low amount of the tetrameric form. Recently, it has been reported that the tetramers are the only enzymatically active forms of NA [34]. NA becomes less stable after removal of the Tm domain, losing activity within a short period of time under the assayed conditions. Deroo *et al.* [7] expressed a soluble NA that was also partially recovered as an authentic tetrameric protein from the culture medium at levels of 6 mg/l. The authors engineered the N2 neuraminidase gene of A/Victoria/3/75 influenza virus to encode a secretable protein by replacing the natural N-terminal membrane anchor sequence with the cleavable signal sequence of the corresponding influenza hemagglutinin gene. These authors also demonstrated that the recombinant NA was immunogenic and generated protective antibodies after vaccination trials in mice. On the other hand, Schmidt *et al.* [34] reported that the soluble domain of NA can be efficiently expressed in insect cell culture supernatants by the addition of a foreign tetramerization domain.

Finally, in our experience, the yield of biologically active full-length NA produced in 1 liter of culture (2×10^9 cells) was 9.97 ± 0.74 mg in the intracellular fraction and 6.57 ± 0.51 mg in the supernatant. Thus, to produce 1 mg of secreted NA, 153 ml of suspension culture would be necessary. Under the same conditions, the expression of the biological active Tm-less NA variant was 0.72 ± 0.12 mg in the cell lysates and 1.95 ± 0.06 mg in the supernatant. This poor biological activity of the Tm-less variant was related to a decreased amount of NA tetramer.

Taking these results into account, we selected the full-length NA construction to obtain high level expression of functional NA in cell cultures. In this paper, we achieved NA expression level similar with that reported by Deroo, without any engineering strategy. In our case, intact enzymatic activity was essential to follow and quantify NA expression level. In addition, the conservation of an intact native conformation protein assures the antigenicity. Anyway, it is possible that the other strategy that expresses a Tm-less variant allows obtaining NA monomers that could be used as antigens.

Expression of NA in *R. nu* larvae

A low-cost alternative to cell culture-based protein production is the use of live insect larvae as "mini bioreactors". After the successful expression of full-length NA variant in cultured insect cells, the direct expression of NA in *R. nu* larvae was studied.

As described in the 'Materials and Methods' section, four groups of five larvae (N = 20) were infected with different doses of the recombinant baculovirus in independent assays and NA bands obtained in SDS-PAGE were measured by densitometry. Figure 4 shows NA expression level in arbitrary units at 96h p.i. The dose of 5×10^4 pfu/larvae was the best option to obtain a high level of expression and no larval mortality. With a higher inoculum, larval mortality was too high (50%).

The amount of recombinant full-length NA was 1.2 ± 0.20 mg of NA/g of larvae (1 larva produced 173 ± 44 μg NA) on day 4 p.i. Therefore, to produce 1 mg of recombinant protein, approximately six larvae will be needed. The concentration of total soluble protein in the extract was 12 mg/ml. Recombinant NA accounts for approximately 2% of the crude extract total protein, 4.5 times higher than the yield obtained in culture supernatants (0.44% of the total protein). In contrast, the yield of the Tm-less NA variant was low. An amount of 0.055 ± 0.006 mg of Tm-less NA/g of larvae was obtained (1 larva produced 7.88 ± 0.71 μg NA).

The adsorption of the full-length NA to a ConA matrix evidenced the presence of glycosylated groups in the secreted molecule as judged by Western Blot (not shown). The pattern obtained in *R. nu* extracts showed two additional bands ranging 35-40 kDa, thus indicating that some degree of degradation because of protease activity was had occurred. Clearly the degraded NA is glycosylated and for this reason is adsorbed on ConA matrix. Although SDS-PAGE revealed that most of the proteins of the crude extract were removed, the hexamerin, a glycoprotein of 70-80 kDa that is present in high concentration in the hemolymph,

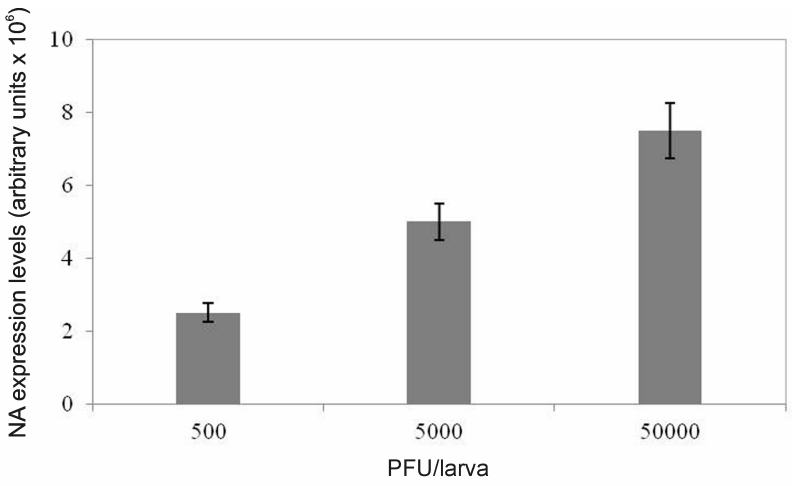

Figure 4. NA expression in *Rachiplusia nu* larvae infected with different baculovirus doses. The graphic represents the recombinant full-length NA expression levels (N = 4, mean ± SEM) between doses as determined by densitometry of reacting bands at 96 h post infection.

appeared contaminating the purified NA (data not shown). Further purification studies may be conducted to separate the hexamerin from NA.

The greatest challenge in the manufacture of influenza vaccines is that the vaccine requires adjustments on an annual basis. The time available to make these adjustments is extremely short (a few months). The current egg-based method can take between 6 to 9 months to produce the vaccine and cannot respond swiftly in the case of a pandemic. Thus, it is of interest to investigate alternatives that can result in timely delivery of the new antigens. Production of recombinant influenza viral antigens (HA and NA) using the baculovirus has been proposed as an alternative to traditional egg-based technology [35]. Likewise, when producing recombinant proteins in lepidopteran larvae, recombinant proteins secreted to the hemolymph are preferred because the protein could be extracted by using a mild extraction buffer (which does not lyse the insect cells) in a "cleaner" way, thus avoiding the presence of contaminating proteins and proteases from the intracellular fraction. Other authors have focused on the expression of the soluble domains of HA and NA [34, 36]. Recently, Schmidt and col. [34] demonstrated that the addition of artificial tetramerization domains drove the formation of catalytically active NA. This construction could be expressed in larvae too. In the present work, we studied two different strategies for the expression of secreted NA. This is the first time that the expression of NA is studied in *R. nu* larvae. To our knowledge, there are no comparative studies of such NA recombinant production yields in larvae. It is important to point out that we did not study the potential antigenicity of the Tm-less NA variant.

In Biotechnology, the choice of a particular production strategy is primarily dictated by the scale of operation, the localization of the target protein and its yield. It might be interesting to investigate the use of insect larvae as a potential biotechnological platform for influenza antigen expression. Thereafter, this low-cost manufacturing capacity could be established in geographic regions that have no possibilities for vaccine production. We believe that the use of *R. nu* larvae in an improved system for recombinant NA expression may be an important step towards meeting seasonal and pandemic demands with minimal investment in manufacturing infrastructure. However, when larva-derived NA is intended to be used in humans, the purification level required will be very high. So, further studies regarding its purification should be carried out. The purified antigen produced by this system can also be available for other applications or research use.

CONCLUSION

Although full-length NA is not completely secreted, it is the best expression option to achieve biologically

active NA. The high yield of NA obtained (1.2 mg/g of larva) makes *R. nu* larvae a very attractive cost-effective alternative to conventional cell culture-based methods for the expression of this important influenza antigen.

The adsoption of NA to the ConA matrix demonstrated that the NA produced in larvae was glycosylated and thus provides a first approach to purification from insect larvae. However, further efforts must be made to improve the downstream processing of the recombinant NA produced with this platform.

In conclusion, our results demonstrated that the full-length NA produced in insect cells and whole insect *R. nu* larvae was glycosylated and biologically active and that *R. nu* larvae yielded high amounts of recombinant NA functionally similar to the authentic viral antigen in a short time.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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