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Potential Use of the Hemagglutinin-Neuraminidase Glycoprotein of Newcastle Disease Virus Expressed in *Rachiplusia nu* Larvae as an Immunogen for Chickens▽

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The hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus (NDV) was obtained as a recombinant antigen in *Rachiplusia nu* larvae. When it was used as an immunogen in chickens, a solid immune response, including neutralizing antibodies, was detected, demonstrating the potential use of this simple and economic strategy in the design of recombinant anti-NDV vaccines.

Insect cell-based baculovirus expression systems have been extensively exploited for the large-scale production of recombinant proteins (12, 14, 19). The main disadvantage of this system is its high cost, which may become prohibitive if large volumes are required. Insect larvae infected with recombinant baculoviruses can be an extremely useful alternative due to the high levels of protein expression achieved, the lower costs for large-scale production, and a higher efficiency of certain types of posttranslational modifications (19). In the last 10 years, larvae have been successfully used to produce different recombinant proteins (3, 5, 7, 13, 16, 21, 22).

Newcastle disease virus (NDV), an avian paramyxovirus, is the causative agent of an economically important and highly contagious disease of poultry. Several reports describe the use of different expression systems to obtain recombinant immunogens based on hemagglutinin-neuraminidase (HN) or F glycoproteins of NDV with various degrees of success (2, 9, 10, 17, 24). For instance, Kamiya and coworkers demonstrated that complete protection was achieved when either HN or F protein of a virulent strain or HN protein from an avirulent strain was used as an immunogen (9).

The aim of the present work was to obtain a recombinant immunogen against NDV by infection of lepidopteran larvae with a recombinant baculovirus for HN glycoprotein.

The HN coding region was amplified from cDNA synthesized from genomic NDV RNA (La Sota strain, provided by the National Service for Animal Health [SENASA]) isolated using TRIzol (Life Technologies, Grand Island, NY), following the manufacturer's instructions. Amplification of the HN coding region was done using oligonucleotides 5′-AAAGGATCC GCTAGCACACCTAGCGAT-3′ and 5′-AGGCTCTAGATT GCCAGACCTGGC-3′, which amplify a 1,585-bp fragment, excluding the first 150 bp coding for 50 amino acids of the protein predicted as the signal peptide and the transmembrane domain (6). Oligonucleotides included BamHI and XbaI restriction sites for cloning into pBacPAK8 (Clontech, Mountain View, CA) transfer plasmid (underlined). The PCR product was cloned into the transfer plasmid, and recombinant plasmids were isolated following classical protocols (15). Sf9 insect cells were cotransfected with the recombinant vector and linearized *Autographa californica* multiple nucleopolyhedrosis virus (AcNPV) DNA, following the BaculoGold kit instructions

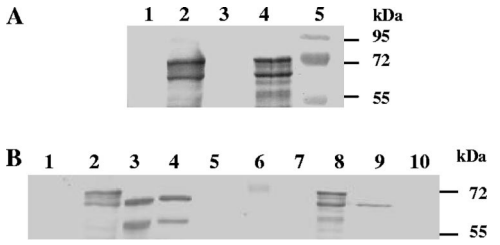

FIG. 1. Expression and glycosylation of recombinant HN protein in Sf9 cells and *Rachiplusia nu* larvae. (A) HN protein expression. Total proteins of extracts from Sf9 cells or *Rachiplusia nu* larvae infected with the recombinant baculovirus AcNPV/HN were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the recombinant protein was detected by Western blotting using an anti-HN polyclonal serum, kindly provided by Darrell Kapczynski from the Southeast Poultry Research Laboratory, U.S. Department of Agriculture. Lane 1, extracts of mock-infected Sf9 cells; lane 2, extracts of Sf9 cells infected with AcNPV/HN; lane 3, extracts of mock-infected *Rachiplusia nu* larvae; lane 4, extracts of *Rachiplusia nu* larvae infected with AcNPV/HN; lane 5, molecular mass standards (Page Ruler; Fermentas, Glen Burnie, MD). (B) Evaluation of N glycosylation. Total proteins of extracts of Sf9 cells infected with the recombinant baculovirus AcNPV/HN and treated with tunicamycin, as well as extracts of infected Sf9 cells and *Rachiplusia nu* larvae treated with PNGase F, were evaluated by Western blotting with anti-HN polyclonal serum. Lane 1, mock-infected Sf9 cells; lane 2, Sf9 cells infected with AcNPV/HN; lane 3, Sf9 cells infected with AcNPV/HN treated with tunicamycin during infection; lane 4, Sf9 cells infected with AcNPV/HN treated with PNGase F after extraction; lane 5, mock-infected Sf9 cells treated with PNGase F; lane 6, molecular mass standards (Page Ruler; Fermentas, Glen Burnie, MD); lane 7, mock-infected *R. nu* larvae; lane 8, *R. nu* larvae infected with AcNPV/HN; lane 9, *R. nu* larvae infected with AcNPV/HN treated with PNGase F after extraction; lane 10, mock-infected *R. nu* larvae treated with PNGase F.

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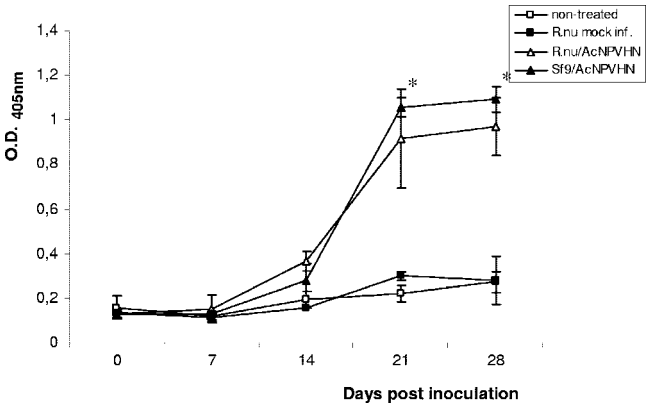

FIG. 2. IgG response against NDV in serum samples. Chickens were vaccinated at days 0 and 14 with the experimental immunogen based on *R. nu* larvae infected with AcNPV/HN (△), with the negative control based on mock-infected *R. nu* larvae (■), or with the positive control based on Sf9 cells infected with AcNPV/HN (▲). Unvaccinated animals are also shown (□). Serum samples were collected weekly and analyzed by ELISA using purified NDV as antigen. Each value represents the mean of individual determinations of each group ± the standard deviation. Data were analyzed by the Student *t* test, and means were compared by the Bonferroni test using Statistixs version 7.0 analytical software. ∗, result significantly different in comparison with the negative-control group ($P < 0.05$).

TABLE 2. Viral neutralization activity individually evaluated in serum samples collected at 28 days postinoculation[a]

AcNPV/HN-infected cell type	Individual VN titer at 28 dpi for sample no.:					Mean	SD
	1	2	3	4	5		
R. nu	2.44	2.48	1.82	2.3	1.75	2.158	0.347
Sf9	2.94	2.91	3.00	2.95	2.88	2.936[b]	0.045

[a] The data obtained in the seroneutralizing assay were analyzed by the Student *t* test, and means were compared by the Bonferroni test using Statistixs version 7.0 analytical software. VN, viral neutralization; dpi, days postinoculation.
[b] Statistically significant ($P < 0.05$).

(Pharmingen, San Diego, CA). Recombinant baculoviruses were purified (19), and selected clones were amplified by three passages in Sf9 cells. Finally, viral stock (AcNPV/HN) was titrated and HN expression was characterized by Western blot assay. Briefly, monolayers of Sf9 cells were infected with the viral inoculum at a multiplicity of infection of 5. After 72 h, cells were collected, washed with phosphate-buffered saline (PBS), pH 6.2, and resuspended in cracking buffer. Cellular extracts were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the presence of the recombinant protein was confirmed by Western blotting, following standard procedures (8). Figure 1A shows a double band slightly lower than 72 kDa, in agreement with the fact that the recombinant protein did not include the first 50 amino acids of HN, and because of that, its molecular mass is lower than that of the one corresponding to HN glycoprotein from purified NDV (data not shown). When HN expression was evaluated in extracts of infected *Rachiplusia nu* larvae (7, 13), a similar pattern was detected (Fig. 1A). Considering that HN glycosylation has a relevant role in its antigenicity and immunogenicity (20), we evaluated the ability of our system to render a correctly glycosylated protein. Sf9 cells were infected with AcNPV/HN (multiplicity of infection, 5). After the adsorption period, the viral inoculum was removed and replaced by fresh medium containing 5 μg/ml tunicamycin (Sigma-Aldrich, St. Louis, MO). After 72 h, cells were collected and processed for Western blot analysis. Results of this treatment demonstrated that the double band pattern corresponded to the glycosylated and nonglycosylated forms of the recombinant HN protein (Fig. 1B). To confirm our observation, both cellular and larval extracts were treated with PNGase F following the manufacturer's indications (New England BioLabs). PNGase F is an endoglycosidase that cleaves oligosaccharides from N-linked glycoproteins. A reduction in the apparent molecular size of the protein was observed in both systems, indicating that N glycosylation occurs in vitro and in vivo (Fig. 1B).

To evaluate the immunizing ability of the recombinant protein obtained, 15-day-old specific-pathogen-free White Leghorn chickens (Rosenbusch, Argentina) were divided into groups of five animals and intramuscularly immunized on days 0 and 14. Each dose consisted of 0.2 ml of the extract formulated in incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO), containing approximately 2 μg of recombinant antigen as estimated by comparison with a bovine serum albumin standard curve stained with Coomassie brilliant blue. The positive-control group consisted of chickens receiving two doses of infected Sf9 cells (9). Feed and water were provided ad libitum. Blood samples were collected weekly from a wing vein, allowed to clot, incubated at 4°C, and centrifuged. Twenty-eight days after the first vaccination, animals were sacrificed and intestine and ceca were dissected. The organs were

TABLE 1. Evaluation of the activity of the antibodies induced by vaccination with recombinant HN[a]

Sample group	Titer at dpi									
	VN					HI				
	0	7	14	21	28	0	7	14	21	28
Nontreated	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
R. nu, mock infected	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
R. nu, AcNPV/HN infected	<0.6	<0.6	<0.6	1.60	2.07	<0.6	<0.6	0.6	1.8	2.1
Sf9, AcNPV/HN infected	<0.6	<0.6	<0.6	1.76	2.96	<0.6	<0.6	1.2	2.7	2.7

[a] Serum samples collected weekly were evaluated for the presence of antibodies with neutralizing activity and antibodies that were inhibitors of hemagglutination. Viral neutralization (VN) assays were performed in chicken embryo fibroblast primary cell culture with serial fourfold dilutions of serum samples and 100 PFU of NDV strain Beaudette. VN titer was calculated as the $\log_{10}$ of the inverse of the dilution that reduced the formation of viral plaques by 50%. HI assays were performed using four hemagglutinin units of NDV strain La Sota. HI titers were expressed as the $\log_{10}$ of the inverse of the highest dilution of sample showing complete inhibition of agglutination. Both VN and HI tests were performed using pools of all the samples of each group. dpi, day postinoculation.

immersed in PBS, cut in small pieces, and washed intensively. Tissue samples were incubated at 41°C and 5% CO_2 in RPMI 1640 medium (Gibco, Carlsbad, CA) and 10% fetal calf serum. Two days later, supernatants were collected and stored at −20°C until used. An indirect enzyme-linked immunosorbent assay (ELISA) was used to measure chicken immunoglobulin G (IgG) against NDV. Briefly, 96-well plates (Maxisorp Nunc) were coated with purified La Sota NDV (1) in 0.1 M bicarbonate buffer, pH 9.6, overnight at 4°C. After blocking with 4% skim milk in PBS–T-ENS (0.05% Tween 20, 0.5% equine normal serum), the plates were subsequently incubated with samples and goat anti-chicken IgG antibodies coupled to horseradish peroxidase (Bethyl Laboratories, Inc.) and ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt]-H_2O_2 in citric acid buffer, pH 5. The analysis showed that HN recombinant immunogen was able to elicit an antibody response detectable from day 14 postinoculation, exhibiting significant differences ($P < 0.05$) from negative-control groups on days 21 and 28 postvaccination (Fig. 2). The kinetics of antibodies induced by HN antigen from insect larvae was essentially similar to that of antibodies induced by the antigen from infected cells (Fig. 2). To evaluate neutralizing activity, pools of serum samples were inactivated for 30 min at 56°C and serially fourfold diluted in 199 medium (Gibco, Carlsbad, CA). Dilutions were incubated in the presence of 100 PFU of NDV strain Beaudette (provided by SENASA) for 1 h at room temperature and 30 min at 4°C. Then, each mix was added to chicken embryo fibroblast primary cell culture. Cells were incubated for 1 h at 37°C before being overlaid with minimum essential medium (Gibco, Carlsbad, CA) containing 2% fetal bovine serum, 25 mM HEPES, 10% antibiotic-antimycotic, and 0.8% low-gelling-temperature agarose. The plates were incubated for 4 days at 37°C with 5% CO_2 before being fixed with 10% Formol and stained with crystal violet. To evaluate hemagglutination inhibition (HI) activity, a conventional microtiter method was used (11). The immunization with infected larva extracts elicited the production of neutralizing antibodies that could be detected from day 21 postvaccination, showing the ability of this immunogen to elicit a solid neutralizing immune response (Table 1). Viral neutralization assay results were, overall, in concordance with the ELISA results. Similar results were obtained when the HI assay was performed, but in this case, activity was detected from day 14 postvaccination (Table 1). When samples from day 28 were individually analyzed, we observed that there were statistical differences between *R. nu* and Sf9 groups infected with AcNPV/HN, the cell culture antigen being the one which gave better neutralizing titers. However, both immunogens induced a solid neutralizing immune response in chickens (Table 2), and the titers obtained were significantly higher than the ones obtained for the negative-control groups. Previous reports showed a high correlation between the presence of neutralizing antibodies to NDV and protective immunity and demonstrated that the presence of specific antibodies determined by the viral neutralization test was imperative for protection (23). Also, an association between neutralizing antibody responses to HN protein and protection against NDV was reported with recombinant vaccinia virus (18). Moreover, Kamiya et al., (9) reported such a correlation using recombinant baculoviruses expressing HN and F proteins. For these reasons, we assumed

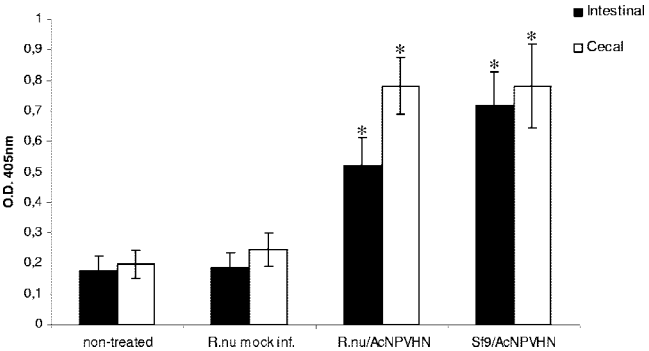

FIG. 3. IgG response against NDV in tissue culture supernatants. Chickens were vaccinated at days 0 and 14 with the experimental immunogen (*R. nu* with AcNPV/HN) and the corresponding controls. On day 28, chickens were sacrificed and guts and ceca were excised. The organs were washed intensively and cut in small pieces which were cultured for 48 h at 41°C and 5% CO_2. The presence of specific antibodies in supernatants of intestinal and cecal fragment cultures was evaluated by ELISA. Each value represents an average ± the standard deviation from five samples. Data were analyzed by the Student *t* test, and means were compared by the Bonferroni test using Statistixs version 7.0 analytical software. *, result significantly different in comparison with the negative-control groups ($P < 0.05$).

that the solid neutralizing response induced by the recombinant immunogen described in this report constitutes an encouraging sign of the protection that could be conferred by it.

In order to evaluate the local production of specific IgG in guts and ceca, supernatants of tissue fragment cultures were analyzed by ELISA. No antibody response was observed in samples of culture supernatants taken at time zero (data not shown), allowing a consistent analysis of the in situ antibody production. A solid anti-NDV specific IgG antibody response ($P < 0.05$) was detected in the supernatants of cultured intestine and cecum fragments of chickens immunized with both cell extracts and larval extracts expressing the HN glycoprotein (Fig. 3), demonstrating local intestinal mucosal immune responses. These results resemble the ones obtained when conventional vaccines are used (4). These antibodies may have a role in the protection of mucosa, where NDV begins its infection cycle.

This study shows the efficacy of infected larval extracts as an NDV recombinant immunogen and shows that the extracts constitute an easier and less expensive approach for the production of recombinant antigens, since production in *R. nu* larvae was nearly 400 times more economical than the expression of the same protein in Sf9 cells (22). In our hands, a single infected larva yields quantities of recombinant HN protein similar to those yielded by 1.5×10^6 infected Sf9 cells (data not shown). The larval expression system may be a very useful tool in the veterinary industry for the inexpensive production of effective recombinant immunogens.

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