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Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV

D.M. Pérez-Filgueira [a,c], P. Resino-Talaván [a], C. Cubillos [b], I. Angulo [b], M.G. Barderas [a], J. Barcena [b], J.M. Escribano [a,*]

[a] *Departamento de Biotecnología, INIA. Autovía A6 Km. 7.5, Madrid (28040), Spain*
[b] *Centro de Investigación en Sanidad Animal, INIA. Valdeolmos, Madrid (28130), Spain*
[c] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)- Buenos Aires, Argentina*

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Abstract

Vaccine antigens against rabbit hemorrhagic disease virus (RHDV) are currently derived from inactivated RHDV obtained from livers of experimentally infected rabbits. Several RHDV-derived recombinant immunogens have been reported. However, their application in vaccines has been restricted due to their high production costs. In this paper, we describe the development of an inexpensive, safe, stable vaccine antigen for RHDV. A baculovirus expressing a recombinant RHDV capsid protein (VP60r) was used to infect *Trichoplusia ni* insect larvae. It reached an expression efficiency of 12.5% of total soluble protein, i.e. ~2 mg of VP60r per larva. Preservation of the antigenicity and immunogenicity of the VP60r was confirmed by immunological and immunization experiments. Lyophilized crude larvae extracts, containing VP60r, were stable, at room temperature, for at least 800 days. In all cases, rabbits immunized with a single dose of VP60r by the intramuscular route were protected against RHDV challenge. Doses used were as low as 2 μg of VP60r in the presence of adjuvant or 100 μg without one. Orally administered VP60r in the absence of an adjuvant gave no protection. The potential costs of an RHDV vaccine made using this technology would be reduced considerably compared with producing the same protein in insect cells maintained by fermentation. In conclusion, the larva expression system may provide a broad-based strategy for production of recombinant subunit antigens (insectigens) for human or animal medicines, especially when production costs restrain their use.
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Keywords: RHDV; Recombinant vaccine; Calicivirus; Baculovirus; Larvae; Low-cost; Insect expression system

Introduction

Rabbit hemorrhagic disease (RHD) is a highly contagious and lethal infection which affects both wild and domestic rabbits (*Oryctolagus cuniculus*). It is a major threat to wildlife and to trade in rabbit-derived products. Its etiological agent, the rabbit hemorrhagic disease virus (RHDV) is a small non-enveloped virus, prototype of the *Lagovirus* genus included in the *Caliciviridae* family (Ohlinger et al., 1990; Parra and Prieto, 1990). RHDV particles consist of a 7.5 kb single-stranded positive-sense RNA genome in a small icosahedrical capsid ~38 nm in diameter. The RHDV coat protein (VP60) has an apparent molecular weight of 60 kDa. One hundred and eighty copies of this protein are assembled to produce native virus capsids (Barcena et al., 2004).

The disease was first described in China in 1984 (Liu et al., 1984. However, within a few years it had spread to Korea (Park et al., 1991) and continental Europe (Mitro and Krauss, 1993). Further outbreaks were reported in Northern Africa, the Middle East, Russia, India, Oceania, Cuba, the USA, Mexico and recently in Uruguay (Gregg et al., 1991; Bouslama et al., 1996; OIE, 2004). Typically, RHD is resolved within 48 h to 72 h post-infection and it is fatal in ~80% of adult animals as a result of acute liver damage and disseminated intravascular coagulation (Ueda et al., 1992; Ramiro-Ibanez et al., 1999). The disease is highly contagious and animals can acquire the virus through oral, nasal or conjunctival routes. Surviving animals can shed the virus for up to 4 weeks after infection. The RHDV is resistant to

* Corresponding author.
E-mail address: mperez@inia.es (J.M. Escribano).

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adverse external conditions, including low pH, high temperature and repeated freeze-thaw cycles. This allows particles to remain stable in the natural environment (Moss et al., 2002).

The lack of an efficient *in vitro* propagation system for RHDV has hindered large-scale production of the virus as source of vaccine antigens. Currently, vaccines are produced by the chemical inactivation of crude virus preparations obtained from the livers of infected rabbits (Argüello-Villares, 1991). This strategy raises serious concerns about biological safety, contaminant residues and animal welfare issues.

Early reports showed that recombinant versions of VP60 can induce protective humoral responses against an RHDV challenge (Laurent et al., 1994). Several heterologous systems have been used to produce recombinant versions of VP60, including bacteria (Boga et al., 1994), yeasts (Boga et al., 1997; Farnos et al., 2005), plants (Castanon et al., 1999; Fernandez-Fernandez et al., 2001; Martin-Alonso et al., 2003; Gil et al., 2006), poxvirus-based vectors (Bertagnoli et al., 1996a,1996b; Fischer et al., 1997) and insects cells using recombinant baculovirus (Laurent et al., 1994; Marin et al., 1995; Nagesha et al., 1995; Plana-Duran et al., 1996; Gromadzka et al., 2006). The latter strategy has also been used to study RHDV coat protein structure and assembly (Barcena et al., 2000). However, biosafety risks and high costs associated with these methods have restricted their use for the commercial production of a recombinant RHDV vaccine.

An important criterion for an ideal vaccine against RHDV is low cost: vaccines for use with rabbits must sell for a few cents a dose. As mentioned above, high yields of recombinant VP60 were obtained using a baculovirus system in insect cell lines. However, the scaling up of insect cell lines in fermentors is both difficult and expensive. Compared with this method, insect larvae do not require sterile conditions or growth media during the production process, thus dramatically reducing production cost. Furthermore, scaling up is immediate once larval infection conditions are established. A wide variety of recombinant proteins have been expressed in insects, including enzymes (Medin et al., 1990), antibodies (Reis et al., 1992), hormones (Mathavan et al., 1995; Sumathy et al., 1996), vaccine antigens (Johansson et al., 1995; Zhou et al., 1995; Matsuoka et al., 1996; Choi et al., 2000; Barderas et al., 2001; Pang et al., 2002), cytokines (Maeda et al., 1985; Muneta et al., 2004; Nagaya et al., 2004) and diagnostic proteins (Ahmad et al., 1993; Katz et al., 1995; Ismail et al., 1995; Perez-Filgueira et al., 2006).

The results of this work show production of recombinant VP60 derived from RHDV AST/89 strain expressed in *Trichoplusia ni* larvae using a recombinant baculovirus carrying the full length VP60 gene. Immunization experiments using both intramuscular and oral routes were conducted to study its immunogenic properties and determine the minimum protective dose in each case. Additional experiments were carried out to determine the stability of the recombinant VP60 (VP60r) over time. Our results show the feasibility of producing commercial vaccines against RHDV using this technology. This would allow its application to animal species with a low relative value such as rabbits and opens the possibility for their commercial use.

Results

Production of recombinant VP60 in T. ni larvae

Experiments were carried out to establish optimal conditions for the production of VP60r in *T. ni* larvae and quantify the recombinant product obtained. Fourth instars were inoculated with three different doses of VP60BAC and samples were processed at different times post-inoculation and analyzed by SDS-PAGE, Western blot and ELISA. Coomassie blue-stained gels showed that VP60r was easily identified as a major band with mobility around 60 kDa. Accumulation in inoculated larvae was time-dependent (Fig. 1b). This was confirmed by immunoblotting (Fig. 1c). It also identified two minor immunoreactive bands with higher mobility (∼55 kDa and 50 kDa). This was probably the result of partial proteolytic processing of VP60r. ELISA allowed a more detailed comparison among samples and immunodetection of accumulated recombinant products basically resembled that of the stained gels and the Western blot assays (Fig. 1c) There was no significant difference in accumulation between the 2.5×10^4 and 10^5 pfu/larva doses. However, percentage of associated deaths of inoculated larvae at the lower dose was less than half those of the higher dose (8.3% vs. 19.5%). Accumulation of VP60r at the highest inoculation dose (4×10^5 pfu/larva) was similar to that of the lower doses up to 84 hpi. However, the damage to larvae, by infection, at this dose most probably affected VP60r accumulation 96 hpi (Figs. 1a–c). Therefore, based on VP60r accumulation levels and death rates for each treatment, a dose of 2.5×10^4 pfu/larva and an infection period of 96 h were selected for the production of recombinant antigen to be tested in vaccination experiments.

Recombinant antigen for immunization experiments was produced under these conditions and was further analyzed by capillary electrophoresis for immunogen characterization. Fig. 2 shows that VP60r was detected as single species with a mobility of 61.51 kDa and reached 12.5% of TSP extracted. Based on the mean TSP extracted per animal (∼16 mg), VP60r accumulated at ∼2 mg per infected larvae.

Intramuscular immunization of rabbits with VP60r

To investigate the immunogenicity of larvae-derived VP60r, different experimental vaccines carrying VP60r were use to immunize rabbits by the IM route. Experimental vaccines carrying 5 different doses of crude larvae extracts containing VP60r – ranging from 40 μg to 2 μg of VP60r per dose – were formulated as water-in-oil emulsions with FCA and 2 more groups were immunized with 100 μg and 40 μg of VP60r in PBS without adjuvant. All rabbits received a single dose of the corresponding vaccine and they were challenged 30 dpi. Blood samples were taken at 0, 15, 30 and 45 dpi and RHDV-specific humoral responses were analyzed by VP60r VLPs-based ELISA. Since most animals rapidly developed strong anti-VP60 humoral responses to allow better comparison among formulations during the experiment, mean OD values (Fig. 3a) are given for sera samples diluted 1/2,700. Fig. 3 shows that

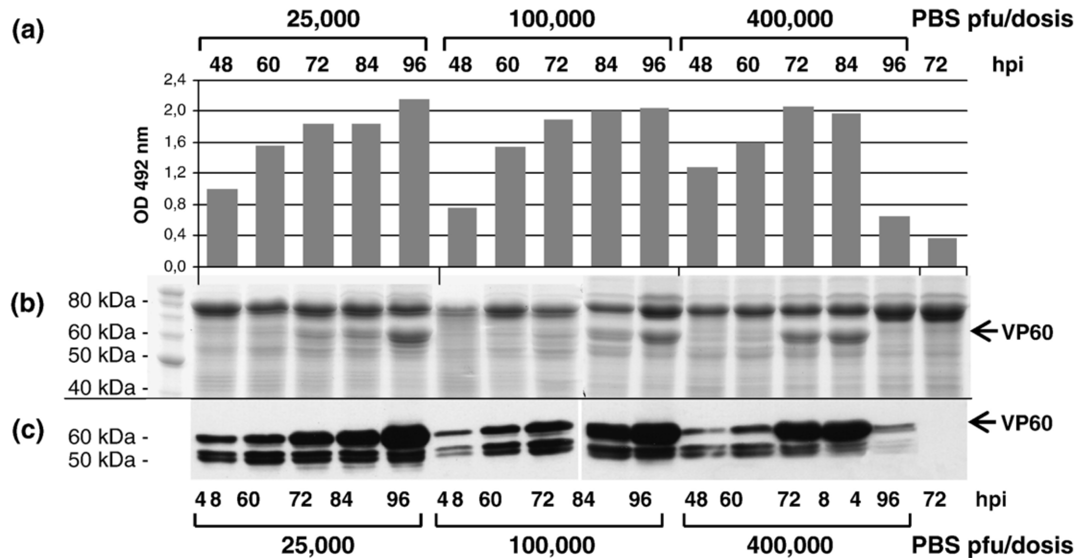

Fig. 1. Production of recombinant VP60 in *Trichoplusia ni* larvae. Fourth-instar larvae were inoculated with different doses of recombinant VP60BAC. Infections progressed for 48, 60, 72, 84 or 96 h post-infection and total protein extracts were taken at the times indicated. Negative controls were inoculated with PBS. (a) Extracts were used to coat ELISA plates and VP60r was detected using mAb 2E11 as a probe. Crude preparations were also resolved by SDS-PAGE (20 μg of TSP/lane) and analyzed by Coomassie brilliant blue staining (b) and Western blot (c) using the same antibody probe as for the ELISA tests. Arrows in panels (b) and (c) indicate the position of VP60r.

antibody levels before challenge were roughly correlated to the amount of inoculated VP60r and the adjuvant. For most immunogens, the humoral responses increased over time. This was more evident for preparations with a low VP60r dose or without FCA (Fig. 3). All rabbits immunized with VP60r vaccines survived a lethal challenge with virulent RHDV at 30 dpi, except for one rabbit which received 40 μg of VP60r without adjuvant. Rabbits immunized with the highest doses of VP60r+ FCA (40 μg and 20 μg) did not significantly increase their antibody levels after RHDV challenge. All control rabbits vaccinated with extracts containing p54r+FCA died 48–72 h after the challenge and showed clinical signs of RHDV.

Oral immunization of rabbits with VP60r

Two different protocols were used to study oral immunization of rabbits with VP60r. A first group of rabbits received 2 doses of 400, 200, 50 or 20 μg of VP60r administered at 0 and 15 dpi. In a second protocol, rabbits were initially immunized with 400, 200, 100 or 50 μg of VP60r and 7 weeks later animals were boosted with 10 μg of VP60r in PBS by the IM route. Animals were challenged 54 days after initial immunization. Serum samples were taken at 25, 53 and 63 dpi days after initial immunization and analyzed by VP60r VLPs-based ELISA (Fig. 4).

Fig. 4 shows that in approximately half the rabbits immunized following the first protocol (rabbits #1 to #12) anti-VP60 antibody levels at 25 dpi were at least double that in the negative controls. However, most rabbits did not significantly increase their anti-RHDV titers by 53 dpi and consequently only one rabbit developed protective levels of RHDV-specific antibodies to survive a lethal challenge (rabbit #3). The second group of rabbits (rabbits #13 to #24), which were orally primed with different doses of lyophilized VP60r and boosted with a sub-immunogenic IM dose of VP60r also had a poor protective responses (Fig. 4). Seven of 12 immunized rabbits had antibody levels at least double that of negative control animals, after the booster, but of these, only 3 attained antibody titers capable of conferring protection (rabbits 13, 19 and 20; Fig. 4). In contrast to the report of Plana-Duran et al. (1996), 2 out of 3 immunized animals (rabbits #25 to #27, Fig. 4) orally immunized with purified RHDV VLPs produced in insect cells by VP60BAC, also failed to induce protective antibody levels The positive

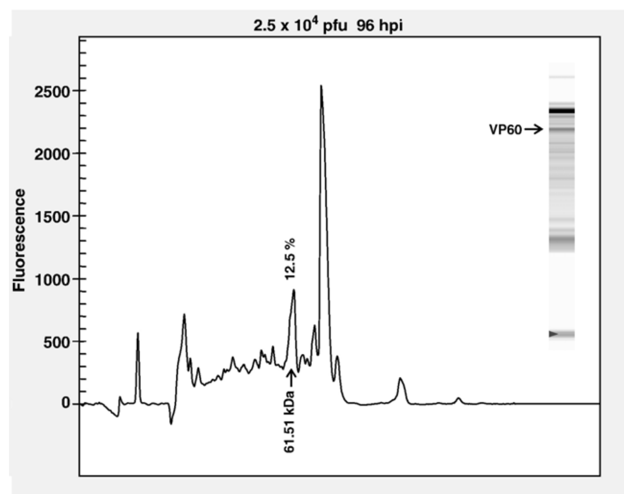

Fig. 2. Characterization of VP60r by capillary electrophoresis. Larvae were inoculated with 10^5 pfu/dose, processed at 72 hpi and crude protein extracts analyzed using the Experion electrophoresis system. The figure shows the resulting electropherogram and simulated gel view inside. The peak corresponding to VP60r is marked in the electropherogram indicating its electrophoretic mobility and percentage of total protein. The position of VP60r in the virtual gel view is also indicated.

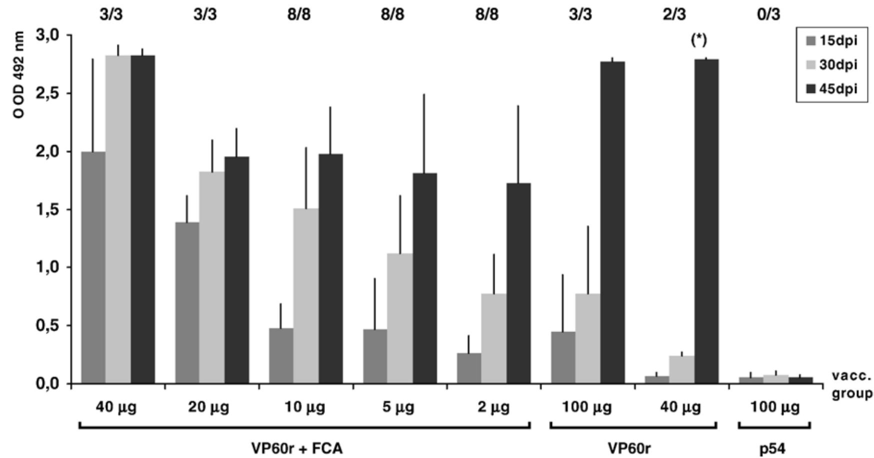

Fig. 3. Humoral response to VP60r in rabbits inoculated IM. Animals were immunized with different amounts of VP60r in an oil formulation with FCA (VP60r+FCA) or aqueous formulations in PBS without an adjuvants (VP60r). An additional control group of rabbits was immunized with an oil vaccine containing p54r from ASFV emulsified with FCA. Animals received a single dose and were challenged with infective RHDV at 30 dpi. Serum samples were taken at 15, 30 and 45 dpi and analyzed by VP60r VLPs-based ELISA. Bars indicate mean OD values obtained for sera diluted 1/2700. Vertical lines in each bar are the standard deviation. Fractions above each immunization group are the number of rabbits protected/number of rabbits challenged. Asterisk indicates that mean OD reading at 45 dpi for this group included only 2 animals.

control group, including rabbits immunized using a single dose of 100 µg of VP60 in FCA, developed an early, sustained and strong anti-RHDV response and survived the virus challenge at 54 dpi (rabbits #30 and #31, Fig. 4).

Stability of VP60r in crude larvae extracts

Lyophilized crude larvae protein extracts were kept at 4 °C or 25 °C for up to 1000 dpp and the antigenic preservation of VP60r was studied by SDS-PAGE, Western blot and ELISA. Coomassie blue-stained gels revealed the presence of a band of the expected size of VP60r, with similar intensity, in all samples. Immunodetection using 2E11 mAb confirmed that VP60r was preserved up to 1000 dpp with minor degradations either at 4 °C or 25 °C (Fig. 5a). ELISA using 2E11 mAb or polyclonal rabbit sera confirmed that both the amount and antigenicity of VP60r were stable in the freeze-dried extracts, with no changes up to at least 800 dpp at both storage temperatures (Fig. 5b). There was a moderate decrease in antigen integrity in samples kept at 25 °C and 4 °C for 1000 dpp, with values around 70% and 80% of the controls, respectively (Fig. 5b).

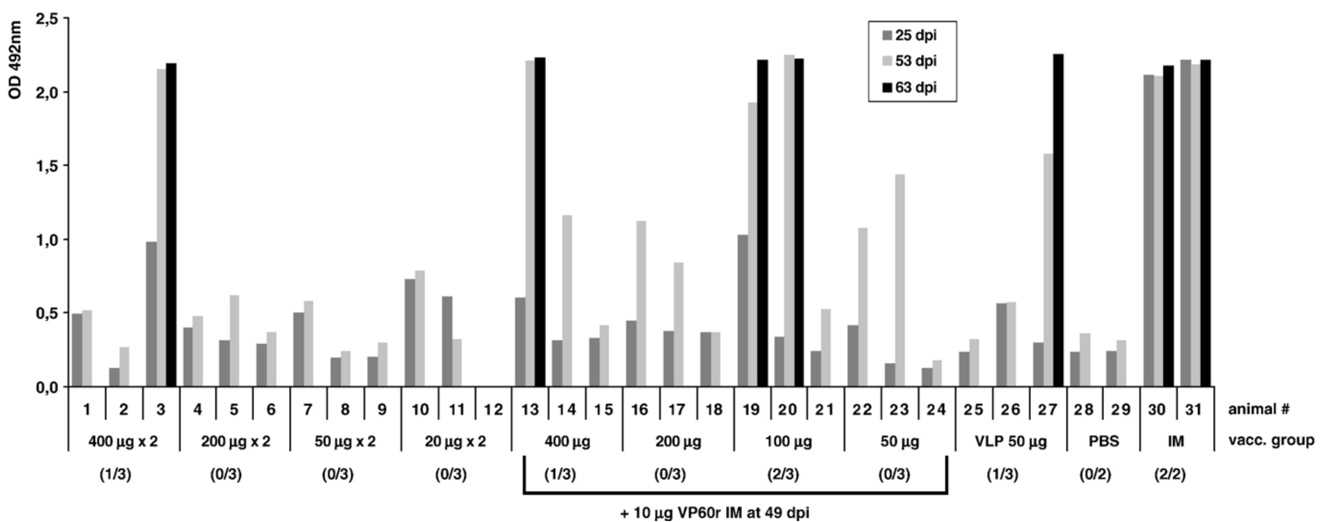

Fig. 4. Humoral response to VP60r in rabbits inoculated by orally. Animals immunized with capsules containing different amounts of lyophilized VP60r without adjuvants. Rabbits 1–12 had 2 doses of the indicated amounts of VP60r at 0 and 15 dpi. Animals 13–24 had a single dose of encapsulated VP60r as indicated and a subsequent IM booster of 10 µg of VP60r in PBS at 49 dpi. VP60r VLPs were used to immunize animals 25–28 as described above. Control rabbits were given PBS orally (#28 and 29) or 100 µg of VP60r in FCA by IM (#30 and 31). All animals challenged with infective RHDV at 53 dpi. Serum samples taken at 26, 53 and 10 days after challenge (63 dpi); animal #12 died before the first bleeding. Bars indicate OD readings for samples diluted 1/100 and fractions below each immunization group show the number of rabbits protected/number of rabbits challenged.

Discussion

Since being described by Laurent et al. (1994), several papers reported the development of immunization strategies against RHDV, predominantly based on recombinant versions of the capsid protein produced by a wide variety of strategies and expression systems (Boga et al., 1994, 1997; Laurent et al., 1994; Plana-Duran et al., 1996; Castanon et al., 1999; Barcena et al., 2000; Fernandez-Fernandez et al., 2001; Jiang et al., 2002; Martin-Alonso et al., 2003; Farnos et al., 2005; Gil et al., 2006). However, recombinant vaccines for use with low value animals such as rabbits are seriously restricted in their production costs.

Production of recombinant proteins in insect larvae has been reported as a suitable method of inexpensive production of large amounts of recombinant proteins for a wide range of fields. Although this technology can be applied to practically all proteins which can be expressed in insect cell lines by recombinant baculoviruses, few papers have reported expression of recombinant vaccine antigens in insect larvae (Johansson et al., 1995; Matsuoka et al., 1996; Choi et al., 2000; Barderas et al., 2001; Pang et al., 2002).

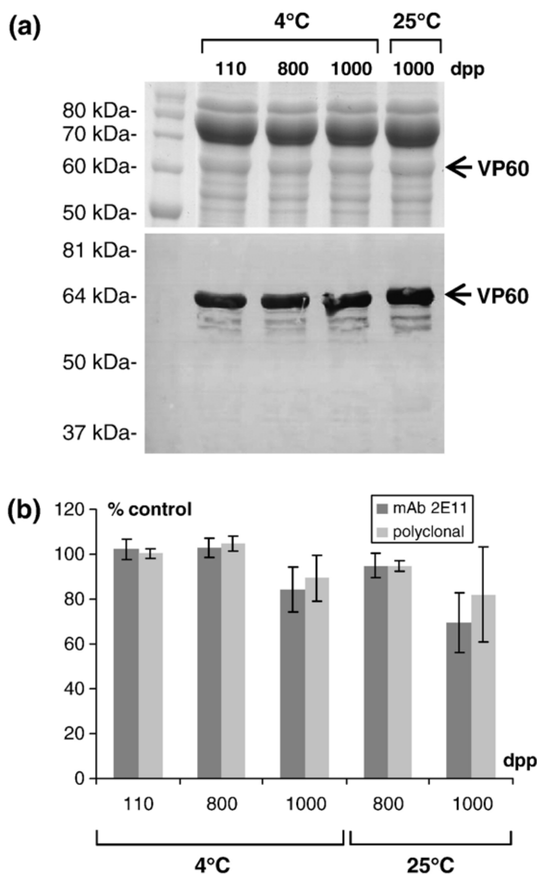

Fig. 5. Stability of VP60r in lyophilized larvae crude extracts. Crude protein extracts were obtained from VP60BAC infected larvae. Aliquots were kept at 4 °C or 25 °C over the indicated time (4 fractions per time and storage temperature). They were then analyzed by SDS-PAGE and ELISA. (a) A representative Coomassie blue-stained gel and corresponding Western blot assay using mAb 2E11 as a probe. In both cases, arrows indicate the position of VP60r. (b) ELISA results indicating the percentage of mean OD obtained by the 4 fractions at each point in time and temperature compared with the control fractions stored at −20 °C. VP60r was detected using mAb 2E11 (dark bars) or a pool of polyclonal sera from rabbits immunized with conventional RHDV vaccine (light bars) as probes. Standard deviations are the vertical lines in each bar.

In this work we present a recombinant version of VP60 from RHDV strain AST/89 which was readily produced in *T. ni* larvae as detected by different electrophoretic and immunological methods. The sizing and quantification of larval produced VP60r, determined by capillary electrophoresis indicated that the recombinant product was mainly a single species with an electrophoretic mobility of 61.5 kDa and reached 12.5% of total extracted protein. Immunoblotting revealed the presence of two smaller bands with mobilities of between 50 and 55 kDa which were recognized by mAb 2E11 reacting against the N-terminal half of the VP60. Proteolytic processing of recombinant versions of VP60 has also been reported in other expression systems, such as plants (Fernandez-Fernandez et al., 2001; Gil et al., 2006). The fragments detected were probably derived from proteolytic cleavages in the larvae as they differed in size from those of plants and other naturally occurring fragments described for RHDV (Granzow et al., 1996).

Yields of VP60r in larvae correspond to ~2 mg of recombinant protein per infected animal. We suspect, but did not confirm, that these high yields for VP60r in larvae may be supported to some extent by multimerization of the protein that prevents degradation by cellular proteases. Supporting this, the results for larval extracts processed through sucrose gradients showed positive signals for VP60 at different densities along the gradient (data not shown). Further work is needed to substantiate this hypothesis.

The amount of VP60r obtained from 10 infected larvae corresponded to 1 l (10^9 cells) of an infected Sf9 culture (Laurent et al., 1994) or 20 to 40 l of recombinant *Saccharomyces cerevisiae* (Boga et al., 1997). Farnos et al. (2005) reported expression of a recombinant VP60 from RHDV in *Pichia pastoris*, with levels around 1.5 g/l of culture media. However, recovery of vaccinal VP60 antigens from *P. pastoris* cultures involved a significant amount of post-production processing which including an enrichment step of denaturation and renaturation, and an enzymatic deglycosylation of VP60-enriched preparations. This gave a ~60% decrease in VP60 antigen compared to the unprocessed cultures.

In contrast, VP60r antigen from larvae results from a fairly simple extraction protocol and retain the antigenic features of native VP60. More importantly, they are suitable for immunization as crude preparations. Intramuscular immunization showed that a single dose of an experimental oil vaccine formulated with classical complete Freund adjuvant protected 100% of rabbits (8/8) with a dose of only 2 μg of VP60r. Animals rapidly developed a systemic anti-RHDV response; rabbits receiving 2 μg of VP60r+FCA had significant RHDV-specific serum antibody titers by 7 dpi with mean antibody levels (n=8) more than 260 times higher than p54-vaccinated controls (ELISA titer=2.42±0.31, data not shown). In all cases (30/30) larger doses of VP60r, in this formulation, were also protective against RHDV challenge at 30 dpi. As RHDV mostly affects systemic

tissues, rapid induction of high levels of specific serum antibodies gave subsequent protection against a lethal dose of RHDV. This clearly shows the effectiveness of VP60r administered IM. Rabbits immunized with 40 μg and 20 μg VP60+ FCA did not increase their antibody levels after an RHDV challenge. This probably indicates that virus replication ceased or was significantly reduced in these animals. Only one animal which had been immunized with a crude extract containing 40 μg of VP60r in PBS succumbed to viral challenge. In all cases (3/3) rabbits which received 100 μg of VP60, without adjuvant, were protected.

Based on production yields and IM immunization results, the amount to VP60r accumulated in a single larva would give ~1000 IM vaccine doses. For an annual production of 10^6 vaccine doses, we estimate a cost of 0.000044 €/dose, including rearing larvae, inoculation and post-harvesting processing of recombinant protein. The average cost of insect cell media is ~\$250 l^{-1} and some of them also require addition of fetal calf serum. Production of 10^6 vaccine doses using a VP60r protein produced in Sf9 cells by fermentation (about 100 l), represents an estimated cost of 0.021 €/dose, a 477 times higher production cost.

Oral vaccination gives a strategy for controlling diseases that persist among wildlife. Oral immunization with encapsulated VP60r extracts through was less reliable. While some rabbits developed specific antibodies against RHDV, the humoral responses were insufficient to provide protection to a virus challenge for most of the rabbits. Only one animal, which had received two doses of 400 μg VP60r survived to RHDV infection. A second set of experiments with oral priming and an IM boost with a subimmunogenic dose of VP60r, gave 3 surviving rabbits out of 12. However, there was no clear correlation between protection and the oral immunization dose in this group. It is possible that the observed protection relied on the injected VP60r rather than the orally administered antigen as VP60r is highly immunogenic when administered IM. Similarly, oral immunization using RHDV VLPs produced in insect cell lines using the same VP60BAC vector did not induce a protective humoral response in 2 out of 3 immunized rabbits. This was despite immunization doses (50 μg) which were 10 times those reported by Plana-Duran et al. (1996) to protect rabbits. These observed results may be due to a combination of factors such a poor delivery of the antigen and oral tolerance induced by VP60r when it is administered this way. Although the oral immunization assays did not included adjuvants and alternative immunization protocols were not tested, these results agree with reports of problems in obtaining systemic antibody responses in rabbits when orally immunized with subunit-based immunogens (Martin-Alonso et al., 2003; Molina et al., 2005). Further work is required to determine if oral immunization is an adequate route for general rabbit vaccination or not.

Finally, the stability experiments showed that the integrity and antigenicity of VP60r, in lyophilized extracts, were not significantly affected by storage for more that 2 years when stored at 4 °C or 25 °C. There was only a small decrease in antigenicity in samples kept at RT for over 32 months. These results are important as proteins in complex extracts are commonly exposed to proteolytic degradation by cellular proteases in the preparation. This extended stability would favor production and storage of the antigen as well as handling of the recombinant immunogen during vaccine manufacturing and distribution.

In conclusion, an immunogenically active recombinant VP60 protein from RHDV was produced by the baculovirus-insect expression system. The protein yields obtained would make it possible for use as a commercial vaccine. This strategy would avoid utilization expensive fermentation procedures, facilitate the scaling-up of production through fairly inexpensive technology. More importantly, its use could replace older production methods still used in RHDV vaccine production, eliminate the chance of viral escape and occurrence of animal infection reservoirs. Along with this, a lack of crossed infection agents between *T. ni* and vertebrates generally would facilitate its approval and application in veterinary medicine. Overall, our results show that the baculovirus–larvae system may be of considerable value for inexpensive production of vaccinal antigens with low associated biosafety risks. This supports the development of this technology for other disease models.

Materials and methods

Recombinant baculovirus

The generation of recombinant baculoviruses expressing full length VP60 protein from RHDV AST/89 strain (VP60BAC; Barcena et al., 2000) and p54 protein from African swine fever virus E70 strain (p54BAC) was previously described (Oviedo et al., 1997). Recombinant baculoviruses were propagated in sf21 insect cells to reach infective titers of ~10^8 pfu/ml (O'Reilly et al., 1992). Working stocks were kept at 4 °C.

Insect growth conditions and inoculation

T. ni (cabbage looper) larvae were reared under non-sterile, level-2 biosafety, conditions as described in Perez-Filgueira et al. (2006). Fourth instar larvae were intracoelemically injected near the proleg with 10 μl of recombinant baculovirus diluted to reach the number of pfu per dose selected for each experiment. Inoculated larvae were kept in growth chambers at 28 °C and collected at the indicated times and frozen immediately and kept at −20 °C until processed.

Preparation of insect protein extracts

The VP60r crude protein extracts for all experiments were obtained as described by Perez-Filgueira et al. (2006). Briefly, frozen material was homogenized in protein extraction buffer [phosphate buffered saline, pH 7.2, 0.01% Triton X-100, 1% sodium dodecyl sulfate, 2.5 mM dithiothreitol, 10 mM β-mercaptoethanol and a protease inhibitor cocktail (Complete, Roche, Germany)]. The resulting suspension was clarified by centrifuging at 10,000×*g* for 10 min. The supernatant was filtered through Whatman paper, and recentrifuged. The amount of total soluble proteins (TSP) was quantified by the Bradford

assay (Bradford, 1976). Aliquots of bulk extracts were freeze dried overnight in a Telstar Cryodos Freeze-Dryer (Telstar, Spain) and fractions stored at 4 °C until use.

Time–dose assays

Groups of 75 larvae were inoculated with 2.5×10^4, 10^5 or 4×10^5 pfu of VP60BAC and further separated into 5 groups of 15 insects each that were processed at 48, 60, 72, 84 and 96 h post-inoculation (hpi). Samples were analyzed by SDS-PAGE, Western blot, ELISA and capillary electrophoresis. Crude total protein extracts were obtained as described above and 20 μg of TSP per lane was resolved in 10% SDS-polyacrylamide gels for Coomassie blue staining or transference onto nitrocellulose membranes (Bio-Rad, USA) for immunoblotting analysis. Blotted membranes were blocked overnight with PBS-0.05% Tween 20 (PBST) 4% skim milk and were subsequently incubated at room temperature (RT) with a VP60-specific mAb (2E11, Martinez-Torrecuadrada et al., 1998) and anti-mouse IgG-horseradish peroxidase (HRP)-labeled conjugate as a secondary antibody (Amersham, USA), both diluted 1/2000 in blocking buffer. Protein bands were detected using the ECL Western blotting detection system on Hyperfilm ECL films (Amersham, USA). ELISA assays were conducted by coating Maxisorp plates (Nunc, Denmark) with 100 ng of TSP from each sample diluted in 50 mM carbonate/bicarbonate buffer at pH 9.6. Plates were incubated overnight at 4 °C and next day were washed with PBST and blocked for 1 h at RT using PBST 2% bovine serum albumin (BSA). The VP60r was detected using 2E11 mAb and anti-mouse IgG-HRP conjugate (Amersham, USA); both diluted as for the immunoblotting assays but using PBST 2% BSA as a dilutent instead. The reaction was developed using a color substrate containing 4 mg/ml *o*-phenylenediamine (OPD)-0.08% H_2O_2 in 0.05 M phosphate-citrate buffer pH 5.0. The peroxidase reaction was allowed to develop for 2 min at RT. It was stopped by the addition of H_2SO_4 3N and read at 492 nm in an ELISA microplate reader (Multiskan EX; Thermo Electron Corp.). Quantification experiments were performed by capillary electrophoresis using the Experion system (Bio-Rad, USA) following the manufacturer's instructions. Briefly, crude protein samples (1 μl) were mixed with sample buffer provided, heated and diluted to 1/90 in deionized water, and 6 μl of each diluted sample was loaded in Pro260 chips (Bio-Rad, USA) for analysis.

Animals and immunizations

Two-month old New Zealand White rabbits, free from anti-RHDV antibodies, were obtained from a local commercial breeder and intramuscular (IM) and oral immunization experiments were conducted.

In the first set of experiments, 36 rabbits were given a single IM immunization of different amounts of crude larvae extract. Thirty rabbits were vaccinated with VP60r-containing extracts which had been emulsified with Freund's complete adjuvant (FCA) in a final volume of 1 ml. Vaccinated animals were given vaccines containing 40 μg ($n=3$), 20 μg ($n=3$), 10 μg ($n=8$), 5 μg ($n=8$) or 2 μg ($n=8$) of VP60r. Six more rabbits were immunized with crude VP60BAC extracts from infected larvae without FCA. In this case, the doses were 100 μg ($n=3$) or 40 μg ($n=3$) of VP60r. As negative controls, 3 rabbits were immunized with larvae extracts containing 100 μg of a recombinant p54 protein (p54r) from African Swine Fever Virus (ASFV) emulsified in FCA. All rabbits were challenged, intramuscularly, with 100 LD50 of RHDV (Spanish isolate AST/89) 30 days post immunization (dpi). Blood samples were collected from the marginal ear vein of each animal prior to immunizations and at 15 and 30 dpi and 15 days after challenge.

For oral immunization, different amounts of lyophilized crude extract were placed into 300 mm^3 capsules. A group of 11 rabbits received two doses (at 0 and 15 dpi) of encapsulated larval extract containing 400 μg ($n=3$), 200 μg ($n=3$), 50 μg ($n=3$) and 20 μg ($n=2$) of VP60r. A second group of rabbits (4 groups of 3 animals) was orally primed with each of the 4 doses and was boosted intramuscularly at 39 dpi with crude larval extract containing 10 μg VP60r in PBS (1 ml final volume). Three rabbits were immunized orally twice (at 0 and 15 dpi) with 50 μg of VP60 virus-like particles (VLPs) treated with 5 mM binary ethylenimine as described by Bahnemann (1990) and Plana-Duran et al. (1996). Two control rabbits were orally immunized once with 400 μg of encapsulated larval extract containing ASFV p54 and 2 animals with a single IM inoculation of a vaccine containing 100 μg of VP60r in FCA. All rabbits were challenged at 54 dpi as in the IM immunizations and blood samples were taken at 25 and 53 dpi and 10 days after challenge.

ELISA for RHDV-antibodies

To evaluate the serological response of rabbits immunized with VP60r larval extract, VP60r virus-like particles (VLPs) were used as an antigen. VP60r VLPs were obtained from *T. ni*-derived H5 cell cultures (Invitrogen) infected with VP60BAC baculovirus and purified and quantified as described by Barcena et al. (2000). ELISA plates (Polysorp, Nunc, Denmark) were coated with 0.1 μg/well VP60r VLPs and incubated overnight at 4 °C. Plates were blocked with PBS-5% skim milk for 2 h at 37 °C, washed five times with PBST and incubated with serial dilutions of sera samples. After several washes with PBST, plates were incubated at 37 °C for 1 h with protein A-HRP conjugate (Sigma) at 1/1000 dilution. Finally, plates were extensively washed with PBST and color was developed and detected as in the time–dose experiments. In some experiments, ELISA titers were expressed as log10 of the reciprocal of the highest serum dilution that gave OD readings which were at least twice the mean OD produced by sera from control rabbits (p54r-immunized animals, $n=3$) at the same level of dilution.

VP60 stability control

Protein extracts from VP60-infected larvae ($n=30$) were taken and quantified for total soluble protein by the Bradford assay. The extract was fractionated in 1 ml aliquots. One third of the aliquots were kept at −20 °C, while the remainder were lyophilized. Half the lyophilized samples were stored at 4 °C and the rest were

maintained at a RT of 25 °C. Four lyophilized samples kept at 4 °C or RT were reconstituted in distilled water at 110, 800 and 1000 days post-production (dpp). The presence of VP60 in the samples was detected by SDS-PAGE, Western blot and ELISA which were compared to the frozen control aliquots. The SDS-PAGE and immunoblotting assays were conducted as described for time–dose experiments but 10 μg of TSP was loaded per lane. ELISA was performed by coating plates with crude extracts (50 ng TSP/well) and detection used 2E11 mAb or a pool of polyclonal sera from rabbits ($n=3$) vaccinated with a conventional RHDV vaccine. Assays using rabbit sera were carried out as described previously for mAb 2E11, except that anti-rabbit IgG-HRP (Amersham, USA) was used as a secondary antibody. Results, in both cases, were expressed as the percent of the mean OD from each sample compared to that of the control fractions.

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