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Oral immunization of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease

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Summary. Rabbit hemorrhagic disease virus (RHDV) causes more than 90% mortality in adult rabbits. In this study, the cDNA of the VP60 coding sequence of RHDV was cloned under the control of the polyhedrin and p10 promoters of baculovirus to be expressed in insect cells. The expression of RHDV VP60 under the control of the p10 promoter was 5–10 times higher than using the polyhedrin promoter. The p10-derived VP60 was able to assemble into virus-like particles (VLPs). RHDV VLPs were successfully used to protect rabbits against the disease even at doses as low as 0.5 μg when injected intramuscularly or sub-cutaneously. The ability to elicit an immune response was independent of the adjuvant or the route of immunization. Remarkably, oral administration of RHDV VLPs efficiently induced protecting antibodies to RHD at doses as low as 3 μg. The use of binary ethylenimine for the stabilization of the VLPs was decisive for eliciting a good oral immunity. This report demonstrates the potential use of these procapsids in obtaining RHD oral vaccines and opens the door to the use of these capsids for the prevention of the disease in wild animals. Therefore, a new, and potentially important application of recombinant VLPs in the induction of protective immunity by the oral route is foreseen.

Introduction

Rabbit hemorrhagic disease virus (RHDV) is the causative agent of a highly contagious and fatal disease in rabbits. The disease was first described ten years ago in China [9], and subsequently has appeared in most European countries [15, 19]. RHDV is a member of the family *Caliciviridae* [16, 18], that also includes feline calicivirus, San Miguel sealion virus, Norwalk virus

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and swine vesicular exanthema virus. Studies on RHDV have been hampered by the fact that the virus does not replicate in stable cell lines, and therefore it has to be obtained by in vivo propagation in rabbits. In fact, current vaccines are based on formalin-inactivated liver homogenates of infected animals [17].

RHDV is a non-enveloped virus with a capsid of icosahedral symmetry and 40 nm in diameter [16]. The genome consists of a single-standed RNA molecule of positive polarity of about 8 kb with a short 3′ poly(A) tail. The whole viral genome has been recently cloned and sequenced [13]. It contains one long open reading frame that represents 86% of the viral genome, in which the 5′ and 3′ regions encode non-structural and structural viral proteins, respectively. It has been reported that RHDV, like other caliciviruses, specifies a subgenomic RNA of approximately 2.2 kb, in addition to the genomic RNA [14]. Both RNAs are protein-linked and packed into particles, conferring protection from nuclease degradation. The major RHDV structural component of the capsid has been identified as a protein of 60 KDa, hereafter called VP60. Active immunization with purified viral VP60 conferred total protection after a virus challenge [18]. Recently, the VP60 encoding cDNA has been cloned and expressed in *E. coli* [2] and the baculovirus expression system [8]. The fusion protein expressed in *E. coli* was highly insoluble and of low immunogenicity. In contrast, the protein expressed in the baculovirus system was soluble, partially secreted to the culture medium and able to assemble into virus-like particles (VLPs). However, from the previous report [8], it was difficult to value the immunogenicity of these capsids in terms of vaccine development, since the immunization experiments were limited to a high amount of antigen (100 μg/animal) that would render the vaccine very expensive from a practical point of view. Also, since one of the most devastating aspects of this disease is its incidence on wild animals, we consider necessary to study the immunogenicity of these particles by oral route as an alternative to parenteral immunizations in field trials. At the present time, oral vaccination is the only economical and practical way to administer a vaccine for mass immunization of wild animals [3, 4].

In this report we have tried to study in more detail the production and immunogenicity of RHDV VLPs. To this end we have expressed the VP60 protein under the control of the polyhedrin and p10 promoter of baculovirus, the latter being optimal for the expression of parvovirus VLPs [5]. With the product obtained, a study focussed on dose and immunization route (parenteral and oral) has been carried out to investigate the cost-effectiveness of this vaccine in rabbits and its possible application to wild animals.

Materials and methods

Cells and viruses

The *Spodoptera frugiperda* cell line Sf9 (ATCC # CRL 1711) was used to propagate parental and recombinant baculoviruses. Sf9 cells were grown in suspension or as monolayer cultures in TNM-FH medium [24] supplemented with 5% fetal calf serum and antibiotics. *Auto-*

grapha californica nuclear polyhedrosis virus (AcNPV) wild type (wt) (a gift from Dr. Summers, Texas A&M University, Texas, USA) was used as parental for the transfections based on the p10 promoter. AcRP23-lacZ$^+$ strain of AcNPV [20], kindly provided by Dr. Possee (IVEM, Oxford), was the parental baculovirus when the polyhedrin promoter was used.

The RHDV isolate used for these studies was obtained from the livers of infected rabbits in Olot (Spain) [19] and then passaged three times more in rabbits before preparation of viral stocks as crude liver homogenates. RHDV Olot/89 was propagated in outbred rabbits, after intranasal inoculation with filtered liver homogenate.

RHDV purification, RNA preparation and cDNA synthesis

RHDV virions obtained from the liver of infected animals, 3 to 4 days post-infection, were partially purified as described by Ohlinger et al. [16]. After a 15–30% sucrose density gradient, two major viral fractions were obtained. Band I (corresponding to 175S particles) showed the highest degree of virion homogeneity and purity, as determined by electron microscopy, and was the starting material for RNA preparation. A volume of 200 µl of virions (Band I) was treated with 1% SDS, 0.5 mg/ml proteinase K and 2 mg/ml pronase for 30 min at 37 °C and then extracted twice with phenol: chloroform prior to ethanol precipitation. For cDNA synthesis, 1 µg of poly(A)$^+$-RNA was incubated with 0.8 µg phosphorylated oligo $d(T)_{12}$ and 40 units of AMV reverse transcriptase in the presence of 1 mM dNTPs (including α-[^{32}P]dCTP as tracer) and 25 units of RNAsin for 1 h at 42 °C. The second strand synthesis was carried out with *E. coli* DNA polymerase for 1 h at 22 °C and 10 min at 65 °C, the cDNA was blunt-ended with T4 DNA polymerase, phenol-extracted and ethanol-precipitated. The total cDNA was loaded in 1% agarose gels and only those molecules comprised between 2 and 5 kbp -which was the range estimated to contain the VP60 gene- were selected. The cDNA was recovered from the agarose gels using DEAE-cellulose membranes (Schleicher & Schuell, FRG) according to the manufacturer's protocols and finally resuspended in TE buffer.

cDNA cloning and construction of baculovirus transfer vectors

RHDV cDNA was cloned into *Sma*I-digested pMTL25 and transformed into *E. coli* XL1 blue cells using standard procedures. Positive clones were identified by restriction endonuclease analysis and confirmed by DNA sequencing [23] and comparison with a previously reported RHDV sequence [13].

One of the selected clones, called pRHDV24, contained an insert of 2134 bp, which started 5 bp downstream of the initiation codon of the VP60 gene. Thus, to provide this gene with a new initiation codon, the cDNA fragment was released from pRHDV24 by partial *Bam*HI digestion and subcloned into the *Bgl*II site of the multiple cloning site of pMTL22. This new plasmid was called pRHDV24′. To facilitate further cloning in *Bam*HI sites and to remove the 3′ non-coding region, the fragment was digested with *Nco*I and *Eco*RI, repaired with Klenow and ligated into *Sma*I-digested alkaline phosphatase-treated pMTL25. This clone was called pRHDV7. The modified VP60 gene was removed from pRHDV7 by partial *Bam*HI digestion and ligated into the baculovirus transfer vectors pAcYM1 [10] and pAcAs3 [24], under control of polyhedrin and p10 promoters, respectively. Positive recombinants were identified by restriction mapping and sequencing across the *Bam*HI insertion sites to confirm correct gene orientation and sequence integrity after the successive modifications (Fig. 1).

Cotransfection of insect cells and selection of recombinant baculoviruses

Sf9 cells were transfected with a mixture of 500 ng of AcNPV wt or linearized AcRP23-lacZ$^+$ DNA and 2 μg of the transfer vector DNA using the lipofectine technique [6]. Culture supernatants from the cotransfection were harvested 7 days post-transfection and used for plaque assays. The recombinant viruses were selected by their blue-white phenotypes. The efficiency of recombination was variable, with more than 30% white (recombinant) plaques for the polyhedrin selection and much lower for the p10 promoter (blue plaques). Recombinant baculoviruses were plaque-purified before high-titer viral stocks were prepared for each virus.

Analysis and purification of recombinant RHDV VP60 particles

Sf9 cells were infected with the recombinant baculoviruses at a m.o.i. of 1 pfu/cell. At 3 days post-infection, cells were collected, suspended in lysis buffer (5% SDS, 1% 2-mercaptoethanol and 17.4% glycerol) and heated at 100 °C for 5 min. Proteins were separated by 9% SDS-PAGE electrophoresis and visualized with Coomassie blue. The levels of expression were estimated by comparison with known amounts of bovine serum albumin run in parallel lanes. For immunoblotting, proteins were transferred onto nitrocellulose membranes using a semidry device (BioRad) at 22 V for 30 min. For detection of RHDV proteins, the membranes were incubated with rabbit serum anti-RHDV (dilution 1:100) and the bound antibodies were detected using a peroxidase-labelled Protein A (Sigma) and 4-chloronapthol (Sigma) as substrate. The reaction was stopped by rinsing the strips with distilled water.

RHDV VP60 particles were purified from insect cells according to the procedures described previously for canine parvovirus VP2 particles [10]. Purity of VP60 preparations was usually higher than 95% (Fig. 2c). For the immunization experiments, protein concentration was quantified by a Bradford assay (BioRad).

ELISA

For detection of anti-RHDV antibodies, a new ELISA based on the recombinant VLPs was set up. Microtiter plates (Polysorb, Nunc, Denmark) were coated with 0.25 μg of recombinant VP60 particles in 0.05 M carbonate buffer (pH 9.6) at 4 °C overnight. Rabbit sera were serially diluted in 0.1% skimmed milk in PBS and incubated at 37 °C for 1 h. Washes between consecutive steps were carried out with 0.05% Tween 20 in PBS. After washing, plates were incubated with peroxidase-conjugated Protein A (dilution 1:4 000) in the same buffer at room temperature for 1 h. Plates were washed, and 2,2′-Azinobis-(3- ethylbenzothiazoline-sulfonic Acid) (ABTS) (Sigma) was used as substrate. Reaction was stopped with 1% SDS after 10 min and the absorbance measured at 405 nm. Cutoff values for the RHDV ELISA were established previously by evaluating more than 50 negative serum samples. Rabbit sera with ELISA values greater than four times the mean value of the absorbance for known negative sera were considered positive.

Rabbit immunization and challenge

Five groups of New Zealand White female rabbits (5–6 weeks old), seronegative to RHDV by inhibition of hemagglutination using human erythrocytes, were immunized with different doses of purified VP60 particles from 50 to 0.5 μg (Table 1, Groups A to E). Two animals per dose were injected intramuscularly with 1 ml of vaccine, consisting of an emulsion of the purified VP60 particles in PBS with oil adjuvant at a ratio 50:50 (V/V). Oil adjuvant consisted of a mixture of 82.3% marcol-82 (ESSO), 6.5% Emulgin-48 (Henkel), 10% montanide-80

(Seppic) and 1.2% Benzylic alcohol (Elf Atochem). As positive control, two animals were vaccinated with a commercially available inactivated RHDV vaccine (CYLAP HVD, Cyanamid) (Group F). Four animals were used as negative controls immunized with a placebo containing only the oil adjuvant and one animal was left as sentinel (Groups G and H). The booster was given 21 days after the first immunization. Rabbits were bled by cardiac puncture at days 0, 14, 21 and 35 from the primary immunization. In parallel, the same number of animals was immunized by subcutaneous route, to check the effect of the route of administration. In other set of immunizations, two groups of animals were immunized with two doses of antigen (0.5 and 25 μg, respectively) using alumina and Quil A as adjuvants, by subcutaneous and intramuscular route.

Thirty six days after the primary immunization, all the rabbits were challenged by intranasal route with 3.6×10^4 LD_{50} of virulent RHDV in 0.5 ml of PBS. Animals were daily observed, and clinical manifestations of the disease were monitored until day 14 post-infection (p.i.). Animals that survived the infection were bled at days 6 and 14 p.i. and, finally, euthanized on day 14 p.i. The livers from the vaccinated and control animals were homogenized immediately after death and tested for the presence of RHDV by hemagglutination (HA) as described previously [16].

Oral immunization and challenge

Fifteen outbred rabbits were used for the experiments. Five rabbits were immunized with 3 μg of recombinant RHDV VLPs, treated with 5 mM binary ethylenimine for 3 days at 37 °C [1], in 1 ml of PBS at days 0 and 21. The immunization was done with a syringe by oral route. Another group of five rabbits was immunized likewise but using non-treated VLPs. Finally, there was a control group of five rabbits treated only with PBS. The challenge with the virulent virus was done in the same conditions as described above.

Results

Construction of recombinant baculoviruses

Clone pRHDV24 was used for the isolation and subsequent expression of the RHDV VP60 gene. Since the first 5 bp of the VP60 gene were lost in this construct, we engineered a small fusion protein to obtain VP60 expression by subcloning the cDNA into the polylinker of the plasmid pMTL22. The new construct resulted in a fusion protein containing 5 extra amino acids (Ala-Cys-Ile-Asp-Arg) in the N-terminus of the VP60 (Fig. 1). Afterwards, the modified VP60 gene was manipulated to remove the 3′ non coding sequence and, finally, ligated into the baculovirus transfer vectors pAcYM1 for polyhedrin and pAcAs3 for p10 promoter. Sequencing of the recombinant clones containing the 1.7 kbp insert in the correct orientation confirmed that no mutations had been introduced. A 95% homology was found between our nucleotide sequence and that previously reported for the German isolate [13]. The similarity increased up to 97.8% when the VP60 amino acid sequences were compared. The selected recombinant clones were called pAcRHDV-710 and pAcRHDV- 729 for polyhedrin and p10 promoter, respectively. The distance between the *Bam*HI site and the initiation of transcription was only of 2 bp (Fig. 1).

Recombinant baculoviruses were derived by cotransfection of Sf9 cells with DNA from AcRP23-lacZ$^+$ or wt and the respective transfer vectors. The

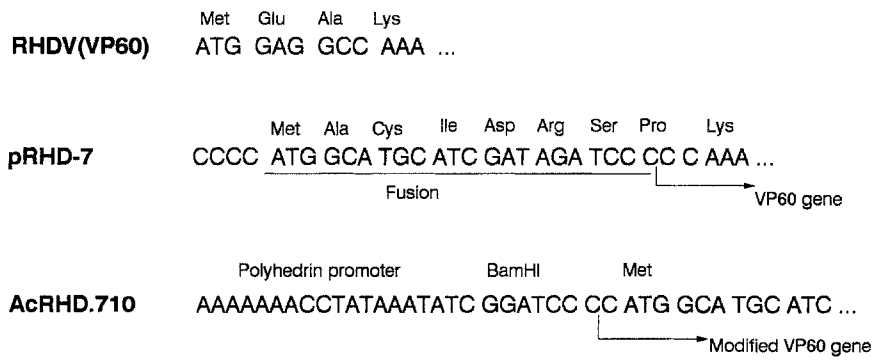

Fig. 1. Sequences of the original and modified N-terminal region of the RHDV VP60. The 5′ junction sequence in the AcRHD-710 is shown underneath, indicating the position of the modified VP60 gene relative to the polyhedrin promoter

efficiency of recombination and production of recombinant baculoviruses was 10 times higher when the linearized AcRP23-lacZ$^+$ was used. After successive rounds of plaque purification, high-titer stocks ($> 10^8$ ufp/ml) were prepared for the recombinant baculoviruses AcRHDV710 and AcRHDV729, which made use of the polyhedrin and p10 promoter, respectively.

Expression of RHDV VP60 by recombinant baculoviruses

Sf9 cells infected with both recombinant baculoviruses were analyzed by gel electrophoresis and immunoblotting to investigate the expression of the VP60 protein. A protein similar in size to that expected for VP60 was readily visualized by Coomassie blue staining (Fig. 2a) in both cases. When longer electrophoresis were made, the recombinant modified VP60 showed a slightly slower migration than the viral VP60 as a consequence of the 5 amino acids fused at the N-terminus. The cellular location of the recombinant VP60 was investigated lysing the infected cells by osmotic shock with a 25 mM sodium bicarbonate solution. The nuclei and cellular debris were sedimented by centrifugation. The different fractions were analyzed by SDS-PAGE and the recombinant protein was mainly found in the cytoplasmic fraction. Identity of this protein was confirmed as RHDV VP60 by immunoblot analysis (Fig. 2b), using RHDV-specific polyclonal rabbit antisera. The levels of expression were moderate, between 20 and 40 $\mu g/10^6$ cells, with the polyhedrin promoter. But, as expected, there was a large increase in the expression, around 200 $\mu g/10^6$ cells for the p10 promoter.

In both cases, the recombinant VP60 was partially released to the culture medium as detected by HA analysis of the culture supernatants and purified preparations of VLPs cell-associated. However, the proportion of recombinant VP60 present in the supernatant of the culture was 15 times lower than that associated to the cellular fraction. Since this low proportion, it is possible that the release occurs only after the cellular lysis. Therefore, only VP60 particles

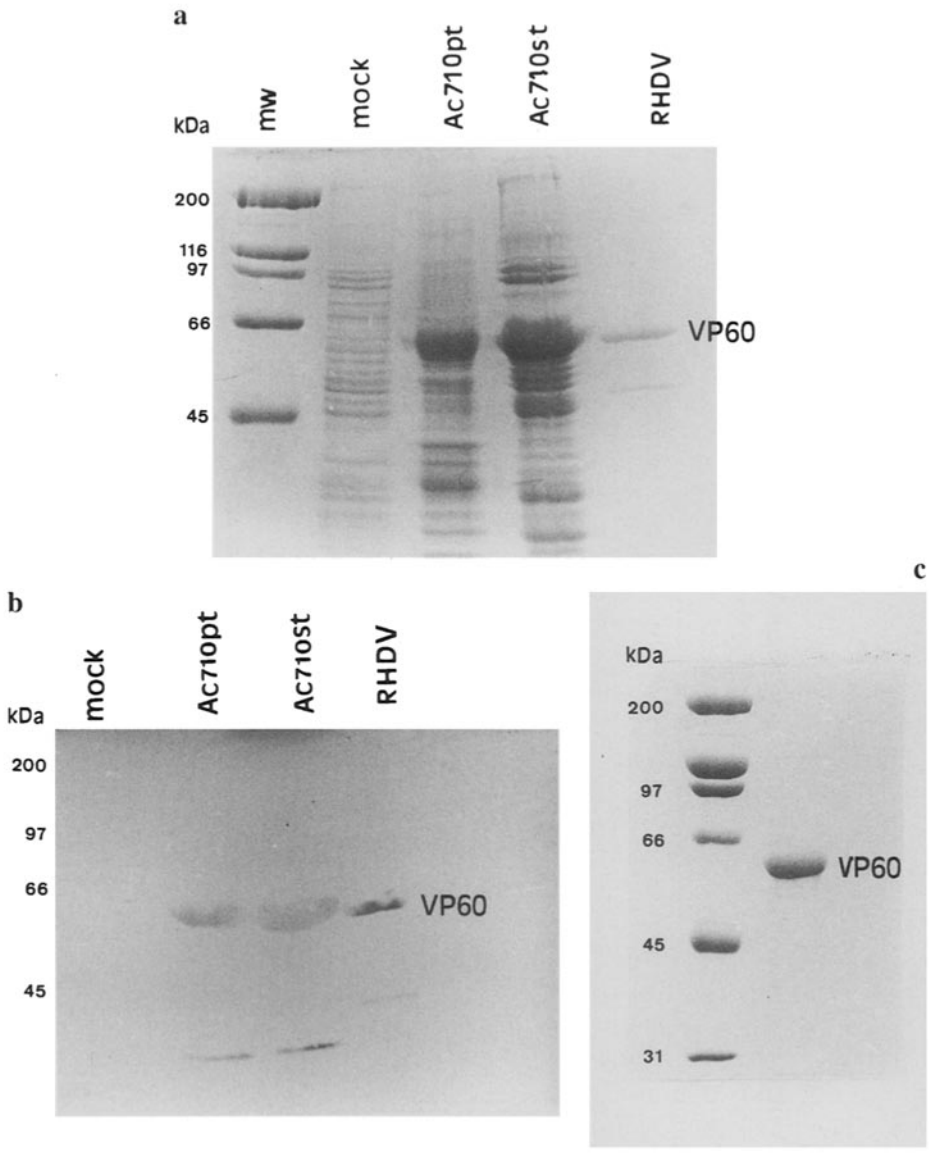

Fig. 2. Analysis of VP60 expression in insect cells. **a** Coomassie blue stained SDS-polyacrylamide gels of AcRHD710 insect cells harvested at 72 h.p.i. *MW* Molecular weight markers, *mock* total extract of mock infected Sf9 cells, *Ac710pt* pellet from infected cells after sonication, *Ac710st* supernatant from infected cells lysed by sonication, *RHDV* RHDV virions (Band I). **b** Western blot analysis of recombinant proteins. Bound antibody was detected as described in Materials and methods. **c** Coomassie blue stained SDS-polyacrylamide gels of RHDV VP60 VLPs purified by cell lysis and 20% ammonium sulfate precipitation

obtained from the cytoplasmic fraction of infected cells were used for immunization studies. Figure 2c shows a typical example of purified VP60 obtained after osmotic cell lysis and precipitation of the supernatant with 20% ammonium sulfate.

Table 1. Serum ELISA titers of rabbits immunized with recombinant VP60 and challenged with RHDV

Dose	Group	No. of animals	Days post immunization				Days post challenge	
			0	14	21	35	6	14
0.5 μg	A	2	–[a]	–	–	6,600	45,000	160,000[b]
3 μg	B	2	–	–	–	4,000	12,500	600,000
10 μg	C	2	–	300	3200	30,000	80,000	480,000
25 μg	D	2	–	2400	9600	60,000	40,000	160,000
50 μg	E	2	–	6400	128000	80,000	240,000	240,000
Commercial	F	2	–	1200	8000	40,000	60,000	160,000
Oil adjuvant	G	4	–	–	–	400	†[c]	†
None	H	1	–	–	–	–	†	†

Rabbits were immunized at days 0 and 21 with the indicated doses of RHDV VP60 in oil adjuvant by intramuscular route. They were challenged 2 weeks later with 3.6×10^4 LD_{50} of RHDV

[a] ELISA titer <200. Titers are expressed as reciprocals of the highest dilutions of serum that gave four times the value of the blank. Table 1 shows the arithmetic means of the titers of the animals vaccinated

[b] Corresponds to a single animal

[c] Animals dead after challenge

Immunogenicity of recombinant VP60 capsids

The immunogenicity of the recombinant VP60 particles was studied by vaccinating rabbits with five different doses (0.5, 3, 10, 25, and 50 μg). Serum samples were analyzed for the presence of VP60-specific antibodies by ELISA using recombinant VP60 particles as coating antigen.

All of the rabbits immunized with the recombinant antigen exhibited VP60-specific antibodies. Tables 1 shows the ELISA titers for the rabbits immunized by intramuscular route. However, no significant differences were observed in the antibody response when the rabbits were injected subcutaneously or intramuscularly. Antibody levels increased with the antigen dose, those receiving a dose $\geqslant 10$ μg developed antibodies as soon as 14 days post-vaccination (Table 1). Antibody titers corresponding to doses to 10 μg and higher were equal or superior to those obtained with the commercial vaccine. Nonetheless, doses as low as 0.5 μg were able to induce a significant antibody response after the booster. The antibody response increased until day 35, except for the highest dose of recombinant VP60 (50 μg) where a plateau of antibody titers were observed between 21 and 35 days p.i. This is probably due to antigenic saturation at high doses of antigen. With larger amounts of VLPs (10–50 μg), one dose seems sufficient to properly immunize the animals. When these sera were analyzed by hemagglutination inhibition, instead of ELISA, the serum titers followed a similar pattern, although the assay sensitivity was approximately 5 times lower than the ELISA (data not shown). Dut to the high sensitivity of this ELISA, we observed a slight increase in the anti-RHDV titers in the sera of control animals injected only with oil adjuvant. The response to the adjuvant was probably polyclonal and unspecific. This effect was not observed in the unvaccinated animal (Table 1).

The role of the adjuvant was also investigated. An adjuvant containing alumina and Quil A was used with the doses of recombinant antigen (0.5 and 25 μg). The antibody response was similar to that observed with oil adjuvant, with a slight increase in the serum titers of the vaccinated animals and no unspecific response in the control animals (data not shown). These results could be explained because the conformation of the particulate antigens is more preserved when adsorbed to alumina than when emulsified with oil adjuvant. No side effects were observed in the animals during the immunization experiments described here.

Protection against RHD

Rabbits were challenged 15 days after the booster by intranasal inoculation with virulent RHDV. Vaccinated animals were clinically healthy during the entire challenge period and, as expected, neither clinical symptoms of disease were observed nor was RHDV detected in liver. Only one animal receiving a 0.5 μg dose of recombinant antigen died on day 7 post challenge. However, we believe that this death was probably accidental, due to the bleeding, and not toRHDV infection, as no virus was detected in the liver. By day 14 post-challenge, the levels

of antibodies increased in all the groups (Table 1), although this effect was more evident in the case of the animals vaccinated with lower doses. This increase may be explained by the booster effect of the challenge virus. By contrast, non vaccinated animals or mock-vaccinated died between 3–4 days post-infection, showing the characteristic pathologic lesions of the disease, e.g. hemorrhages in lung and hyperemic liver. RHDV was detected in the liver of these animals, with HA titers ranging from 16,384 to 65,536 HA units per 50 µl of liver extract.

Oral immunization and protection

To study the immunogenicity of these capsids by oral route and their applicability for vaccination of wild animals in field conditions, two groups of rabbits were fed with 3 µg of capsids. In one group the capsids were treated with binary ethylenimine, an alkylating reagent traditionally used to inactivate viruses and other pathogens that also confers some stability to the product. The other group of rabbits was orally immunized with untreated VLPs.

The results obtained are shown in Table 2. The best results were obtained when the rabbits were fed with the ethylenimine-treated VLPs, 4 out of 5 animals survived the challenge with virulent virus. Surprisingly, the antibody titers were, in some cases, superior to those obtained with a subcutaneous injection of the same dose of antigen. The non-protected animal did not develop antibody titer at any moment, which could suggest some problems in the administration of the antigen. The protected animals did not shown RHDV in liver, except in one case that showed a very low titer (8 HA units). Rabbits fed with untreated VLPs or placebo succumbed to the virus and died within seven days of challenge, except one animal fed with untreated VLPs that survived. In these two groups, all the other animals hardly developed antibodies, confirming a good correlation between presence of anti-RHDV antibodies and protection.

Discussion

The production of RHDV VLPs by expression of the VP60 in insect cells under control of the polyhedrin promoter was published recently [8]. However, the feasibility of using these VLPs in a vaccine campaign was uncertain, since the dose reported (100 µg/animal), the purification procedures and the immunization conditions were far from being cost-effective. In this report we have tried to learn more about better conditions of expression and purification of these VLPs and their suitability for vaccination. Specially, the ability of these capsids to confer protection by oral immunization has been investigated.

To learn more about the effect of the promoter on the levels of expression, the VP60 gene was inserted into recombinant baculoviruses well under the control of polyhedrin or p10 promoters. In a previous work, we demonstrated the superior performance of the p10 promoter to prepare parvovirus VLPs [5]. Again, as it happened with parvovirus, RHDV VLPs derived from the p10 promoter were obtained in higher yields (5–10 times more) and the preparations looked more homogeneous under the electron microscope (data not shown). The

Table 2. Serological results and virus detection after oral vaccination with RHDV VLPs and subsequent challenge

Group	Animal	ELISA titers				Surv.[a]	Virus[b]
		0	21	35	43		
Treated VLPs	1	–[c]	1600	400	800	S	–
	2	–	12800	12800	6400	S	–
	3	–	800	400	400	S	–
	4	–	25600	6400	6400	S	8
	5	–	–	–	†	†	8192
Untreated VLPs	6	–	–	–	†	†	8192
	7	–	6400	3200	3200	S	4
	8	–	–	–	†	†	4096
	9	–	–	*[d]	*	*	*
	10	800	800	800	†	†	64
Placebo	11	–	–	*	*	*	*
	12	–	–	–	†	†	8192
	13	400	–	–	†	†	16384
	14	800	–	–	†	†	8192
	15	800	400	400	†	†	8192

Rabbits were fed at days 0 and 21 with 3 μg of binary ethylenimine-treated or untreated RHDV in PBS by oral route. They were challenged 2 weeks later with 3.6×10^4 LD_{50} of RHDV

[a] Animals that survived throughout the experiment. † Dead from RHDV disease

[b] The presence of virus in the livers of the infected animals was detected by HA. Not detectable

[c] ELISA titer <200. Titers are expressed as in Table 1

[d] Dead from bleeding

reasons for the superior performance of the p10 promoter for expression of particulate antigens remain unclear at the moment. The presence of polyhedrin crystals in the infected cells constitutes also a good marker to indicate the optimal collection time for harvesting the cultures. The coexpression of polyhedrin in these cells seems not to constitute any drawback to obtain high levels of expression of the recombinant proteins.

The presence of 5 extra residues in the N-terminus of the VP60 neither affected the antigenicity nor the ability of the recombinant VP60 to assemble into empty capsids, which is indicative for some permissiveness in this position for the insertion of foreign epitopes. This recombinant antigen can also be used for the diagnosis of RHDV. The rapid ELISA we have developed is economical, safe and simple. The combination of vaccine and diagnosis will render these VLPs a major tool in the control and eradication of RHDV.

In this report, we have demonstrated that RHDV VLPs can be used very efficiently as synthetic vaccines, either by the parenteral route or by oral route. Our results show a complete protection of rabbits against this important disease. The high immunogenicity of RHDV VLPs was remarkable. Doses as low as 0.5 μg were able to evoke a significant immune response sufficient to render all the immunized animals protected. The protection obtained was reproducible and effective in all the vaccinated animals (26 of 26). The high immunogenicity seems to be independent of the route of immunization and the type of adjuvant used in the vaccine. In addition, the very high levels of expression obtained with the p10 promoter (200 μg/10^6 cells), together with the easy scheme of purification here described, make this system very adequate for vaccine production.

One of the most devastating aspects of RHDV is its incidence on wild-life, being highly virulent for wild rabbits. In order to find ways for the application of this vaccine to wild animals, our next goal was to investigate the ability of these capsids to induce oral immunity. Oral vaccination of free-ranging wildlife has been successfully used to control enzootic rabies in Europe [4]. However, all oral vaccines in use or under clinical trials are based on live attenuated or live recombinant viruses [3, 22]. Expression systems, such as baculovirus, which produce large amounts of protein could provide a cheap source of antigen devoid of the risks associated with live viruses. A demonstration of the feasibility of this approach was shown recently by Fu et al. [7], who successfully protected raccoons against rabies virus using the glycoprotein G of the virus, administered orally. However, they needed large amounts (300 μg of G protein) to confer protection. In contrast, it is remarkable that amounts as small as 3 μg of RHDV VLPs (one hundred times less) were able to induce significant titers of serum IgG antibodies conferring 80% protection to the vaccinated rabbits, without the use of any adjuvant. This is, to our knowledge, the first report where such a low amount of antigen is able to induce an immune response by the oral route. The major difference is the particulate structure of this antigen. Each particle contains 180 copies of the same protein, highly organized in a regular structure of 40 nm, which makes it an ideal candidate for the stimulation of different branches of the immune system. Because of the mucous membrane tropism of this virus, this protein might show adhesive properties which have shown to be a major reason for the high immunogenicity at the mucosal level [26]. Therefore, the immunogenicity of these VLPs is comparable to extremely immunoenhancing molecules such as cholera toxin [12]. Probably, another reason for the superior immunogenicity of RHDV VLPs was the treatment with binary ethylenimine. As reported in other cases [21], the treatment with reagents traditionally used for chemical inactivation of pathogens could help to the stabilization of the antigens and enable them to pass the acidic and proteolytic barriers of the gut, without affecting the immunogenicity. These results open new and unexpected possibilities to the use of virus-like particles in vaccination.

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