A single dose immunization with rabbit haemorrhagic disease virus major capsid protein produced in Saccharomyces cerevisiae induces protection

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Rabbit haemorrhagic disease virus (RHDV), a member of the Caliciviridae (Ohlinger et al., 1990; Parra & Prieto, 1990), is an important pathogen that causes a highly contagious disease in wild and domestic rabbits (Liu et al., 1984). The RHDV virion is 30–40 nm in diameter, shows a characteristic morphology and is composed of a major protein of 60 kDa (VP60). The 7.5 kb positive-sense ssRNA genome encodes a large precursor polypeptide which undergoes proteolytic cleavage to yield mature proteins (Martín-Alonso et al., 1996; Wirblich et al., 1996). The C-terminal region of the polyprotein gives rise to the polypeptide p60, which is detected by antibodies against the capsid protein. Furthermore, the presence of an abundant encapsidated subgenomic mRNA with coding capacity for VP60 (Meyers et al., 1991; Boga et al., 1992; Parra et al., 1993) suggests that the major capsid protein could be obtained either by proteolytic processing of the polyprotein or by translation of the subgenomic mRNA (Sibilia et al., 1995).

Commercial vaccines against RHD are prepared from tissues collected from experimentally infected rabbits, because of the lack of a cell culture system allowing virus replication (Argüello Villares, 1991). To obviate the need to handle infectious material for vaccine preparation, several authors have expressed the capsid protein in different heterologous systems. To date, the RHDV VP60 has been produced in Escherichia coli and in Sf9 insect cells; the recombinant protein obtained in either system is able to induce protection in rabbits against a lethal challenge with RHDV (Boga et al., 1994; Laurent et al., 1994; Marín et al., 1995; Sibilia et al., 1995).

Synthesis of foreign proteins in yeast has proven to be a useful technique (Romanos et al., 1992). High levels of expression can be obtained in this host, in addition to post-translational processing of heterologous proteins. The yeast Saccharomyces cerevisiae has been shown to be an excellent host for the production of mammalian polypeptides with clinical applications. A key development in the utilization of S. cerevisiae for the production of biomedical products was the synthesis of hepatitis B virus surface antigen (HBsAg) particles in yeast (Valenzuela et al., 1982), the only recombinant vaccine used for humans. The success and safety of the yeast system has prompted us to explore this host as a candidate for the production of a biologically safe and low-cost vaccine for prevention of RHD.

For this purpose, the VP60 coding region was inserted between the 5′ and 3′ control regions of the efficiently expressed yeast phosphoglycerate kinase gene (PGK) contained in the expression vector pMA91 (Mellor et al., 1983). In brief, an 884 bp fragment corresponding to the 5′ region of the VP60-coding sequence, amplified by PCR (Boga et al., 1994), was digested with BglII and BstZI. The resulting 415 bp fragment was inserted into the cDNA clone pT35 (Boga et al., 1994), previously digested with BglII and BstZI. The resulting vector, pT35Δ4955–5305, was digested with HindIII, blunt-ended by Klenow and ligated to BglII linkers. The digestion with BglII yielded a 1.8 kb fragment (nt 5305–7125), including the complete VP60 coding region, which was inserted into the unique BglII site of the pMA91 yeast expression vector, yielding pMADV60. This construct should allow the constitutive expression of recombinant VP60.

Yeast strain DBY-476 (MATα leu2-3,112 his3Δ1 ura3-52 hpr1-289) was transformed with the recombinant vector pMADV60 as well as pMA91 as negative control (Klebe et al.,
1983). Clonal isolates were grown at 28 °C for 48–60 h in 0.67% yeast nitrogen base medium without amino acids, supplemented with adenine, histidine, tryptophan and uracil (30 mg/l), and containing 2% glucose. After harvesting, the cell pellets were broken by vortexing with glass beads for 4×1 min periods, with intermittent cooling on ice. The cell extracts were clarified by centrifugation (10000 g, 10 min).

Polypeptides in the soluble fraction from whole cells may represent individual proteins, or higher structures resulting from specific interactions. To examine the form in which VP60 antigen is made in yeast, the cell-free extracts were subjected to zonal sedimentation through a 30% sucrose cushion with a Kontron TST28-38 rotor at 26000 r.p.m. for 2 h; the pellets were suspended in PBS. Most of the VP60 was found in the sediments, indicating the particulate nature of the antigen. The pellets from yeast cells transformed with pMAVP60 and pMA91 were solubilized in sample dissociation buffer containing 62.5 mM Tris–HCl pH 6.8, 2% SDS, 0.625% β-mercaptoethanol, 8.75% glycerol and 0.01% bromophenol blue, and the mixture was heated at 100 °C for 5 min. The samples were then analysed by electrophoresis in a 10% SDS–PAGE. After Coomassie blue staining, the track corresponding to the yeast transformed with pMAVP60 showed a major protein band similar in size to the viral VP60 protein from purified RHDV analysed in a parallel track (Fig. 1a). This protein band was absent from the lane corresponding to yeast cells transformed with pMA91. To confirm the identity of this protein, a similar gel was electrophoretically transferred onto a nitrocellulose membrane. The filters were blocked with PBS containing 0.1% Tween 20 and 1% gelatine (PTG) and probed with a 1:200 dilution of an anti-RHDV rabbit serum. After washing, peroxidase-conjugated protein A at a dilution of 1:2000 in PTG was added and the filters were developed with 4-chloro-1-naphthol as substrate. The results showed that the 60 kDa polypeptide present in the yeast transformed with pMAVP60 was antigenically similar to viral VP60 since its reactivity with the polyclonal serum was comparable to that of VP60 from RHDV (Fig. 1b). Furthermore, a larger and less abundant polypeptide was also identified by the polyclonal serum. The molecular mass of this polypeptide was not altered after endoglycosidase F treatment under denaturing conditions, indicating that it did not contain N-glycan chains. The nature of the post-translational modification of this protein remains to be elucidated.

The sedimentation behaviour of VP60 produced in yeast was determined in order to investigate the structure of the particulate material obtained, which could be in the form of VLPs as has been shown for the VP60 from different isolates of RHDV expressed by baculovirus (Laurent et al., 1994; Sibilia et al., 1995). Pellets from the sucrose cushions were adsorbed onto formvar-coated grids, stained with 2% phosphotungstic acid and examined with a Jeol 2000 EX-II electron microscope. Under these conditions, VLPs were observed only in the sediments from the yeast transformed with pMAVP60 (Fig. 2a). These structures were not seen in the pellets from yeast cells transformed with pMA91. The size of the observed VLPs was relatively uniform and, on average, was similar to the native virions. Nevertheless, the absence of an electron-dense core suggested that they did not contain RNA, in contrast to purified virions (Fig. 2b). Furthermore, the absence of RNA from the particles was also deduced from their buoyant density in CsCl gradients, which was 1.31 g/cm³, similar to that reported for baculovirus-produced VLPs (Laurent et al., 1994), and lower than the 1.36 g/cm³ found for RHDV virions (Parra & Prieto, 1990). These results support the idea that recombinant VP60 is able to self-assemble and that no other viral component is required to form virus-like particles. It should also be mentioned that the yeast expression construct contains

![Fig. 1. SDS–PAGE analysis of the pellets obtained by ultracentrifugation of yeast cell-free extracts: (a) stained with Coomassie blue; (b) immunodetected with a hyperimmune anti-RHDV rabbit serum.](Image)

![Fig. 2. Negative-stained electron micrographs: (a) ultracentrifuge pellets from pMAVP60-transformed yeast cell extracts; (b) purified RHDV. Bar, 50 nm.](Image)
part of RHDV ORF2. From our data the particles expressed in yeast cells do not contain a carboxy-terminally truncated form of the ORF2 gene product (based on Western blot analysis with an anti-ORF2 protein polyclonal serum; data not shown).

To investigate the immunogenicity of the recombinant protein, a group of four rabbits was injected subcutaneously with aliquots from the ultracentrifugation sediments, containing about 400 µg of total protein measured by the Bio-Rad protein assay method. The relative amount of recombinant VP60 in these samples was about 10% of the total protein as estimated by SDS–PAGE (Fig. 1a) and densitometry, with BSA as the standard. Blood samples were taken from each animal prior to the immunization and 1 or 2 weeks afterwards. The specificity and levels of anti-VP60 antibodies in the sera from vaccinated or naive control rabbits were measured with an ELISA test. Microtitre plates (Fastbinder, Costar) were coated overnight at room temperature with appropriate amounts of CsCl-purified RHDV diluted in PBS. The plates were then blocked with PTG for 1 h at room temperature. The rabbit sera were added (1:50 dilution in PTG) and incubated for 2 h at room temperature. After several washings with PT, peroxidase-conjugated protein A (diluted 1:2000 in PTG) was added and the plates were incubated for 1 h at room temperature. After further washings with PT, the plates were developed with o-phenylenediamine (OPD) as the substrate. The reaction was stopped with 3 M sulphuric acid. The absorbance at 450 nm was measured in a Titertek Multiskan (Flow Laboratories) spectrophotometer. The resulting anti-VP60 antibody levels (Fig. 3) indicated a gradual seroconversion to VP60 of the immunized animals in a time-dependent manner. For comparative purposes, a hyperimmune anti-RHDV serum was also analysed in these experiments. Two weeks post-immunization, the vaccinated animals and four control naive rabbits were challenged with a lethal dose of purified RHDV (100 LD50 per animal), administered intramuscularly. All control rabbits died 24 h after the challenge showing clinical signs of RHD, whereas the immunized animals survived and showed a high specific antibody level 7 days after the challenge (Fig. 3).

In this work, we have demonstrated the high immunogenicity of the VP60 produced by the yeast *S. cerevisiae*, which is able to protect rabbits with a single immunization in the absence of conventional adjuvants. This is probably due to the similarity in structure of the yeast VLPs to RHDV virions. Although the yield of VLPs produced by yeast was estimated to be 0.5–1 mg/l of culture, much lower than the 20 mg/l reported for the baculovirus system (Laurent et al., 1994), the value of the yeast VP60 antigen resides in that it can be produced in the complete absence of infectious agents. Furthermore, optimum conditions for production of yeast VP60 are currently being established which can increase the protein yield reported in this work. It should be also mentioned that in veterinary medicine the simplicity of the procedures and the cost per dose is a crucial aspect that can limit the practical use of vaccines. In consequence, the yeast system provides a simple, biologically safe and low-price alternative for production of an efficient vaccine against RHD.

VP60 VLPs can be formed in other heterologous systems (Laurent et al., 1994; Sibilia et al., 1995), but it remains to be determined where within the cell the VP60 particle is assembled, and what biochemical processes accompany this. An advantage of the yeast system is that the physiological and genetic requirements for particle assembly may be systematically explored, making use of yeast mutants conditionally defective for the secretory pathway and for other aspects of cellular metabolism.

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**References**


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