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Protection of rabbits against rabbit viral haemorrhagic disease with a vaccinia-RHDV recombinant virus

S. Bertagnoli*§, J. Gelfi*, F. Petit*, J.F. Vautherot†, D. Rasschaert†, S. Laurent†, G. Le Gall‡, E. Boilletot‡, J. Chantal* and C. Boucraut-Baralon*

In order to protect domestic and wild rabbits against RVHD, we constructed a recombinant vaccinia-RHDV virus, using the Copenhagen strain of the vaccinia virus. This recombinant virus expressed the RHDV capsid protein (VP60). Analysis of the expressed product showed that the recombinant protein, which is 60 kDa in size, was antigenic as revealed by its reactions in immunoprecipitation and indirect immunofluorescence with the antibodies raised against RHDV. The recombinant virus induced high level of RHDV specific antibodies in rabbits following immunization. Inoculations by both the intradermal and oral routes allow protection of animals against a challenge with virulent RHDV.

Keywords: RVHD; recombinant vaccinia virus; vaccination

Rabbit Viral Haemorrhagic Disease (RVHD), a highly contagious disease in wild and domestic rabbits, was first reported in the People's Republic of China in 1984[1]. RVHD spread throughout Europe during the years 1987 to 1989[2]. Infected rabbits—adults or young animals older than 2 months—usually died within 48 to 72 h of necrotizing hepatitis and haemorrhagic syndrome[3]. The disease is responsible for high economic losses in rabbitries, as well as for high mortality rate in wild rabbits. Recently, the causative agent was characterized as a member of the *Caliciviridae* family[4,5]. The genome of the Rabbit Haemorrhagic Disease Virus (RHDV) is a 7.5 kb single-stranded positive RNA with only two open reading frames (ORFs). ORF1 extends from nucleotide 10 to nucleotide 7042, and encodes for a polyprotein that is cleaved into non-structural proteins and the unique capsid protein VP60[6,7]. ORF2 overlaps the 3′ end of ORF1 by 17 nucleotides and encodes for a 12 kDa protein[5,6].

Both active immunization with VP60 alone and passive immunization with anti-VP60 antibodies were reported to afford protection against a viral challenge[5,8,9]. However, because there is no cell culture system to grow RHDV *in vitro*, the source of vaccinal antigen is still the liver of infected animals[10].

*Laboratoire INRA/ENVT de microbiologie moléculaire, ENVT, 23 chemin des capelles, 31076 Toulouse cedex, France. †Unité de virologie et d'immunologie moléculaires, INRA, 78350 Jouy en Josas, France. ‡Laboratoire central de recherches avicole et porcine, CNEVA, Beausemaine, B.P. 53, 22440 Ploufragan, France. §To whom correspondence should be addressed. (Received 23 June 1995; revised 21 September 1995; accepted 19 October 1995)

Recently, the full-length genome of a french RHDV isolate collected from a wild rabbit has been cloned and sequenced[7]. Laurent *et al.*[8] also showed that a recombinant capsid protein VP60 expressed in the Baculovirus/Sf9 cells expression system self-assembled into virus like particles (VLPs). These VLPs turned out to be highly immunogenic and to induce a good protection against the disease[8].

In order to protect domestic and wild rabbits against RVHD, we have constructed a recombinant vaccinia virus expressing RHDV VP60. The ability of this recombinant virus to protect animals against a challenge with virulent RHDV was tested both by intradermal (i.d.) and oral vaccinations.

MATERIALS AND METHODS

Cells and viruses

Vaccinia virus (Copenhagen strain) and recombinant viruses were propagated in RK13 cells grown in DMEM (Dulbecco's minimal Eagle's medium) supplemented with 2% foetal calf serum (FCS).

Construction of recombinant viruses

The 3′ terminal 2133 nucleotides (nt) of the RHDV genome, covering the VP60 gene and the ORF2 (VP12), previously cloned[7] in pSK$^+$ (bluescript, Stratagene), were digested by SacI and SmaI, blunt-ended, and ligated into the transfert vector pSC11[11] (kindly provided by G. Sutter) at the SmaI restriction site, to produce the pSVV plasmid. In pSVV the Thymidine Kinase (TK) gene of vaccinia virus was interrupted by

an insert of 5.6 kb containing the *lac Z* gene under the control of the vaccinia virus P11 promoter and the VP60+VP12 sequences of RHDV under the control of the vaccinia virus P7.5 promoter. The good orientation of the VP60 gene was determined both by polymerase chain reaction (PCR) and restriction analysis (not shown).

The procedure used to isolate recombinant viruses was adapted from one previously used for vaccinia virus (VV)[11,12]. Recombinant virus RecV-VP60 was amplified by infection of RK13 cells.

Pulsed field gel electrophoresis (PFGE) and Southern blot analysis of recombinant virus RecV-VP60

The cytoplasmic extract from one 175 cm^2 flask RK13 culture infected with RecV-VP60 virus was obtained by resuspending the pelleted cells in 1 ml TE (Tris 10 mM, EDTA 1 mM, pH 8) followed by a Dounce homogenization. The nuclei were removed by a centrifugation at 1000*g* for 10 min, and the supernatant was mixed with an equal volume of 1.5% Low Melting Agarose (LMA), and further processed as described by Boucraut-Baralon *et al.*[13] Briefly, agarose blocks were incubated, at 4°C, in 1 ml of 1 × restriction buffer of the desired enzyme. After 15 min, the buffer was removed and replaced by 1 ml of fresh buffer containing the restriction enzyme (80 U of *Hind*III). The incubation was performed for 16 h at 37°C.

Then digested blocks were electrophoresed in a gel containing 1% agarose in 0.5 × TBE, on the CHEF (Pharmacia LKB gene navigator) pulsed-field system at 275 V (18 V cm^{-1}), 10°C. Switch time was ramped at 1.5 s for 6 h 30.

After ethidium bromide staining, a Southern transfer was performed to positively charged nylon membrane. The DNA fragments were then hybridized to digoxigenin-labelled DNA probes. Full-length vaccinia virus DNA and internal VP60 (830 nt in length) probes were prepared as described by the manufacturer (Boehringer Mannheim).

Immunoprecipitation of the radiolabelled expressed protein

RK13 cells were infected with VV or recombinant virus RecV-VP60 at about 30 p.f.u. per cell. The infected cells were labelled for 5 h 30 in medium containing [^{35}S]methionine (100 mCi ml^{-1}). The infected labelled cells were treated with lysis buffer (Tris 10 mM, Triton X100 2%, NaCl 15 mM, KCl 600 mM, $MgCl_2$ 0.4 mM, proteases inhibitors, pH 8) for 2 h on ice bed and centrifuged at 17000*g* for 90 min (4°C). The proteins contained in the supernatant of the radiolabelled cell lysate were immunoprecipitated by using either a monoclonal antibody (mAb) specific for the VP60 (4E3)[8], or a hyperimmune antiserum and proteine A sepharose. Proteins were resolved by 8.75% SDS–PAGE, then visualized by autoradiography.

Indirect immunofluorescence of recombinant proteins

The 4E3 mAb was used to detect VP60 in infected cell monolayers by indirect immunofluorescence test (IF). RK13 cells infected with wild or recombinant viruses were fixed in 50% acetone–50% ethanol 20 h after infection and stained according to standard procedures.

Immunization of rabbits with RecV-VP60 virus and challenge with RHDV

To determine whether the recombinant expressing VP60 vaccinia virus could induce a protective immune response against RHDV, 20 5-week-old New Zealand white rabbits (*Oryctolagus cuniculus*) were injected i.d. on the external part of the ear with 0.1 ml dose of virus (10^8 p.f.u. in DMEM), and 20 others were inoculated per os (10^9 p.f.u. in DMEM), in a protected rabbitry at the CNEVA laboratory of Ploufragan. The serological responses were evaluated by an ELISA test 5, 10 and 15 days after immunization. Briefly, ELISA plate wells (Becton Dickinson, Falcon probind) were coated with 1 μg of recombinant baculovirus-purified VP60. The specific RHDV antibodies binding was visualized by incubating the plate wells with alkaline phosphatase antibody conjugate (Sigma). The absorbance of each sample was measured at 405 nm using a spectrophotometer 15 min after addition of substrate solution (pNPP in 10% diethanolamine, pH 9.8) (Sigma).

All the rabbits were then challenged intramuscularly with 1000 LD_{50} of RHDV 15 days after immunization. The rabbits were clinically examinated daily for 7 days after the challenge.

RESULTS

RecV-VP60 virus genome profile

To confirm the position of the foreign DNA insert within the vaccinia-RHDV recombinant virus genome, a vaccinia virus DNA probe was used to hybridize with the *Hind*III restriction fragments of RecV-VP60 virus. The profile of the recombinant virus DNA was modified as follows. A 5 kb fragment disappeared (corresponding to the *Hind*III-J fragment of the Copenhagen strain containing the TK gene[14]), and a fragment of 6 kb appeared (*Figure 1*, lanes 1A–2A).

Hybridization with an internal VP60 probe confirmed the data from restriction profile. The new 6 kb *Hind* III fragment hybridized with the VP60 probe. The insert was present at an unique site of the recombinant virus genome (*Figure 1*, lane 2B).

Synthesis of VP60 in cells infected with recombinant virus

To determine whether the VP60 protein was produced by the RecV-VP60 virus in infected cells, immunofluorescence and immunoprecipitation experiments were performed.

Cells infected with recombinant virus showed cytoplasmic fluorescence staining (*Figure 2*) whereas no fluorescence was detected in cells infected with vaccinia virus.

As judged by immunoprecipitation experiments using [^{35}S]methionine labelled lysates of RK13 cells infected with vaccinia or vaccinia recombinant viruses, the protein expressed in RecV-VP60 virus exhibit some characteristics of the authentic VP60 protein of RHDV.

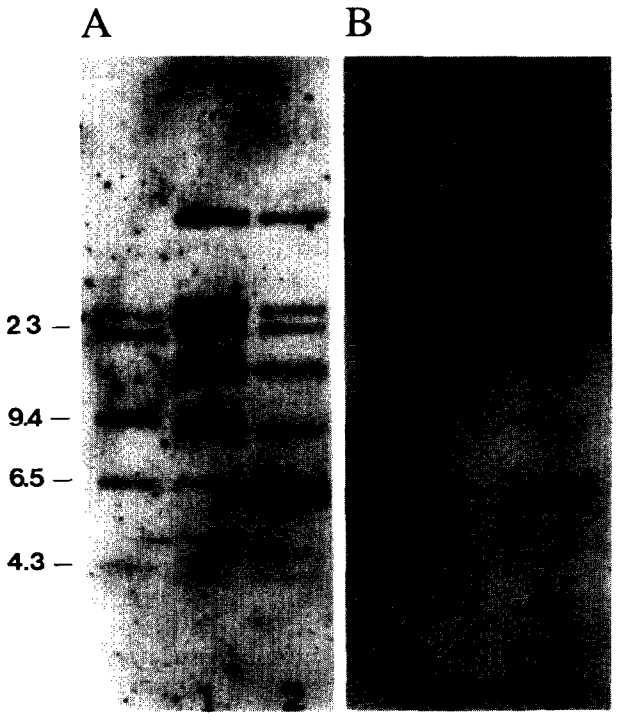

Figure 1 Southern-blot analysis of *Hind*III digested vaccinia (Copenhagen strain) and vaccinia recombinant (RecV-VP60) viruses DNA. The digested genomic DNA from wild-type Copenhagen vaccinia virus (lane 1) and recombinant RecV-VP60 virus (lane 2) were separated by PFGE. The DNA fragments were hybridized with either vaccinia virus DNA probe (A) or VP60 DNA probe (B). *Hind*III-digested λ DNA was included in the far left lane of (A) and (B) as molecular size markers. The arrow in (A) indicates the position of the *Hind*III-J fragment of wild-type vaccinia DNA

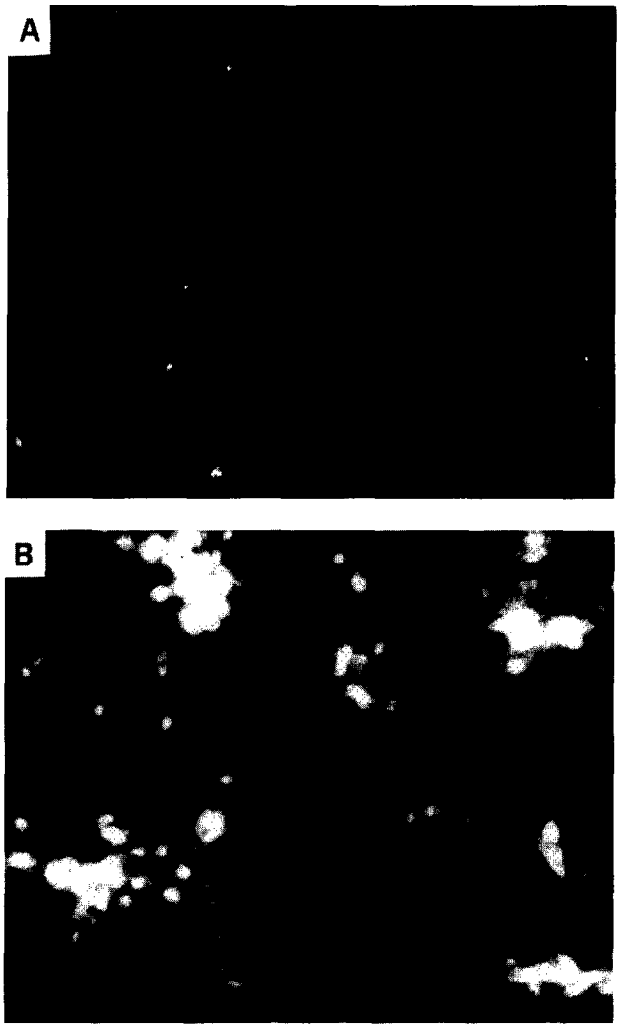

Figure 2 RHDV VP60 expressed in RK13 cells infected with RecV-VP60 virus detected by indirect fluorescent antibody assay using a monoclonal antibody that reacts specifically with VP60. (A) RK13 cells infected with Copenhagen strain of VV (negative control). (B) RK13 cells infected with RecV-VP60 virus

The 4E3 mAb and the specific antiserum chosen were previously used for western blot analysis with purified RHDV and recombinant baculovirus VP60 protein[8].

Both mAb and specific antiserum immunoprecipitated a protein with a calculated apparent molecular mass (M_r) of 60 kDa (*Figure 3*, lanes 2A–3A), corresponding to the monomeric form of the VP60 capsid protein, in agreement with published values[4–6,8,15]. The minor amount of protein precipitated with the negative antiserum (lane 1A) migrating at the same molecular mass as VP60 could result from traces of anti RHDV antibodies (it seems difficult to obtain totally negative wild rabbit antiserum).

A minor band migrating close to the recombinant VP60 was also immunoprecipitated by both polyclonal and monoclonal antibodies. A similar result has been described for the VP60 of RHDV expressed in the baculovirus system[8]. But, contrary to what was previously described[6,8,15], we have not detected any proteins migrating with a M_r of 30–40 kDa. As in recombinant baculovirus infected cells[8], no detectable expression of the low molecular weight protein corresponding to ORF 2 could be observed in recombinant vaccinia virus infected cells. No recombinant VP60 in the supernatant of infected RK13 cells could be detected, contrary to what could be observed in the baculovirus system[8].

Immune response and protection induced by recombinant virus

To determine whether the VP60 expressed in the RecV-VP60 virus was immunogenic for young rabbits, serial serum samples were monitored for the presence of anti-VP60 antibodies by ELISA.

When the anti-VP60 response was tested, antibodies were detected as soon as 5 days post RecV-VP60 administration in orally as well as i.d. treated groups (*Figure 4*). Both groups progressively developed high anti-VP60 antibody titres while animals vaccinated with the Copenhagen strain of VV and the unvaccinated controls remained seronegative for RHDV.

To investigate the protection induced by the recombinant VP60 protein, vaccinated and control animals were challenged with 1000 LD_{50} of RHDV 15 days after vaccination.

All rabbits vaccinated with RecV-VP60 virus survived without any clinical sign (*Table 1*). No RHDV could be detected by ELISA in the livers collected from all immunized animals, which were killed 10 days after challenge.

In contrast, 26 out of 30 animals belonging to the control group and the VV vaccinated group died between 48 and 72 h after challenge. RHDV could be detected in all liver homogenates from these animals (data not shown).

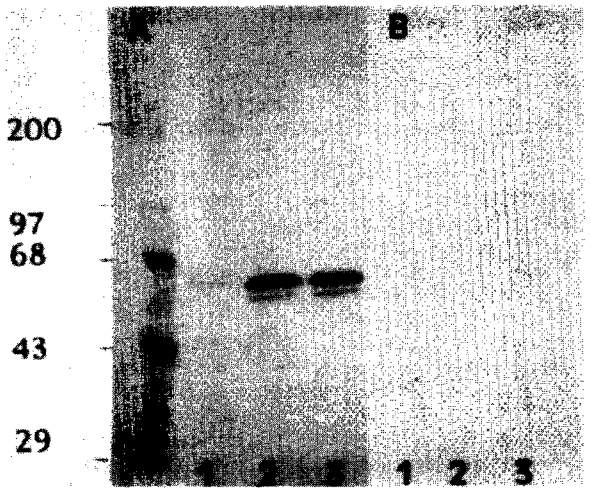

Figure 3 Expression of VP60 in vaccinia recombinant (RecV-VP60)-infected RK13 cells. Autoradiographs of SDS-polyacrylamide gels showing ^{35}S-labelled, immunoprecipitated VP60 from infected RK13 cells (RecV-VP60 virus in A; VV Copenhagen in B). Lanes 1, negative antiserum; lanes 2, RHDV antiserum and lanes 3, anti-VP60 monoclonal antibody

DISCUSSION

The use of poxviruses, such as the vaccinia virus, as viral vectors for foreign genes in a vaccinal aim has been described for about 10 years[16]. These expression systems have proven their efficacy in many diseases[17–21]. One of the best examples is the vaccinia–rabies recombinant virus, the Copenhagen strain of vaccinia virus expressing the rabies G glycoprotein[16,22]. Until now, it is the only recombinant vaccinia virus used for routine vaccination (oral vaccination of foxes in France and Belgium)[23,24].

RVHD is a new acute disease of domestic and wild rabbits present in Europe since 1987. The causative agent (RHDV) is a member of the *Caliciviridae* family. The lack of cell culture system to grow RHDV still does not allow production of virus *in vitro*. So, until now, all the commercially available vaccines against RHDV are produced from livers of infected rabbits[10]. A major structural protein, the capsid protein VP60, has been identified. The recombinant protein either produced in *Escherichia coli*[9] or in the Baculovirus/Sf9 expression system[8] induces protective immunity, as it has been previously reported[8,9].

Table 1 Protection against RVHD challenge of rabbits vaccinated orally or i.d. with vaccinia or vaccinia recombinant (RecV-VP60) viruses

Vaccine	Inoculation route	Dose (p.f.u.)	Mortality[a]
RecV-VP60	i.d.	10^8	0/9
VV Copenhagen	i.d.	10^8	10/10
RecV-VP60	Oral	10^9	0/9
VV Copenhagen	Oral	10^9	8/10
Not vaccinated	—	—	8/10

[a]Rabbits were challenged with 1000 LD_{50} of RHDV 2 weeks post-vaccination

To improve vaccination of rabbits, we have expressed this unique capsid protein of RHDV, VP60, in the vaccinia virus recombinant system.

In our experiments, we have vaccinated seronegative animals at weaning in order to check whether such a recombinant vaccine could be used early in the rabbit breeding schedule. In industrial and traditional breeding conditions, specific maternal antibodies could block the effect of an inactivated vaccine used at weaning. The use of an attenuated recombinant virus could resolve this kind of problem. One i.d. injection of recombinant vaccinia-RHDV virus was able to protect young rabbits against a virulent RHDV challenge, under the conditions used for vaccine controls for older animals in France[8]. Furthermore, we have shown that the protection induced by the oral vaccination was as complete as the one induced by the i.d. vaccination.

A protective immunity was rapidly established since all the rabbits vaccinated (i.d. or orally) 15 days before challenge were protected. The same results was previously reported, using inactivated RHDV[10], VLP[8], or VP60 expressed in *E. coli*[9] by parenteral route.

In our hands, as it was observed with the VP60 expressed in the Baculovirus/Sf9 expression system[8], anti VP60 antibodies could be detected as early as 5 days after vaccination and the titres progressively increased until the day of challenge. It was previously shown that the humoral response plays a key role in the protection against the disease[5], and that the protective immunity

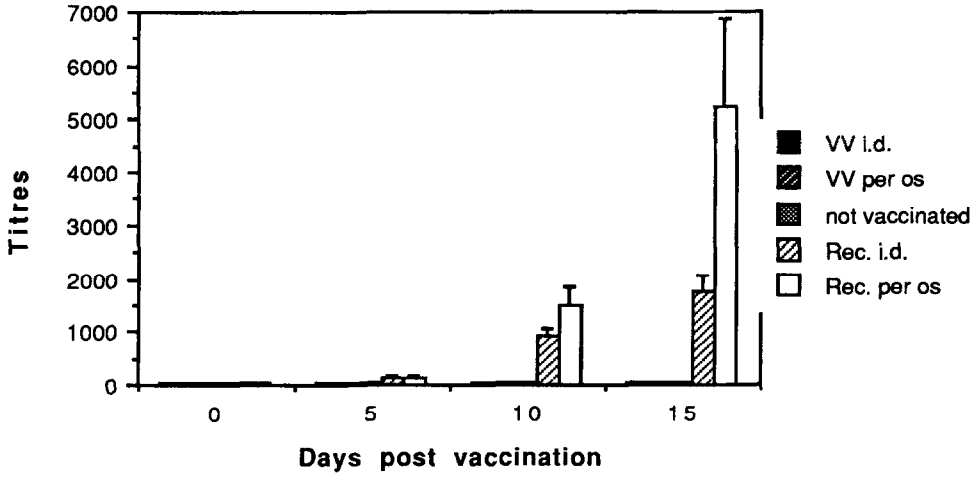

Figure 4 Serum anti-VP60 antibody response (ELISA test) in rabbits immunized i.d. or orally with vaccinia virus or vaccinia recombinant virus that expresses the RHDV VP60. Antibody titres are reported as reciprocal geometric mean titres plus standard error of the means (n=9)

was efficient as soon as antibodies against VP60 could be detected in animal sera[5,8,9]. So, we can reasonably think that our recombinant virus may protect animals against challenge as soon as five days after vaccine administration.

Thus, in young domestic rabbits, an early and efficient protection against RVHD may be obtained by vaccinia-RHDV recombinant virus administration, either by i.d. route or by oral route, which is a new fact compared with protection induced by the VP60 expressed in *E. coli* or in the baculovirus system. The efficiency of oral route gives rise to the opportunity of wild rabbit vaccination by field distribution of baits containing the recombinant virus.

The use of vaccinia virus as live vector might lead, like all live vaccine, to the emergence of disease. In general, all members of the *orthopoxvirus* genus, except rabbitpoxvirus (RPV) and buffalopoxvirus, are relatively non-pathogenic in rabbits[25]. The RPV induces a systemic and frequently lethal infection. Although never observed in the wild, half a dozen epidemics of the disease have been reported in rabbits colonies (for example at Rockefeller university in 1930, 1932 and 1967). The intradermal and oral administration of vaccinia-RHDV recombinant virus, based on vaccinia attenuated strain and less pathogenic thymidine kinase-negative variant, should not create lethal strains like RPV in rabbits colonies. Nevertheless, to improve safety of poxvirus based recombinant RHDV vaccine, it could be interesting to test the potency of highly attenuated vaccinia strains (like NYVAC strain[26]) or other poxviruses (avipoxvirus, leporipoxvirus) as new vectors for rabbit vaccination.

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