

Protection against Myxomatosis and Rabbit Viral Hemorrhagic Disease with Recombinant Myxoma Viruses Expressing Rabbit Hemorrhagic Disease Virus Capsid Protein

STEPHANE BERTAGNOLI,¹ JACQUELINE GELFI,¹ GHISLAINE LE GALL,² ERIC BOILLETOT,²
JEAN-FRANCOIS VAUTHEROT,³ DENIS RASSCHAERT,³ SYLVIE LAURENT,³
FREDERIQUE PETIT,¹ CORINE BOUCRAUT-BARALON,¹
AND ALAIN MILON^{1*}

Laboratoire Associé de Microbiologie Moléculaire, Institut National de la Recherche Agronomique and Ecole Nationale Vétérinaire, Toulouse,¹ Laboratoire central de recherches avicole et porcine, Centre National d'Etudes Vétérinaires et Alimentaires, Ploufragan,² and Unité de virologie et d'immunologie moléculaires, Institut National de la Recherche Agronomique, Jouy en Josas,³ France

Received 8 December 1995/Accepted 26 April 1996

Two myxoma virus-rabbit hemorrhagic disease virus (RHDV) recombinant viruses were constructed with the SG33 strain of myxoma virus to protect rabbits against myxomatosis and rabbit viral hemorrhagic disease. These recombinant viruses expressed the RHDV capsid protein (VP60). The recombinant protein, which is 60 kDa in size, was antigenic, as revealed by its reaction in immunoprecipitation with antibodies raised against RHDV. Both recombinant viruses induced high levels of RHDV- and myxoma virus-specific antibodies in rabbits after immunization. Inoculations by the intradermal route protected animals against virulent RHDV and myxoma virus challenges.

Two major viral diseases affect European rabbit populations (*Oryctolagus cuniculus*) in continental Europe: myxomatosis and rabbit viral hemorrhagic disease (RVHD). The wild rabbit population is an important reservoir for both viruses.

The etiological agent of myxomatosis, myxoma virus (MV), is a large double-stranded DNA virus belonging to the *Leporipoxvirus* genus of the *Poxviridae* family (12). It induces a mild disease in cottontail rabbits (*Sylvilagus* spp.) but a systemic and usually fatal one in European rabbits (11).

Control of the disease is very difficult because of its epidemiological characteristics. Only vaccination can reduce the spread of myxomatosis in breeding animals. In France, primary vaccination is done with Shope fibroma virus, another leporipoxvirus, and a cell culture-attenuated strain of MV (SG33) is used for booster injections (34). Nevertheless, the SG33 strain may at times show residual pathogenicity for young rabbits (7).

RVHD, a highly contagious disease in wild and domestic rabbits, was first described in the People's Republic of China in 1984 (19). RVHD spread throughout Europe between 1987 and 1989 (22). Infected rabbits—adults or young animals older than two months—usually die within 48 to 72 h of necrotizing hepatitis and hemorrhagic syndrome (20). The disease is responsible for high economic losses in rabbitries as well as for high mortality rates in wild rabbits. The causative agent, the rabbit hemorrhagic disease virus (RHDV), was characterized as a member of the *Caliciviridae* family (25, 28), with a genome of 7.5-kb single-stranded, positive-sense RNA with two open reading frames (ORFs). ORF1 extends from nucleotide (nt) 10 to 7042 and encodes a polyprotein that is cleaved into non-structural proteins and the unique capsid protein VP60 (21,

30). ORF2 overlaps the 3' end of ORF1 by 17 nt and encodes a 12-kDa protein (21, 28).

Both active immunization with VP60 alone and passive immunization with anti-VP60 antibodies were reported to afford protection against a viral challenge (4, 18, 28). However, because there is no cell culture system to produce RHDV in vitro, the source of vaccinal antigen remains the livers of infected animals (1).

The full-length genome of an RHDV isolate collected from a wild rabbit in France has recently been cloned and sequenced (30). Laurent et al. (18) showed that a recombinant capsid protein VP60 expressed in the baculovirus/Sf9 cell expression system self-assembled into virus-like particles. These virus-like particles turned out to be highly immunogenic and to induce good protection against the disease (18). We then showed that a vaccinia virus-RHDV recombinant virus expressing the VP60 also provided total protection against RVHD when administered either intradermally (i.d.) or orally (2).

To simultaneously protect rabbits against myxomatosis and RVHD, we constructed recombinant MVs based on the attenuated SG33 myxoma strain that express the RHDV VP60 protein. We also tried to improve the safety of SG33 virus by deleting its thymidine kinase (TK) gene (15) or ORFs coding for the previously identified pathogenesis factors MGF (myxoma growth factor) (26, 36) and M11L (14, 26). The abilities of these recombinant viruses to protect animals against challenges with virulent RHDV and MV were tested.

MATERIALS AND METHODS

Cells and viruses. MV SG33 and recombinant viruses were propagated in RK13 cells grown in OptiMEM (Gibco Life Technologies) supplemented with 2% fetal calf serum (FCS [Gibco Life Technologies]).

Amplification and cloning of SG33 virus M11L-MGF and TK genes. Partially overlapping M11L and MGF ORFs are located approximately 13 kb from the left terminus of the MV DNA genome (26).

PCR (30 cycles; 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C) was used to amplify these two ORFs (1,060 bp) with SG33 DNA as the template and primers 5'-GTAGATCCGTAACAAGTGTAATATA-3' (located upstream of the

* Corresponding author. Mailing address: Laboratoire associé de microbiologie moléculaire, Institut National de la Recherche Agronomique and Ecole Nationale Vétérinaire, Ecole Nationale Vétérinaire de Toulouse, 23 chemin des capelles, F-31076 Toulouse cedex, France. Phone: (33) 61 19 38 84. Fax: (33) 61 19 39 75.

M11L major RNA 5' initiation site) and 5'-CCCGGATGTTTTTCGCGATGT GA-3' (located after the stop codon of MGF).

The TK gene (700 bp) was also obtained by PCR (same parameters) with SG33 DNA as the template and primers 5'-GGTGTGGATAAGGAAGTT ACG-3' (located 112 bp upstream from the start codon of the TK gene) and 5'-GAGGTCGCTGTCGGAGACG-3' (located 43 bp downstream from the TK gene putative early transcript termination signal).

The amplification products were ligated into the cloning vector pGEM-T (Promega), and the M11L-MGF-amplified products were then subcloned into Bluescript vector (pSK⁺; Stratagene).

Construction of the recombinant transfer vectors pSM1 and pSM2. The 3'-terminal 2,133 nt of the RHDV genome, covering the VP60 gene and ORF2 (VP12), which were previously cloned (30) in pSK⁺ (Bluescript; Stratagene), were digested by *SacI* and *SmaI*, blunt ended with Klenow polymerase, and ligated into the transfer vector pSC11 (9) (kindly provided by G. Sutter) at the *SmaI* restriction site to produce the pSVV plasmid.

In pSVV, the TK gene of vaccinia virus was interrupted by an insertion of 5.6 kb containing the *lacZ* gene under the control of the vaccinia virus p11 promoter and the VP60 and VP12 sequences of RHDV under the control of the vaccinia virus p7.5 promoter. Correct orientation of the VP60 gene was determined both by PCR and by restriction enzyme analysis (not shown). First, pSVV was digested by *NotI* and then partially digested by *PstI*. A 5.6-kb restriction fragment, containing the *lacZ* gene and the VP60 and VP12 genes, was subcloned in pSK⁺ to produce pSM0. The same 5.6-kb digested *NotI-KpnI* fragment was blunt ended and ligated into the pGEMT-TK plasmid at the *EcoRV* restriction site of the TK ORF to produce pSM2.

A 5.3-kb digested *HindIII* fragment of pSM0, containing the *lacZ* and VP60 genes, was ligated into the pSK⁺ (M11L-MGF) plasmid at the *HindIII* and *BsaBI* restriction sites of the M11L and MGF ORFs to produce pSM1. Correct orientation of the VP60 gene (3' end toward the 3' end of disrupted genes) for the pSM1 and pSM2 plasmids was confirmed by PCR and restriction enzyme analysis (data not shown).

Isolation of recombinant viruses. The procedure used to isolate recombinant MVs was adapted from a method previously used for vaccinia virus (9, 27). Briefly, 90% confluent RK13 cells, in 60-mm petri dishes, were infected with SG33 virus at a multiplicity of infection of 0.03 to 0.04 PFU per cell. After 2 h of adsorption at 37°C, the inoculum was removed and the cells were washed twice with OptiMEM without FCS. For transfection, 10 µg of pSM1 or pSM2 plasmid DNA was first mixed with 40 µg of lipofectamine at room temperature for 20 min. The lipofectamine-DNA complex was then added to 3 ml of OptiMEM serum-free medium and this mix added to the cells. After 5 h and 30 min at 37°C, the medium was supplemented with 3 ml of OptiMEM plus 5% FCS. Forty-eight hours later, the cells were frozen and thawed three times, sonicated (20 s), and applied to fresh RK13 cells. Two days later, the medium was removed and fresh medium containing 1% agar and 2.5% FCS was added. After 48 h, a new medium containing 1% agar, 1% neutral red, and 330 µg of X-Gal (5-bromo-4 chloro-3-indolyl-β-galactoside) per ml was added. The following day, blue plaques were picked and five additional plaque purification cycles were performed under the same conditions. Recombinant viruses RecA (TK site) and RecB (M11L-MGF site) were amplified by infection of RK13 cells.

The genome profiles (*PstI* restriction enzyme digestion) of the two recombinant viruses were determined by pulsed-field gel electrophoresis (PFGE) and Southern blot analysis, as previously described (29) (data not shown). The DNA fragments were hybridized to digoxigenin-labeled DNA probes. DNA probes from the SG33 TK and M11L-MGF genes and an internal fragment (830 nt in length) of VP60 were prepared as described by the manufacturer (Boehringer Mannheim) by the random-priming technique.

Immunoprecipitation of the radiolabeled expressed proteins. RK13 cells were infected with SG33 or recombinant viruses at about 30 PFU per cell. The infected cells were labeled for 5 h and 30 min in medium containing [³⁵S]methionine (100 µCi/ml). The infected labeled cells were treated with lysis buffer (10 mM Tris, 2% Triton X-100, 15 mM NaCl, 600 mM KCl, 0.4 mM MgCl₂, protease inhibitors [pH 8]) for 2 h on ice and were centrifuged at 17,000 × g for 90 min (4°C). The proteins contained in the supernatant of the radiolabeled cell lysate were immunoprecipitated by using either a monoclonal antibody (MAb) specific for the VP60 (4E3) (18) or a hyperimmune antiserum and protein A-Sepharose. Proteins, eluted from protein A-Sepharose, were resolved by Sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis and then visualized by autoradiography.

Immunization of rabbits with SG33, RecA, and RecB viruses and challenges with MV and RHDV. Male New Zealand White rabbits (*O. cuniculus*) (4 to 5 weeks old) were obtained from a local supplier. The rabbits were observed for 3 days to confirm that they were healthy and were then injected once i.d. on the external part of the ear with a 0.1-ml dose of virus (5 × 10³ PFU) in minimum essential medium. The rabbits were monitored daily for 2 weeks. The serological responses against MV and RHDV VP60 were evaluated by use of enzyme-linked immunosorbent assays (ELISA) (recombinant-purified VP60 or semipurified MV as antigens) 5, 10, 20, 30, and 44 days after immunization. Briefly, ELISA plate wells (Falcon probind; Becton Dickinson) were coated with 1 µg of recombinant baculovirus-purified VP60 or 1 µg of semipurified MV. Binding of the RHDV or MV antibodies was visualized by incubation with goat anti-rabbit immunoglobulin G serum conjugated to alkaline phosphatase (Sigma). The A₄₀₅

of each sample was measured 15 min after the addition of substrate solution (*p*-nitrophenyl-phosphate in 10% diethanolamine [pH 9.8]) (Sigma). Serological responses against MV were also evaluated by use of a serum neutralization test 30 and 44 days after immunization. Serum samples were diluted in 96-well microtiter plates, and an equal volume (50 µl containing 750 PFU) of MV (T1 strain) was added to each well. The plates were incubated for 1 h at 37°C and 1 h at 4°C. RK13 cells (10⁴ cells in 50 µl) were then added to each well, and the plates were incubated at 37°C for 3 days in a CO₂ incubator. The plates were read by examining the unfixed and unstained cell monolayers under an inverted-phase light microscope.

Rabbits were challenged i.d. on day 44 with virulent MV strain T1 (5 × 10³ PFU) and were then monitored daily for symptoms of myxomatosis. One month later (75 days after the vaccination), anti-MV antibodies were measured by ELISA.

To determine whether the recombinant MVs which expressed VP60 could induce a protective immune response against RHDV, 36 5-week-old New Zealand White rabbits were injected i.d. with viruses (5 × 10³ PFU in minimum essential medium) and 18 others were injected subcutaneously (s.c.) with inactivated classical vaccine in a protected rabbitry at the Centre National d'Etudes Vétérinaires et Alimentaires (CNEVA) laboratory of Ploufragan, France. One group of rabbits was then challenged intramuscularly with 1,000 50% lethal doses of RHDV 5 days after immunization, and a second group was challenged 15 days after immunization. The rabbits were clinically examined daily for 7 days after the challenge. One month later (35 and 45 days, respectively, after the vaccination), anti-VP60 antibodies were measured by ELISA.

RESULTS

Construction of the RecA and RecB recombinant viruses. Homologous recombination occurred between the sequences flanking the foreign gene of both the pSM1 and pSM2 insertion vectors and the corresponding sequences of the infecting MV (SG33 strain) genome so that two recombinant genomes containing the foreign DNA were formed.

In the first recombinant MV-RHDV, RecA virus, the TK gene of the SG33 strain was interrupted by an insertion of 5.6 kb containing the *lacZ* gene under the control of the vaccinia virus p11 promoter and the VP60 and VP12 sequences of RHDV under the control of the vaccinia virus p7.5 promoter. In the RecB virus, 356 nt were deleted at the 3' end of the M11L gene and the 5' end of the overlapping MGF gene of the SG33 strain and were replaced by *lacZ* and VP60 (without VP12) sequences (Fig. 1). To confirm the positions of the foreign DNA inserts within the recombinant viruses genomes, DNA from infected cells was digested with *PstI* restriction endonuclease and analyzed by PFGE and Southern blotting. The results of the hybridization experiments were analyzed with respect to the genome structure of MV (Lausanne strain) described by Russell and Robbins (33), and to the restriction map of the SG33 strain. This attenuated strain has a deletion at the right end of the DNA and lacks a fragment of approximately 13 kb (5) compared with the DNA of the Lausanne strain. The hybridization reactions with the SG33 and VP60 probes confirmed that the inserts were present in a unique target site in each recombinant virus genome (data not shown).

Expression of VP60 in cells infected by recombinant viruses. Immunoprecipitation experiments with [³⁵S]methionine-labeled lysates of RK13 cells infected with SG33 or recombinant viruses showed that the proteins expressed in the RecA and RecB viruses exhibited some characteristics of the authentic VP60 protein of RHDV. The 4E3 MAb and the specific antiserum chosen had previously been used for Western blot (immunoblot) analysis with purified RHDV and recombinant baculovirus VP60 protein (18).

Both MAB and specific antiserum immunoprecipitated a protein with an apparent molecular mass of 60 kDa (Fig. 2A and B, lanes b), corresponding to the monomeric form of the VP60 capsid protein, in agreement with published values (18, 21, 25, 28, 31). A minor band migrating close to the recombinant VP60 was also immunoprecipitated by the polyclonal and MABs.

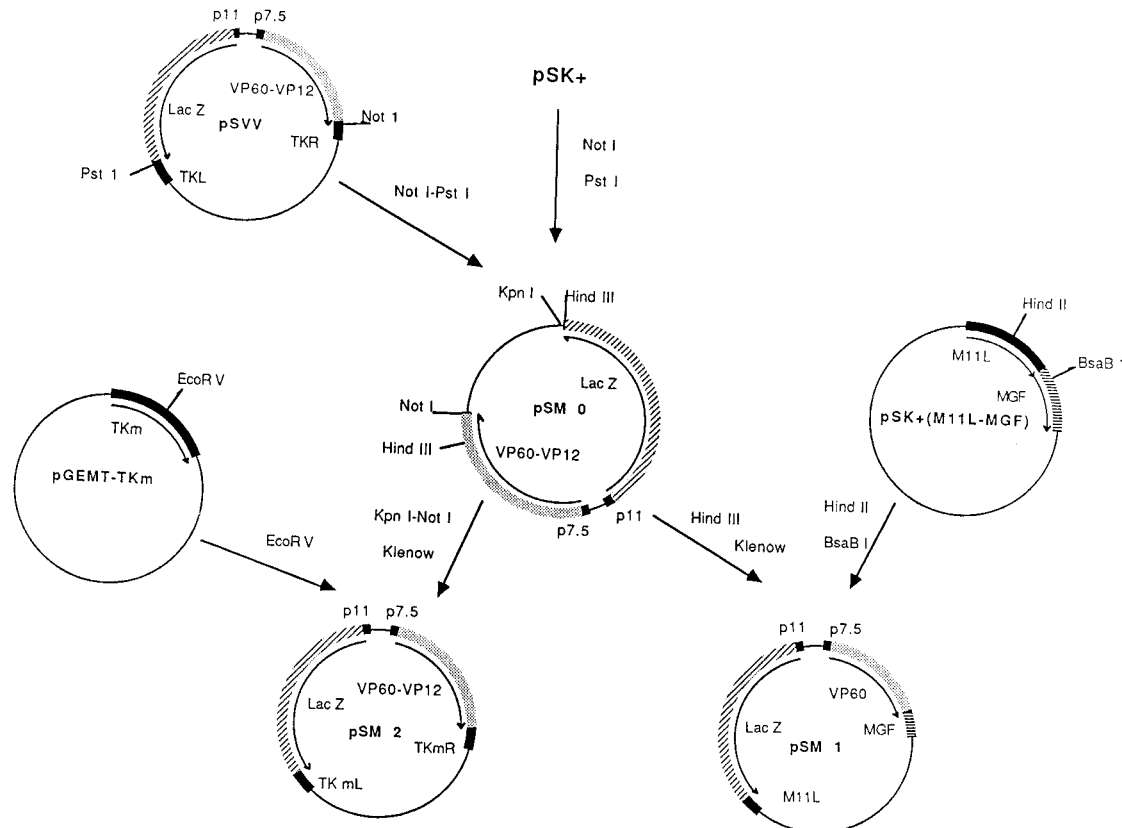


FIG. 1. Construction of MV insertion vectors (see Materials and Methods for details). TKL, left part of the vaccinia virus TK gene; TKR, right part of the VV TK gene; TKmL, left part of the MV (SG33 strain) TK gene; TKmR, right part of the MV (SG33 strain) TK gene. M11L and MGF are genes of the SG33 strain. The pSM 1 and pSM 2 insertion vectors were used to obtain RecB and RecA recombinant viruses, respectively.

Immune responses and protection induced by recombinant viruses. After i.d. administration of both recombinant viruses or the SG33 strain, only a localized primary tumor on the external part of the ears of the rabbits could be observed. All induced tumors were similar in terms of size and time frame. We used this observation to confirm the safety of these viruses for young rabbits.

To determine whether the VP60 expressed in the RecA and RecB recombinant MVs was immunogenic for young rabbits, serial serum samples were monitored for the presence of anti-VP60 antibodies by ELISA. At the same time, anti-MV antibodies were detected by ELISA and by a neutralization test. A specific response was observed with both recombinant viruses when the anti-MV response was tested (Fig. 3 and 4), although this was lower than the response observed with the SG33 strain. The differences noted between the anti-MV antibody titers induced by the RecA and RecB viruses were not significant ($P = 0.43$ by the Mann-Whitney test at day 44).

Anti-VP60 antibodies were detected as early as 5 days post-administration of the recombinant viruses (titers ranging from 50 to 100). Both groups progressively developed high anti-VP60 antibody titers, while animals vaccinated with the SG33 strain of MV and the nonvaccinated rabbits remained seronegative for RHDV (Fig. 5). The RecB virus induced a better anti-VP60 antibody response than the RecA virus, and this time the differences were highly significant ($P = 0.0004$ by the Mann-Whitney test at day 44).

To see whether previously vaccinated rabbits could withstand a virulent infection by MV, the animals were challenged 44 days postvaccination with the T1 strain of MV. The propor-

tions of rabbits that survived infection were equivalent in all three groups, and some of these rabbits showed clinical signs of myxomatosis without fatality (Table 1). Thus, the recombinant MV-RHDVs, like the SG33 strain of MV, virus, induced a par-

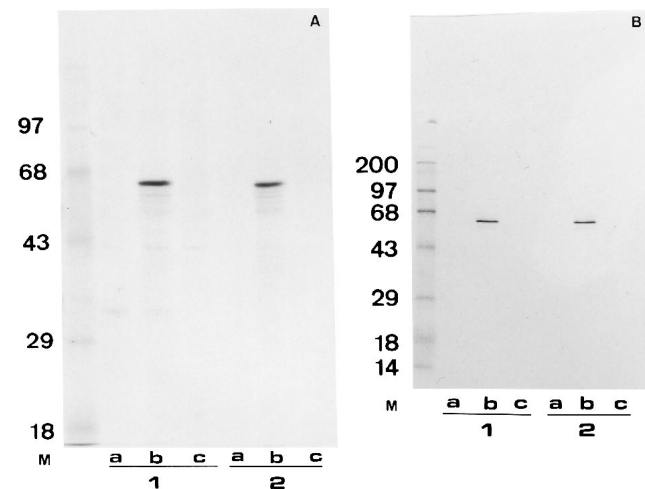


FIG. 2. Expression of VP60 in MV-RHDV recombinant virus-infected cells; autoradiographs of SDS-polyacrylamide gels showing ^{35}S -labeled, immunoprecipitated VP60 from infected RK13 cells. (A) a, b, and c, SG33-infected cells, RecA-infected cells, and noninfected cells, respectively; (B) a, b, and c, SG33-infected cells, RecB-infected cells, and noninfected cells, respectively. Group 1, anti-VP60 MAb; group 2, RHDV antiserum. M, molecular mass markers (in kilodaltons).

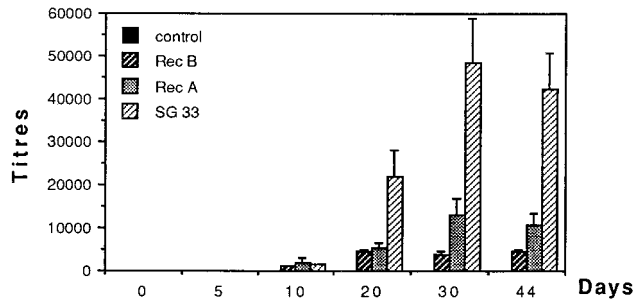


FIG. 3. Serum anti-MV antibody responses (ELISA) in rabbits immunized i.d. with MV-RHDV recombinant viruses (RecA or RecB) or SG33 virus. The serological responses were evaluated before challenge and 5, 10, 20, 30, and 44 days after immunization. Antibody titers are reported as reciprocal geometric mean titers plus standard errors of the means ($n = 12, 15, 22,$ and $12,$ respectively, for the RecA group, RecB group, SG33 group, and control group). The control group contained nonvaccinated animals.

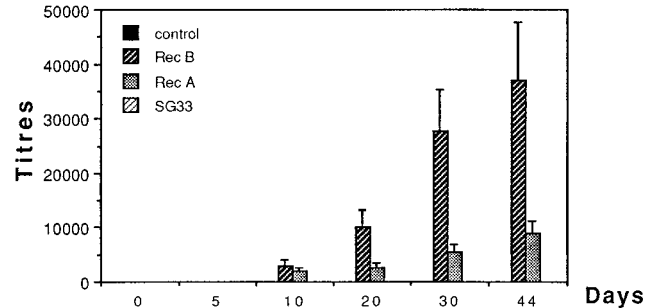


FIG. 5. Serum anti-VP60 antibody response (ELISA) in rabbits vaccinated i.d. with recombinant MVs (RecA or RecB) that express RHDV VP60. Antibody titers are reported as reciprocal geometric mean titers plus standard errors of the means ($n = 12, 15, 22,$ and 12 for RecA, RecB, the SG33 group, and the control group, respectively). The control group contained nonvaccinated animals. The serological responses were evaluated 5, 10, 20, 30, and 44 days after immunization. None of the rabbits in these groups were challenged with RHDV.

tially protective antimyxomatosis primary immune response. The postchallenge ELISA titers to MV of rabbits vaccinated with RecA or RecB virus were very similar, ranging from 80,000 to 300,000.

To investigate the protection induced by the recombinant VP60 protein, six other groups of young rabbits were vaccinated with either RecA virus or RecB virus or a commercially available inactivated anti-RVHD vaccine at the CNEVA Ploufragan laboratory 5 or 15 days before a challenge with 1,000 50% lethal doses of RHDV. Nonvaccinated animals (control groups) were also challenged at the same time.

All vaccinated rabbits survived, regardless of the day of challenge, without clinical signs of disease (Table 2). The rabbits challenged 5 days after vaccination with one of the two recombinant viruses had anti-VP60 antibody titers ranging from 50 to 100 on the day of challenge. Titers of the other groups of vaccinated rabbits ranged from 500 to 2,000 on the day of challenge (15 days postvaccination).

No RHDV could be detected by ELISA in the livers collected from vaccinated animals which were sacrificed 30 days after challenge. In contrast, all animals in the control groups died between 48 and 72 h after challenge and had anti-VP60 antibody titers less than the detection limit of the test. RHDV could be detected in all liver homogenates from these animals (data not shown).

The postchallenge anti-VP60 antibody titers of rabbits vac-

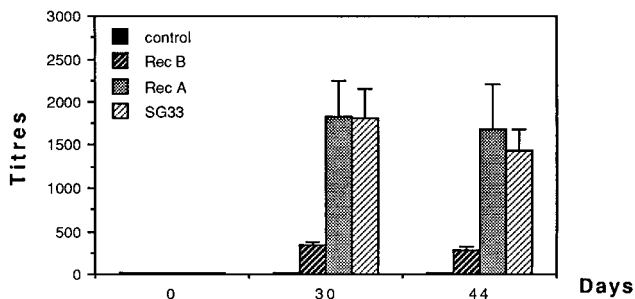


FIG. 4. MV-neutralizing antibody titers in rabbits after i.d. vaccination with MV-RHDV recombinant viruses (RecA or RecB) or SG33 virus. Serological responses were evaluated before challenge and 30 and 44 days after immunization. Antibodies titers are reported as reciprocal geometric mean titers plus standard errors of the means ($n = 12, 15, 22,$ and 12 for the RecA group, RecB group, SG33 group, and control group, respectively). The control group contained nonvaccinated animals.

inated with RecA or RecB virus were in the ranges 20,000 to 300,000 and 40,000 to 300,000, respectively.

DISCUSSION

RVHD is a new acute disease of domestic and wild rabbits that has been present in Europe since 1987 (22). The causative agent (RHDV) is a member of the *Caliciviridae* family, and a major structural protein, the capsid protein VP60, has been identified. The recombinant VP60 protein produced either in *Escherichia coli* (4) or in the baculovirus/Sf9 expression system (18) or by a vaccinia virus-RHDV recombinant virus (2) induces protective immunity.

The use of poxviruses such as the vaccinia virus as viral vectors for foreign genes for purposes of vaccination was described more than 10 years ago (17). These expression systems have proven their efficacy against many diseases (10, 13, 16, 23, 35). The vaccinia virus-rabies virus recombinant virus, the Copenhagen strain of vaccinia virus expressing the rabies virus G glycoprotein (17, 37), for example, has been used successfully for routine vaccination against rabies in the wild (oral vaccination of foxes in France and Belgium) (3, 6).

Moreover, some poxviruses used as viral vectors have an advantage over the vaccinia virus-based recombinant vaccines in that they also protect against vector-induced diseases. For example, recombinant capripoxvirus can protect cattle against lumpy skin disease (32) and recombinant fowlpoxvirus can

TABLE 1. Protection against MV challenge of rabbits vaccinated with SG33 or MV-RHDV recombinant viruses (RecA or RecB virus)^a

Vaccine	No. of rabbits affected/ n			Mortality
	Localized primary tumor	Primary and secondary tumors	Primary and secondary tumors with debilitation	
RecA	12/12	6/12	4/12	1/12
RecB	15/15	14/15	7/15	3/15
SG33	22/22	16/22	9/22	3/22
Nonvaccinated	12/12	12/12	12/12	12/12

^a Rabbits were challenged i.d. with 5×10^3 PFU of MV strain T1 44 days postvaccination. Clinical signs from a localized primary tumor to fatality were classified. Some of the rabbits that developed generalized symptoms died of myxomatosis.

TABLE 2. Protection against RVHD of rabbits vaccinated s.c. with inactivated anti-RVHD vaccine or i.d. with MV-RHDV recombinant viruses (RecA or RecB virus)^a

Vaccine	Inoculation route	Dose	No. of dead animals/no. of animals challenged at day 5	No. of dead animals/no. of animals challenged at day 15
RecA	i.d.	5×10^3 PFU	0/8	0/9
RecB	i.d.	5×10^3 PFU	0/9	0/9
Classical vaccine	s.c.	1 ml	0/9	0/9
Nonvaccinated			5/5	5/5

^a Rabbits were challenged intramuscularly 5 or 15 days postvaccination with 1,000 50% lethal doses of RHDV.

protect chickens against challenge with virulent fowlpoxvirus (24).

Myxomatosis is still a major viral disease of both wild and domestic rabbits. The causative agent belongs to the *Leporipoxvirus* genus of the *Poxviridae* family and shares the characteristics required to construct recombinant vaccines. Therefore, we tried to produce a bivalent vaccine by means of a new type of recombinant virus so that rabbits could be vaccinated simultaneously against these two diseases. Two recombinant MVs expressing RHDV VP60 were constructed. We used the SG33 MV strain, an attenuated French strain, and attempted to attenuate its residual pathogenicity for young rabbits by inactivating either TK (15) or M11L (13) and MGF (36) genes which encode pathogenesis factors. It has previously been reported that deletions in the M11L and MGF genes dramatically decrease the virulence of wild-type MV (26) and that inactivation of the vaccinia virus TK gene attenuates pathogenicity in mice (8).

The foreign VP60 in both recombinant viruses was expressed to a high level, but we also observed a minor band migrating close to the recombinant VP60. A similar result has been described for the VP60 of RHDV expressed in the baculovirus system (18) or by a vaccinia virus-RHDV recombinant virus (2). However, in contrast to previous reports (18, 21, 31), we did not detect any proteins migrating with molecular masses of 30 to 40 kDa. As in recombinant baculovirus (18) and vaccinia virus-RHDV-infected cells (2), no detectable expression of the low-molecular-mass protein corresponding to ORF2 (VP12) could be observed in recombinant RecA virus-infected cells. No recombinant VP60 could be detected in the supernatant of infected RK13 cells, in contrast to that observed in the baculovirus system (18).

One i.d. injection of both MV-RHDV recombinant viruses was able to protect young rabbits against a virulent RHDV challenge as efficiently as the commercial vaccines (1) under the conditions used for vaccine controls in France. On the whole, the protective immunity against RHDV was rapidly established, since all of the rabbits vaccinated 5 or 15 days before challenge were protected. Similar results had been reported previously with inactivated RHDV (1), virus-like particle (18), VP60 expressed in *E. coli* (4), or vaccinia virus-RHDV recombinant virus (2). It is likely that the humoral response plays a key role in protection against the disease (28) and that the protective immunity is efficient as soon as antibodies against VP60 can be detected in animal sera (4, 18, 28). As was observed with the VP60 expressed in the baculovirus/Sf9 expression system (18) or with a vaccinia virus-RHDV recombinant virus (2), we found that anti-VP60 antibodies could be detected as early as 5 days after vaccination and

correlated with resistance to challenge. However, nonspecific host responses to the live recombinant viruses cannot be ruled out as effectors of protection in such an early result. On the contrary, it seems likely that anti-VP60 antibodies might be fully responsible for the protection of animals that were challenged 15 days after vaccination. At that time, antibodies had already risen to moderate to high levels, and previous results obtained with i.d. inoculation of vaccinia virus 15 days before RHDV challenge showed no induced protection of rabbits against RVHD, suggesting that nonspecific responses did not intervene under these conditions (2). Although rabbits vaccinated with RecA or RecB virus were completely protected against RVHD after the challenge without any clinical sign or traces of RHDV, a rapid and more-than-10-fold increase in anti-VP60 antibody ELISA titers was observed. This might indicate an anamnestic response to RVHD due to challenge exposure, as well as a good priming effect by the recombinant VP60 protein.

The differences observed in antibody responses to the recombinant VP60 protein in rabbits that received RecA or RecB have not been explained to date. Whether the two recombinant MVs allowed the same antigen presentation of the foreign gene product remains to be investigated.

We vaccinated seronegative animals at weaning to check whether such recombinant viruses could be used early in the rabbit breeding schedule. Under industrial and traditional breeding conditions, maternal RHDV-specific antibodies may block the effect of an inactivated vaccine administered at weaning. The use of an attenuated recombinant virus may help to resolve this kind of problem, and specific data should be gathered by testing the recombinant viruses in young rabbits born from vaccinated does.

A single i.d. injection of a classical dose of either MV-RHDV recombinant virus gave the same level of partial protection against myxomatosis as the SG33 virus strain. Thus, the immunogenicity of MV as a live vaccine was not significantly impaired by the insertion of a foreign gene into the TK or M11L-MGF genes of the SG33 virus strain. We did not observe any signs of residual pathogenicity for young rabbits after administration of both recombinant viruses in our experiments, but this result needs to be confirmed under breeding conditions. The partial protection against myxomatosis induced by both RecA and RecB viruses and the very high increase in ELISA titers to MV may indicate replication of challenge T1 MV, a sign of nonsterilizing immunity. This result is not surprising, since a single SG33 administration is known to be insufficient to establish complete protective immunity against myxomatosis in rabbit.

Early and efficient protection against RVHD and myxomatosis may be obtained in young domestic rabbits by i.d. administration of MV-RHDV recombinant viruses. Such MV-RHDV recombinant vaccines have several advantages over the vaccinia virus-based RVHD recombinant vaccine. First, they protect rabbits against both RVHD and myxomatosis. Second, the geographical distributions of RHDV and MV are very similar, so that the use of recombinant vaccines based on MV will not entail the introduction of viruses that do not already exist in a particular area. Third, leporipoxviruses, unlike vaccinia virus, are very restricted in their host range and are not pathogenic for human beings. The wide host range of vaccinia virus has in fact been suggested as a possible cause of problems, e.g., transmission from target animals to nontarget species with the possible associated problem of recombination with other poxviruses. The choice of a vector with a small host range, such as MV, may further limit spread of the vector, thus

reducing the chance of an exchange of genes with wild-type pathogens.

We now need to control the efficiency of the oral route so as to explore the possibility of wild rabbit vaccination. MV-based recombinant vaccines seem to be good candidates for use in the control of RVHD in Europe.

ACKNOWLEDGMENTS

We thank G. Sutter for providing pSC11 plasmid and R. Py, J. Loupias, B. Moreno, H. Marcus, C. Héry, and M. Moulignié for excellent technical assistance.

This work was partially supported by a grant from ONC (Office National de la Chasse) and from the region Midi-Pyrénées.

REFERENCES

- Argüello Villares, J. L. 1991. Viral haemorrhagic disease of rabbits: vaccination and immune response. *Rev. sci. Tech. Off. Int. Epiz.* **10**:471-480.
- Bertagnoli, S., J. Gelfi, F. Petit, J. F. Vautherot, D. Rasschaert, S. Laurent, G. Le Gall, E. Boilletot, J. Chantal, and C. Boucraut-Baralon. Protection of rabbits against rabbit viral haemorrhagic disease with a vaccinia-RHDV recombinant virus. *Vaccine*, in press.
- Blancou, J., M. P. Kieny, R. Lathe, J.-P. Lecocq, P.-P. Pastoret, J. P. Soulebot, and J. P. Desmetre. 1986. Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. *Nature (London)* **322**:373-375.
- Boga, J. A., R. Casais, M. S. Marin, J. M. Martin-Alonso, R. S. Carmenes, M. Prieto, and F. Para. 1994. Molecular cloning, sequencing and expression in *Escherichia coli* of the capsid protein gene from rabbit haemorrhagic disease virus (Spanish isolate AST/89). *J. Gen. Virol.* **75**:2409-2413.
- Boucraut-Baralon, C., F. Petit, R. Py, S. Bertagnoli, and J. Chantal. 1994. Epidémiologie moléculaire du virus de la myxomatose par électrophorèse en champs pulsés, p. 1-8. In *INRA-ITVAI, VIème Journées de la Recherche Cunicole*, La Rochelle, France, 6 and 7 December 1994.
- Brochier, B., M. P. Kieny, F. Costy, P. Coffens, B. Bauduin, J.-P. Lecocq, B. Languet, G. Chappuis, J. P. Desmetre, K. Afiademomyo, R. Lobois, and P.-P. Pastoret. 1991. Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine. *Nature (London)* **354**:520-522.
- Brun, A., A. Godard, and Y. Moreau. 1981. La vaccination contre la myxomatose, vaccins hétérologues et homologues. *Bull. Soc. Sci. Vet. Med. Comp.* **83**:251-254. (In French.)
- Buller, R. M. L., G. L. Smith, K. Cremer, A. L. Notkins, and B. Moss. 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature (London)* **317**:813-815.
- Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell. Biol.* **5**:3403-3409.
- Drillien, R., D. Spehner, A. Kirn, P. Giraudon, R. Buckland, F. Wild, and J.-P. Lecocq. 1988. Protection of mice from fatal measles encephalitis by vaccination with vaccinia virus recombinants encoding either the hemagglutinin or the fusion protein. *Proc. Natl. Acad. Sci. USA* **85**:1252-1256.
- Fenner, F., and F. N. Ratcliffe. 1965. *Myxomatosis*. Cambridge University Press, London.
- Francki, R. I. B., C. M. Fauquet, D. L. Knudson, and F. Brown (ed.). 1991. Classification and nomenclature of viruses. Fifth Report of the International Committee on Taxonomy of Viruses. *Arch. Virol.* **1991**(Suppl. 2):1-450.
- Giavedoni, L., L. Jones, C. Mebus, and T. Yilma. 1991. A vaccinia virus double recombinant expressing F and H genes of rinderpest virus protects cattle against rinderpest and causes no pock lesions. *Proc. Natl. Acad. Sci. USA* **88**:8011-8015.
- Graham, K. A., A. Opgenorth, C. Upton, and G. McFadden. 1992. Myxoma virus M11L ORF encodes a protein for which cell surface localization is critical in manifestation of viral virulence. *Virology* **191**:112-124.
- Jackson, R. J., and H. G. Bults. 1992. The myxoma virus thymidine kinase gene: sequence and transcriptional mapping. *J. Gen. Virol.* **73**:323-328.
- Jones, L., L. Giavedoni, J. T. Saliki, C. Brown, C. Mebus, and T. Yilma. 1993. Protection of goats against peste des petits ruminants with a vaccinia virus double recombinant expressing the F and the H genes of rinderpest virus. *Vaccine* **11**:961-964.
- Kieny, M. P., R. Lathe, R. Drillien, D. Spehner, S. Skory, D. Schmitt, T. Wiktor, H. Koprowski, and J. P. Lecocq. 1984. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. *Nature (London)* **312**:163-166.
- Laurent, S., J.-F. Vautherot, M.-F. Madelaine, G. Le Gall, and D. Rasschaert. 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into virus-like particles and induces protection. *J. Virol.* **68**:6794-6798.
- Liu, S. J., H. P. Xue, B. Q. Pu, and S. H. Qian. 1984. A new viral disease in rabbits. *Anim. Husb. Vet. Med.* **16**:253-255. (In Chinese.)
- Marcato, P. S., C. Benazzi, G. Vecchi, M. Galeotti, L. Della Salda, G. Sarli, and P. Lucidi. 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and of the European brown hare syndrome. *Rev. Sci. Tech. Off. Int. Epiz.* **10**:371-392.
- Meyers, G., C. Wirblich, and H. J. Thiel. 1991. Rabbit hemorrhagic disease virus—molecular cloning and nucleotide sequencing of a calicivirus genome. *Virology* **184**:664-676.
- Morisse, J.-P., G. Le Gall, and E. Boilletot. 1991. Hepatitis of viral origin in Leporidae: introduction and aetiological hypotheses. *Rev. Sci. Tech. Off. Int. Epiz.* **10**:283-295.
- Moss, B., G. L. Smith, J. L. Gerin, and R. H. Purcell. 1984. Live recombinant vaccinia virus protects chimpanzees against hepatitis B. *Nature (London)* **311**:67-69.
- Nagy, E., P. J. Krell, R. A. Heckert, and J. B. Derbyshire. 1993. Vaccination of chickens with the hemagglutinin-neuraminidase gene of newcastle disease virus under the control of the fowlpox virus thymidine kinase promoter. *Can. J. Vet. Res.* **57**:306-308.
- Ohlinger, V. F., B. Haas, G. Meyers, F. Weiland, and H. J. Thiel. 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. *J. Virol.* **64**:3331-3336.
- Opgenorth, A., K. A. Graham, N. Nation, D. Strayer, and G. McFadden. 1992. Deletion analysis of two tandemly arranged virulence genes in myxoma virus M11L and myxoma growth factor. *J. Virol.* **66**:4720-4731.
- Panicali, D., and E. Paoletti. 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. *Proc. Natl. Acad. Sci. USA* **79**:4927-4931.
- Parra, F., and M. Prieto. 1990. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. *J. Virol.* **64**:4013-4015.
- Petit, F., C. Boucraut-Baralon, R. Py, and S. Bertagnoli. Analysis of myxoma virus genome using pulsed field gel electrophoresis. *Vet. Microbiol.*, in press.
- Rasschaert, D., S. Huguet, M. F. Madelaine, and J. F. Vautherot. 1995. Sequence and genomic organization of a rabbit hemorrhagic disease virus isolated from a wild rabbit. *Virus Genes* **9**:121-132.
- Rodak, L., B. Smid, L. Valicek, T. Vesely, J. Stepanek, J. Hampl, and E. Jurak. 1990. Enzyme-linked immunosorbent assay of antibodies to rabbit haemorrhagic disease virus and determination of its major structural proteins. *J. Gen. Virol.* **71**:1075-1080.
- Romero, C. H., T. Barrett, S. A. Evans, R. P. Kitching, P. D. Gershon, C. Bostock, and D. N. Black. 1993. Single capripoxvirus recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. *Vaccine* **11**:737-742.
- Russell, R. J., and S. J. Robbins. 1989. Cloning and molecular characterization of the myxoma virus genome. *Virology* **170**:147-159.
- Saurat, P., Y. Gilbert, and J.-P. Ganière. 1978. Etude d'une souche de virus myxomateux modifié. *Rev. Med. Vet.* **129**:415-451. (In French.)
- Smith, G. L., B. R. Murphy, and B. Moss. 1983. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proc. Natl. Acad. Sci. USA* **80**:7155-7159.
- Upton, C., J. L. Macen, and G. McFadden. 1987. Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. *J. Virol.* **61**:1271-1275.
- Wiktor, T. J., R. I. Macfarlan, K. Reagan, B. Dietzschold, P. Curtis, W. H. Wunner, M. P. Kieny, R. Lathe, J.-P. Lecocq, M. Macket, B. Moss, and H. Koprowski. 1984. Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. *Proc. Natl. Acad. Sci. USA* **81**:7194-7198.