First field trial of a transmissible recombinant vaccine against myxomatosis and rabbit hemorrhagic disease

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Abstract

As a novel approach for immunisation of wild rabbits, we have recently developed a transmissible vaccine against myxomatosis and rabbit hemorrhagic disease (RHD) based on a recombinant myxoma virus (MV) expressing the RHDV capsid protein [J. Virol. 74 (2000) 1114]. The efficacy and safety of the vaccine have been extensively evaluated under laboratory conditions. In this study, we report the first limited field trial of the candidate vaccine that was undertaken in an island of 34 Has containing a population of around 300 rabbits. Following administration by the subcutaneous route to 76 rabbits, the vaccine induced specific antibody responses against both myxomatosis and RHDV in all the inoculated rabbits. Furthermore, the recombinant virus exhibited a limited horizontal transmission capacity, promoting seroconversion of around 50% of the uninoculated rabbit population. No evidence of undesirable effects due to the recombinant virus field release was detected. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Field evaluation; Myxoma-RHDV; Transmissible vaccine

1. Introduction

Myxomatosis and rabbit hemorrhagic disease (RHD) are considered the major viral diseases affecting the European rabbit (Oryctolagus cuniculus). Myxoma virus (MV), the causative agent of myxomatosis, belongs to the Leporipoxvirus genus of the Poxviridae family [1]. In its natural host, Sylvilagus rabbits in the Americas, MV induces a mild benign infection. In European rabbits, however, MV causes the systemic and lethal infection known as myxomatosis [2,3]. The disease is endemic in the entire rabbit range in Europe since the deliberate release of MV in France (1952) as a biological control agent of wild rabbit populations.

RHD was first reported in domestic rabbits in China [4]. It subsequently reached other countries, primarily by trade of contaminated rabbit products, spreading throughout rabbit populations in Europe between 1987 and 1989 [5]. RHD is responsible for high economic losses in rabbitries as well as heavy mortality among wild rabbits [6–10]. The etiological agent, rabbit hemorrhagic disease virus (RHDV), is a member of the Caliciviridae family [11]. The RHDV virions are non-enveloped and icosahedral, with capsids composed of a major protein component of 60 kDa (VP60). In the last years, the VP60 gene has been successfully expressed in several heterologous systems [12–20] and has been shown to induce full protection of rabbits against a lethal challenge with RHDV.

A number of vaccines are available to protect rabbits against myxomatosis and RHD [21–23], which are useful for immunising domestic rabbits. However, these vaccines are not suited to immunise wild rabbit populations, as they need to be delivered individually, which is not a feasible approach to vaccinate free-ranging animals. As an alternative approach, we have explored the
possibility of developing a ‘transmissible vaccine’. In order to protect wild rabbits against both myxomatosis and RHD, we constructed a recombinant virus based on the naturally attenuated MV field strain 6918 [24] that expressed the RHDV VP60 protein. Direct administration of the recombinant virus (6918VP60-T2) by the subcutaneous, intradermal or oral routes protected rabbits against lethal RHDV and MV challenges. Furthermore, the recombinant 6918VP60-T2 virus showed a limited horizontal transmission capacity, either by direct contact or in a flea-mediated process, promoting immunisation of contact uninoculated animals [25].

The results obtained so far suggest that the transmissible vaccine could be effective for wild rabbit immunisation. However, before considering the environmental release of the candidate vaccine, considerations regarding safety issues should be thoroughly addressed. For this reason, potential risks of vaccine administration were evaluated in the laboratory. Results indicated that vaccine administration was safe even at a 100-fold overdose, and no undesirable effects were detected upon administration to immunosuppressed or pregnant rabbits [26].

As a next step towards the field use of the transmissible vaccine, the recombinant virus was subjected to the mandatory risk assessment process relative to the environmental release of genetically modified organisms. On the basis of the efficacy and safety data previously reported [24–26], a limited field trial was authorised by the Spanish competent authorities in a small island containing a population of about 300 wild rabbits. In this paper, we report the first results of the trial covering an observation period of 32 days after vaccination, concerning some aspects on the safety and efficacy of the vaccine under controlled field conditions.

2. Materials and methods

2.1. Vaccine virus and cells

Recombinant virus 6918VP60-T2 was propagated in RK-13 (rabbit kidney) cell line grown in Dulbecco’s minimum essential medium (DMEM) supplemented with 5% foetal bovine serum (FBS), 2 mM l-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. SIRC (rabbit cornea) cells were used for viral titer determination on plaque assay. Both rabbit cell lines were obtained from the American Type Culture Collection (ATCC).

2.2. Experimental area

The experiment took place in Isla del Aire, an island of 34 Has located 1 km to the east of Menorca (Balearic Islands). The island holds an important colony of seagulls and a population of European rabbits (Oryctolagus cuniculus). It has no natural predators for rabbits. There is no human activity on the island other than sporadic visits and hunting, but during the course of the experiment, it was guarded in order to avoid any kind of human presence with the exception of the personnel involved in the experiment.

2.3. Experimental design

A total of 147 adult rabbits (over 2 months old) were caught using live-traps baited with dried alfalfa, random and uniformly distributed over the entire surface of the experimental area. The captured animals were tagged with a microchip (AVID), placed subcutaneously in the dorsal region of the neck, and a blood sample (2 ml) was extracted from the jugular vein of each rabbit. Subsequently, the animals were randomly allocated in one of two groups (A and B) of 76 and 71 animals, respectively. The same day, rabbits from group A were inoculated at the back by the subcutaneous (s.c.) route with one dose of the vaccine (10⁴ pfu of 6918VP60-T2 recombinant virus), while animals from group B were used as contact non-vaccinated rabbits. Following this, the rabbits from both groups (A and B) were released near their point of capture. At days 8, 24 and 32 post-vaccination, a number of rabbits were captured until reaching a minimum of 15 animals (see Table 1) of each group in order to evaluate the development of clinical signs. In addition, a blood sample was extracted from the jugular vein of the rabbits captured 32 days post vaccination (dpv). A serum sample was drawn of each blood sample and frozen until subsequent analysis in the laboratory.

2.4. Estimation of the rabbit population number on the experimental area

The total rabbit population of the island was estimated by a method based on the frequencies of rabbit recaptures. This method is based on the assumption that all individuals have the same chance of being captured; therefore, microchip-marked and non-marked rabbits would be captured with the same prob-

<table>
<thead>
<tr>
<th>Number of rabbits captured per day</th>
<th>0 dpv</th>
<th>8 dpv</th>
<th>24 dpv</th>
<th>32 dpv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>76</td>
<td>22</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Group B</td>
<td>71</td>
<td>22</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Non-marked (NM)</td>
<td>46</td>
<td>51</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>147</td>
<td>90</td>
<td>86</td>
<td>92</td>
</tr>
</tbody>
</table>
ability. It is also assumed that no significant variations in the total rabbit population will occur during the 8 day period between two captures (if these were to occur, dead animals would be detected on the island). On the first day (0 dpv), 147 rabbits were captured and marked with a microchip. Eight days later, 44 marked animals and 46 that had not been marked previously, were captured (see Table 1). Accordingly, the total rabbit population of the island was estimated in 300 rabbits as follows:

\[
\text{Total rabbit population} = 147 \times \left( \frac{44 + 46}{44} \right) \geq 300 \text{ rabbits.}
\]

2.5. Evolution of the relative density of rabbit population during the trial

The commonly used method for estimating relative and absolute densities of wild rabbit populations described by Taylor et al. [27], based on the measurement of the number of fecal pellets, was considered unsuitable for this trial. Preliminary work evidenced that pellet counts varied from day to day by factors unrelated to the number of rabbits, probably due to the strong winds present in the island. As an alternative approach to estimate variations in the relative population density during the trial, a method based on the visual inspection of the experimental area was used. Density estimates were performed on days −3, −2, 3, 6, 9, 12, 15 and 31 dpv. For this purpose, the island was divided into five areas, A, B, C, D and E and three people inspected each area at the same time, spending 30 min in an area before moving into the next one. The number of rabbits detected by each person was annotated, and after all the areas had been inspected, the total number was recorded. Data obtained during the days prior to the recombinant virus release (101 rabbits at −3 dpv and 110 rabbits at −2 dpv) showed that the animal counts by visual inspection did not vary significantly from day to day, indicating that the results of relative density obtained by this method were reliable. Data obtained on days 3, 6, 9, 12, 15 and 31 dpv showed that the number of animals counted by visual inspection remained statistically constant during the first 32 days following vaccination. In addition to the density estimates performed on the indicated days, substantial visual inspection of the island also took place on the days of rabbit capture (0, 8, 24, 32 dpv). This systematic inspection of the island throughout the observation period allowed the detection of ill or injured animals and any dead body. All the rabbit carcasses found were collected for post-mortem examination and virus determination studies.

2.6. Immune response evaluation

Serum samples extracted from rabbits on days 0 and 32 after vaccination were used to evaluate the antibody response against MV and RHDV, using an enzyme-linked immunosorbent assay (ELISA) previously described [25]. Antibody titres were defined as the reciprocal of the highest dilution giving an A₄₀₅ value twofold over the background level (negative control rabbit sera).

2.7. Virus detection

The presence of MV virus in tissue samples extracted from rabbits was determined by polymerase chain reaction (PCR) using oligonucleotides: MV1 and MV2, derived from the MV genomic sequence flanking the VP60 gene insertion site, and oligonucleotide VP60-1, which hybridises with the 5’ region of the VP60 gene, as previously described [25].

2.8. Statistical analysis

Data were analysed using Student’s t-test for non-paired variants. Significance was considered when \( P < 0.05 \).

3. Results

3.1. Effects induced by vaccine virus administration

To evaluate the effects of delivering the transmissible vaccine to rabbits under field conditions, a total of 147 adult rabbits were captured using live traps distributed over the experimental area. Seventy-six of these animals (group A) were tagged with a microchip and inoculated by s.c. route with a vaccinal dose of 6918VP60-T2 recombinant virus. The other 71 animals (group B) were microchip-tagged and used as contact non-vaccinated rabbits. All the rabbits were released near their point of capture.

On days 8, 24 and 32 post-vaccination, rabbits were captured and thoroughly examined for clinical signs. The number of rabbits captured from each group is summarised in Table 1.

In order to obtain a semi-quantitative measure to allow an objective comparison, the classical myxomatosis symptoms were ranked from 1 to 6 points. The results registered during the observation period according to this ranking are summarised in Table 2. About 65% of the inoculated rabbits (Group A) captured 8 and 24 dpv showed clinical signs associated with the vaccine (14 out of 22 captured rabbits 8 dpv, and 10 out of 15 captured rabbits 24 dpv). These consisted of a localised primary nodule at the inoculation site and,
in some rabbits (less than 30%), scanty secondary skin lesions in the form of small discrete nodules, usually less than 0.5 cm in diameter, in the back, face or ears (see Table 2). No clinical signs were observed in inoculated rabbits (Group A) captured 32 dpv. Furthermore, lesions observed in four animals captured 24 dpv had completely disappeared when they were re-captured 32 dpv. None of the inoculated rabbits exhibited classical severe myxomatosis symptoms like closure of the eyes, generalised oedema, or respiratory syndrome (Table 2), and the overall health of the rabbits was largely unaffected. None of the contact uninoculated rabbits, either marked (Group B) or not marked (NM), showed any symptomatology associated with myxomatosis (Table 2).

Given the size and the orographic conditions of the island, visual inspection was considered a good way of estimating the relative rabbit population. Variations in the number of animals counted should reflect changes in the total rabbit population. During the trial, the number of animals counted by visual inspection remained statistically constant, indicating that vaccine administration had no significant influence over the total number of individuals of the studied rabbit population during the observation period.

Additional information about the overall health status of the rabbit population was obtained by the visual inspection of the experimental area. Any abnormal observations like ill animals or detection of dead bodies were registered. No ill rabbits presenting myxomatosis symptoms were detected. Only two vaccinated animals (Group A) were found dead throughout the trial. These rabbits were recovered on days 15 and 24 post-vaccination. Although the corpses presented a certain degree of decomposition, no evidence of clinical signs attributable to myxomatosis were detected. PCR virus determinations revealed the presence of the vaccinal virus in the skin of the animal recovered 15 dpv, but not in the rabbit recovered 24 dpv. One marked non-vaccinated rabbit (Group B) and one non-marked rabbit (NM) were found dead on day 6 following vaccination. The marked rabbit (Group B) probably died due to Tyzzer disease (*Bacillus piliformis*), and the non-marked rabbit (NM) had a traumatic lesion in the lumbar area. Finally, two marked non-vaccinated (Group B) animals were found dead on day 32. One of them died during blood extraction, probably due to the stress suffered during the capture and manipulation; the other was found in advanced state of decomposition and partially eaten by seagulls. The PCR analysis performed to detect the vaccinal MV virus was negative in these four non-vaccinated rabbits (not shown).

### 3.2. Antibody response induced in vaccinated rabbits

To evaluate the immune response elicited by the vaccine in rabbits after s.c. inoculation, sera samples from inoculated rabbits obtained 32 dpv were analysed for the presence of anti-MV and anti-RHDV antibodies by ELISA, and compared with the sera samples obtained before vaccine administration (0 dpv). Results are represented as a frequency distribution of five antibody titre intervals in Fig. 1. At 0 dpv, more than 80% of rabbits from Group A (vaccinated rabbits) were seronegative (titre <10) for both MV (Fig. 1A) and RHDV (Fig. 1B), and only a few animals (less than 20%) showed low anti-MV or anti-RHDV antibody titres (10–100). At 32 dpv, 100% of the rabbits from group A showed anti-MV antibody titres ranging from 1000 to 100000 (Fig. 1A) and anti-RHDV antibody titres ranging from 100 to 100000 for (Fig. 1B). It is important to remark that all the rabbits from group A, which were analysed 32 days after vaccination, were doubly seropositive (MV +, RHDV +).
3.3. Antibody response induced in contact non-vaccinated rabbits

In order to analyse the ability of the vaccinal virus to disseminate among the rabbit population and induce an immune response in contact rabbits, the antibody response against both MV and RHDV was also evaluated in contact non-vaccinated rabbits. For this purpose, sera samples from microchip-marked uninoculated rabbits (Group B) obtained 32 dpv were analysed by ELISA for the presence of anti-MV and anti-RHDV antibodies and compared with the sera obtained before vaccination (0 dpv). Results are represented as a frequency distribution of five antibody titre intervals in Fig. 1C and D. At 0 dpv, more than 80% of the rabbits were seronegative (titre <10) for both MV and RHDV, and only a few animals (less than 20%) had a low anti-RHDV and/or anti-MV antibody titres (10–100). On day 32 dpv, more than 75% of the rabbits from group B exhibited anti-MV (Fig. 1C) and anti-RHDV (Fig. 1D) antibody titres ranging from 10–10000.

The number of double seropositive animals (MV+, RHDV+) in contact non-vaccinated rabbits from group B increased from 8% (0 dpv) to 64% (32 dpv), while the number of single seropositive animals (MV+, RHDV−) increased from 0% (0 dpv) to 12% (32 dpv). According to this, 56% of the contact non-vaccinated rabbits from Group B developed significant antibody titres against both MV and RHDV upon vaccinal virus release (although the titres observed in this group of animals were consistently lower than those of directly vaccinated rabbits from group A), and 12% of the animals developed antibodies against MV but not against RHDV.

Similar results were obtained when the serological study was extended to the totality of the contact non-vaccinated rabbits captured 32 dpv, including microchip-marked (Group B) and non-marked rabbits (NM). The number of non-vaccinated rabbits captured 32 dpv (71) was large enough to be considered representative of the total non-vaccinated rabbit population in the experimental area, which was estimated in 230 rabbits. Anti-MV and anti-RHDV antibody titres rep-

![Graphs showing antibody response](image)

Fig. 1. Serum antibody response (ELISA) in rabbits inoculated by the s.c. route (Group A) with a single dose (10⁴ pfu) of 6918VP60-T2 recombinant virus (A and B) or in contact uninoculated (Group B) rabbits (C and D). Anti-MV (A and C) and anti-RHDV (B and D) antibody titres before vaccination (0 dpv) and 32 dpv are represented as a frequency distribution of five antibody titre intervals in which bars represent the percentage of rabbits with the indicated titre value.
contact rabbit population (Group B + NM) and in the total rabbit population (Group A + Group B + NM), upon vaccinal virus release.

Finally, Fig. 3 compares the serological status of the contact rabbit population (Group B + NM) and the total rabbit population (Group A + Group B + NM) by day 32 post-vaccination, with the serological situation of the total rabbit population (Group A + Group B) before vaccination (0 dpv). Of the contact rabbit population, 58.6% were double seropositive for both MV and RHDV, and 11.4% were single positive for MV. Since only 8.8% of the total rabbit population were seropositive for MV before vaccination (0 dpv), we can conclude that the vaccinal virus disseminated among rabbits by horizontal transmission inducing an antibody response against both MV and RHDV in about 50% of the contact rabbits.

Considering together directly vaccinated rabbits (Group A) and contact rabbits (Group B + NM), the serological situation of the total rabbit population in the experimental area 32 days after vaccination was as follows: 68.1% of the rabbits were seropositive to both MV and RHDV, 8.8% were seropositive only for MV, 19.8% were seropositive only for RHDV, and only 3.3% of the rabbits were seronegative for both MV and RHDV (Fig. 3).

4. Discussion

Since the proposed use of 6918VP60-T2 involves the environmental release of a recombinant virus, considerations regarding safety issues are as important as the potential efficacy of the candidate vaccine. For this reason, safety concerns have been at the core of the rational design of the proposed immunisation strategy.

The biological characteristics of MV make it a good candidate as a vaccine vector in terms of safety considerations, as it exhibits a very restricted host range, infecting exclusively rabbits. Safety aspects were also

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**Fig. 2.** Serum antibody response (ELISA) 32 dpv in uninoculated rabbit population (Group B + NM) or total rabbit population (Group A + Group B + NM), compared with the serum antibody titres of the rabbits captured 0 dpv (Group A + Group B). Anti-MV (A) and anti-RHDV (B) antibody titres from rabbits captured 0 dpv and 32 dpv are represented as a frequency distribution of five antibody titre intervals in which bars represent the percentage of rabbits with the indicated titre value.

**Fig. 3.** Seroprevalence of specific Anti-MV and anti-RHDV antibodies in the contact rabbit population (Group B + NM) and in the total rabbit population (Group A + Group B + NM) at 32 dpv compared with that of the rabbits captured 0 dpv (Group A + Group B). The percentage of rabbits doubly seropositive for both MV and RHDV (M+H+), single positive for MV (M+R−) or RHDV (M−R+), and doubly seronegative (M−R−) are represented as proportional bars.
considered in the choice of the parental MV strain. It was decided to use a naturally attenuated MV field strain already circulating among wild rabbit populations. Strain 6918 was selected from a field survey of MV strains circulating in Spain [24]. This strain causes a non-pathogenic infection comparable to that of cell culture-attenuated vaccinal strains, yet retaining a limited capacity of horizontal spreading [24].

Extensive laboratory testing reported previously indicated that the administration of either 6918 MV or recombinant 6918VP60-T2 virus to healthy rabbits is safe. Inoculated rabbits exhibit only mild clinical symptoms and rapidly recover [24,25]. The safety assessment of the vaccine was further extended by analysing the potential risks of vaccine administration under a varied range of situations that might occur if the recombinant virus is used for large-scale field immunisation of rabbits [26]. The overall results obtained demonstrate a notable lack of adverse effects attributable to the recombinant virus, regardless of dose, route or life history stage of individuals. In addition, the biological and genetic stability of the recombinant virus was demonstrated after 15 passages in cultured cells or 10 passages in vivo [25,26].

Despite all the safety data obtained under laboratory conditions, the response to the live attenuated vaccine could be expected to be different in the field given the greater variability in health or immune status among individuals, due to biological and environmental conditions, as well as varying levels of pressure from parasites, pathogens, competitors and predators. In order to address these safety concerns, a limited field trial under controlled conditions was performed.

The first environmental release of a vaccine based on a genetically engineered organism should necessarily be conducted in relative biocontainment conditions, such as on an island. The geographic isolation of the Isla del Aire rabbit population was conducive for an intensive study of the rabbits exposed to the vaccine, thereby enabling a detailed evaluation of the effects associated to the recombinant virus release.

Under field conditions, the rabbits directly inoculated with the vaccine (76 animals) exhibited only small transient lesions similar to those previously observed in the laboratory (Table 2). None of the contact rabbits showed clinical signs associated with myxomatosis (Table 2). During the observation period, six rabbits were found dead in the experimental area: two vaccinated rabbits, three marked contact rabbits and one non-marked rabbit. These numbers can be considered normal given the length of the observation period (32 days) and a total population estimated at around 300 rabbits. Post-mortem examinations concluded that none of the rabbit deaths could be attributed to myxomatosis. Moreover, the lack of statistically significant differences in the total rabbit population of the island during the first 32 days following vaccination indicated that there was no detectable increase in mortality that could be associated with the vaccine release.

In conclusion, the overall results obtained demonstrate a remarkable lack of undesirable effects in the rabbit population attributable to the recombinant virus release, and no adverse phenomena were observed in wildlife throughout the observation period. The data presented in this paper add to the extensive body of knowledge regarding the recombinant Myxoma-RHDV transmissible vaccine safety and extend it to include evaluation in field conditions, in a relatively simple ecosystem.

Vaccine efficacy was another objective of the field evaluation. The substantial live trapping conducted during this study generated extensive serological data suitable to address this question. Direct immunisation of rabbits by a single s.c. inoculation of the vaccine induced a high antibody response against both MV and RHDV (Fig. 1A and B). Furthermore, the viral virus disseminated among the rabbit population by horizontal transmission inducing a lower but significant antibody response against both MV and RHD in about 50% of the contact uninoculated rabbits (Fig. 1C and D, 2 and 3). This limited horizontal transmission capacity exhibited by the vaccinal virus in the field was similar to that previously reported in the laboratory [25].

The relatively low antibody titres against MV and RHD found in contact rabbits might raise questions about the potential efficacy of the transmissible vaccine in the field. However, in previous experiments conducted under laboratory conditions [25], similar antibody titres were detected in contact rabbits, which in turn survived to lethal MV or RHDV challenges. Indeed, several studies have shown that protective immunity against RHDV is efficient as soon as there are detectable antibody levels against VP60 in rabbit sera [12,13,17,25]. Moreover, we have previously shown that infection of immunised rabbits with virulent MV or RHDV induces a high increase in the respective antibody titres [25]. This result indicates that the immunity evoked by 6918VP60-T2 is readily reinforced by exposure to virulent virus. In areas where myxomatosis or RHD is endemic, vaccinated rabbits will be readily re-exposed to the viruses. Therefore, a high level of immunity is expected to be maintained in vaccinated rabbits over a prolonged period of time.

In this paper, we have reported the preliminary results of the first field trial of the recombinant Myxoma-RHDV transmissible vaccine. Additional observations regarding vaccine safety and efficacy will soon be reported.
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References


