Safety evaluation of a recombinant myxoma-RHDV virus inducing horizontal transmissible protection against myxomatosis and rabbit haemorrhagic disease

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Abstract

We have recently developed a transmissible vaccine to immunize rabbits against myxomatosis and rabbit haemorrhagic disease based on a recombinant myxoma virus (MV) expressing the rabbit haemorrhagic disease virus (RHDV) capsid protein [Bárcena et al. Horizontal transmissible protection against myxomatosis and rabbit haemorrhagic disease using a recombinant myxoma virus. J. Virol. 2000;74:1114–23]. Administration of the recombinant virus protects rabbits against lethal RHDV and MV challenges. Furthermore, the recombinant virus is capable of horizontal spreading promoting protection of contact animals, thus providing the opportunity to immunize wild rabbit populations. However, potential risks must be extensively evaluated before considering its field use. In this study several safety issues concerning the proposed vaccine have been evaluated under laboratory conditions. Results indicated that vaccine administration is safe even at a 100-fold overdose. No undesirable effects were detected upon administration to immunosuppressed or pregnant rabbits. The recombinant virus maintained its attenuated phenotype after 10 passages in vivo.

Keywords: Safety; Myxoma-RHDV; Transmissible vaccine

1. Introduction

Myxomatosis and rabbit haemorrhagic 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 Poxxviridae 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. Immunization of domestic rabbits against myxomatosis is currently achieved using heterologous vaccines based on Shope fibroma virus, a less virulent Leporipoxvirus, or homologous vaccines based on cell culture-attenuated strains of MV [4,5].

RHD was first reported in the People’s Republic of China [6]. The disease spread throughout Europe between 1987 and 1989 [7] and is endemic since then. Infected rabbits usually die within 48–72 h of necrotising hepatitis. RHD is responsible for high economic losses in rabbitries as well as high mortality rates in wild rabbit populations [8–12]. The etiological agent, rabbit haemorrhagic disease virus (RHDV), is a member of the Caliciviridae family.
The RHDV virions are non-enveloped and icosahedral, with capsids composed of a major protein component of 60 kDa (VP60). Commercial vaccines against RHD are prepared from the livers of experimentally infected rabbits [14], since in vitro systems are not available for efficient virus propagation. In the last years, the VP60 gene has been successfully expressed in several heterologous systems [15–23] and has been shown to induce full protection of rabbits against a lethal challenge with RHDV.

While the currently available vaccines against myxomatosis and RHD have proven effective in the control of these diseases in domestic rabbits, they are not suited to immunize wild rabbit populations, as vaccines need to be delivered individually by conventional veterinary practices, which is not a feasible approach to vaccinate free ranging animals.

As a novel approach for wildlife vaccination, we have explored the possibility of developing “transmissible vaccines” by the use of viral vectors capable of spreading within an animal population. 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 [25]. A linear epitope tag from the nucleoprotein of porcine transmissible gastroenteritis coronavirus (TGEV) was included within the recombinant VP60 protein to allow monitoring the spread of the recombinant virus in the environment. Following inoculation of rabbits, the recombinant virus (6918VP60-T2) induced specific antibody responses against MV, RHDV as well as for the TGEV tag. Administration of 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 immunization of contact un inoculated animals [25].

The promising results obtained so far under laboratory conditions suggest the recombinant 6918VP60-T2 could be used in large-scale immunization schemes for the control of myxomatosis and RHD in wild rabbit populations. However, before considering its environmental release, vaccine safety considerations should be extensively evaluated. Potential risks with regard to vaccine dose (i.e., accidental administration of an overdose), age, physiological condition (i.e., pregnant does) and immune status of exposed individuals, should be taken into account. Biological stability is another important aspect to evaluate in a recombinant virus intended for environmental release. In the present study, we report the safety evaluation under laboratory conditions of recombinant 6918VP60-T2 virus concerning the above mentioned issues.

2. Materials and methods

2.1. Cells and viruses

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 penicillin, and 100 µg/ml streptomycin. SIRC (rabbit cornea) cells were used for viral titre determination on plaque assay. Both rabbit cell lines were obtained from the American Type Culture Collection (ATCC).

2.2. Rabbits

Common rabbits (brown coloured) free from anti-MV and anti-RHDV antibodies, were provided by a commercial breeder. These rabbits are routinely used for restocking in the field and from now on will be referred to as “wild rabbits”.

2.3. Administration of an overdose of 6918VP60-T2 virus

Groups of eight wild rabbits (2 month old, weighing around 0.8 kg) free from MV and RHDV antibodies, were inoculated at the back by intradermic (i.d.) or subcutaneous (s.c.) route with different doses of the vaccine (10^4, 10^5, 10^6 pfu of 6918VP60-T2 recombinant virus). Rabbits were observed daily for 21 days and clinical symptoms were recorded. Weight and temperature determinations were made on each animal until the 21st day. Serum samples extracted from the marginal ear vein of the rabbits on days 0 and 21 after immunization were used to evaluate the serological responses against MV and RHDV, by using an enzyme-linked immunosorbent assay (ELISA), as previously described [25]. Antibody titres were defined as the reciprocal of the highest dilution giving an A405 value two-fold over the background level (negative control rabbit sera).

2.4. Administration of 6918VP60-T2 virus to immunosuppressed rabbits

Groups of eight wild rabbits (2 month old, weighing around 0.8 kg) were immunosuppressed by treatment with prednisolone (2 mg per animal per day) for 3 days before vaccination and 2 days after vaccination. Prednisolone treated rabbits were inoculated by i.d. or s.c. route at the back with 10^5 pfu of 6918VP60-T2

virus. Control rabbits were vaccinated but not treated with prednisolone. Rabbits were observed daily for a period of 21 days and clinical symptoms were recorded. Weight and temperature determinations were made on each animal until the 21st day. Serum samples extracted 0 and 21 days after immunization were used to evaluate the serological responses against MV and RHDV by ELISA. Antibody titres were defined as described above.

2.5. Administration of 6918VP60-T2 virus to pregnant does

Groups of six pregnant does were inoculated at different times of gestation (days 7, 14, 21 and 28) by s.c. route at the back with 10⁴ pfu of 6918VP60-T2 virus. Control does were inoculated at the same days of gestation with 0.5 ml of phosphate-buffered saline (PBS). Animals were observed daily and general clinical symptoms were recorded. No body weight and temperature determinations were performed in order to minimise the handling-induced stress in does, which are specially sensible during gestation. The following reproductive parameters were recorded both at first and second parturition: number of animals born alive per litter; number of animals born dead per litter; number of living animals per litter 8 days postparturition (dpp), and weight of each litter at 8 dpp.

2.6. Analysis of 6918VP60-T2 virus biologic stability

Two rabbits (2 month old, weighing around 0.8 kg) were inoculated by i.d. route at the back with 10⁴ pfu of 6918VP60-T2 virus. Seven to 9 days postvaccination the inoculation site nodule was extracted, homogenated in PBS, and reinoculated into two new rabbits. This procedure was repeated up to 10 passages. The virus obtained from the last passage was titrated and the effects of inoculating 10⁴ pfu by s.c. in a group of eight rabbits were evaluated as described above and compared with those of the original recombinant virus. Serum samples extracted 0 and 21 days after immunization were used to evaluate the serological responses against MV and RHDV by ELISA. Antibody titres were defined as described above. In order to evaluate the genetic stability of 6918VP60-T2 virus after 10 passages in rabbits, DNA extracted from the nodules at the inoculation site was analysed by PCR. The oligonucleotides used were MV1 and MV2, which are derived from the MV genomic sequence flanking the foreign gene insertion site [25]. The amplification of a 3.3-kb PCR product, instead of the 1.0-kb product obtained from wild-type MV, was indicative of the presence of the inserted VP60 gene construct.

2.7. Statistical analysis

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

3. Results

3.1. Effects induced by the administration of an overdose of 6918VP60-T2 virus

Previous work showed 10⁴ pfu was an efficient vaccine dose to ensure horizontal transmissible protection against myxomatosis and RHD, either by direct contact or in a flea-mediated process [25]. To evaluate the effects of administering an overdose of the vaccine, wild rabbits were inoculated by i.d. or s.c. route with different doses of 6918VP60-T2 virus (10⁴, 10⁵ and 10⁶ pfu).

In order to obtain a semi-quantitative measure to allow graphic representation and objective comparison, the classical myxomatosis symptoms were classified in a ranking of 1 to 6 points (see Table 1), and the results registered during the observation period were represented (Fig. 1). Rabbits inoculated by i.d. route displayed similar clinical signs at all vaccine doses tested. These consisted of a localised primary nodule at the inoculation site and, in some rabbits, scanty secondary skin lesions in the form of small discrete nodules, usually less than 0.5 cm in diameter, in face, ears or eyelids. Lesions appeared 5–7 days postinoculation (dpi) and completely resolved in all rabbits normally by 15 dpi. None of the infected rabbits exhibited classical severe myxomatosis symptoms like closure of the eyes, generalised oedema, or respiratory syndrome (Fig. 1). Rabbits inoculated by s.c. route showed similar clinical symptoms but these were consistently

Fig. 1. Effects of administering different doses of 6918VP60-T2 virus. Groups of eight wild rabbits were inoculated by i.d. route with 10⁴ (●), 10⁵ (■), or 10⁶ (▲) pfu. Rabbits were observed daily for a period of 18 days and the clinical signs due to virus infection of each animal were ranked from 0 to 6 according to Table 1.
milder: there were less secondary nodules, which were slightly smaller and resolved earlier (results not shown). No febrile response or loss of body weight was detected. Table 2 shows temperature increases registered from 0 to 2 dpi and from 0 to 4 dpi, as well as the weight increase from day 0 to day 21. No significant differences in the increases of body temperature or body weight were observed in recombinant virus-infected rabbits as compared with control rabbits, regardless of virus dose or inoculation route.

To evaluate the immune responses elicited by the inoculated rabbits, sera samples obtained 21 dpi were monitored by ELISA for the presence of anti-MV and anti-RHDV antibodies. The inoculated rabbits developed high anti-MV and anti-RHDV antibody titres, which increased with the vaccine dose (Table 2). There was no gross difference in the antibody titres induced by vaccine administration by i.d. or s.c. inoculation routes.

3.2. Effects induced by the administration of 6918VP60-T2 virus to immunosuppressed rabbits

To evaluate the effects of recombinant virus infection on immunocompromised animals, rabbits were immunosuppressed by treatment with prednisolone. Treated rabbits were inoculated (by s.c or i.d. route) with 10^4 pfu of 6918VP60-T2 virus, and clinical signs due to virus infection were compared with those induced in control rabbits, which were vaccinated but not treated with prednisolone (Fig 2, Table 3). Results indicated that administration of 6918VP60-T2 virus to immunocompromised animals was safe (either by i.d. or s.c routes), as prednisolone treated rabbits exhibited only mild clinical symptoms and were all completely recovered by 18 dpi. Fig. 2 shows a graphic representation of the symptomatology observed in rabbits inoculated by i.d. route, according to the ranking of myxomatosis clinical signs established in Table 1. After i.d. inoculation, immunosuppressed rabbits exhibited similar local lesions to those observed in control non-immunosuppressed rabbits. Lesions appeared at the same time (5–7 dpi) in both cases but showed a subtle tendency to resolve later in immunosuppressed rabbits (15–18 dpi vs. 15 dpi). Results obtained with rabbits inoculated by the s.c route were essentially the same (data not shown). No significant differences in body temperature increase or body weight increase were observed when immunosuppressed rabbits were compared with control rabbits (Table 3). The humoral immune responses elicited 21 dpi in both prednisolone treated and control rabbits were similar. All vaccinated rabbits developed high anti-MV and anti-RHDV antibody titres (Table 3).

Table 1

<table>
<thead>
<tr>
<th>Point value</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Non apparent lesions</td>
</tr>
<tr>
<td>1</td>
<td>A localised primary nodule at the inoculation site</td>
</tr>
<tr>
<td>2</td>
<td>Secondary skin lesions in the form of small discrete nodules near the inoculation site, in face, or ears</td>
</tr>
<tr>
<td>3</td>
<td>Small discrete nodules in eyelids</td>
</tr>
<tr>
<td>4</td>
<td>Small nodules in genitals, limbs, and other parts of the body</td>
</tr>
<tr>
<td>5</td>
<td>Severe myxomatosis symptoms like closure of the eyes, generalised oedema, or respiratory syndrome</td>
</tr>
<tr>
<td>6</td>
<td>Death</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination route</th>
<th>Mean body temperature increase (°C)</th>
<th>Mean body weight increase (g, 0–21 dpi)</th>
<th>Mean antibody titres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–2 dpi^a</td>
<td>0–4 dpi^a</td>
<td>Anti-MV</td>
</tr>
<tr>
<td>Vaccinated with 1 dose (10^4 pfu)</td>
<td>s.c.</td>
<td>0.2 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>206 ± 93</td>
</tr>
<tr>
<td>Vaccinated with 1 dose (10^4 pfu)</td>
<td>i.d.</td>
<td>0.3 ± 0.2</td>
<td>0.0 ± 0.3</td>
<td>200 ± 69</td>
</tr>
<tr>
<td>Vaccinated with 10 doses (10^4 pfu)</td>
<td>s.c.</td>
<td>0.3 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>217 ± 84</td>
</tr>
<tr>
<td>Vaccinated with 10 doses (10^4 pfu)</td>
<td>i.d.</td>
<td>0.2 ± 0.4</td>
<td>0.2 ± 0.4</td>
<td>198 ± 71</td>
</tr>
<tr>
<td>Vaccinated with 100 doses (10^5 pfu)</td>
<td>s.c.</td>
<td>0.3 ± 0.5</td>
<td>0.3 ± 0.5</td>
<td>181 ± 89</td>
</tr>
<tr>
<td>Vaccinated with 100 doses (10^5 pfu)</td>
<td>i.d.</td>
<td>0.4 ± 0.6</td>
<td>0.2 ± 0.4</td>
<td>168 ± 91</td>
</tr>
<tr>
<td>Control (not vaccinated)</td>
<td></td>
<td>0.2 ± 0.2</td>
<td>−0.1 ± 0.3</td>
<td>220 ± 77</td>
</tr>
</tbody>
</table>

^a dpi: Days postvaccination.

^b N.D.: not detected.
3.3. Effects induced by the administration of 6918VP60-T2 virus to pregnant does

To evaluate the effects of recombinant virus infection on reproduction, pregnant does were inoculated at different times of gestation (days 7, 14, 21 and 28) by s.c. route. The daily observation of the animals showed a total absence of general clinical symptoms in all inoculated animals. Reproductive parameters such as number of animals born alive per litter, number of animals born dead per litter, number of living animals per litter 8 dpp, and average weight of each litter at 8 dpp, for both first and second parturition, have been summarised in Table 4. The overall results showed that recombinant virus infection did not induce any alteration during reproduction. Pregnant does infected at different days of gestation showed reproductive values being in the expected range for rabbits, and no differences were observed when recombinant virus-infected does were compared with control does inoculated with PBS at the same day of gestation. The absence of alterations in reproductive parameters was maintained in the following parturition (Table 4). Furthermore, none of the rabbits born from 6918VP60-T2 virus-infected does showed any symptomatology associated with myxomatosis.

3.4. Analysis of the biological stability of 6918VP60-T2 virus

The biological stability of the recombinant virus, and therefore its potential to evolve to a virulent state were evaluated by comparing the effects of rabbit infection with “Passage 0” virus (the same virus stock used in all the experiments reported in this paper), with the effects of rabbit infection with the virus obtained after 10 serial passages in rabbits (Passage 10 virus). Fig. 3 shows a graphic representation of the symptomatology observed in rabbits infected with either Passage 0 or Passage 10 virus, according to the ranking of myxomatosis clinical signs established in Table 1. Rabbits infected with Passage 10 virus exhibited the same mild clinical signs as those infected with Passage 0 virus. Symptoms appeared 5–7 dpi and completely resolved by 15 dpi in both cases. None of the infected rabbits exhibited classical severe myxomatosis symptoms. Table 5 shows temperature increases from 0 to 2 dpi and from 0 to 4 dpi, as well as weight

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination route</th>
<th>Mean body temperature increase (°C)</th>
<th>Mean body weight increase (g) (0–21 dpv)</th>
<th>Mean antibody titre Anti-MV</th>
<th>Anti-RHDV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–2 dpv *</td>
<td>0–4 dpv *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunosuppressed rabbits</td>
<td>s.c.</td>
<td>0.0±0.3</td>
<td>0.2±0.4</td>
<td>203±83</td>
<td>10000</td>
</tr>
<tr>
<td>Immunosuppressed rabbits</td>
<td>i.d.</td>
<td>0.3±0.4</td>
<td>0.0±0.2</td>
<td>233±86</td>
<td>10000</td>
</tr>
<tr>
<td>Non-immunosuppressed rabbits</td>
<td>s.c.</td>
<td>0.2±0.3</td>
<td>0.1±0.3</td>
<td>206±93</td>
<td>8800</td>
</tr>
<tr>
<td>Non-immunosuppressed rabbits</td>
<td>i.d.</td>
<td>0.3±0.2</td>
<td>0.0±0.3</td>
<td>200±69</td>
<td>9500</td>
</tr>
</tbody>
</table>

* dpv: Days post vaccination.
increases from day 0 to 21. No significant differences in body temperature increase or body weight increase were observed when rabbits infected with Passage 10 virus were compared with rabbits infected with Passage 0 virus or control uninfected rabbits. The humoral responses elicited by rabbits infected with Passage 0 or Passage 10 virus were similar. All infected rabbits developed high anti-MV and anti-RHDV antibody titres.

The genomic stability of 6918VP60-T2 virus was analysed by PCR using oligonucleotide primers external to the insertion site of the VP60 gene. After 10 serial passages in rabbits, a product of 3.3 kb (the expected size for the recombinant virus) was amplified by PCR with no detection of the corresponding wild-type MV 1.0 kb product (not shown), indicating that the VP60 gene was stably integrated in the MV genome.

### 4. Discussion

A number of vaccines are available to protect rabbits against myxomatosis and RHD [4,5,14] which are useful for immunizing domestic rabbits. However, control of both diseases among wild rabbit populations remains an unsolved problem of great concern. In this regard it should be noted that the European rabbit plays a key ecological role in Mediterranean ecosystems. In addition, rabbits are among the most important small game species in several European countries.

Immunization of wildlife is difficult to achieve because direct delivery of vaccines to free ranging animals is not possible. The oral route is considered a feasible way of vaccine administration. For example, oral vaccination is being used to control enzootic sylvatic rabies in Europe and North America by means of a recombinant vaccinia-rabies vaccine delivered by baiting [26]. An alternative strategy is the use of "transmissible vaccines", i.e., viral vectors capable of spreading within an animal population. Hopefully, the administration of a recombinant vaccine of this characteristics to a small number of captured individuals, would eventually lead to the immunization of a fraction of animals within a given population, which is sufficient to reduce the spread of the target disease. This approach might be useful, especially when the distribution, size, and turnover rate of a population precludes capture or baiting techniques as the only means for antigen delivery. The European rabbit is an example of such a population. With this in mind, we have developed a transmissible vaccine against both myxomatosis and RHD based on a recombinant MV-VP60 virus capable of spreading through rabbit populations [25]. The results obtained under laboratory conditions suggest the recombinant virus might be effective for wild rabbit immunization. However, 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. It is for this reason that safety concerns have been at the core of the rational design of the proposed immunization strategy.

The biological characteristics of MV make it a good candidate as a vaccine vector in terms of safety considerations. MV exhibits a very restricted host range, infecting exclusively rabbits (both Sylvilagus and Oryctolagus spp.). The virus has been widely distributed throughout Europe, Australia and

### Table 4
Effects induced by 6918VP60-T2 virus infection in pregnant does

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of inoculation</th>
<th>First parturition</th>
<th>Second parturition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Animals/litter</td>
<td>W/litter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Mean ± SD)</td>
<td>(g) (Mean ± SD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alive</td>
<td>Dead</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 dpp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8 dpp&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vaccinated with 10&lt;sup&gt;4&lt;/sup&gt; pfu (s.c.)</td>
<td>7</td>
<td>9.2 ± 1.5</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>8.7 ± 1.4</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>9.2 ± 1.5</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>8.8 ± 1.2</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>Inoculated with PBS (s.c.)</td>
<td>7</td>
<td>8.7 ± 1.5</td>
<td>0.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>10.7 ± 3.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>9.3 ± 2.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>9.3 ± 2.5</td>
<td>1.0 ± 1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> SD: standard deviation.
<sup>b</sup> dpp: Days postparturition.
the Americas for nearly 50 years with no evidence of infection of other species. Thus, the host restricted nature of MV minimizes the risk of recombinant vaccine spreading to non-target species in nature. On the other hand, given the current widespread geographic distribution of MV, which is similar to the distribution of RHDV, the field use of a recombinant MV-VP60 vaccine would normally not involve the introduction of a virus species that does not already exist in a particular area.

Safety aspects were also considered in the choice of the parental MV strain. It was decided not to use one of the available vaccinal strains, obtained by cell culture-attenuation of virulent MV strains, as this would involve the release of a new strain to the environment, which might undergo reversion to virulence in nature. Instead, we decided to use an attenuated MV field strain which was already circulating among wild rabbit populations. Strain 6918 was selected from a field survey of MV strains circulating in Spain, which were analysed for virulence and transmissibility. This strain exhibited adequate biological characteristics for the development of a recombinant transmissible vaccine, as it caused a non-pathogenic infection comparable to that of cell culture-attenuated vaccinal strains, yet retaining the capacity of horizontal spreading.

Since preservation of the valuable biological properties of strain 6918 was of major importance in the development of the recombinant virus, the foreign gene was inserted in the intergenic site between ORFs MJ2 and MJ2a, as recombinant MVs with insertions at this site have been shown to retain overall parental biological characteristics [21]. Moreover, the VP60 expression cassette was inserted into the MV genome using the TDS two-step selection system [28]. This procedure enables the construction of recombinant poxviruses without any marker genes inserted in the final recombinant viral genome. Thus, the recombinant 6918VP60-T2 does not harbour selectable markers such as antibiotic resistance genes, the widespread of which is currently regarded as a major health and environmental threat.

Considering the potential risks associated with the DNA sequence inserted, it should be noted that the VP60 gene has been cloned in a wide range of heterologous systems [15–20] and no indication of toxicity or side effects associated to the expression of VP60 have been reported.

Previous results indicated that administration of either 6918 MV or recombinant 6918VP60-T2 virus to healthy rabbits under laboratory conditions by standard procedures is safe, as all rabbits exhibited only mild clinical symptoms and rapidly recovered [24,25]. In this report, we have extended the safety assessment of the vaccine by analysing the potential effects induced by 6918VP60-T2 virus infection after 10 passages in vivo.

Table 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination route</th>
<th>Vaccination route Mean body temperature increase (°C)</th>
<th>Mean body weight increase (g) (0–21 dpv)a</th>
<th>Mean antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–2 dpva</td>
<td>0–4 dpva</td>
<td>Anti–MV</td>
</tr>
<tr>
<td>Vaccinated with 10⁴ pfu of 6918VP60-T2 (Passage 10) s.c.</td>
<td>0.4 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>214 ± 66</td>
<td>10000</td>
</tr>
<tr>
<td>Vaccinated with 10⁴ pfu of 6918VP60-T2 (Passage 0) s.c.</td>
<td>0.2 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>206 ± 93</td>
<td>8800</td>
</tr>
<tr>
<td>Control (Unvaccinated)</td>
<td>0.2 ± 0.2</td>
<td>−0.1 ± 0.3</td>
<td>220 ± 77</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

a dpv: Days postvaccination.

b N.D.: not detected.

risks of vaccine administration under a varied range of situations that might occur if the recombinant virus is used for large-scale field immunization of rabbits.

Concerning vaccine dosage and the possibility of accidental administration of an overdose, the results demonstrated vaccine safety even when a 100-fold overdose (10^6 PFU) was inoculated (Fig. 1, Table 2). Assessment of vaccine effects in immunosuppressed rabbits was considered relevant, given the incidence in nature of immunocompromised individuals due to infections, environmental or genetic causes. For this reason we assayed the effect of vaccine administration in rabbits treated with prednisolone, a potent immunosuppressor. This treatment induces depletion of circulating eosinophils and mononuclear cells, causing a strong decrease of the T-cell response with only a slight effect on B-cell function [29]. It is a commonly used procedure for the safety evaluation of veterinary vaccines [30–32]. Results showed that prednisolone treated rabbits exhibited similar symptoms to those observed in control rabbits (Fig. 2, Table 3). The only remarkable observation was that immunosuppressed rabbits showed a subtle tendency to delay the resolution of local lesions: 16–18 dpi vs. 15 dpi (Fig. 2).

Another important aspect addressed was the effect of 6918VP60-T2 virus infection in reproduction. Results showed that recombinant virus inoculation did not alter the reproduction parameters and none of the rabbits born from vaccinated does showed myxomatosis-associated clinical signs (Table 4). In conclusion, 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 (i.e., neonate, young, pregnant does or immunocompromised).

Finally the biological stability of the recombinant virus was analysed. The environmental release of recombinant 6918VP60-T2 virus would involve a certain number of serial passages in its natural host, even when this capability seemed to be limited to only two serial passages under laboratory conditions [25]. Should there be a tendency for the virus to evolve to a virulent state, serial passage in rabbits would cause it to do so. Accordingly, the biological stability of 6918VP60-T2 was studied by subjecting the virus to 10 serial passages in rabbits, and the results obtained (Fig. 3, Table 5) indicated the recombinant virus maintained grossly the same biological characteristics through the passages. Thus, the attenuated nature of 6918VP60-T2 seems to be a stable trait. On the other hand, the genetic analysis indicated that the VP60 gene remained stably integrated in the MV genome after serial passage in rabbits, in agreement with the previously reported results obtained after 15 serial passages of 6918VP60-T2 virus in RK-13 cell monolayers [25].

On the basis of the results previously reported [24,25] and those presented in this paper, along with experimental data addressing further safety and efficacy issues (to be published elsewhere), the recombinant 6918VP60-T2 has been subjected to the mandatory risk assessment process relative to the release of genetically-modified organisms. A limited field trial authorised by the Spanish competent authorities is in course. This trial will assess the efficacy and safety of the vaccine under controlled field conditions, in the perspective of its use in a large-scale program for the control of myxomatosis and RHD among wild rabbit populations.

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References


