Arch Virol (2000) 145: 759–771

Archives of Virology

Isolation of an attenuated myxoma virus field strain that can confer protection against myxomatosis on contacts of vaccinates

J. Bárcena[1], A. Pagès-Manté[2], R. March[2], M. Morales[1], M. A. Ramírez[1], J. M. Sánchez-Vizcaíno[1], and J. M. Torres[1]

[1]Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, Spain
[2]Laboratorios Hipra, S.A., Amer, Girona, Spain

Accepted October 7, 1999

Summary. Twenty MV strains obtained from a survey of field strains currently circulating throughout Spain were analyzed for their virulence and horizontal spreading among rabbits by contact transmission. A virus strain with suitable characteristics to be used as a potential vaccine against myxomatosis in wild rabbit populations was selected. Following inoculation, the selected MV strain elicited high levels of MV specific antibodies and induced protection of rabbits against a virulent MV challenge. Furthermore, the attenuated MV was transmitted to 9 out of 16 uninoculated rabbits by contact, inducing protection against myxomatosis.

Introduction

Myxoma virus (MV), the causative agent of myxomatosis, is a large virus with a double-stranded DNA genome of 163 kb which replicates in the cytoplasm of infected cells. MV belongs to the *Leporipoxvirus* genus of the *Poxviridae* family [20]. In the natural hosts (*Sylvilagus brasiliensis* and *Sylvilagus bachmani* rabbits in the Americas), MV induces a benign cutaneous fibroma. In European rabbits (*Oryctolagus cuniculus*), however, MV causes myxomatosis, a systemic and usually fatal disease. The virus was deliberately released as a biological control agent for the European rabbit initially in Australia (1950) and soon after in France (1952), from where it rapidly spread across the entire rabbit range in Europe, and has become endemic since then. Following initial release, devasting epizootics occurred in both continents with a mortality rate around 99.5% of the infected rabbits. Rabbit populations were reduced by more than 90% as a whole and in some areas rabbits completely disappeared. The evolution of attenuated viral strains along with the development of host resistance led to a diminished incidence of the disease [10, 11]. Nevertheless, recent studies carried out in Europe

and Australia indicate that myxomatosis still controls rabbit population numbers in both continents [27, 29].

MV spreads within rabbit populations by contact transmission and by blood-feeding arthropod vectors, such as fleas or mosquitoes. The seasonal cycle of myxomatosis epidemics shows a main peak in summer/autumn and a minor peak in spring [24] although this is subject to regional variations. Once myxomatosis is present, its prevalence in the population depends on the availability of vectors and a sufficient density of susceptible rabbits. For recent reviews on myxomatosis see Fenner and Ross [11], and Kerr and Best [17].

Currently there are commercially available vaccines to immunize rabbits against myxomatosis. Some of these are heterologous vaccines based on the use as an immunizing agent of Shope fibroma virus (SFV), a less virulent leporipoxvirus. However, vaccines based on SFV present certain disadvantages, as the virus fails to immunize consistently and the resulting immunity is short lived [12, 13]. Homologous vaccines are based on cell culture attenuated-strains of MV [1, 18, 19, 22, 26, 28]. These vaccines are being used with successful results [23], but some of them show residual pathogenicity for young rabbits [2] and there have been reports of reversion to virulence [15]. Despite considerable efforts, all attempts to produce an effective inactivated myxoma virus vaccine have proved unsuccessful [16]. This is not surprising in view of the failure to produce an effective inactivated smallpox vaccine [7].

While the above mentioned vaccines are useful for protecting domestic rabbits against myxomatosis, they are not suitable to be used among wild rabbit populations, as these vaccines need to be inoculated by conventional veterinary means in each animal, and this is not a feasible approach to immunize free ranging wild rabbits. One possibility would be to use as a vaccine an attenuated MV strain capable of spreading within a rabbit population. Hopefully, the capture, direct vaccination and release of a small number of rabbits could eventually lead to the protection of a sufficient proportion of animals in the populatin to reduce the spread of the disease.

Results obtained so far by several authors indicate that it is unlikely to obtain a MV strain with adequate characteristics (i.e., non-pathogenic and transmissible) by cell culture passage-attenuation of virulent MV strains, as usually these attenuated viruses have lost the ability to disseminate among rabbits by contact or arthropod vectored transmission [22, 25, 26]. With this in mind, we decided to undertake a survey among the currently circulating strains of MV in Spain, in search of a field MV strain with the desired characteristics. The rationale was that if we were able to find an adequate strain in terms of marginal pathogenicity, it should be able of some extent of horizontal transmission among rabbits, since the virus was circulating in nature.

In this paper we report the isolation of an attenuated MV field strain capable of horizontal contact transmission among rabbits, and describe experiments carried out to evaluate its possible use for protecting wild rabbit populations against myxomatosis.

Materials and methods

Virus strains

The viral isolates analyzed in this paper were obtained from samples of infected wild rabbits collected in 12 regions throughout Spain between the years 1992–1995. The reference name and the origin of the viral isolates is indicated in Table 1. As specimens were unavoidably kept at environmental temperatures for several days between their collection in the field and receipt in the laboratory, the skin lesion material was passed 2–4 times in cell culture before the virus was tested for virulence and transmissibility. Rabbit cell line RK-13 (obtained from the ATCC), grown in DMEM supplemented with 5% fetal bovine serum was used for virus propagation. The viral stocks were titrated by plaque assay in RK-13 cells before use, virus titres being usually in the range of 10^5 pfu per ml. The MV strain 6918 was cloned by three successive plaque purification cycles in monolayers of RK-13 cells.

Rabbits

New Zealand White rabbits provided by a local supplier were used for the initial virulence and transmission test of MV field isolates. Further work with the MV strain 6918 was carried out using common rabbits (brown colored) supplied by a commercial breeder. These rabbits are commonly used for restocking in the field and from now on will be regarded as "wild rabbits".

Test for virulence and transmission of MV field isolates

Two groups of 5 New Zealand White rabbits (30 days old, weighing 0.5–0.7 kg) free from MV antibodies were inoculated intradermally (i.d.) in the eyelid with 10^4 pfu of a viral isolate that had been passed 2–4 times in cell culture as described above. Each group of inoculated rabbits was held in the same cage for up to 50 days in contact with a group of 5 uninoculated rabbits. The animals were observed daily for signs of myxomatosis. The clinical symptoms recorded were: appearance of primary lession at the inoculation site (eyelid), development of secondary lessions, (other eyelid, genitals, skin), development of severe myxomatosis symptoms (eye closure, generalized oedema, respiratory syndrome), and survival time.

Immunization of rabbits with MV strain 6918

To analyze the immunogenic potential of MV strain 6918, a group of ten wild rabbits (2 months old, weighing around 1 kg) free from MV antibodies, were inoculated by i.d. route at the back with 10^4 pfu of 6918 MV strain, and another group of ten wild rabbits were inoculated by subcutaneous route (s.c.) at the back with the same dose of the virus. The rabbits were observed daily for a period of 56 days and clinical symptoms due to the virus inoculation were recorded. Weight and temperature determinations were made on each animal, begining on the 2nd day and continuing until the 15^{th} day. Serum samples extracted from the marginal ear vein of the rabbits 0, 21, 35 and 56 days after immunization were used to evaluate the serological responses against MV, by using an enzyme-linked immunosorbent assay (ELISA). For this, MV from infected RK-13 cell extracts was semipurified by centrifugation through a 30% sucrose cushion, and used as antigen to coat ELISA plate wells (Polysorp, Nunc). Binding of MV specific antibodies present in serial dilutions of serum samples was visualized by incubation with protein G conjugated with horseradish peroxidase (Pierce), and subsequent addition of substrate solution (ABTS, Sigma). After ten minutes of substrate incubation the reaction was stopped by addition of 1% SDS. Serum titres were defined as the inverse of the highest dilution giving an A_{405} twofold over the background level (a negative control rabbit serum).

Table 1. Virulence and transmissibility of MV field isolates recovered in Spain during the years 1992–1995

Virulence grade[a]	Strain	Origin	Inoculated rabbits				Contact rabbits				
			mean initial symptoms (dpi)[b]	mean time with symptoms (days)	mean survival time (dpi)[b]	mortality	rabbits with symptoms	mean initial symptoms (dpi)[b]	mean time with symptoms (days)	mean survival time (dpi)[b]	mortality
A	87	Lleida	5.3 ± 0.4	5.5 ± 1.0	9.8 ± 1.0	10/10	10/10	11.7 ± 0.7	8.7 ± 0.7	19.4 ± 1.1	10/10
	6036	Tarragona	4.8 ± 0.8	6.2 ± 1.1	10.0 ± 1.0	10/10	10/10	11.4 ± 1.0	9.2 ± 0.9	22.4 ± 1.0	10/10
	2583	Barcelona	5.3 ± 0.4	6.9 ± 0.5	11.2 ± 0.8	10/10	10/10	13.6 ± 0.8	12.6 ± 2.0	25.2 ± 2.7	10/10
	466	Valencia	5.3 ± 0.4	6.7 ± 1.4	11.0 ± 1.2	10/10	10/10	11.5 ± 0.6	12.8 ± 1.4	23.3 ± 1.6	10/10
	2012	Asturias	5.2 ± 0.3	7.8 ± 1.4	12.0 ± 1.6	10/10	10/10	11.1 ± 0.2	10.5 ± 1.8	20.6 ± 1.9	10/10
	6717	León	4.6 ± 0.7	8.7 ± 0.4	12.3 ± 0.8	10/10	10/10	11.9 ± 0.5	11.5 ± 0.7	23.3 ± 1.6	10/10
	86	Badajoz	5.2 ± 0.3	8.4 ± 0.9	12.6 ± 0.9	10/10	10/10	13.3 ± 0.4	17.1 ± 0.9	29.4 ± 1.0	10/10
	5623	Badajoz	4.0 ± 0.0	9.7 ± 0.9	12.7 ± 0.9	10/10	10/10	12.3 ± 0.4	13.9 ± 0.9	25.2 ± 0.8	10/10
	4554	Barcelona	3.7 ± 0.4	10.2 ± 0.5	12.9 ± 0.5	10/10	10/10	13.5 ± 0.5	14.1 ± 0.7	26.6 ± 0.5	10/10
	2710	Barcelona	3.0 ± 0.0	10.9 ± 0.7	12.9 ± 0.7	10/10	10/10	15.7 ± 0.7	14.3 ± 0.8	29.0 ± 0.6	10/10
B	2788	Albacete	3.9 ± 0.4	10.6 ± 1.0	13.5 ± 1.2	10/10	10/10	14.2 ± 0.5	14.5 ± 1.2	27.7 ± 1.4	10/10
	7514	Pontevedra	4.4 ± 0.6	10.1 ± 0.7	13.5 ± 0.7	10/10	10/10	12.7 ± 0.8	16.8 ± 1.8	28.5 ± 1.8	10/10
	2814	Navarra	4.7 ± 0.4	10.2 ± 1.0	13.9 ± 1.3	10/10	10/10	12.9 ± 0.5	11.6 ± 0.9	23.5 ± 1.4	10/10
	7692	Zaragoza	5.8 ± 0.6	8.6 ± 1.5	14.0 ± 1.2	10/10	10/10	12.7 ± 0.8	16.8 ± 1.8	32.6 ± 2.0	10/10
	1312	La Rioja	5.6 ± 0.7	10.5 ± 0.7	15.1 ± 1.1	10/10	10/10	12.5 ± 0.6	16.3 ± 1.8	27.8 ± 2.4	10/10
C	7411	Canarias	4.7 ± 0.4	14.7 ± 0.8	18.4 ± 1.1	10/10	10/10	18.0 ± 1.4	18.9 ± 1.3	35.9 ± 1.9	10/10
	6994	Málaga	5.2 ± 0.5	20.6 ± 3.0	24.8 ± 3.3	10/10	10/10	16.6 ± 0.7	17.9 ± 1.5	33.5 ± 2.1	10/10
D	5206	Girona	4.2 ± 0.7	26.7 ± 0.9[c]	29.7 ± 0.9[c]	3/10	10/10	18.4 ± 0.6	11.4 ± 1.9[c]	28.5 ± 1.8[c]	8/10
E	6918	Girona	4.2 ± 0.8	29.8 ± 0.8[d]	N.A.	0/10	10/10	22.2 ± 0.7	14.6 ± 1.1[d]	N.A.	0/10
	4604	Lleida	4.6 ± 0.6	39.2 ± 3.6[d]	N.A.	0/10	0/10	N.A.	N.A.	N.A.	0/10

[a]Virulence grade groups were define acording to the mean survival time (MST) and the case mortality rate (M) exhibited by the inoculated rabbits (adapted from Fenner and Marshall [9]). A: MST <13 days; B: MST 13–16 days; C: MST 17–28 days; D: M<50%; E: M = 0%. The data is expressed as mean ± standard deviation

[b]Days post infection (dpi). Refers to the number of days after the experimental infection of the inoculated rabbits

[c]Corresponds only to the the animals that died from myxomatosis (survivors not included)

[d]Indicates the mean time with clinical signs of myxomatosis until full recovery of the rabbits

N.A. Not aplicable

Transmissible immunization of rabbits and challenge with virulent MV

Two groups (Inoculated A and B groups) of 8 wild rabbits (2 months old weighing around 1 kg) free from MV antibodies were inoculated by s.c. route with 10^4 pfu of 6918 MV strain. Three days after, each rabbit group was placed in the same cage with a group of 8 rabbits free from MV antibodies (1st Passage A and B groups, respectively), for six days. Subsequently, rabbits from the 1st passage groups were separated from the 6918-strain inoculated animals and were held in contact with another group of 8 rabbits free from MV antibodies (2nd passage A and B groups, respectively), for another six days. The mentioned groups of rabbits were then separated in different cages and 35 days after the initial inoculation with 6918 MV strain, a serum sample was extracted from each rabbit. The same day, rabbits from the three B groups together with a group of 4 control rabbits, were challenged by i.d. injection with 10^3 pfu of virulent MV (Laussane strain). Finally, the rabbits were held up to 56 days from the initial immunization and a serum sample was extracted from all the survivors. The serological responses against MV were evaluated by ELISA as described above. Throughout the experiment, the animals were observed daily and clinical symptoms caused by inoculation of 6918 MV strain, as well as myxomatosis signs due to the virulent MV infection were recorded.

Results

Analysis of the virulence and transmissibility of MV field isolates

20 MV field isolates collected from naturally infected wild rabbits from 12 different regions throughout Spain, over the period 1992–1995, were analyzed in terms of virulence grade and horizontal spread by contact transmission (Table 1). Since we were interested in detecting any residual pathogenic potential of the MV strains tested, we adopted in this study several modifications of the standardized experimental conditions used in previous MV virulence studies [5, 9] so as to promote the most severe course of myxomatosis infection possible. The experimental conditions used are detailed under Materials and methods.

Initial myxomatosis symptoms appeared during the first 3–6 days post infection (dpi) in all inoculated rabbits. 17 out of the 20 field isolates tested killed 100% of the inoculated rabbits, although the mean survival time greatly varied among the different isolates, spanning from 9.8 to 24.8 dpi. Isolate 5206 killed 30% of the inoculated rabbits, and two isolates, 6918 and 4604, produced a non-lethal myxomatosis disease when inoculated into rabbits. According to the mean survival time and the case mortality rate exhibited by the inoculated rabbits, the 20 field isolates were allocated to one of five groups, their virulence being described as grades A, B, C, D or E (adapted from ref. [9], see Table 1). Grade A viruses were the most virulent, killing all inoculated rabbits with an average survival time of less than 13 days; Grade B and C viruses killed all the inoculated rabbits with mean survival times of 13–16 and 17–28 days, respectively. Grade D was used to classify virus isolates which caused a mortality rate less than 50% of inoculated rabbits, and non-lethal viruses were grouped in Grade E.

Regarding horizontal transmissibility, the field isolates classified in the virulence grade groups A–C, which produced 100% mortality in inoculated rabbits, efficiently infected all the contact control rabbits. Initial detectable clinical symp-

toms appeared after an average of 11.1–18.0 days from the beginning of the test and all the contact infected rabbits finally died of myxomatosis with a mean survival time of 19.4–35.9 days from the start of the test. Interestingly, the average length of the disease caused by these viruses, estimated as mean number of days with apparent clinical signs of myxomatosis (see Table 1), was, with only one exception (strain 6994), significantly longer in the group of contact infected rabbits than in the correspondent inoculated group. The increase in the average disease length was in the range of 1.4 (strain 2814) to 8.7 days (strain 86). This result indicated that the stringent experimental conditions used with the inoculated rabbits produced an enhanced severity of the disease, as compared to that observed in the contact infected rabbits. The latter probably reflect more closely the situation in the field, since contact transmission is one of the natural means of MV infection.

The opposite result was observed with strain 5206, the only virus classified as virulence grade D. Upon inoculation with this virus only 3 out of 10 rabbits died from myxomatosis, showing apparent clinical signs of disease for an average of 29.7 days. In contrast, 8 of the 10 contact infected rabbits died of myxomatosis after an average of only 11.4 days of clinical symptoms. This result suggested a virulence increase of this viral strain as it was passed in rabbits by contact transmission.

Considering the virus strains classified as virulence grade E (non-lethal in inoculated rabbits), strain 6918 was efficiently transmitted to the contact control rabbits, as all the animals developed detectable clinical symptoms. Strain 4604 induced no apparent clinical symptoms in the contact control rabbits, suggesting this MV strain did not spread by contact transmission.

In view of the results, the isolate 6918 appeared as a promising candidate to be used as an attenuated-virus transmissible vaccine. All the rabbits infected either by direct inoculation or by contact, suffered a non-lethal myxomatosis disease followed by complete recovery. In the inoculated animals, a local lesion developed on the eyelid at the inoculation site 3–5 dpi. Secondary signs appeared 3–5 days later (6–10 dpi) and consisted of a localized lesion in the other eyelid and nodules (10 mm) scattered through the face, around the mouth and ears. About 8–12 dpi, some small nodules appeared in genitals, limbs, and other parts of the body. None of the rabbits exhibited classical severe myxomatosis symptoms like closure of the eyes, generalized oedema, or respiratory syndrome. The disease developed slowly and ran a relatively benign course, with gradual regression of lesions and full recovery of all the rabbits by 35–37 dpi. Clinical signs in rabbits infected by contact were much milder. They lasted 13–17 days and consisted almost exclusively of small localized discrete nodules on the eyelids.

Further characterization of MV strain 6918

MV strain 6918 was selected for further characterization regarding its pathogenicity and immunogenic potential. As a first step, the virus isolate was cloned by 3 cycles of plaque purification in monolayers of RK-13 cells. This was decided in order to perform all following work with an homogeneous material, as previous

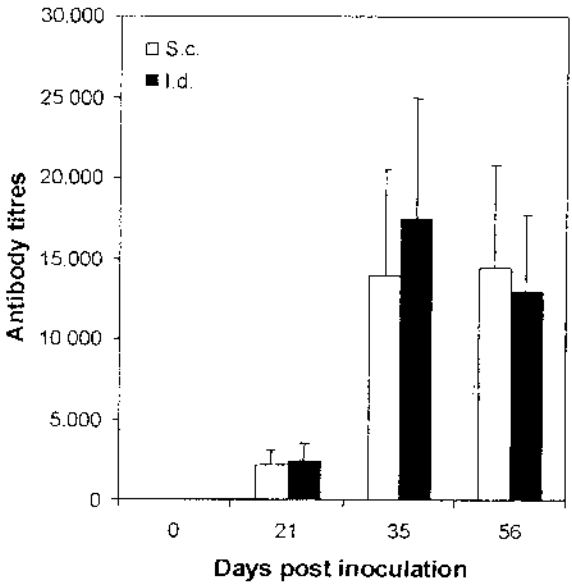

Fig. 1. Serum anti-MV antibody responses (ELISA) in rabbits immunized by i.d. or s.c. route with MV strain 6918. Antibody titres are reported as mean titres plus standard error of the means (n = 10). Serum titres were defined as the inverse of the highest dilution giving an A_{405} twofold over the background level (a negative control rabbit serum)

studies with attenuated MV field isolates have reported variability and cases of reversion to virulence due to the presence of mixtures of MV strains of different virulence grades [5, 9, 14]. Subsequently, the cloned virus was tested under experimental conditions similar to those in which a vaccine against myxomatosis intended for wild rabbit immunization in the field would be used. Two groups of wild rabbits were inoculated with strain 6918 at the back by either i.d. or s.c. route, and clinical signs as well as the anti-MV antibody response were monitored up to 56 dpi. Clinical symptoms after i.d. administration of cloned 6918 MV strain were mild and transient. Initial signs were detected at the inoculation site by 6 dpi and were completely resolved in all inoculated rabbits by 13–15 dpi. The symptomatology observed consisted of a localized primary nodule at the inoculation site and, in some inoculated rabbits, scanty secondary skin lesions in the form of small discrete nodules, usually less than 5 mm in diameter, in eyelids, face or ears. No febrile response or loss of body weight was detected in the inoculated rabbits, and the overall health of the rabbits was largely unaffected. Rabbits inoculated by s.c. route exhibited quite similar clinical symptoms but these were consistently milder: there were less secondary nodules, and these were slightly smaller and resolved earlier.

To evaluate the immune response elicited by the inoculated rabbits, the serum samples obtained at different times post infection were monitored for the presence of anti-MV antibodies by ELISA. As seen in Fig. 1, both groups of rabbits progressively developed anti-MV antibody titres which maintained high up to 56 days post vaccination. There was no gross difference in the antibody titres observed in the i.d. or the s.c inoculated rabbits. In a parallel experiment carried out with domestic rabbits (New Zealand White) the results obtained were essentially the same (data not shown).

Table 2. Transmissible protection induced by 6918 strain against a virulent MV challenge

Group	No. of rabbits	Immunization (6918 MV strain)	Myxoma challenge (35 dpi)	Mean antibody titre (35 dpi)[a]	Mean antibody titre (56 dpi)[a]	Survival
Control	4	–	10^3 pfu (i.d.)	ND	NT	0/4
Inoculated A	8	10^4 pfu (s.c.)	–	7.500 (8/8)	6.250 (8/8)	8/8
Inoculated B	8	10^4 pfu (s.c.)	10^3 pfu (i.d.)	8.750 (8/8)	20.560 (8/8)	8/8
1st Passage A	8	Contact with inoculated rabbits A	–	275 (4/8)	300 (4/8)	8/8
1st Passage B	8	Contact with inoculated rabbits B	10^3 pfu (i.d.)	115 (5/8)	9500 (5/8)	5/8
2nd Passage A	8	Contact with 1st Passage rabbits A	–	25 (1/8)	50 (1/8)	8/8
2nd Passage B	8	Contact with 1st Passage rabbits B	10^3 pfu (i.d.)	5 (1/8)	NT	0/8

[a]Antibody titres are reported as mean titres of the seropositive rabbits. In brakets: no of seropositive rabbits/no of total rabbits

N.D. Not detected

N.T. Not tested, all rabbits had died before

Transmissible protection against myxomatosis induced by MV strain 6918

To determine whether administration of strain 6918 could protect wild rabbits from a virulent MV infection, and if this protection could be transmitted to uninoculated rabbits by contact transmission, a challenge experiment was conducted as described under Materials and methods. In brief, two groups of rabbits (Inoculated A and B groups) were inoculated with cloned 6918 MV strain. Each of these rabbit groups was placed in the same cage with another group of rabbits for 6 days (1st Passage A and B groups, respectively). Subsequently, the "1st Passage" groups were placed in the same cage with a new group of rabbits (2nd Passage A and B groups, respectively) for another 6 days, at the end of which the different rabbit groups were separated. Finally, 35 days after the initial inoculation with strain 6918, the animals of the three B groups were challenged with the virulent MV Laussane strain.

The results are shown in Table 2. Upon inoculation with 6918 MV strain, small nodules were first detected 5–7 dpi and were completely resolved by day 15 post inoculation. After challenge with virulent MV all rabbits previously immunized with 6918 MV strain survived, exhibiting only a transient small lesion at the virulent MV inoculation site. In contrast, the 4 control rabbits died from myxomatosis. Thus, strain 6918 induced a protective anti-myxomatosis primary immune response, demonstrating its efficacy in protecting directly immunized animals.

No clinical symptoms were detected in rabbits of the 1st passage A group throughout the experiment or in the 1st Passage B group before the challenge

infection. After challenge with virulent MV, 5 out of 8 rabbits survived, showing only a transient nodule at the inoculation site. The 2nd passage rabbits showed no clinical signs prior to the experimental infection with the virulent virus, and upon challenge none of the 8 infected rabbits survived.

Data from the serological analyses were in good agreement with the protection results (see Table 2). Rabbits inoculated with 6918 strain elicited high titres of anti-MV antibodies by day 35 post inoculation. Upon challenge, a remarkable increase in the antibody titres was observed (compare antibody titres 56 dpi of Inoculated A and B groups). 9 out 16 rabbits of the 1st passage groups presented anti-MV antibodies 35 dpi, indicating they had been infected with 6918 MV strain by contact transmission without apparent clinical symptoms. The anti-MV antibody titres of these rabbits were low, but this level of immune response was sufficient for protection, as all seropositive rabbits from the 1st passage group B withstood the challenge infection. In contrast, seronegative rabbits died of myxomatosis. The postchallenge antibody titres of the survivors showed a marked increased as compared with the titres exhibited by the seropositive rabbits from the unchallenged 1st passage group. Finally, 2 out of 16 rabbits from the 2nd passage groups had detectable levels of anti-MV antibodies by day 35, but this immune response was not enough for protection as none of the 8 challenged rabbits survived.

Discussion

A number of vaccines have been developed against myxomatosis [1, 12, 13, 18, 19, 22, 26, 28]. Although the vaccines are effective in protecting domestic rabbits against myxomatosis, control of the disease 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 the Mediterranean ecosystems, as it is the staple prey of a wide variety of predators [4]. These include severely endangered carnivores like the Iberian lynx (*Lynx pardinus*) and several birds of prey (*Aquila adalberti, Hieraetus fasciatus*). In addition, rabbits are one of the most important small game species in Mediterranean countries.

As a first step towards the development of a new strategy for immunizing wild rabbits against myxomatosis, we carried out a survey among MV field strains in search of a candidate vaccinal strain, that is, a non-pathogenic and horizontally transmissible MV strain. For this purpose, 20 field isolates collected from infected wild rabbits from 12 regions throughout Spain, over the period 1992–1995, were analyzed in terms of virulence and transmissibility. Although the number of isolates is small, it is the first report of these characteristics carried out in Spain. The results showed that a wide range of viruses of differing properties coexist. Their virulence ranges from very high (100% mortality in less than 10 days of experimental infection) to non-lethal strains. Geographically, strains of differing virulence and lesion types are scattered widely throughout the country. The field isolates studied were classified in 5 virulence grades, A–E, which were almost equivalent to the virulence grades I–V defined by Fenner and Marshall [9]. According to this classification, 50% of the isolates were grouped as virulence grade

A viruses, 25% as virulence grade B, 10% as virulence grade C, 5% as virulence grade D, and 10% as virulence grade E viruses.

The figures obtained differ somewhat from those reported in the last surveys carried out in Australia and Great Britain [11], where there are virtually no strains of virulence grade I circulating (1.9 and 0.0% respectively), some strains of grade II (3.3 and 35.8% respectively), and the prevalent circulating strains belong to the virulence grade III group (67.0 and 62.6 respectively). The differences observed may in part be attributed to differences in the experimental procedures. In order to detect any residual pathogenicity, we decided to use extreme conditions known to affect the course of myxomatosis infection. Thus, we used in this study young rabbits (30 days old) which are reportedly much more susceptible to myxomatosis, even to attenuated strains, than are adult rabbits [8]. In contrast, rabbits at least 4 months old were used in other virulence studies [5, 9]. The route and site of inoculation are also important aspects influencing virulence [3]. We used i.d. inoculation in the eyelid, which has been shown to promote the most severe symptoms [16]. Finally, a high dose of virus was used, 10^4 pfu per rabbit, by far out of the range of the virus loads that originate natural infections by arthropod vectors, which are normally in the range of 1–10 infective particles [6] and was the dosage adopted in previous studies [5, 9]. The fact that in most of the cases the mean survival time of the contact control rabbits was consistently longer than that of the correspondent inoculated rabbits, further confirmed the stringency of the experimental conditions adopted. Thus, the results obtained with the contact control rabbits reflect more closely the real situation in the field and are better suited for comparison with the mentioned reports from Australia and Great Britain.

From this study a MV strain arose, isolate 6918, which potentially fulfilled the desired characteristics of low pathogenicity along with horizontal transmissibility. It is important to note that this MV strain (as well as the rest of the strains tested) was subjected to 2 cell culture passages in RK-13 cell line prior to the virulence test, in order to obtain a small titrated virus stock enough to perform the experimental inoculations. It is unlikely that such a low number of cell culture passages could be responsible of the attenuated phenotype exhibited by strain 6918, in view of the high number of cell culture passages (in the range of 40–200) that have been necessary to obtain attenuated viruses derived from fully virulent field strains [1, 18, 26, 25].

The candidate vaccinal strain was subsequently cloned by plaque purification and its potential as immunizing agent against myxomatosis in the field was tested. Wild rabbits inoculated by both i.d. and s.c. routes with strain 6918, developed similar antibody titres against MV (Fig. 1). This immune response efficiently protected rabbits against infection with virulent MV as all the inoculated animals resisted a lethal challenge (Table 2). Furthermore, protection against myxomatosis could be transmitted to other rabbits by contact transmission. 9 out 16 rabbits from the 1st passage rabbit groups elicited antibody responses against MV, and 5 out of 8 resisted the lethal MV challenge (Table 2). However, protection was not achieved in a second contact passage, although low anti-MV antibody titres were detected in 2 out of 16 rabbits from the 2nd passage groups.

Infection with virulent MV of previously immunized rabbits induced a high increase in the anti-MV antibody titres, indicating certain degree of virulent virus replication, a sign of nonsterilizing immunity. On the other hand, this result shows that the immunity evoked by 6918 MV strain is readily reinforced by exposure to virulent virus. In areas where myxomatosis is endemic, vaccinated rabbits will be constantly re-exposed to MV virus. Therefore, in these regions a high level of immunity is likely to be maintained in immunized rabbits over a prolonged period of time. Finally, it should be borne in mind that the virulent challenges were carried out using a high dose (1.000 pfu) of the Laussane strain (virulence grade I), which is the MV strain initially released in France, from which all European MV strains are derived, and it is considered the most virulent MV strain [11]. The challenge conditions used in the experiment were thus far in excess to those occurring in the field.

Regarding safety issues, the serial transmission experiment suggests that the attenuated nature of 6918 is a stable trait. Should there be a tendency for the virus to revert to a virulent state, serial contact transmission in rabbits would cause it to do so. In fact, this seemed to be the case for isolate 5206, which caused a mortality rate of only 30% of the inoculated rabbits but this increased to 80% of the contact transmitted rabbits, along with a significant enhanced severeness of the symptoms in the survivors. In the case of 6918, however, symptoms declined with passage of the virus in rabbits.

On the basis of the experimental results presented in this paper, we conclude that the MV strain 6918 presents adequate characteristics for the development of immunization schemes for the control of myxomatosis among wild rabbit populations, that are not feasible with the currently available myxomatosis vaccines. To further asses the potential of MV strain 6918 for vaccination of wild rabbits in the field, work is in progress to determine if it can confer transmissible protection against myxomatosis by arthropod-vectored transmission, as well as the efficiency of immunization by the oral route.

Acknowledgement

This work was supported by a grant from the "Fundación para el Estudio y Defensa de la Naturaleza y la Caza" (FEDENCA).

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Authors' address: Dr. J. M. Torres Trillo, Centro de Investigación en Sanidad Animal (CISA-INIA) Valdeolmos, 28130 Madrid, Spain.

Received June 10, 1999