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Deliberate release of a recombinant vaccinia-rabies virus for vaccination of wild animals against rabies

Paul-Pierre Pastoret, Bernard Brochier, Paul Coppens

Department of Virology-Immunology, Faculty of Veterinary Medicine, University of Liège, B43, Sart Tilman, B-4000 Liège, Belgium

Abstract. Since 1978, several European countries have conducted, at different times, large-scale field trials of oral vaccination of foxes (*Vulpes vulpes*) against rabies, using the SAD, standard or B19-modified attenuated strains of rabies virus. The use of attenuated strains of rabies virus remains controversial as far as safety and stability are concerned, since these virus strains retain pathogenicity for rodents or other wildlife species and are heat-sensitive. To improve both safety and stability of the vaccine used in the field, a recombinant vaccinia virus expressing the immunogenic G protein of rabies virus has been developed and released in the field. The first safety-efficacy results are very encouraging.

Key words: Rabies – Oral vaccination – Recombinant vaccinia-rabies virus – Safety – Efficacy

Introduction

Control of infections in wild animals is difficult to achieve, mainly because such animals are free ranging, making them difficult to handle by conventional veterinary means. Vaccination is the most appropriate way of controlling infections at a population level, but in these cases the only practical route of administration is the oral one.

Sylvatic rabies provides the best example of control of a wildlife infection. In northern countries, rabies is a zoonotic disease, the epidemiology of which is solely linked to a wildlife reservoir. In western Europe, the main vector is the red fox (*Vulpes vulpes*), whereas in North America, it is either the red fox, the raccoon (*Procyon lotor*) or the striped skunk (*Mephitis mephitis*). In this respect, "vector" refers to the animal host that is most susceptible to rabies in a region at a given time and that is solely responsible for maintaining the infection. The control of the infection within the vector species thus permits overall control of the infection and, most importantly, eliminates the risk of transmission to man (Pastoret and Brochier 1991).

In western Europe, control measures to reduce fox populations were only temporarily effective and did not prevent the disease from spreading. For this reason, other methods such as oral immunization of foxes needed to be assessed. Research has focused on oral vaccination, the only means allowing the immunization of a sufficient proportion (75%) of wild foxes, through the distribution of vaccine baits. Since 1978, several European countries have organized large-scale field trials of oral vaccination of foxes using the standard or modified (B19) attenuated strain of rabies virus, Street Alabama Dufferin (SAD) (Blancou et al. 1988). The promising results obtained from these vaccination campaigns attest to the feasibility and the efficacy of the method. However, the use of attenuated rabies virus remains controversial as far as safety and stability are concerned, as these virus strains are still pathogenic for laboratory and wild rodents or wildlife species such as the chacma baboon (*Papio ursinus*) (Bingham et al. 1992) and target species such as the striped skunk (Leblois and Flamand 1988); moreover, these strains may still be pathogenic to man. Pathogenicity of attenuated rabies virus strains can be abolished by mutating Arg residues at position 333 of the glycoprotein, although such strains can still revert to virulence (Tuffereau et al. 1989).

Thus, in order to improve both the safety and stability of the vaccines used, a recombinant vaccinia virus which expresses the immunizing glycoprotein of rabies virus has been developed and tested in the field for oral vaccination of wildlife vectors against rabies.

Construction of the recombinant vaccinia-rabies virus

The glycoprotein of rabies virus is the sole viral protein present on the external surface of the membrane. It is the only viral antigen capable of eliciting the formation of rabies virus-neutralizing antibodies and has been shown to be capable of conferring immunity to rabies. Thus, the rabies virus glycoprotein is an ideal candidate for use in the construction of subunit vaccines.

Correspondence to: P.-P. Pastoret

Nucleotide sequence analysis of the glycoprotein gene reveals an open reading frame of 524 amino acids. Expression of exogenous protein-coding sequences in vaccinia virus involves essentially two steps. First, the exogenous coding sequence is aligned with a vaccinia promoter and inserted in vitro at a site within a (non-essential) segment of vaccinia DNA cloned into a suitable bacterial plasmid replicon. Second, the flanking vaccinia sequences permit homologous recombination in vivo between the plasmid and the viral genome. Double reciprocal recombination results in transfer of the DNA insert from the plasmid to the viral genome, wherein it is propagated and expressed. The rabies virus glycoprotein gene has been inserted into the thymidine-kinase (TK) gene of vaccinia virus in this way, generating a selectable TK^- virus (Kieny et al. 1984; Wiktor et al. 1984) known as VVT Gg RAB.

Efficacy of the virus in target species

VVT Gg RAB has been tested for efficacy and safety in the main target species in western Europe and North America: fox, raccoon and striped skunk (Blancou et al. 1986; Tolson et al. 1987; Rupprecht et al. 1988; Desmettre et al. 1990).

The results of tests for efficacy in foxes can be summarized as follows: all but one of 26 adult foxes inoculated by various routes developed high titres of rabies-neutralizing antibodies and resisted wild rabies virus challenge on day 28 after vaccination (Blancou et al. 1986). The duration of immunity conferred by VVT Gg RAB, a minimum of 12 months in cubs and 18 months in adult animals, corresponds to the length of protection required for fox vaccination in the field, due to the high population turnover of the fox population (Brochier et al. 1988; Blancou et al. 1989). Foxes receiving less than the recommended dose showed a clear dose-dependent response. A second administration of VVT Gg RAB induces an increase of rabies-neutralizing antibodies (booster effect). Oral administration is the only route appropriate for the vaccination of wild animals. Accordingly, the vaccine must be presented in a form suitable for ingestion.

The efficacy of VVT Gg RAB (10^8 $TCID_{50}$) contained in a machine-made baiting system has been tested (Brochier et al. 1990). Twenty-two young foxes were divided into three experimental groups of six and a control group of four animals. Foxes in the first three groups were fed one, two and three vaccine baits, respectively, on successive days. The four unvaccinated foxes were housed separately. As shown by the incorporation of a tetracycline biomarker into their bones, all the baited foxes ingested at least one bait. Thirty days after baiting, seroconversion to rabies was observed in 15 of 18 foxes and seroconversion to vaccinia in 14 of 18. Of the 18 baited foxes, 16 resisted a wild rabies virus challenge 90 days after baiting. One cub was protected against rabies despite the absence of detectable anti-rabies antibodies. These results demonstrate that the baiting-sachet system used permits an efficient release of the virus suspension into the mouth.

Safety of the virus

A vaccine virus most not only be efficacious but also innocuous for the target species. VVT Gg RAB was observed to be non-pathogenic in the fox whatever the dose of inoculation (10^2–10^{10} $TCID_{50}$), or route of administration (oral, intramuscular, intraduodenal, subcutaneous, intradermic, conjunctival or intranasal).

It is preferable that a vaccine virus used for oral vaccination of wildlife not be horizontally transmitted to unvaccinated animals. In order to test for horizontal transmission, unvaccinated control animals have been held in close contact with vaccinated ones. Using this assessment method, no transmission of immunizing amounts of VVT Gg RAB was found to occur in adult or young foxes, with the exception of one adult fox bitten by a freshly inoculated animal.

It is also of major importance to preclude epizootiological risks, such as the emergence of asymptomatic carriers of wild rabies virus (Brochier et al. 1989a). This situation could occur in the field by the vaccination of naturally infected animals during the incubation period. The influence of vaccination with VVT Gg RAB on the onset of the disease and on the delay before death in foxes previously infected with wild rabies virus, has been investigated. All foxes, vaccinated or not, died from rabies. Animals vaccinated early after challenge died after a shorter period of incubation than unvaccinated controls. On the other hand, animals vaccinated belatedly after challenge died after the controls. The results show that "early" and "late" death phenomena occur as a consequence of interactions between oral vaccination with VVT Gg RAB and rabies infection, but preclude the risk of the emergence of asymptomatic carriers of wild-rabies virus after vaccination.

Similar results have been obtained with the raccoon and the skunk. The safety of VVT Gg RAB for administration to pregnant female raccoons has been evaluated, revealing an absence of epigenetic transmission to offspring, as well as the absence of the recombinant in the cerebrospinal fluid (Hanlon et al. 1989). There was no evidence of active in utero or lactogenic transmission of the recombinant. These results are in contrast with those obtained with attenuated strains of rabies virus in the skunk (Rupprecht et al. 1990).

Field trials with attenuated strains of rabies virus have shown that several non-target wildlife species compete with foxes for bait consumption. It must also be taken into account that, within the orthopoxvirus group, vaccinia virus has a wide range of host species. In fact, bait uptake surveillance and tetracycline (biomarker) detection controls, performed after vaccination campaigns, proved that mustelids, wild boars (*Sus scrofa*) and domestic carnivores can ingest the vaccine baits. Moreover, a significant proportion of the baits are partly eaten by small mammals. It is important, therefore, to verify the safety of VVT Gg RAB for non-target species (both domestic and wild).

Several non-target wild species have been chosen for testing in Europe because of their opportunistic feeding behaviour and their presence in the areas where the vac-

cine must be distributed (Brochier et al. 1989b). Safety of the vaccine has been tested in Daubenton's bat (*Myotis daubentoni*), wild boar, Eurasian badger (*Meles meles*) wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*), bank vole (*Clethrionomys glareolus*), common vole (*Microtus arvalis*), field vole (*Microtus agrestis*), water vole (*Arvicola terrestris*), common buzzard (*Buteo buteo*), kestrel (*Falco tinnunculus*), carrion crow (*Corvus corone*), magpie (*Pica pica*) and jay (*Garrulus glandarius*). Clinical signs of rabies and/or pox-inflicted lesions were never observed in the vaccinated animals during the observation period (28 days minimum after vaccination).

Similar experiments have been carried out with wild species from North America (Artois et al. 1990), including meadow vole (*Microtus pennsylvanicus*), woodchuck (*Marmota monax*), grey squirrel (*Sciurus carolinensis*), ring-billed gull (*Larus delawarensis*), red-tailed hawk (*Buteo jamaicensis*), great horned owl (*Bubo virginianus*) and coyote (*Canis latrans*). Comparable results were observed in these studies. Recent experiments have also shown that the recombinant virus, administered either by scarification or by the oral route, is also safe for squirrel monkeys (*Saimiri sciureus*) and for chimpanzees (*Pan troglodytes*) (Rupprecht et al. 1992).

Multiplication site of the virus in foxes

When assessing a recombinant virus for use in vaccination, it is also of great importance to detect any variation in tissue specificity compared with the parental vector strain. Experiments have been designed to determine the multiplication site in foxes of the recombinant virus, compared with the parental strain of vaccinia virus, by virus isolation, titration and indirect immunofluorescence. The polymerase chain reaction was also used to detect specific virus DNA in several fox organs (Thomas et al. 1990). Foxes were fed with 10^8 $TCID_{50}$ of either VVT Gg RAB or vaccinia virus, and were killed 12, 24, 48 or 96 h after inoculation by the oral route.

Using these different techniques, VVT Gg RAB or vaccinia virus could be detected during the first 48 h following vaccination by the oral route, but only in the tonsils, buccal mucosa and soft palate. Similar results have been obtained in raccoons using virus isolation (Rupprecht et al. 1988). Results of other experiments demonstrate that tonsillectomy of foxes does not completely impede seroconversion (I. Thomas et al., unpublished data).

As no virus could be detected in the salivary glands of foxes (parotid or maxillary), the risk of transmission from one animal to another through saliva can be assumed to be negligible. Furthermore, the fact that VVT Gg RAB only multiplies in restricted sites minimizes the potential risk of recombination with other wild orthopoxviruses. In these experiments, no difference was observed between the multiplication sites of VVT Gg RAB and vaccinia virus, demonstrating that the recombination event did not modify the tropism of the virus. Additionally, virus was never detected in the brain. These results are in agreement with those from studies on raccoons vaccinated orally with VVT Gg RAB (Hanlon et al. 1989) that report the absence of detectable cytological abnormalities in the cerebrospinal fluid.

Deliberate release of the virus

Taking into account all the available experimental data concerning the safety of the VVT Gg RAB for target and non-target species and its efficacy in foxes, initial limited field trials of fox vaccination were authorized first by the Belgian (Pastoret et al. 1988) and then by the French public health authorities. In the Belgian trial (17–18 October 1987) a total of 250 vaccine baits (chicken heads) were delivered manually on a 6 km^2 area situated in the central part of a military zone.

With the safety of the VVT Gg RAB being confirmed by this small trial, the Belgian authorities agreed for an enlarged open field trial (Brochier et al. 1990). This was conducted in a 435 km^2 area in the southern part of the country, chosen because it has the lowest average human population density in the country (42 inhabitants km^{-2}) as well as high rabies incidence in foxes. Furthermore, the region is characterized by various habitats housing most of the animal species liable to consume bait. Each bait contained a suspension of 10^8 $TCID_{50}$ of VVT Gg RAB (2.2 ml by volume) within a plastic sachet and 150 mg tetracycline as a long-term biomarker of bait uptake. After the vaccination campaign, 222 dead wild animals belonging to 19 species were collected in the vaccination area. After necropsy, the following tissues were removed: brain for rabies diagnosis, jaws for tetracycline detection and blood for the titration of vaccinia and rabies antibodies.

Tetracycline was detected in foxes (61%), stone martens (*Martes foina*; 40%), domestic feral cats (*Felis catus*; 27%), wood mice (*Apodemus* sp.; 10%), wild boars (47%) and carrion crows (10%), showing that wild boars, mustelids and feral cats are strong competitors of the foxes for bait uptake (Brochier et al. 1990b). Twelve months of monitoring failed to detect any ecological hazard or public health risk. The vaccine was very stable even following natural freezing and thawing cycles. The VVT Gg RAB vaccine has been shown to retain its capacity to immunize for at least 1 month in field conditions, a period which corresponds to the delay of uptake that many baits may undergo in the field (Brochier et al. 1990b).

Following this enlarged trial, three fox-vaccination campaigns using VVT Gg RAB were then carried out in Belgium in November 1989, April 1990 and October 1990 in order to check for efficacy in an area of 2200 km^2 with a mean baiting density of 15 baits km^{-2} (Brochier et al. 1991a). The 25000 baits were dropped by helicopter. After each vaccination campaign, foxes found dead (or shot by hunters) were collected for rabies diagnosis and bone tetracycline analysis. The three collection periods permitted the collection of 10 rabid and 178 healthy foxes. The bait uptake rate determined from tetracycline status in 23 adult foxes (9 rabid, 14 healthy)

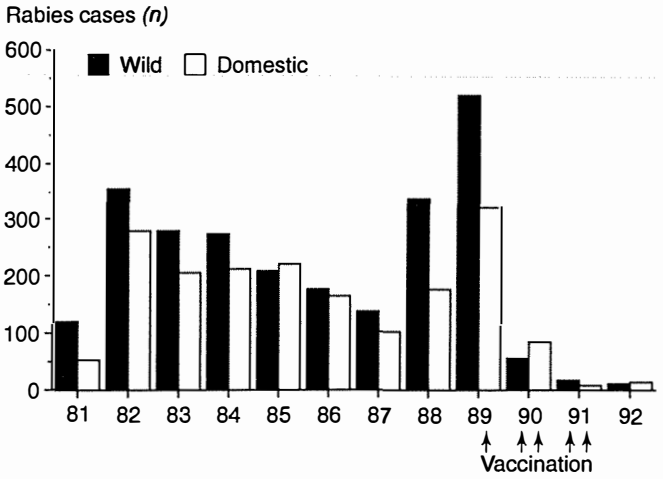

Fig. 1. Evolution of animal rabies in Belgium, 1981–1992. *Arrows,* full campaigns of fox vaccination in the infected area (10000 km^2)

during the first period was 74%; 6 of the 9 rabid and 11 of the 14 healthy foxes were positive for tetracycline. During the second collection period bait-uptake rates were 80% (25/31) in adult foxes but only 49% (27/55) in juveniles. During spring dispersal of baits, most cubs (born principally in March) would be expected to be confined to the immediate surroundings of the breeding den, and thus less likely to ingest baits. No rabid fox was recorded during this period (0/86). After the third phase of vaccination (October 1990), 81% (64/79) of inspected animals were tetracycline positive. The one rabid animal recorded, at the periphery of the baited area, was tetracycline-negative. Serological data are currently misunderstood because of strong discordances in the results obtained with the rapid fluorescent foci inhibition test (RFFIT) and the ELISA technique. The poor quality of blood samples collected from dead animals generally induces non-specific reactions. Serological results would be reliable when using fresh sera only (Brochier et al. 1990b).

Despite the dramatic decrease in the number of rabid foxes recorded after vaccine-bait distribution, the efficacy of the vaccination campaign was difficult to evaluate because systematic collection of foxes is not logistically feasible. Because transmission of rabies to domestic animals occurs through the bite of a rabid wild animal, however, and because notification of cases of rabies in cattle and sheep is mandatory in Belgium, the incidence of rabies in domestic livestock provides a reliable indicator of the prevalence of rabies in the wild. No case of livestock rabies has been recorded in the study zone since the second phase of vaccination. Our results therefore indicate that the dispersion of live recombinant vaccinia virus VVT Gg RAB can be an effective means of controlling rabies.

In autumn 1989, a first campaign of fox vaccination was carried out in the whole infected area of Belgium (10000 km^2). Similar full campaigns were then repeated four times: in spring and autumn 1990 and spring and autumn 1991. Starting in 1990 the VVT Gg RAB was used because of its efficacy, stability and safety. During each campaign, 150000 vaccine baits were dropped by aircraft (mean 15 baits/km^{-2}).

As shown in Fig. 1, these campaigns have brought about a drastic decrease in the incidence of rabies, both in foxes and in domestic animals. From April 1991 to the time of writing, that is to say for more than a year, all rabies cases were recorded in a 2000-km^2 region along the border with France. Rabies has disappeared from the majority of the initial infected area. In light of this epidemiological situation a new spatial strategy for bait dispersal was initiated in 1992, namely formation of an immune belt along international borders. Two "defence" campaigns were carried out in April and October 1992 (Brochier et al. 1991b; Coppens et al. 1992).

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