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Authors: Brochier, Bernard, Blancou, Jean, Thomas, Isabelle, Languet, Bernard, Artois, Marc, et al.

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USE OF RECOMBINANT VACCINIA-RABIES GLYCOPROTEIN VIRUS FOR ORAL VACCINATION OF WILDLIFE AGAINST RABIES: INNOCUITY TO SEVERAL NON-TARGET BAIT CONSUMING SPECIES

Bernard Brochier,¹ Jean Blancou,² Isabelle Thomas,¹ Bernard Languet,³ Marc Artois,² Marie-Paule Kieny,⁴ Jean-Pierre Lecocq,⁴ Françoise Costy,⁵ Philippe Desmettre,³ Gilles Chappuis,³ and Paul-Pierre Pastoret¹

¹ Department of Virology and Immunology, Faculty of Veterinary Medicine, University of Liège, B-1070 Brussels, Belgium

² C.N.E.V.A. - Centre National d'Etudes sur la Rage et la Pathologie des Animaux Sauvages, F-54200 Malzéville, France

³ Laboratoire Iffa, Rhône-Mérieux, F-69007 Lyon, France

⁴ Transgene, S.A., F-67082 Strasbourg, France

⁵ Department of Rabies, Pasteur Institute of Brabant, B-1040 Brussels, Belgium

ABSTRACT: The pathogenicity of a vaccinia recombinant virus expressing the rabies glycoprotein (VVTGgRAB) was tested in several wild animal species which could compete with the natural rabies host, the red fox (*Vulpes vulpes*) in consuming vaccine baits in Europe. The following species were included in this study: wild boar (*Sus scrofa*), 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*). During the observation period, the 107 animals given the VVTGgRAB vaccine orally did not show any clinical signs. Daily monitoring for 28 days and postmortem examination did not result in the detection of pox lesions in the oral mucosa or the skin in mammals or the unfeathered portions of birds. VVTGgRAB seems to multiply in the mammalian species tested, since rabies seroconversion was observed in all of them. Birds failed to develop demonstrable rabies virus-neutralizing antibody. A seroconversion against vaccinia virus was observed in two of four wild boars. Serological results obtained in badgers and wild boars also demonstrates the absence of direct or indirect horizontal transmission of the recombinant virus. The potential of the recombinant virus for the immunization of badgers against rabies also was investigated. Only 50% of the badgers orally administered with $1 \times 10^{8.3}$ TCID₅₀ of this vaccine were protected against rabies.

Key words: Rabies, oral vaccination, wildlife, vaccinia-rabies recombinant virus, innocuity, badger (*Meles meles*), red fox (*Vulpes vulpes*), field trial.

INTRODUCTION

Since 1978, several European countries have conducted at different times large scale field trials of oral vaccination of foxes (*Vulpes vulpes*) using the SAD, standard or B19 modified, live attenuated strain of rabies virus (Steck et al., 1982; Schneider and Cox, 1983; Artois et al., 1987; Frisch et al., 1987; Brochier et al., 1988b). The promising results obtained from these vaccination campaigns attest to the feasibility and efficacy of the method. However, the use of attenuated rabies virus still remains controversial as far as innocuity and stability are concerned, since these virus strains retain pathogenicity for laboratory

and wild rodents (Wandeler et al., 1982; Schneider and Cox, 1983; Leblois and Flahmand, 1988) and are heat-sensitive. Furthermore, conventional rabies vaccines, when administered orally, are inefficient in rabies vectors in North America such as raccoons (*Procyon lotor*) and striped skunks (*Mephitis mephitis*) (Rupprecht et al., 1986; Tolson et al., 1987).

A recombinant vaccinia virus expressing the immunizing G glycoprotein of rabies virus has been developed (Kieny et al., 1984). A gene coding for the glycoprotein of strain ERA of rabies virus has been inserted within the coding sequence of the vaccinia virus thymidine kinase (TK) gene.

The oral administration of this live recombinant vaccinia-rabies virus (VVTGgRAB) to young and adult foxes, raccoons and striped skunks elicits high levels of rabies virus neutralizing antibodies, confers long-term protection against rabies and is free of any pathogenicity (Blancou et al., 1986; Rupprecht et al., 1986; Tolson et al., 1987; Brochier et al., 1988a).

The absence of pathogenicity of VVTGgRAB administered by the oral route also has been demonstrated in the laboratory mouse, rabbit, ferret (*Putorius furo*), cattle, cat, dog and sheep (Wiktor et al., 1984, 1985; Soria Baltazar et al., 1987; Blancou et al., 1989). Field trials conducted in Europe have shown that several wildlife non-target species compete with foxes in consuming "Tübingen" baits consisting of fat and fish meal (W.H.O. Collaborating Centre for Rabies Surveillance and Research, Tübingen, Federal Republic of Germany). Bait uptake and tetracycline detection controls performed after vaccination campaigns have proven that mustelids and wild boars could ingest the vaccine bait (Kalpers et al., 1987; Brochier et al., 1988b; Paquot et al., 1988). A significant proportion of the baits are partially eaten by small mammals. Despite this competition, further uptake by foxes is generally not precluded. However, when baits persist in the field, rodents may eat the whole coating, perforate the plastic capsule and swallow the vaccine liquid (Brochier et al., 1988b). No case of vaccine-induced rabies has been reported in rodents trapped within vaccination areas (Wachendorfer et al., 1985; Schneider and Cox, 1988). Nevertheless, the method of rabies virus isolation in captured small mammals cannot be considered as an appropriate tool for safety control. This method requires many more specimens per hectare for the detection of rodents with prepatent rabies. When considering the behaviour of rabid animals and the action of predators and scavengers, the trapping method does not help to detect patent or dead specimens.

The use of a recombinant vaccinia virus may help to solve some of the problems related to the use of a live modified rabies virus. Nevertheless, the safety problems of live vaccines (required for oral immunization) must still be investigated when using a recombinant orthopox virus.

Therefore, it is important to verify the absence of pathogenicity, excretion and transmission of VVTGgRAB in both target and non-target animal species. The following non-target species have been chosen for testing, because of their opportunist food habits and presence in the areas where this vaccine may be distributed: wild boar (*Sus scrofa*), wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*), bank vole (*Clethrionomis 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*).

The present study also investigates the innocuity and efficacy of VVTGgRAB in the immunization and protection of badgers (*Meles meles*) against rabies virus. In Europe, badgers also are considered as a target species for rabies vaccination since they are often infected. This species is strongly susceptible to the vulpine strain of rabies virus and can excrete high amounts of virus in its saliva (Wandeler et al., 1974; Moegle and Knorpp, 1978). Furthermore, the usual co-occurrence of foxes and badgers in sympatric habitats results in frequent infection of the latter species.

MATERIALS AND METHODS

Animals

Ten Eurasian badgers were captured from their sett by digging in a rabies-free area of France (Moulin, Department of Allier). Seven wild boars were purchased in the nature reserve of Han-sur-Lesse (Belgium). All manipulations of wild boars and badgers were conducted under anaesthesia. Wild boars were handled under anaesthesia induced by intramuscular injection of Fentanyl® + Azaperone® (Janssen Pharmaceu-

tica, Beerse, Belgium, 10 mg/kg + 0.3 mg/kg). Nine of 10 badgers were tranquilized with ketamine hydrochloride (Imalgène 500[®], Rhône-Mérieux, Lyon, France, 20 mg/kg). Thirty-four wood mice, eight yellow-necked mice, five *Apodemus* sp., three bank voles, one field vole, two common voles and five water voles were trapped in the province of Luxembourg (Belgium) and the Department of Meurthe et Moselle (France). Fifteen bank voles were purchased in a laboratory breeding unit (University of Brussels, Brussels, Belgium). Twenty carrion crows, 10 magpies and two jays were trapped in a rabies-free area of Belgium (La Hulpe, Belgium). Eight buzzards and four kestrels were kindly supplied by the "Groupement d'Etudes et de Conservation de la Nature en Lorraine" (Velaine en Haye, France). These birds had been found injured and had no chance of survival in the wild.

Badgers, wild boars, carrion crows and magpies were housed in familial cages or boxes. Rodents, jays and birds of prey were housed in individual cages. Captive animals were raised either in the Pasteur Institute of Brabant (Brussels, Belgium) or in the experimental centrum of Atton (Nancy, France). Before being included in this study, they were sexed, marked and wild specimens were put through a 10 day period of conditioning to captivity.

Viruses

A live recombinant vaccinia virus (Copenhagen strain) expressing the rabies glycoprotein (ERA strain) (VVTGgRAB-26D3 187XP strain) (Kieny et al., 1984) was used as vaccine. VVTGgRAB was propagated in a VERO green monkey kidney cell line. A master stock of the VVTGgRAB was established and was preserved by freeze drying. All the vaccine preparations originate from this stock, within the limit of a maximum of five cell passages. The diluted viral suspension is available either deep-frozen or freeze dried associated with a stabilizer. Each batch of vaccine preparation was subjected to a titration on VERO cell line and an activity test by vaccination of mice, followed by a challenge with virulent rabies virus. The titer of the vaccine preparation used in this study was 1×10^8 or $1 \times 10^{8.5}$ TCID₅₀ per 1 ml. The challenge virus suspension was prepared as previously described (Blancou et al., 1979). It consisted of an homogenate of salivary glands of foxes dead of natural rabies (wild strain GS7). The titer was determined by intracerebral (i.c.) inoculation of 21-day-old mice. Badgers were inoculated intramuscularly with 1 ml of virus suspension titrating $1 \times 10^{8.2}$ mouse i.c. LD₅₀/ml.

Experimental protocol

The experimental protocol for each species is detailed in Table 1. On day 0, vaccine was ad-

ministered by direct application into the oral cavity either via a needleless syringe (volume: 1 ml in wild boars, badgers and birds) or via a 100 µl tip (volume: 30 µl in small mammals).

As shown in Table 1, unvaccinated animals were either separately housed and used as controls or held in close contact with vaccinated animals. They were given an equal volume of PBS (phosphate buffered saline, pH 7.4). Animals were observed daily for a period of 28 days or more postvaccination. Potential clinical signs and/or pox lesions were recorded. For rabies and vaccinia serological analysis, blood was collected, on days 0 and 28, from the jugular vein of anaesthetized badgers and wild boars and from the brachial vein of crows and magpies. Five of eight buzzards, three of four kestrels and jays were bled in the brachial vein approximately 1 mo (from 28 to 45 days) postvaccination for rabies and/or vaccinia virus-neutralizing antibody determination. On day 28 postvaccination, rodents were anaesthetized by fluothane[®] (ICI Pharma, Destelbergen, Belgium) inhalation, bled in the heart and subsequently euthanatized with a higher dosage of fluothane[®]. All other animals were humanely euthanatized by intravenous injection of T 61[®] (Hoechst, Unterschleißheim, Federal Republic of Germany) approximately 1 mo postvaccination (except boars on day 88). Postmortem examination was performed on all animals after euthanasia.

Badgers were challenged by inoculation of 1 ml of the virus suspension in the right temporal muscle on day 45 postvaccination. They were observed for rabies for a 45 day period.

Serological analysis

Rabies virus neutralizing antibodies were determined by the inhibition of fluorescence technique (RFFIT) (Smith et al., 1974). Titers are expressed in IU (International Units) per ml as determined by comparison with a standard serum (titer: 4.4 IU/ml). The arbitrarily defined level of 0.5 IU/ml in humans is considered indicative of successful rabies immunization.

Vaccinia virus-neutralizing antibodies were determined by a technique of seroneutralization on VERO cells. Titers are expressed as the logarithm of the dilution capable of neutralizing 30 TCID₅₀ of vaccinia virus and are considered indicative of the presence of anti-vaccinia antibodies when exceeding a value of 1.

Rabies diagnosis

The presence of rabies virus in the brain of dead badgers was detected by the analytical techniques recommended by the World Health Organization: immunofluorescence and i.c. inoculation of mice (Dean and Abelseth, 1974; Koprowski, 1974).

TABLE 1. Experimental protocol of VVTGgRAB oral administration to several non-target wild animal species.

Species	Number of tested animals	VVTGgRAB dose (TCID ₅₀)	Volume (ml)	Number of unvaccinated animals		Observation period (days)
				Control	Contact*	
European badger (<i>Meles meles</i>)	6	$1 \times 10^{8.3}$	1	2	2	45
Wild boar (<i>Sus scrofa</i>)	4	$1 \times 10^{8.3}$	1	2	1	88
Wood mouse (<i>Apodemus sylvaticus</i>)	27	$2-3 \times 10^6$	0.03	7	—	28–43
Yellow-necked mouse (<i>Apodemus flavicollis</i>)	7	3×10^6	0.03	1	—	41
<i>Apodemus</i> sp.	4	2×10^6	0.03	1	—	41
Bank vole (<i>Clethrionomys glareolus</i>)	13	2×10^6	0.03	5	—	28
Common vole (<i>Microtus arvalis</i>)	2	3×10^6	0.03	—	—	35
Field vole (<i>Microtus agrestis</i>)	1	3×10^6	0.03	—	—	35
Water vole (<i>Arvicola terrestris</i>)	5	3×10^6	0.03	—	—	41
Common buzzard (<i>Buteo buteo</i>)	8	1×10^6	1	—	—	30–45
Kestrel (<i>Falco tinnunculus</i>)	4	1×10^6	1	—	—	30–45
Carrion crow (<i>Corvus corone</i>)	17	1×10^6	1	3	—	28
Magpie (<i>Pica pica</i>)	7	1×10^6	1	3	—	28
Jay (<i>Garrulus glandarius</i>)	2	1×10^6	1	—	—	28

* Animals kept in contact with vaccinated animals.

RESULTS

Serological analysis

All the badgers, boars, crows and magpies included in this study were shown to be serologically negative for rabies virus-neutralizing antibodies on day 0. On day 28 postvaccination, rabies virus antibody titers exceeding 0.5 IU/ml appeared in two of four boars (50%), two of six badgers (33%), 16 of 27 wood mice (59%), three of four *Apodemus* sp. (75%), five of seven yellow-necked mice (71%), eight of 13 bank voles (61.5%), one of one field vole (100%), two of two common voles (100%), four of five water voles (80%), none of five buzzards (0%), none of two kestrels (0%), none of 17 crows (0%), none of 7 magpies (0%) and none of two jays (0%). On day 0, the serum of all crows inhibited the vaccinia

virus infection of VERO cells (titers varying from 1.17 to 1.63). Tables 2 and 3 indicate vaccinia and rabies serology in badgers and wild boars, respectively. Anti-vaccinia seroconversion was detected in two of four wild boars (50%). Neither of the unvaccinated wild boars and badgers, kept in contact with vaccinated individuals, elicited any demonstrable rabies virus-neutralizing antibody; neither did control unvaccinated animals of either species show any rabies virus-neutralizing antibody.

Rabies challenge

As shown in Table 2, two of six badgers (animals 8 and 9) administered VVTGgRAB resisted challenge on day 45 postvaccination. Badger 9 was protected

TABLE 2. Vaccinia and rabies virus-neutralizing antibodies and protection from rabies challenge in badgers orally administered with $1 \times 10^{3.1}$ TCID₅₀ of VVTGgRAB.

Treatment	Animal number	VNA titers*				Resistance to challenge ^c
		Rabies		Vaccinia		
		Day 0 ^b	Day 28	Day 0	Day 28	
Unvaccinated control	1	<0.5	<0.5	1	<0.6	D (34) ^d
	2	<0.5	<0.5	<0.4	0.5	D (20)
Unvaccinated contact	3	<0.5	<0.5	0.7	0.8	D (21)
	4	<0.5	<0.5	<0.6	0.8	D (18)
Vaccinated	5	<0.5	<0.5	<0.6	<0.4	D (27)
	6	<0.5	<0.5	<0.4	<0.5	D (21)
	7	<0.5	<0.5	<0.5	<0.5	D (30)
	8	<0.5	4.8	<0.7	<0.7	S
	9	<0.5	<0.5	0.7	<0.4	S
	10	<0.5	3.8	<0.4	<0.4	ND

* Titers of rabies and vaccinia virus-neutralizing antibodies are expressed, respectively, in International Units/ml and in logarithm of the dilution neutralizing 30 TCID₅₀ of vaccinia virus.

^b Day 0, day of vaccination.

^c S, survived; D, died; ND, accidentally died on day 45.

^d Day of death after challenge is in parentheses.

despite the absence of demonstrable rabies antibodies. The vaccinated badger 10 had produced rabies antibodies, but died accidentally before challenge. The three other vaccinated and all the unvaccinated badgers died from rabies as confirmed by immunofluorescence and i.c. inoculation of mice.

Clinical monitoring and necropsy

Clinical signs and/or pox lesions were not observed in any of the vaccinated animals during the observation period (28 days minimum after vaccination). At necropsy, all carcasses were in normal condition and lesions indicative of poxvirus infection were not detected.

DISCUSSION

Rabies serologies demonstrate that VVTGgRAB, when orally administered, can elicit seroconversion in all the species of mammals included in this study. Despite the unknown serological status of rodents on day 0, seroconversion could be attested since none of the control unvaccinated trapped animals possessed specific antibody. Furthermore, most of the bank

voles were born and raised in a laboratory breeding unit free of rabies infection. The absence of rabies and/or vaccinia seroconversion in birds suggests that the recombinant virus does not replicate in these avian species. On day 0, the sera of all carrion crows had a neutralizing activity on the vaccinia virus infection of VERO cells. This serological reaction is probably non-specific, since vaccinia and/or rabies neutralizing antibody titers did not increase in vaccinated crows.

During a minimum of 28 days postvaccination, neither clinical signs nor lesions were observed in any of the 107 vaccinated animals. This period of observation has been chosen because it exceeds the delay of recovery from vaccinia infection in humans (Fenner, 1985). The absence of mortality, especially in rodents, is a notable advantage of the recombinant virus as compared to the SAD live-modified strains of rabies virus, which retain pathogenicity in small mammals. The absence of seroconversion in unvaccinated boars and badgers kept in close contact with vaccinated animals indicate that excretion and horizontal transmission of immunizing amounts

TABLE 3. Vaccinia and rabies virus-neutralizing antibodies in wild boars orally administered with $1 \times 10^{6.3}$ TCID₅₀ of VVTGgRAB.

Treatment	Animal number	VNA titers ^a				
		Rabies		Vaccinia		
		Day 0 ^b	Day 28	Day 0	Day 28	Day 90
Vaccinated	1	<0.45	4.99	0.58	0.82	1.98
Vaccinated	2	<0.45	<0.45	0.58	<0.35	0.70
Vaccinated	3	<0.45	5.46	0.47	0.82	1.40
Vaccinated	4	<0.45	<0.45	0.82	<0.35	0.47
Unvaccinated control	5	<0.45	<0.45	0.82	<0.35	<0.35
Unvaccinated control	6	<0.45	<0.45	0.70	<0.35	0.58
Unvaccinated contact	7	<0.45	<0.45	0.58	<0.35	<0.35

^a Titers of rabies and vaccinia virus-neutralizing antibodies are expressed, respectively, in International Units/ml and in logarithm of the dilution neutralizing 30 TCID₅₀ of vaccinia virus.

^b Day 0, day of inoculation.

of VVTGgRAB vaccine did not occur in those animals. Similar results have been obtained in foxes, dogs and cats (Blancou et al., 1989). This observation is also an important feature as an indication of vaccine safety. It precludes the epidemiological risks possibly associated with the use of a recombinant strain of orthopox virus. The innocuity of this VVTGgRAB vaccine seems to be established in these wild species as it has been in domestic, laboratory and target wild animal species.

Our results confirm the predicted safety linked to this recombinant virus. Inactivation of the thymidine kinase (TK) gene by the insertion of a c-DNA sequence coding for rabies glycoprotein affects the pathogenesis of vaccinia virus and markedly attenuates its virulence (Buller et al., 1985).

When including the unchallenged badger 10, which had produced rabies antibodies, it can be attested that only 50% of vaccinated badgers are protected against rabies infection. The efficacy of VVTGgRAB in the immunization and protection of badgers against rabies is weak when compared to the protection level conferred to other wild target species. Higher percentages of protected animals were obtained in similarly inoculated (route and dose) red foxes, raccoons and

striped skunks (Blancou et al., 1986; Rupprecht et al., 1986; Tolson et al., 1987; Brochier et al., 1988a). Badgers previously have been shown to be poorly responsive to some antigens (Higgins and Gatrill, 1984), for example *Mycobacterium bovis* (Morris et al., 1978). As suggested by Higgins and Gatrill (1984), the repeated use of ketamine hydrochloride might affect the immune response of badgers as it has been demonstrated in monkeys (Thomas et al., 1982). Nevertheless, the possibly immunosuppressive effect of this drug cannot be incriminated in this experiment, since badger 7, which was never anaesthetized, failed to develop a protective immune response. It has been reported (Higgins and Gatrill, 1984) also that badgers are only weakly responsive to T cell-dependent antigens. This poor immunological responsiveness could be linked to a circumvented macrophage processing of T-dependent antigens. A further experiment including a larger number of badgers will be conducted in order to confirm these results.

Because of its efficacy, complete innocuity from the rabies aspect, and heat-stability, this recombinant virus would seem to offer an excellent alternative to the attenuated strains of rabies virus currently used in the field. A first field trial of fox vaccination with the VVTGgRAB was

conducted in Belgium in October 1987 (Pastoret et al., 1988).

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