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Target and Non-Target Effects of a Recombinant Vaccinia-Rabies Virus Developed for Fox Vaccination against Rabies

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Rabies: a World-wide Problem

Rabies is a much-feared disease that is still prevalent in much of the world. It is maintained in two not necessarily related cycles, urban and sylvatic. Urban rabies, which affects stray and feral dogs and cats, is by far the more dangerous to man, and accounts for an estimated 99 per cent of all recorded human cases and for 92 per cent of all human post-exposure treatments. Sylvatic rabies mainly involves one or two wild species in specific locations, a pattern which has remained stable over many years [1].

Wild animal species involved in maintaining infection may vary in different geographical and ecological conditions. For instance, in Latin America there is a sylvatic rabies cycle linked to vampire bats, mainly *Desmodus rotundus.* In North America, wildlife species that play a distinct role include the raccoon (*Procyon lotor*), the striped skunk (*Mephitis mephitis*), the red fox (*Vulpes vulpes*), the coyote (*Canis latrans)* and the Arctic fox (*Alopex lagopus*). The present European terrestrial sylvatic epizootic of rabies, which originated in Poland, has spread westward some 1 400 km since 1939. For several years, the front advanced from 20 to 60 km per year [2]. While this epizootic involves all susceptible species, both wild and domestic, the red fox (*Vulpes vulpes*), which is both the vector of the disease and its reservoir, is involved in more than 75 per cent of cases. The red fox plays a key role in maintaining the disease but does not usually transmit it directly to humans, who are mainly at risk from affected domestic animals, such as cattle and cats. However, because the fox seems to be the only species maintaining the present epizootic, if rabies is eliminated from the fox population it will cease to be a problem in other wildlife or domestic species.

The control of fox rabies will be discussed below, but it must be remembered that there are many different epidemiological cycles, both urban and sylvatic, in the world. These cycles involve many different animal species, and control measures (e.g. through vaccination) that can be applied in as many situations as possible are needed.

Rabies Virus

Rabies virus belongs to the family *Rhabdoviridae*, which are enveloped RNA viruses characterised by their shape (Greek *rhabdos*: rod) and by the presence of infectious helical nucleocapsids enclosed in a lipid envelope with surface projections. The genome consists of a single molecule of negative single-stranded RNA which is non-infectious and is transcribed into five mRNAs, each of which codes for a single protein. The rabies virus genome, which has been completely sequenced, contains 11,932 nucleotides [3] The presence of a pseudogene between the G and L cistrons indicates that rabies virus is intermediate, in evolutionary terms, in the *Rhabdoviridae* family.

The helical core of the ribonucleoprotein (RNP) contains RNA complexed with about 1,800 molecules of nucleoprotein N, 30 to 60 molecules of transcriptase L and 950 molecules of phosphoprotein NS. The nucleocapsid structure is surrounded by an envelope of about 1,500 molecules of membrane protein M, through which protrude 1,800 molecules of surface spikes of the transmembrane protein [4].

The pathogenicity of rabies virus is partially related to the G protein, since the introduction of a mutation at arginine 333 of this protein (selection of mutants resisting neutralisation by appropriate monoclonal antibodies) renders the virus avirulent for mice and other species [5,6].

Rabies has been known for centuries as a disease of man and animals and, for many years, it was thought that there was a single rabies virus. It is now clear that antigenic variation exists within rabies virus strains, as monoclonal antibodies show, and the existence of several distinct rabies-related viruses is now fully recognised [7].

Recent developments in molecular biology have led to the increased use of genetic typing as an epidemiological tool. Application of these techniques to rabies epidemiology is still in the early stages; no consensus has yet been reached on the area of focus in the genome or on how much sequence information is necessary. Nevertheless, it is clear that there are biological variants of the virus adapted to different species. For instance, Blancou and co-workers have shown that the rabies virus strain that now prevails in Western Europe is well adapted to the fox and far less to the dog [8,9], as dogs rarely excrete fox rabies virus.

Control of Fox Rabies

Prophylactic measures taken in the past, such as the destruction of foxes, did not prevent the spread of the epizootic. During recent years, most of the research on the control of fox rabies has concentrated on developing methods of

oral vaccination of foxes [10], a method which has been extensively used in all the contaminated countries of the European Community. Research has focused on oral vaccination through the distribution of vaccine baits, because it is the only way to immunise a sufficient proportion (75 per cent) of wild foxes. This has meant that only attenuated strains of rabies virus or live vectored vaccines could be used. Inactivated rabies vaccines are ineffective when administered orally [11].

In 1986, in order to develop a common European strategy, a co-ordinated trial of oral vaccination of foxes was undertaken in several European countries using the SAD (Street Alabama Dufferin) B19 attenuated strain of rabies virus. The goal was to assess both the efficacy and the feasibility of the method [12]. The results of these campaigns confirmed the efficacy of fox vaccination for the control of sylvatic rabies.

However, the use of attenuated rabies virus remains controversial, as these virus strains are still pathogenic for laboratory and wild rodents [13-15] or wildlife species such as the chacma baboon (*Papio ursinus*) [16] and target species such as the striped skunk (*Mephitis mephitis*) [17]. Moreover, these strains may still be pathogenic to humans. Human beings exposed to SAD-derived attenuated strains of rabies must be treated with a conventional inactivated rabies vaccine, which elicits good cross-protective immunity (P. Sureau, personal communication). The SAD-derived attenuated strains may also be inefficient for some rabies vectors such as the raccoon (*Procyon lotor*) in North America [18]. Due to their residual pathogenicity [19], attenuated strains of rabies virus are no longer used to vaccinate domestic animals in Western Europe.

As already mentioned, pathogenicity of attenuated rabies virus strains can be eliminated by mutating the arginine residue at amino acid position 333 of the rabies virus glycoprotein. This has led to the development of a new attenuated vaccine strain already used in the field [20]. However, heat sensitivity is a further disadvantage of attenuated rabies virus strains (Languet, personal communication), as it reduces their potential efficacy in field conditions. Thus, to improve both the safety and stability of the vaccines used for fox vaccination in the field, a recombinant vaccinia virus which expresses the immunising glycoprotein of rabies virus has been developed and field-tested for oral vaccination of foxes against rabies [21-24].

Development of a Vaccinia-rabies Vectored Vaccine for Oral Vaccination of Target Wildlife Species Against Rabies

The glycoprotein of rabies virus is the sole viral protein present on the external surface of the viral membrane. It is the only viral antigen capable of eliciting the production of neutralising antibodies and has been shown capable of confering immunity to rabies. Thus, the rabies virus glycoprotein is an ideal candidate for constructing a subunit vaccine.

Nucleotide sequence analysis of the glycoprotein gene reveals an open reading frame of 524 amino acids. The rabies virus glycoprotein gene has been inserted into the thymidine-kinase (TK) gene of vaccinia virus (VV), generating a selec-

table TK-negative virus known as VVTGg RAB [21,22]. VVTGg RAB was tested for efficacy and safety in the main target species in Western Europe and North America: fox, raccoon and striped skunk [23,25,26]. Immunity conferred by VVTGg RAB lasts a minimum of 12 months in cubs [27] and 18 months in adult animals [28]. This is the duration of protection required for field vaccination, due to the high turnover of the fox population.

As oral administration is the only appropriate route, the vaccine had to be given in a form suitable for ingestion. The efficacy of VVTGg RAB ($10^8 TCID_{50}$) contained in baits was therefore tested [29] and shown to be fairly efficacious. The baiting-sachet releases the vaccine virus suspension efficiently into the fox's mouth. A vaccine virus must not only be efficacious but also safe for the target species. VVTGg 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 [24].

It is also extremely important to preclude epizootic risks, such as the emergence of asymptomatic carriers of wild rabies virus. This could occur in the field if naturally infected animals were vaccinated during the incubation period [30]. The influence of vaccination with VVTGg RAB on the onset of the disease and on the delay before death in foxes previously infected with wild rabies virus has been investigated. The results show that both «early» and «late» death occurs as a consequence of interactions between oral vaccination with VVTGg RAB and rabies infection, but asymptomatic carriers of wild rabies virus will never emerge after vaccination [31].

It is also preferable that a vaccine virus used for oral vaccination of wildlife be not horizontally transmitted to unvaccinated animals. No transmission of immunising amounts of VVTGg RAB was found in adult or young foxes, with the exception of one adult fox bitten by another that had been freshly inoculated [23,27]. Other safety studies, like those used in the development of a conventional vaccine, were also conducted [24].

Studies on the Tropism of the Recombinant Vaccinia-rabies Virus in the Target Species

Experiments were designed to determine the multiplication site of the recombinant virus compared with that of the parental strain of the virus, by virus isolation, titration and indirect immunofluorescence. The polymerase chain reaction (PCR) was also used to detect specific viral DNA in several fox organs. Foxes were fed with $10^8 TCID_{50}$ of either VVTGg RAB or VV and were sacrificed 12, 24, 48, or 96 hours after inoculation [32].

Virus isolation

Small amounts of virus (10^2 to $10^{4.3} TCID_{50}$/ml) were recovered from the tonsils of five of the seven foxes inoculated with VVTGg RAB, but only during the first 48 hours. Virus was not detected ($<10^{1.5} TCID_{50}$/ml) in any other organ, serum or faeces of the vaccinated foxes or in any organ of the control animal.

Indirect immunofluorescence

The indirect immunofluorescence test carried out with anti-VV rabbit polyclonal serum confirmed the presence of the virus in the tonsils of four of the five foxes from which VVTGg RAB had been isolated and in the fox from which VV was isolated. The indirect immunofluorescence test carried out with the anti-rabies glycoprotein monoclonal antibody confirmed the presence of virus in the tonsils of three of the five foxes from which the virus was re-isolated. Immunofluorescence was, however, too diffuse to allow precise localisation of virus multiplication in the tonsil.

Polymerase chain reaction

Negative controls consisted of DNA from an uninfected fox. Positive controls were prepared with several dilutions of VVTGg RAB or VV in normal fox DNA; the dilutions ranged from approximately one infectious particle per cell to one infectious particle per 10^6 cells. For each virus (VV or VVTGg RAB) 10 ml of the amplified product of the 10^{-2} dilution (corresponding to 1 infectious particle/100 cells) were used for direct gel analysis. 30 ml of the reaction mixture submitted to two series of 35 PCR cycles were detected by dot blot hybridisation, up to and including the 10^{-6} dilution (1 infectious particle/million cells). No detectable amplification was observed in different samples of normal fox genomic DNA. VVTGg RAB was detected in the tonsils, the buccal mucosa, and the soft palate of foxes. No virus was detected in any other organ or in any of the organs of the control fox. VV was detected in the tonsils and the buccal mucosa only.

Conclusions of the studies on the tropism of VVTGg RAB

Using different techniques, VVTGg RAB or VV were detected during the first 48 hours following vaccination by the oral route, but only in tonsils, buccal mucosa and soft palate. Similar results have also been obtained in raccoons, using virus isolation [26]. Results of other unpublished experiments demonstrate that tonsillectomy of the foxes does not affect the protection conferred by VVTGg RAB. Viraemia was not observed on days 0, 2, 3, 4, 5, 6, 7, 8, and 14 in inoculated foxes.

All these data suggest that orally administered recombinant virus multiplies locally and at a low level. No virus could be detected in salivary glands (parotid and maxillary glands); the risk of transmission by saliva from one animal to another is therefore very low. Furthermore, the fact that VVTGg RAB multiplies only in restricted sites minimises the potential risk of recombination with other orthopoxviruses. These experiments showed no difference in the replication sites of VVTGg RAB as compared to the parental strain of VV, demonstrating that recombination does not modify the tropism of VV [32].

None of these viruses was detected in the brain, suggesting that VVTGg RAB does not multiply in nerve cells. These results were consistent with those of other studies reporting the absence of detectable cytological abnormalities in cerebrospinal fluid from raccoons orally vaccinated with VVTGg RAB [33].

Safety of the Recombinant Vaccinia-rabies Virus for Non-target Species

Field trials with baits have shown that several non-target wildlife species compete with foxes for bait [34]. 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 monitoring and tetracycline (biomarker included within the bait) detection controls, performed after vaccination campaigns, proved that mustelids, wild boars (*Sus scrofa*) and domestic carnivores may ingest the vaccine baits. Moreover, a significant proportion of the baits are partly eaten by small mammals [35,36]. It is therefore important to verify the safety of VVTGg RAB for non-target species (both domestic and wild).

In fact, non-target effects of a vectored vaccine administered orally for the immunisation of wildlife may be considered as a worst-case scenario. Several non-target wild species were chosen for testing in Europe because of their opportunistic feeding behaviour and their presence in the areas where the vaccine was to be distributed [37]. 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 not 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 [15,38], 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*), coyote (*Canis latrans*), gray fox (*Vulpes cinereo argenteus*), white-tailed deer (*Odocoileus virginianus*), bobcat (*Lynx rufus*) common opossum (*Didelphis virginiana*), and river otter (*Lutra canadensis*). Recent experiments have also shown that the recombinant virus, administered either by scarification or orally, is also safe for squirrel monkeys (*Saimiri sciureus*) and for chimpanzees (*Pan troglodytes*) [39].

For several species, experiments were performed with in-contact control animals (including cows) to test for horizontal transmission of VVTGg RAB, always with the same negative results [38].

Studies on the Possibility of Gene Exchange

The only remaining perceived risk to be investigated was the eventual recombination of the recombinant virus with a wild orthopoxvirus. For such an event to occur, both parental viruses must multiply during the same period of time in the same cells of the same animal. This risk can now be disregarded since there is no serological evidence of orthopoxvirus infection in the fox population [Crouch et al, unpublished results]. Moreover, when foxes have been orally inoculated with cowpoxvirus experimentally, the virus only multiplies at a low level for a short period of time in the mouth cavity alone [40].

Taking into account the experimental and epidemiological data, it appears most unlikely that recombination between VVTGg RAB and another orthopoxvirus could occur in vaccinated foxes. It is therefore preferable to choose a recombinant virus such as vaccinia virus which has no wild counterpart. In addition, vaccinia virus has a long history of use in uncontrolled conditions and has never become established in wildlife [41]. A vector virus unknown in wildlife but with a wide host range is, for safety reasons, preferable to one isolated from a target species, such as the raccoonpox virus [42], which is still prevalent in the wild [43], if one wishes to lower the risk of virus recombination.

Deliberate Release of the Vaccinia-rabies Recombinant Virus for Oral Vaccination of Foxes Against Rabies

Given the available experimental data concerning the safety of the VVTGg RAB for target and non-target species and its efficacy in foxes, limited field trials of fox vaccination with the recombinant virus were authorised first by Belgian [44-46] and then by French public health authorities. The Belgian authorisations were preceded by a risk-benefit safety assessment. It showed that the risk presented by rabies could be clearly identified and measured and could be reduced by the use of a vaccine such as the VVTGg RAB, which is more efficacious, owing to its immunogenicity and stability, than those already available. In terms of safety, there are clear and identified risks associated with the use of conventionally attenuated rabies virus strains such as the SAD B19 strain. The safety assessment demonstrated that these risks can be removed by using either the recombinant vaccinia-rabies virus or rabies virus strains that are modified at the level of arginine 333 of the glycoprotein.

Once the safety of the VVTGg RAB was confirmed by these trials, the Belgian authorities agreed to an enlarged open field trial of 435 km^2, using 15 doses of bait/km^2 in the southern part of Belgium [34]. Each bait contained, in a plastic sachet, a suspension of 10^8 $TCID_{50}$ of VVTGg RAB (2.2 ml by volume) and 150 mg tetracycline as a long-term biomarker of bait uptake. The vaccine was very stable even following natural freezing and thawing cycles. The VVTGg RAB vaccine has been shown to retain its capacity to immunise for at least one month in field conditions, a period which corresponds to the delay of uptake of a large number of bait doses in the field. Following this enlarged trial, three fox vaccination campaigns using VVTGg RAB were carried out in Belgium in order to check for efficacy over an even larger area.

Towards Elimination of Rabies?

VVTGg RAB was released over an area of 2,200 km^2 in southern Belgium to test the possibility of eradicating rabies on a sufficiently large scale [47]. Helicopters dropped 25,000 doses of bait containing VVTGg RAB and tetracycline on three occasions (November 1989, April 1990 and October 1990). After the third round, 81 per cent (64/79) of inspected foxes were tetracycline-positive. Only one rabid fox was recorded at the periphery of the baited area and this was tetracycline-negative.

Despite the dramatic decrease in the number of rabid foxes recorded after vaccine-bait distribution, the efficacy of the vaccination campaign remains difficult to evaluate because systematic collection of foxes is not feasible. Nevertheless, because notification of cases of rabies in cattle and sheep is mandatory in Belgium, the incidence of rabies in 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.

The economics of the vaccine-bait dispersal programme were also investigated. The average yearly cost of rabies in Belgium (1980-89), including post-exposure treatments of humans, animal diagnosis, compensation to farmers for the culling of infected livestock, and the culling of wild foxes, is estimated to be ECU 400,000 per 10,000 km^2, or ECU 88,000 a year for the area studied. These figures do not include the cost of vaccination of domestic animals or the salaries of civil servants. In comparison, the overall expenditure during the three campaigns of vaccine-bait distribution is estimated at ECU 118,000. Because after eradication vaccination can, in principle, be halted or limited to the borders of vaccination zone, the long-term maintenance of a rabies-free area by peripheral vaccination with VVTGg RAB is economically justified.

VVTGg RAB is now used throughout the contaminated areas in Belgium and Luxembourg as well as over large areas in France. In Belgium, rabies has nearly been eliminated [48], as only four cases were reported during the second semester of 1992 (three foxes, one cow) and only one (a badger) during 1993, in the area where the last cases of fox rabies were reported in 1992.

The elimination of rabies in Belgium has already had beneficial effects aside from the improvement of animal health. First, the number of human post-exposure treatments decreased with the drop in the incidence of rabies in animals (mainly cattle). Second, it has had a beneficial effect on the survival of threatened wildlife species, such as the Eurasian badger. Estimates of the badger population in the treated area show gradually increasing numbers (Bauduin et al, unpublished results). In fact, Belgium is returning to the situation that existed before 1966 when rabies was re-introduced from Germany.

Conclusions

Today, much of the research required for initiating programmes to control and then eradicate both urban and sylvatic rabies has been completed. What is needed now is education to change public awareness of the hazards of the disease. Control methods must be socially and internationally acceptable, legally enforceable, and economically feasible. No control programme is likely to succeed without international co-operation.

In Western Europe, rabies is considered a source of economic loss and, above all, restricts movement of animals among the different member states of the European Union. This has serious implications for the «open market», since some member states are currently rabies-free and wish to maintain their disease-free status. Therefore, the control of rabies requires a common strategy established within the European Union.

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