REFERENCES

1. Laude, H. (1988). Perspectives d'utilisation des biotechnologies dans la lutte contre les maladies infectieuses du porc. *Journées de la Recherche Porcine en France* **20**, 133-140.
2. Mettenleiter, T.C. (1989). Glycoprotein gIII deletion mutants of pseudorabies virus are impaired in virus entry. *Virology* **171**, 623-625.

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DEVELOPMENT OF A RECOMBINANT VACCINIA-RABIES VACCINE FOR ORAL VACCINATION OF FOXES AGAINST RABIES

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ABSTRACT

To improve both safety and stability of the vaccines used in the field to vaccinate foxes against rabies by the oral route, a recombinant vaccinia virus, expressing the immunizing G protein of rabies virus (VVTGg RAB-26D3 187 X P strain) has been developed. The c-DNA corresponding to the glycoprotein of the ERA strain of rabies virus has been inserted into the thymidine kinase (TK) gene of the vaccinia virus (Copenhagen strain).

The efficacy of this recombinant strain was tested by the oral route, primarily in foxes. The duration of immunity conferred by the VVTGg 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. VVTGg RAB innocuity was tested in foxes and in domestic animals as well as in numerous wild animal species that could compete with the red fox in consuming vaccine baits in Europe. During a minimum of 28 days post vaccination, neither clinical signs nor lesions were observed in any of the vaccinated animals. Morever no transmission of immunizing amounts of the recombinant occurred in the red fox or the other species tested. To study the stability of the vaccine strain, baits containing the vaccine were placed in the field. Despite considerable variations of environmental temperatures, the VVTGg RAB titre remained stable after one month in the field. Since all the baits are taken within one month, it can be assumed that most of the animals taking the baits are effectively vaccinated.

For testing the field efficacy of the recombinant vaccine, large-scale campaigns of fox vaccination were set up in a 2200 Km² region of southern Belgium, an area in which rabies was prevalent. A dramatic decrease in the incidence of rabies was noted after the campaigns.

INTRODUCTION

Rabies infection of domestic and wild animals is a serious problem throughout the world. The major disease vector in Europe is the red fox (*Vulpes vulpes*) and rabies control has focused on vaccinating and/or culling foxes.

In most European countries, control measures for reducing the fox population were only temporarily effective and did not stop the spread of the disease. Other methods such as oral immunization of foxes required assessing. Oral administration is the only route appropriate for the vaccination of a sufficient number of wild foxes (distribution of vaccine baits). Since 1978, several European countries have conducted large scale field trials of oral vaccination of foxes using the SAD standard or B 19 modified, attenuated strain of rabies virus at different times.

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 (1, 2) and are heat-sensitive.

Pathogenicity of attenuated rabies virus strains can be abolished by mutating Arg residue at position 333 of the glycoprotein, although such strains can still revert to virulence (3, 4). To improve both safety and stability of the vaccine used in the field, a recombinant vaccinia virus, expressing the immunizing G glycoprotein of rabies virus (VVTGg RAB-26D3 187 X P strain) has been developed (5). The c-DNA corresponding to the glycoprotein of the ERA strain of rabies virus has been inserted into the thymidine kinase (TK) gene of vaccinia virus (Copenhagen strain). This paper describes the development of this vaccine strain.

CONSTRUCTION OF THE VACCINIA-RABIES RECOMBINANT VIRUS

The glycoprotein of rabies virus is the sole viral protein present on the external surface of the membrane. It is the only antigen capable of eliciting the formation of rabies virus-neutralizing antibodies and has been shown to be capable of conferring immunity against rabies. Thus, the rabies virus glycoprotein seems to be an ideal candidate for use in the construction of subunit vaccines. 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 essentially involves 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, where 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 (5,6), known as VVTGg RAB.

EFFICACY OF THE VVTGg RAB IN THE TARGET SPECIES

VVTGg RAB has been tested for efficacy and safety in the main rabies-target species in western Europe and North America namely the: fox, raccoon and striped skunk (7, 8, 9, 10). The results of tests for efficacy in foxes can be summarized as follows. All but one of 26 adult captive foxes inoculated by various routes developed high titres of rabies neutralizing antibodies, and resisted wild rabies virus challenge on day 28 after vaccination. Immunity conferred by VVTGg RAB (10^8 plaque-forming units; oral route) lasted for at least 18 months, which is more than adequate, as most foxes in the wild are under 24 months of age. Foxes receiving less than the recommended dose showed a clear dose-dependent response. The liquid form of VVTGg RAB seems to be more efficient than the freeze-dried one, when administered by the oral route. A second administration of VVTGg RAB induces an increase of rabies neutralizing titres (booster effect). When administered to fox cubs by the oral route, the vaccine induces significant levels of rabies

virus antibody and protects 92% of foxes (11/12) against rabies, with the duration of immunity exceeding 12 months (11).

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 vaccine vehicles (bait plus container) can affect the contact of the virus with the oral mucosa, and thereafter, if the baiting system is inappropriate, lower the efficacy of the vaccine. The efficacy of VVTGg RAB contained in a new machine-made baiting system has been tested (12).

Twenty-two captive young foxes were divided into three experimental groups of six, and a control group of four. 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 out of 18 of the foxes and seroconversion to vaccinia in 14 out 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 antibody. These results demonstrate that the baiting-sachet system used permits an efficient release of the virus suspension into the mouth.

SAFETY OF THE VVTGg RAB FOR THE TARGET SPECIES

A vaccine virus must not only be efficacious but also innocuous for the target species. VVTGg RAB was observed to be non-pathogenic in the fox whatever the dose (10^2-10^{10} $TCID_{50}$) or route of administration (oral, intramuscular, intraduodenal, subcutaneous, intradermal, conjunctival or intranasal). It is preferable that a vaccine virus used for oral vaccination of wildlife is not transmitted horizontally 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 no transmission of immunizing amounts of VVTGg RAB was found to occur in adult or young foxes, with the exception of one adult fox bitten by a freshly inoculated one.

When assessing a recombinant virus for use in vaccination, it is also of great importance to detect any variations 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 (13).

Foxes were fed with 10^8 $TCID_{50}$ of either VVTGg RAB or vaccinia virus, and were euthanazied 12, 24, 48 or 96 h after inoculation by the oral route. Using these different techniques, VVTGg 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. Results of other experiments demonstrate that tonsillectomy of foxes does not completely impede seroconversion.

As no virus could be detected in the salivary glands of foxes (parotid and maxillary), the risk of transmission from one animal to another through saliva can be neglected. Furthermore, the fact that VVTGg RAB only multiplies in restricted sites minimizes the potential risk of recombination with other wild orthopox

viruses. In these experiments, no difference was observed between the multiplication sites of either VVTGg RAB of vaccinia virus, demonstrating that the recombination event did not modify the tropism of the virus. Additionally, virus was never detected in the brain.

It is also of major importance to preclude epizootiological risks, such as the emergence of asymptomatic carriers of wild rabies virus (14). This situation could occur in the field by the vaccination of naturally infected animals during the incubation period. 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. 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. These results show that «early» and «late» death phenomena occur as a consequence of interactions between oral vaccination with VVTGg RAB and rabies infection, but preclude the risk of the emergence of asymptomatic carriers of wild rabies virus after vaccination.

SAFETY OF THE VVTGg RAB FOR NON-TARGET SPECIES

Field trials with attenuated strains of rabies virus have shown that several wildlife non-target species compete with foxes for bait consumption (15, 16). It must also be taken into account that, within the orthopox virus group, vaccinia virus is a wide-host range species. In fact, bait uptake surveillance and tetracycline (biomarker) detection controls, performed after vaccination campaigns, proved that mustelids, wild boars (*Sus scrofa*) and domestic carnivora can ingest the vaccine baits. Moreover, a significant proportion of the baits are partially eaten by micromammals. It is important therefore to verify the safety of VVTGg 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 vaccine must be distributed (17, 18). Safety of the vaccine has been tested in daubenton 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 and/or pox lesions were never observed in the vaccinated animals during the observation period (28 days minimum after vaccination).

EFFICACY TRIALS IN THE FIELD WITH THE VACCINIA-RABIES RECOMBINANT VIRUS

Taking into account all the available experimental data concerning the safety of the VVTGg 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 (19), and

then by the French Public Health authorities. In the Belgian trial (17-18 oct. 1987) a total of 250 vaccine-baits (chicken heads) were delivered manually over a 6 km^2 area situated in the central part of a military zone.

With the safety of the VVTGg RAB confirmed by this small trial, the Belgian authorities agreed to an enlarged open field trial (20). This was conducted in a 435 km^2 area in the southern part ot the country, chosen because it has the lowest average human population density in the country (42 inhabitants km^{-2}), as well as a high incidence of rabies in foxes. Furthermore, the region is characterized by various biotopes, including most of the animal species susceptible to bait consumption. Each bait contained a suspension of 10^8 $TCID_{50}$ of VVTGg RAB (2,2 ml by volume) within a plastic sachet and 150 mg tatracycline 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 organs were removed: brain for rabies diagnosis, jaws for tatracycline detection and blood for the titration of vaccinia and rabies antibodies. Tetracycline was detected in foxes, stone martens (*Martes foina*), domestic feral cats (*Felus catus*), wood mice (*Apodemus sp.*), wild boars and carrion crows, showing that wild boars, mustelids and feral cats are strong competitors of the foxes for bait uptake. Twelve months of monitoring failed to detect any ecological hazard or public health concern. The vaccine was very stable even following natural freezing and thawing cycles.

Three fox vaccination campaigns using VVTGg 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} (21). 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 (nine rabid, 14 healty) during the first period was 74%. During the second collection period bait uptake rates were 80% (25/31) in adult foxes, but only 49% (27/55) in juveniles. 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.

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. Transmission of rabies to domestic animals occurs through the bite of a rabid wild animal. 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.

CONCLUSIONS

Because of its efficacy, safety and heat stability, VVTGg RAB appears to be an excellent alternative to the attenuated strains of rabies virus currently used in the field. Local eradication of fox rabies may by achieved after three or four vaccina-

tion campaigns, if the following requirements are met: a potent, safe and thermostable vaccine, an efficient and resistant baiting system, a practical and efficacious method of bait dispersal, and an effective spatial and temporal pattern of bait distribution.

REFERENCES

1. Wandeler, A., Bauder, W., Prochaska, S. and Steck F. (1982). Small mammals studies in a SAD Baiting area. *Comp. Immunol. Microbiol. Infect. Dis.* **5**, 173.
2. Leblois, H. and Flamand, A. (1988). *Studies on pathogenicity in mice of rabies virus strains used for oral vaccination of foxes in Europe. In*: Vaccination to control rabies in foxes (Eds Pastoret PP., Brochier B., Thomas I., Blancou J.). Brussels, Belgium, *Office for Official Publications of the European Communities*, Brussels-Luxembourg, pp. 101-104.
3. Tuffereau, C., Leblois, H., Benejean, J., Coulon, P. Lafaye, E. and Flamand, A. (1989). Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice. *Virology* **172**, 206-212.
4. Leblois, H., Tuffereau, C., Blancou, J., Artois, M., Aubert, A. and Flamand, A. (1990). Oral immunization of foxes with avirulent rabies virus mutants. *Vet. Microbiol.* **23**, 259-266.
5. Kieny, M.P., Lathe, R., Drillien, R., Spehner, S., Skory, D., Schmitt, T., Wiktor, T., Koprowski, H., Lecocq, J.P. (1984). Expression of rabies virus glycoprotein from a recombinant vaccinia virus. *Nature* (London) **312**, 163-166.
6. Pastoret P.P., Brochier, B. (1991). Biological control of wild animal infections. *Current Opinion in Biotechnology* **2**, 465-469.
7. Blancou, J., Kieny, M.P., Lathe, R., Lecocq, J.P., Pastoret, P.P., Soulebot, J.P., Desmettre, P. (1986). Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. *Nature* (London) **322**, 373-375.
8. Rupprecht, C.E., Hamir, A.N., Johnston, D.H., Koprowski, H. (1988). Efficacy of a vaccinia - Rabies glycoprotein recombinant virus vaccine in raccoons (*Procyon lotor*). *Rev. infect. Dis.* **10**, 803-809.
9. Tolson, N.D., Charlton, K.M., Stewart, R.B., Campbell, J.B., Wiktor, T.J. (1987). Immune response in skunks to a vaccinia virus recombinant expressing the rabies virus glycoprotein. *Can. J. Vet. Res.* **51**, 363-366.
10. Desmettre, P., Languet, B., Chappuis, G., Brochier, B., Thomas, I., Lecocq, J.P., Kieny, M.P., Blancou, J., Aubert, M., Artois, M., Pastoret, P.P. (1990). Use of vaccinia rabies recombinant for oral vaccination of wildlife. *Vet. Microbiol.* **23**, 227-236.
11. Brochier, B., Languet, B., Blancou, J., Kieny, M.P., Lecocq, J.P., Costy, F., Desmettre, P., Pastoret, P.P. (1988). Use of recombinant vaccinia-rabies virus for oral vaccination of fox cubs (*Vulpes vulpes L.*) against rabies. *Vet. Microbiol.* **18**, 103-108.
12. Brochier, B., Languet, B., Artois, M., Zanker, S., Guittre, C., Blancou, J., Chappuis, G., Desmettre, P., Pastoret, P.P. (1990). Efficacy of a baiting system for fox vaccination against rabies with vaccinia-rabies recombinant virus. *Vet. Rec.* **127**, 165-167.
13. Thomas, I., Brochier, B., Languet, B., Peharpre, D., Desmettre, P., Kieny, M.P., Pastoret, P.P. (1990). Multiplication site of the vaccinia-rabies glycoprotein recombinant virus administered by the oral route in foxes. *J. Gen. Virol.* **71**, 37-42.
14. Brochier, B., Blancou, J., Aubert, M.F.A., Kieny, M.P., Desmettre, P., Pastoret, P.P. (1989). Interaction between rabies infection and oral administration of vaccinia-rabies recombinant virus to foxes (*Vulpes vulpes*). *J. Gen. Virol.* **70**, 1601-1604.
15. Pastoret, P.P., Frisch, R., Blancou, J., Wolff, F., Brochier, B., Schneider L.G. (1987). Campagne internationale de vaccination antirabique du renard par voie orale menée au grand-duché de Luxembourg en Belgique et en France. *Ann. Méd. Vét.* **131**, 441-447.
16. Brochier, B., Thomas, I., Iokem, A., Ginter, A., Kalpers, J., Paquot, A., Costy, F., Pastoret, P.P. (1988). A field trial in Belgium to control fox rabies by oral immunization. *Vet. Rec.* **123**, 618.

17. Brochier, B., Blancou, J., Thomas, I., Languet, B., Artois, M., Kieny, M.P., Lecocq, J.P., Costy, F., Desmettre, P., Chappuis, G., Pastoret, P.P. (1989). Use of recombinant vaccinia-rabies glycoprotein virus for oral vaccination of wildlife against rabies: innocuity to several non-target bait consuming species. *J. Wild. Dis.* **25**, 540-547.
18. Blancou, J., Artois, M., Brochier, B., Thomas, I., Pastoret, P.P., Desmettre, P., Languet, B., Kieny, M.P. (1989). Innocuité et efficacité d'un vaccin antirabique recombinant des virus de la vaccine et de la rage administré par voie orale au renard, au chien et au chat. *Ann. Rech. Vét.* **20**, 195-204.
19. Pastoret, P.P., Brochier, B., Languet, B., Thomas, I., Paquot, A., Bauduin, B., Kieny, M.P., Lecocq, J.P., Debruyn, J., Costy, F., Antoine, H., Desmettre, P. (1988). First field trial of fox vaccination against rabies using a vaccinia-rabies recombinant virus. *Vet. Rec.* **123**, 481-483.
20. Brochier, B., Thomas, I., Bauduin, B., Leveau, T., Pastoret, P.P., Languet, B., Chappuis, G., Desmettre, P., Blancou, J., Artois, M. (1990). Use of vaccinia-rabies recombinant virus for the oral vaccination of foxes against rabies. *Vaccine* **8**, 101-104.
21. Brochier, B., Kieny, M.P., Costy, F., Coppens, P., Bauduin, B., Lecocq, J.P., Languet, B., Chappuis, G., Desmettre, P., Afiademanyo, K., Libois, R., Pastoret, P.P. (1991). Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine. *Nature* (London) **354**, 520-522.