

## Primary multiplication site of the vaccinia–rabies glycoprotein recombinant virus administered to foxes by the oral route

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The primary multiplication site of VVTGgRAB, a recombinant vaccinia virus (VV) expressing the rabies virus G glycoprotein, was studied in comparison with that of the parental VV Copenhagen strain, after oral administration to foxes. Foxes were fed with  $10^8$  TCID<sub>50</sub> of either VVTGgRAB or VV and were sacrificed 12, 24, 48 or 96 h after inoculation. Both viruses were detected by viral isolation in the tonsils during the first 48 h after inoculation at titres between  $10^2$  and  $10^{4.3}$  TCID<sub>50</sub>/ml. Indirect immunofluorescence confirmed the presence of the virus in tonsils of some of the foxes. The polymerase chain reaction allowed the detection of VVTGgRAB in the tonsils of both of two foxes tested after 24 h, three of three foxes

after 48 h, in the buccal mucosa of one of two foxes tested after 24 h and two of three foxes after 48 h and in the soft palate of one of two foxes tested after 24 h and one of three foxes after 48 h. VV was detected in the tonsils of one fox tested after 48 h, in the buccal mucosa of another fox tested after 24 h, and in the first fox after 48 h by the same reaction. Foxes were inoculated with virus isolated from fox tonsils 24 h after oral administration (with or without cell culture amplification) to perform back passages. No virus could be isolated in either case after this passage. The innocuity of VVTGgRAB was also demonstrated when foxes were inoculated with passaged virus.

### Introduction

Sylvan rabies remains a disease of major importance in many parts of the world. The red fox (*Vulpes vulpes*) is currently the major vector of the disease in western Europe whereas striped skunks (*Mephitis mephitis*) and raccoons (*Procyon lotor*) are the main vectors of the disease in North America. For the last 25 years, most of the research on the control of sylvan rabies has been focused on the development of wildlife vaccination by the oral route. Vaccination campaigns carried out in several European countries with an attenuated strain of the rabies virus have shown the feasibility of the method (Steck *et al.*, 1982; Schneider & Cox, 1983; Pastoret *et al.*, 1987; Brochier *et al.*, 1988*a*). However the use of these conventional rabies vaccines remains controversial because they are still pathogenic for laboratory and wild rodents (Wandeler *et al.*, 1972; Schneider & Cox, 1983; Leblois & Flamand, 1988) and are heat-sensitive. Further technical developments are needed. A recom-

binant virus (VVTGgRAB-26D3 187XP strain) derived from vaccinia virus (VV; Copenhagen strain) expressing the immunizing rabies virus glycoprotein has been developed by genetic engineering (Kieny *et al.*, 1984). In this strain the cDNA corresponding to the glycoprotein of the ERA strain of rabies virus has been incorporated under the control of the 7.5K promoter into the thymidine kinase (TK) gene of VV. This virus (VVTGgRAB) was demonstrated to be effective for the oral immunization of foxes, raccoons and skunks, eliciting high titres of rabies virus-neutralizing antibodies and conferring long-term protection against rabies (Blancou *et al.*, 1986; Rupprecht *et al.*, 1986, 1988; Tolson *et al.*, 1987, 1988; Brochier *et al.*, 1988*b*). Oral administration of VVTGgRAB was shown to be perfectly safe for several domestic (Blancou *et al.*, 1989; Soria Baltazar *et al.*, 1987), laboratory (Wiktor *et al.*, 1988) and wildlife species (Brochier *et al.*, 1989; Rupprecht *et al.*, 1986, 1988; Tolson *et al.*, 1987, 1988). Taking into account all the available experimental data

concerning the efficacy and safety of this recombinant, a preliminary restricted field trial of fox vaccination against rabies was carried out in 1987 in Belgium (Pastoret *et al.*, 1988). Nothing was known about the multiplication site of this vaccine when administered orally in foxes, nor about the fate of the parental VV strain administered by the same route. Experiments were therefore designed to determine the multiplication site in foxes of the recombinant virus as compared with that of the parental strain of the virus, by virus isolation, titration and indirect immunofluorescence. The polymerase chain reaction (PCR; Saiki *et al.*, 1985) was also used to detect specific viral DNA in several fox organs. In this paper we also investigate the recovery of the virus from foxes after one passage with and without cell culture amplification.

## Methods

**Animals.** Twenty-six foxes aged from 4 to 8 months, captured in a rabies-free area in France, were used in this study. They were weighed, their sex was determined and they were divided into several experimental groups.

**Viruses.** A live vaccinia (Copenhagen strain)-rabies glycoprotein (ERA strain) recombinant virus (VVTGgRAB-26D3 187XP strain) (Kieny *et al.*, 1984) and the parental VV (Copenhagen strain) propagated in Vero cells were used for the inoculation at a titre of  $10^8$  TCID<sub>50</sub> per ml.

**Experimental protocol.** In the first experiment nine animals were divided into two experimental groups (A and B). In group A, seven foxes were inoculated with 1 ml of recombinant virus. In group B, two foxes received 1 ml of the VV Copenhagen strain. One control animal was given 1 ml of phosphate-buffered saline (PBS). VVTGgRAB, VV and PBS were administered directly into the oral cavity. Foxes were sacrificed at various times after vaccination by intra-cardiac injection of T61 (Hoechst Veterinär), after sedation with Hypnorm (0.1 ml/kg) (Janssen Pharmaceutica). The protocol of experiment 1 is shown in Table 1.

In the second experiment, seven foxes were divided into two experimental groups (A and B). On day 0, the five foxes of group A were inoculated with 1 ml of VVTGgRAB and the two foxes of group B were inoculated with 1 ml of VV. One control fox was given 1 ml of PBS. The protocol of Experiment 2 is detailed in Table 2. Post-mortem examination was performed on all animals after euthanasia.

In the third experiment, four foxes were inoculated with VVTGgRAB isolated from the tonsils of foxes during the first experiment. The titre of the inoculum was  $10^{4.3}$  TCID<sub>50</sub>/ml. Two of them were sacrificed after 24 h and the remaining two were observed over 28 days, after which they were sacrificed and immediately necropsied. The same experiment was repeated with four other foxes inoculated with the same virus, previously amplified on Vero cell culture, at a titre of  $10^{8.5}$  TCID<sub>50</sub>/ml.

**Sampling.** Blood samples were collected from the jugular vein of foxes at the time of death, and faeces were removed at necropsy. The following organs were collected from each animal: brain, buccal mucosa, tonsils, spleen, parotid glands, maxillary glands, soft palate, retropharyngeal lymph nodes, submaxillary lymph nodes and mesenteric lymph nodes. Samples of approximately 1 cm<sup>3</sup> were taken in triplicate, placed in individual Petri dishes and maintained at -70 °C or placed in liquid nitrogen until used.

**Virus isolation and titration.** Samples of each organ were placed in 5 ml of Eagle's MEM containing gentamicin and ground before being centrifuged for 15 min at 1500 r.p.m. The supernatant (0.5 ml) was used for serial dilutions. Virus dilutions were inoculated into micro-wells simultaneously with a Vero cell suspension. After 5 days incubation, positive wells were counted, and virus titres were evaluated using Kärber's methods (Kärber, 1931).

**Detection of viral antigens by indirect immunofluorescence.** Samples of each tissue were mounted on tissue holders and cut with a microtome at a thickness of 3 to 5 µm. A minimum of 15 sections were cut for each sample and transferred to microscope slides covered with water containing albumin. Sections were fixed in acetone at -20 °C for 2 h. Half the slides were stained using a rabbit anti-VV serum (dilution 1:20) (Rhône-Mérieux) and a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1:40) (Flow Laboratories). The other half were stained using an anti-rabies glycoprotein monoclonal antibody (dilution 1:1000) kindly supplied by Dr M. Lafon (Pasteur Institute, Paris, France) and an FITC-conjugated rabbit anti-mouse IgG (dilution 1:60) (Cappel Laboratories). Examination was performed using an epifluorescence microscope (Reichert, model 'Polyvar').

### *Polymerase chain reaction*

**Preparation of samples for PCR.** DNA was prepared by deproteinization of tissue samples by overnight incubation with 2% 2-mercaptoethanol, 10 mM-Tris-HCl pH 8, 100 mM-NaCl, 10 mM-EDTA, pH 8, 0.5% SDS, and proteinase K (0.2 mg/ml) digestion followed by three phenol-chloroform extractions as described previously (Maniatis *et al.*, 1982). DNA from each organ was then mixed with 50 µl of TE buffer (10 mM-Tris-HCl pH 7.5, 1 mM-EDTA) and DNA concentrations were estimated by measuring the absorbance ratio at 260/280 nm.

**Synthesis and purification of oligonucleotides.** Two pairs of oligonucleotide primers chosen following sequence analysis of the VV TK gene (Weir & Moss, 1983) and the rabies virus glycoprotein gene (Anilionis *et al.*, 1981) were synthesized on an automatic DNA synthesizer and purified by PAGE (Fig. 1). The first set (TK1 and TK2), with 24 bp of the TK gene of VV flanking the insert for the rabies virus glycoprotein, allowed the amplification of a 528 bp sequence of VV DNA and a 2347 bp sequence of VVTGgRAB (Fig. 2). The other set (G1 and G2), with 21 bp of the rabies virus glycoprotein gene was used for the amplification of a 621 bp sequence of VVTGgRAB DNA.

**DNA sequence amplification.** Ten µg of DNA was amplified through two series of 35 PCR cycles. The reaction mixture consisted of 10 µl of a 10-fold concentrated reaction buffer containing 500 mM-KCl, 100 mM-Tris-HCl pH 8, 15 mM-MgCl<sub>2</sub>, 0.1% gelatin, 200 µM of each of the four deoxynucleoside triphosphates (dATP, dCTP, dTTP, dGTP), 0.4 µl of Taq polymerase (0.5 units/ml), 1 µg of each primer, water and DNA, to a total volume of 100 µl. The reaction mixture was covered with 50 µl of mineral oil, to prevent evaporation. The amplification reaction was performed in a DNA thermal cycler (Perkin-Elmer/Cetus). The following conditions were found to be optimal: samples were first heated to 93 °C for 2.5 min, then submitted to 35 PCR cycles of 1 min at 93 °C to denature the DNA, 2 min at 55 °C to allow annealing of the primers, and 5 to 10 min at 72 °C to allow primer extension. A final extension step was then performed for 10 min at 72 °C. Ten µl samples were then submitted to a second identical round of 35 PCR cycles.

**Detection of amplified products.** The amplified products were detected by direct gel analysis (for controls) and by dot blot hybridization assay using labelled oligonucleotide probes (for controls and samples). For direct analysis, 10 µl of the reaction mixture was subjected to electrophoresis on a 1 to 1.5% agarose gel and the DNA

was observed by u.v. fluorescence after staining with ethidium bromide.  $M_r$  markers were included in each gel. For dot blot analysis, 30  $\mu$ l of the final product was adjusted to 0.4 M-NaOH, 0.25 mM-EDTA in a 200  $\mu$ l volume, boiled for 2 min at 95 °C, cooled on ice, and immediately applied to a nylon filter membrane (GeneScreen Plus) by vacuum filtration using a Manifold I (Schleicher & Schüll). Wells were rinsed with 20  $\times$  SSPE (1  $\times$  SSPE is 0.18 M-NaCl, 10 mM- $\text{Na}_2\text{HPO}_4$ - $\text{NaH}_2\text{PO}_4$ , 1 mM-EDTA, pH 7.4), and the filters were then dried at room temperature.

Oligonucleotide probes were labelled at their 5' end by phosphorylation with  $^{32}\text{P}$ -labelled ATP (sp. act. > 5000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Amersham). The oligomers were then purified on a 15% polyacrylamide/7 M-urea gel, which allows their separation from their precursors. The nylon membrane was prehybridized in a solution of 5  $\times$  SSPE, 5  $\times$  Denhardt's solution, 0.5% SDS for 1 h at the hybridization temperature (TK1, 29 °C; TK2, 33 °C). Approximately 100 ng of probe ( $2 \times 10^6$  c.p.m./ml) was then added and hybridization was allowed for 1 night at the same temperature. The filters were then rinsed twice in 2  $\times$  SSPE, 0.1% SDS at room temperature, and once for 10 min in 5  $\times$  SSPE containing 0.1% SDS at  $T_m - 1$  °C. Finally, the filters were subjected to autoradiography at -70 °C (Fuji XR) for 4 h or 18 h.

## Results

### *Virus isolation (experiments 1 and 3)*

As shown in Table 1, small amounts of virus ( $10^2$  to  $10^{4.3}$  TCID<sub>50</sub>/ml) were recovered from the tonsils of five of the seven foxes inoculated with VVTGgRAB, during the first 48 h only. In group B, VV was detected in the tonsils of the animal sacrificed after 24 h. Virus was not detected ( $< 10^{1.5}$  TCID<sub>50</sub>/ml) in any other organ, serum or faeces of the vaccinated foxes, nor in any organ of the control animal. VVTGgRAB could not be isolated from foxes inoculated with viral stock recovered after one fox passage nor in foxes inoculated with the same viral stock amplified in cell culture.

### *Indirect immunofluorescence (experiment 1)*

As shown in Table 1, 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 VVTGgRAB 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 localization of the virus in the tonsil.

### *PCR results (experiment 2)*

#### *Determination of the sensitivity of the method*

Negative controls consisted of DNA from an uninfected fox. Positive controls were prepared with several

dilutions of VVTGgRAB or VV in normal fox DNA; the dilutions ranged from approximately one infectious particle per cell to one infectious particle per  $10^6$  cells. TK1 and TK2 oligonucleotides were used for the detection of VV and several combinations of oligonucleotide primers (TK1-TK2, G1-G2, TK1-G2, G1-TK2), (Fig. 1) were used for the detection of VVTGgRAB.

For each virus 10  $\mu$ l of the amplified product of the  $10^{-2}$  dilution (corresponding to 1 infectious particle/100 cells) was detected by direct gel analysis (Fig. 2). For each virus 30  $\mu$ l of the reaction mixture submitted to two series of 35 PCR cycles were detected by dot blot hybridization, up to and including the  $10^{-6}$  dilution (1 infectious particle/million cells) (Fig. 3). No detectable amplification was observed in different samples of normal fox genomic DNA.

### *Detection of VVTGgRAB in DNA of vaccinated foxes*

As shown in Table 2, using TK1-TK2 as primers and G1 as the probe, VVTGgRAB was detected in the tonsils of two out of two inoculated foxes after 24 h and three of three foxes after 48 h, in the buccal mucosa of one of two foxes after 24 h and two of three foxes after 48 h and in the soft palate of one of two foxes after 24 h and one of three foxes after 48 h. No virus was detected from any other organ nor from any of the organs of the control fox.

### *Detection of VV in DNA of vaccinated foxes*

After verification of the specificity of the amplified product in controls, we used TK1 and TK2 as primers and TK1 as probe. VV was detected in the tonsils of the inoculated fox tested after 48 h, and in the buccal mucosa of two other foxes, one tested after 24 h and the other after 48 h. Virus was not detected in any other organ.

## Discussion

Many experiments have been carried out to demonstrate the efficacy and safety of the vaccinia-rabies glycoprotein recombinant virus for vaccination against rabies. This vaccine could offer a suitable alternative to the use of attenuated strains of rabies virus used until now and has the advantage of good thermostability under field conditions. The parental Copenhagen strain of VV was associated with a limited number of human encephalitis complications in immunosuppressed people (Lane *et al.*, 1969). However previous experiments have demonstrated that the introduction of a foreign gene into the TK gene of VV greatly reduces the virulence of this virus, even of neurovirulent strains (Buller *et al.*, 1985). Furthermore no lesion was ever observed in immunosup-

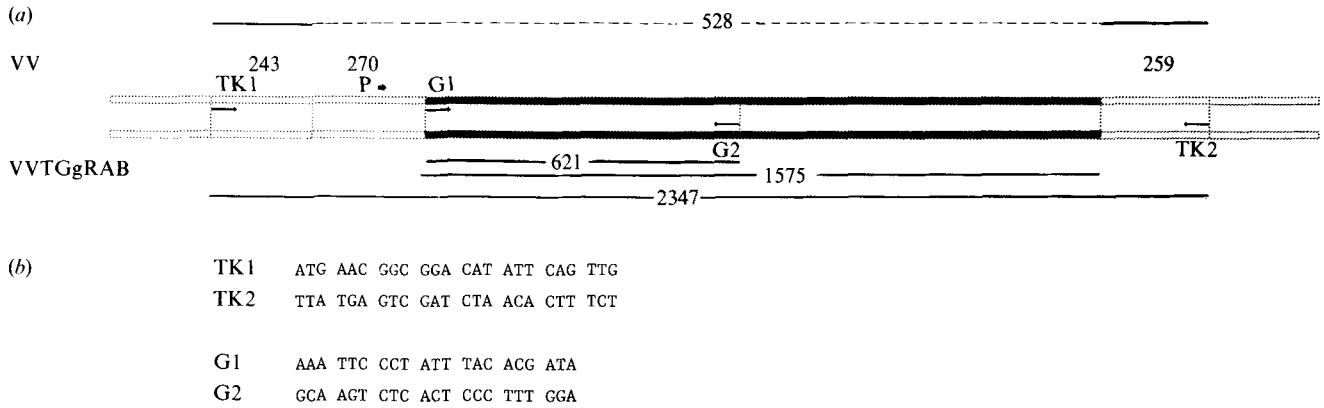


Fig. 1. (a) The cDNA of the rabies glycoprotein gene under the control of a promoter (P) is inserted within the TK gene of the VV genome. Primers TK1 and TK2 are located on the TK gene and primers G1 and G2 are located on the glycoprotein gene. Numbers indicate the size (bp) of the different segments. (b) Sequence of synthetic oligonucleotide primers and complementary oligonucleotide probes.

Table 1. Detection by viral isolation and indirect immunofluorescence of VVTGgRAB and VV in tonsils of foxes vaccinated by the oral route

Fox no.	Treatment									
	VVTGgRAB (Group A)							VV (Group B)		PBS (Control)
	1	2	3	4	6	7	9	5	8	10
Time of organ collection (h)	12	12	24	24	48	48	96	24	48	96
Virus titre*	-	10 <sup>2</sup>	10 <sup>4</sup>	10 <sup>2.8</sup>	10 <sup>4.3</sup>	10 <sup>2.8</sup>	-	10 <sup>4.3</sup>	-	-
Detection of VV antigens by indirect immunofluorescence†	-	-	+	+	+	+	-	+	-	-
Detection of rabies glycoprotein by indirect immunofluorescence	-	-	+	-	+	+	-	-	-	-

\* Virus titres are expressed as TCID<sub>50</sub>/ml.  
† (+), Viral antigen detected; (-), not detected.

Table 2. Detection by PCR of VVTGgRAB or VV in orally vaccinated foxes

Fox no.	Treatment								
	VVTGgRAB (Group A)					VV (Group B)		PBS (Control)	
	11	12	13	14	15	16	17	18	
Time of organ collection (h)	24	24	48	48	48	24	48	24	
Tonsils	+*	+	+	+	+	-†	+	-	
Buccal mucosa	+	-	+	-	+	+	+	-	
Soft palate	+	-	+	-	-	-	-	-	
Brain	-	-	-	-	-	-	-	-	
Spleen	-	-	-	-	-	-	-	-	
Parotid glands	-	-	-	-	-	-	-	-	
Maxillary glands	-	-	-	-	-	-	-	-	
Liver	-	-	-	-	-	-	-	-	
Retropharyngeal lymph nodes	-	-	-	-	-	-	-	-	
Submaxillary lymph nodes	-	-	-	-	-	-	-	-	
Mesenteric lymph nodes	-	-	-	-	-	-	-	-	
Blood	-	-	-	-	-	-	-	-	
Faeces	-	-	-	-	-	-	-	-	

\* (+), Viral DNA detected.  
† (-), Viral DNA not detected.

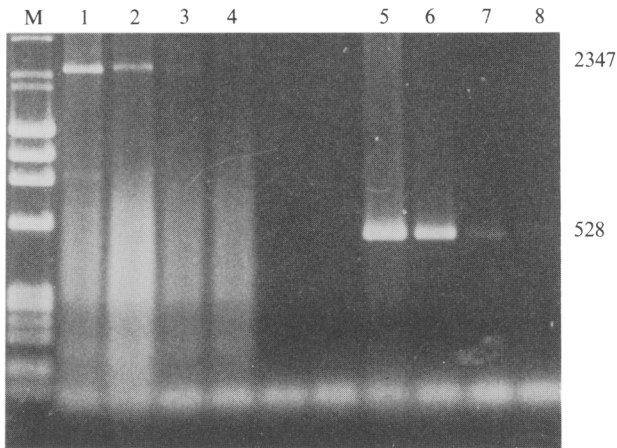


Fig. 2. Electrophoretic gel analysis of PCR products of different dilutions of VVTGgRAB (lane 1, 2, 3, 4) or VV (lane 5, 6, 7, 8) in normal fox DNA. TK1 and TK2 were used as primers. Lane M, size markers (bp) (*Hind*III-digested  $\lambda$  DNA, *Hae*III-digested  $\phi$ X174 RF DNA); lanes 1 and 5, 1 infectious particle/cell; lanes 2 and 6,  $10^{-1}$  infectious particle/cell; lanes 3 and 7,  $10^{-2}$  infectious particle/cell; lanes 4 and 8,  $10^{-3}$  infectious particle/cell.

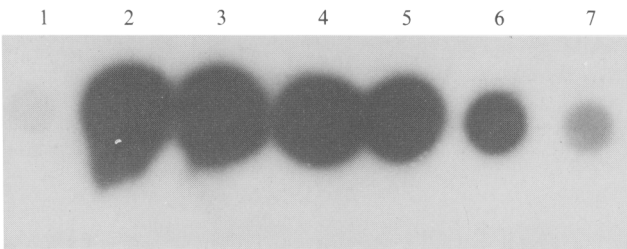


Fig. 3. Dot blot autoradiograph of PCR products of different dilutions of VVTGgRAB in normal fox DNA. Two series of 35 cycles were performed using TK1 and G2 as primers and G1 as probe. Lane 1, negative control; lane 2,  $10^{-1}$  infectious particle/cell; lane 3,  $10^{-2}$  infectious particle/cell; lane 4,  $10^{-3}$  infectious particle/cell; lane 5,  $10^{-4}$  infectious particle/cell; lane 6,  $10^{-5}$  infectious particle/cell; lane 7,  $10^{-6}$  infectious particle/cell.

pressed nude mice inoculated with the VVTGgRAB by different routes (P. Desmettre, unpublished results). Nevertheless in preparation for a large scale release of the recombinant it is necessary to make a thorough study of the pathogenesis of this vaccine and to improve methods for detecting the virus.

Using different techniques (virus isolation, indirect immunofluorescence and the very sensitive PCR method), virus (VVTGgRAB or VV) was 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 by others in raccoons (tonsils, parotid or submandibular lymph nodes, buccal mucosa) using virus isolation (Rupprecht *et al.*, 1988). The virus titres were very low in all cases, suggesting that if there is any viral multiplication, it must take place locally and at a very low level.

To ascertain our PCR results, the sensitivity of the method was analysed. The limit of detection of the test in our experimental conditions was one infectious particle per  $10^6$  cells. On the other hand, as the organ samples contained  $10 \mu\text{g}$  of DNA, the DNA content of about  $1.5 \times 10^6$  cells, any negative result would indicate the absence of virus particles in the sample.

Experiments carried out with VVTGgRAB administered into foxes' stomachs and to skunks intraduodenally (Tolson *et al.*, 1987, 1988) showed that a reduced rate of seroconversion and lower rabies virus-neutralizing antibody titres were obtained by those routes. Furthermore, results of other experiments demonstrate that tonsillectomy of the foxes does not affect the protection conferred by vaccination with VVTGgRAB (I. Thomas *et al.*, unpublished results). All these data suggest that orally administered recombinant virus multiplies at a low level within the oral cavity, in the tonsils and also in other tissues. Viraemia was never observed on days 0, 2, 3, 4, 5, 6, 7, 8 and 14 in foxes inoculated with VVTGgRAB or VV (I. Thomas *et al.*, unpublished results). No virus could be detected in salivary glands (parotid glands and maxillary glands) of foxes; the risk of transmission by saliva from one animal to another is therefore very low. This explains why transmission of the virus from vaccinated animals to unvaccinated controls was shown not to occur in foxes (one case) (Blancou *et al.*, 1986; Tolson *et al.*, 1988; Brochier *et al.*, 1988c), in cats and dogs (Blancou *et al.*, 1986) and in ferrets (Brochier *et al.*, 1989). However in raccoons, some unvaccinated animals held in contact with vaccinated ones had rabies virus-neutralizing antibodies and resisted challenge (Rupprecht *et al.*, 1988).

In our experiments no difference was observed in the replication sites of the recombinant virus as compared to the parental strain of VV, demonstrating that recombination does not modify the tropism of VV. None of these viruses were detected in the brain, suggesting that the recombinant has no predilection for 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 the recombinant virus (Hanlon *et al.*, 1989).

It is very important also to verify that the virus will not adapt to the fox and will not acquire some pathogenicity. In our experiments, virus was not detected in foxes inoculated with virus isolated after one passage, nor in others inoculated with the same virus after cell culture amplification. However, further experiments should be carried out with a higher number of animals to avoid any misinterpretation due to individual variations. In any case, neither lesions nor clinical signs were detected in animals vaccinated with these viruses during a 28-day observation period.

The fact that the recombinant virus multiplies only in restricted sites minimizes the eventual risk of recombination with other orthopoxviruses, bearing in mind that there is no known reservoir of VV in the wild.

All these results represent additional arguments in favour of the use of the recombinant virus in the field. However even if risks appear to be very unlikely it is important to be cautious. In this respect, the PCR technique will be a useful tool for safety controls in field trials because of its very high sensitivity and specificity. Even in samples with a very low viral titre, the presence of the virus was detected. This technique should therefore permit the detection of the recombinant virus in any animal collected in a vaccinated area, suspected of carrying a pox lesion.

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