Epidemiol. Infect. (1999), **123**, 165–175. Printed in the United Kingdom

An update on safety studies of SAD B19 rabies virus vaccine in target and non-target species

A. VOS[1]*, A. NEUBERT[1], O. AYLAN[2], P. SCHUSTER[1], E. POMMERENING[1], T. MÜLLER[3] AND D. CHAI CHIVATSI[4]

[1] *IDT (Impfstoffwerk Dessau-Tornau GmbH), PSF 214, 06855 Rosslau, Germany*
[2] *Etlik Central Veterinary Research and Control Institute, Etlik – Ankara 06020, Turkey*
[3] *Federal Research Centre for Virus Diseases of Animals, 16868 Wusterhausen, Germany*
[4] *Institute of Primate Research, PO Box 24481, Nairobi, Kenya*

(*Accepted 16 March 1999*)

SUMMARY

SAD B19 is an attenuated vaccine virus for oral vaccination of carnivores against rabies. The safety of SAD B19 was investigated in 16 animal species by different routes of administration. During the observation period all animals given the vaccine virus, irrespective of the route of administration, did not show any clinical signs of rabies, with the exception of certain rodent species. In these animals a low residual pathogenicity was observed, however transmission of the vaccine virus to control animals was not demonstrable. No vaccine virus could be detected in the saliva of the six mammal species examined. Furthermore, the genetical stability was shown for SAD B19 through passaging in neural tissue of dogs, foxes and mice. From the results presented here on innocuity and stability, it can be concluded that SAD B19 rabies vaccine is suitable for oral vaccination campaigns for carnivores against rabies.

INTRODUCTION

Oral immunization has been developed into the method of choice in wildlife rabies control in Europe and North America [1]. Oral vaccination of foxes against rabies using a live attenuated virus vaccine was first successfully applied in Switzerland in the late 1970s [2]. Since 1983, over 70 million vaccine baits with the SAD B19 vaccine virus have been distributed in many European countries. No other available oral rabies vaccine has been used so extensively in a variety of environmental and epidemiological conditions as SAD B19. Although no incidents have been reported with SAD B19, the use of this and other live virus vaccines for oral vaccination of wildlife remains controversial as far as innocuity in target and non-target species is concerned [3]. Field studies have shown that many other animal species compete with the target species in consuming the distributed vaccine baits. Therefore, it is important to determine possible pathogenicity, excretion and transmission of the vaccine virus not only in the target species, but also in non-target species. This paper summarizes the latest safety tests with the SAD B19 virus vaccine. The selection of the animal species tested, as well as the study protocols, were established according to the World Health Organization (WHO) and European Pharmacopoeia recommendations and requirements.

* Author for correspondence.

MATERIAL AND METHODS

SAD B19 vaccine virus

All present available modified live virus vaccines for oral vaccination of carnivores are derivatives of the original SAD virus. The SAD (Street-Alabama-

Table 1. *Experimental protocol of the oral vaccination of several carnivore species with SAD B19*

Animal species	Number	SAD B19 dose (FFU)	Period*	Institute
Fox	42	$3{\cdot}0 \times 10^6$	190	IDT†
Dog	16	$2{\cdot}1 \times 10^7$	74	VCRI‡
Dog	8	$3{\cdot}0 \times 10^7$	57	VCRI
Dog	7	$1{\cdot}5 \times 10^8$	57	VCRI
Cat	5	$6{\cdot}0 \times 10^7$	84	VCRI
Cat	7	$3{\cdot}6 \times 10^6$	183	VCRI
Mink	5	$1{\cdot}0 \times 10^8$	90	IDT
Ferret	5	$1{\cdot}0 \times 10^8$	90	IDT
Stonemarten	4	$1{\cdot}0 \times 10^8$	90	IDT

* Observation period post vaccination (days).
† Impfstoffwerk Dessau Tornau GmbH, Rosslau, Germany.
‡ Etlik Central Veterinary Control and Research Institute, Ankara, Turkey.

Dufferin) strain was isolated from a dog at the Centre for Diseases Control (CDC) in Montgomery in 1935 and propagated by passaging in mouse brain cells. The SAD strain was adapted to BHK 21 cells [4]. The original strain was sent to the Federal Research Centre for Virus Diseases of Animals in Tübingen, Germany (BFAV) by the Swiss Rabies Centre in Berne, Switzerland. The SAD B19 strain was derived by adaptation of the SAD strain passaged in mice brain on cloned BSR cells [5].

Safety tests in target and non-target species after a single administration of SAD B19

Carnivores

The following animals were administered SAD B19 by the oral route (p.o.) using different concentrations of the vaccine virus; the red fox (*Vulpes vulpes*), dog (*Canis familiaris*), cat (*Felis catus*), mink (*Mustela vision*), stonemarten (*Martes foina*), domestic ferret (*Mustela putorius furo*). All animals tested seronegative for rabies prior to inoculation; the sera of the animals was examined by seroneutralization on cells – Rapid Fluorescent Focus Inhibition Test (RFFIT). Table 1 provides the experimental protocol for each species.

Rodents

While the SAD strain and its derivatives remained pathogenic for some rodents, the innocuity of SAD B19 in these animals was studied intensively. The vaccine virus was directly applied into the mouth cavity of these wild-caught animals. Of all animals that died during the observation period or that were euthanized afterwards brain samples were tested for rabies by the direct fluorescent antibody test (FAT). Details of the safety tests in rodents are listed in Table 2.

Table 2. *Experimental protocol of the oral vaccination of rodents with SAD B19*

Animal species	Number	Inoculation dose (FFU)	Period*	Institute
O. zibethicus	5	$1{\cdot}0 \times 10^8$	30	IDT†
O. zibethicus	1	$2{\cdot}0 \times 10^7$	30	IDT
R. norvegicus	1	$2{\cdot}6 \times 10^5$	32	PVI‡
R. norvegicus	3	$3{\cdot}6 \times 10^6$	35	PVI
R. norvegicus	5	$4{\cdot}2 \times 10^6$	avr. 37	PVI
M. musculus	9	$1{\cdot}8 \times 10^6$	avr. 46	PVI
M. musculus	8	$2{\cdot}1 \times 10^6$	avr. 27	PVI
A. sylvaticus	28	$1{\cdot}3 \times 10^5$	32	PVI
A. sylvaticus	1	$1{\cdot}8 \times 10^6$	32	PVI
A. agrarius	12	$1{\cdot}3 \times 10^5$	32	PVI
A. agrarius	1	$1{\cdot}8 \times 10^6$	32	PVI
M. epiroticus	3	$1{\cdot}3 \times 10^5$	32	PVI
M. epiroticus	10	$1{\cdot}8 \times 10^6$	avr. 37	PVI

* Observation period post vaccination (days).
† Impfstoffwerk Dessau Tornau GmbH, Rosslau, Germany.
‡ Provincial Veterinary Office, Erenköy-Istanbul, Turkey.

Before vaccine virus administration, the animals were kept under observation for several days in order to confirm the absence of any intercurrent diseases and to give them enough time to adapt to their new state of captivity and feeding. At PVI (Provincial Veterinary Office, Istanbul), the first wild rodents caught, all *M. musculus*, were anaesthetized with ether followed by ketamine-hydrochloride (Ketavet 110 mg/ml, Parke Davis GmbH, Berlin) before inoculation. Two of five animals did not survive the anaesthesia. Therefore, it was decided to administer the vaccine directly into the mouth cavity. When offered directly, most rodents licked the vaccine without problems from the plastic syringe. However, the rats, being difficult to handle, were anaesthetized in an ether jar before inoculation. To test possible virus vaccine transmission, several inoculated animals were placed together with controls for the entire observation period. These 25 vaccinated and 25 control animals were divided over 20 cages.

In another trial at IDT (Impfstoffwerk Dessau-

Table 3. *Experimental study to detect possible vaccine virus in saliva of different animal species after administration of SAD B19*

Animal species	Inoculation route	Number	Inoculation dose (FFU)	Saliva sampling*	Institute
Fox	p.o.	19	$1{\cdot}0 \times 10^8$	2, 24, 48, 72 h	IDT†
Fox (puppies)	p.o.	5	$2{\cdot}0 \times 10^8$	2, 24, 48, 72 h	IDT
Fox	i.c.	4	$2{\cdot}5 \times 10^7$	1, 2, 3, 4, 5, 6, 8, 12, 14 d	IDT
Dog	i.c.	4	$2{\cdot}5 \times 10^7$	1, 3, 5, 8, 16 d	VCRI‡
Dog	p.o.	8	$2{\cdot}0 \times 10^8$	1, 2, 4, 8 d	VCRI
Mink	p.o.	5	$1{\cdot}0 \times 10^8$	2, 24, 48, 72 h	IDT
Ferret	p.o.	5	$1{\cdot}0 \times 10^8$	2, 24, 48, 72 h	IDT
Stonemarten	p.o.	4	$1{\cdot}0 \times 10^8$	2, 24, 48, 72 h	IDT

* Saliva sampling: time of sampling post inoculation: h, hours; d, days.
† Impfstoffwerk Dessau Tornau GmbH, Rosslau, Germany.
‡ Etlik Central Veterinary Control and Research Institute, Ankara, Turkey.

Tornau GmbH, Germany), 11 muskrats (*Ondatra zibethicus*) were housed in a special enclosure at the experimental animal facility. One and five animals were vaccinated orally 0·2 ml (< 500 g bodyweight) and 1·0 ml (> 500 g body weight) with 1×10^8 FFU/ml, respectively. From the time of vaccination onwards a vaccinated and one unvaccinated control animal were in contact with each other for at least 10 min per day by opening of a slide in the enclosure, and then seperated again. In addition, contact between the vaccinated and unvaccinated animal was possible at all other times in the study along the common fence of the enclosures. In one enclosure, two vaccinated animals were placed together in one unit with one control animal. The vaccinated and control animals were euthanized 30 and 60 days after vaccination, respectively. The reisolated 10th passage of SAD B19 in NMRI-mice with a titre of $10^{5{\cdot}2}$ FFU/ml (see section: *in vivo* stability of SAD B19) was examined for its residual pathogenicity in mice after oral, intramuscular and intracerebral administration. For every group 20 NMRI-mice were used and observed for 30 days. The 20 mice inoculated i.c. were placed together with 20 control animals; in every cage one vaccinated animal was housed together with one unvaccinated animal. After the observation period, a blood sample was collected from the control animals and examined for rabies neutralizing antibodies (RFFIT).

Birds

During field studies, it had been observed that certain bird species can come into contact with the vaccine baits when distributed indirectly [6, 7]. Therefore the innocuity of SAD B19 was tested in the following bird species: pigeons (*Columba* sp.), chickens (*Gallus gallus*) and magpies (*Pica pica*). The latter were captured by cage trapping in Ankara, Turkey. All magpies were aged as juveniles and were clipped (one wing). They were administered $6{\cdot}3 \times 10^7$ FFU SAD B19 by the oral route using a needleless syringe. The birds were daily observed and were euthanized, on average, 43 days after inoculation. Furthermore, pigeons ($n = 10$) and 1-day-old SPF-chicks ($n = 10$) were inoculated orally with $1{\cdot}0 \times 10^7$ FFU and $2{\cdot}0 \times 10^6$ FFU SAD B19, respectively. The chickens were observed for 40 days and the pigeons for 90 days. The brains of all animals were analysed for rabies virus (FAT) and also the sera of the animals were examined (RFFIT).

Primates

After an incident with the SAD Berne vaccine virus in baboons [8], the WHO suggested that any candidate vaccine should also be tested in primates [4]. Baboons (*Papio cynocephalus*) were inoculated orally with $2{\cdot}0 \times 10^8$ FFU SAD B19; ten olive baboons (*P.c. anubis*) and two yellow baboons (*P. c. cynocephalus*). For this purpose the animals were sedated with an appropriate ketamine-hydrochloride solution. All animals were clinically healthy and free of infectious diseases and tested seronegative for rabies prior to inoculation (RFFIT). The animals were kept individually in a closed section of the Institute of Primate Research (National Museums of Kenya), in Nairobi, Kenya. At 90 days post vaccination, the

animals were euthanized. Full pathological and histopathological examinations were conducted on all animals.

Safety tests in young animals

Considering that young animals may form an important part of the fox population during vaccination campaigns and, consequently, the probability of contact between the cubs and the vaccine virus, young animals of different age-groups were inoculated with the vaccine virus. Two groups of fox cubs, ten 1-month and fourteen 3-month old cubs, were given $2{\cdot}0 \times 10^7$ FFU SAD B19 orally. The animals were observed for 1 year. Additionally, five fox cubs (6 weeks old) were vaccinated orally with $2{\cdot}0 \times 10^8$ FFU SAD B19. These animals were observed for 90 days.

The vaccine virus SAD B19 is also one of the candidate vaccines for oral vaccination of dogs against rabies. As a result of the high probability of contact between children and puppies, the candidate vaccine virus should not produce disease in dogs less than 10 weeks of age [4]. All dogs used in this study were free-roaming cross-breeds, captured by the local municipality in different neighbourhoods of Ankara, Turkey. All puppies were aged as less than 10 weeks old at the time of vaccination. The mother animals were free of antirabies antibodies (RFFIT). Two different concentrations, $2{\cdot}4 \times 10^7$ and $4{\cdot}2 \times 10^8$ FFU SAD B19, were administered orally by a single instillation in the oral cavity of seven and twelve puppies, respectively, using a needleless syringe. Furthermore, two groups of two and six puppies were administered $2{\cdot}4 \times 10^7$ and $1{\cdot}0 \times 10^8$ FFU by the parenteral route, respectively. At the end of the observation period (average: 65 days) the animals were euthanized and the brains examined for rabies virus (FAT). Also, all puppies that died during the observation period were examined.

Safety tests in immunodeficient animals

Due to the possible risk of unintentional exposure of severely immunocompromised persons to the vaccine virus, the WHO suggested conducting additional safety tests in immuno-deficient laboratory animals [4]. Hence, SAD B19 was tested for their pathogenicity after oral and intramuscular administration in two different strains of immunodeficient mice; Nude-mice (CD1-nu/nu) and SCID-mice (CB17-SCID). All mice received 0·02 ml ($2{\cdot}5 \times 10^7$ FFU) SAD B19. The following data were obtained: (i) determination of mortality; (ii) presence of the vaccine virus in the brain, salivary glands, spleen, lung, oesphagus, oral cavity mucosa and (iii) determination of virus-neutralizing rabies antibodies.

Dissemination and excretion of SAD B19

The possibility of excretion of vaccine virus in the saliva of vaccinated animals should also be examined [4]. Exposure of non-target species (esp. humans) to the vaccine virus may occur through contact with a freshly vaccinated animal; e.g. by licking or biting. The following animal species were tested: fox, dog, mink, ferret and stone marten (Table 3).

Saliva and, on some occasions, nasal secretions were collected by swabbing of the oral and nasal cavity for 1–1·5 min, respectively. Afterwards, the cotton wool cylinder was placed in the holding tube (Cultiplast®, LP Italiana Spa, Mailand). 2 ml of the following medium was added: MEM/SNT (MEM, minimal essential medium; SNT, sera neutralization test) plus antibiotics [gentamicin (50 mg/l) and amphotericine B (2·5 mg/l)]. The suspension was centrifugated for 5 min at 4000 r.p.m. The liquid phase was removed for further testing. For every sample one microflask (25 cm^2) was used. Every microflask contained 10 ml cell suspension BSR Cl 13 ($3{\cdot}0 \times 10^5$ cells per ml) in MEM for rabies mixed with 10% NCS. Of every sample 0·5 ml was added to the microflask. The microflasks were incubated at 35 °C for 6 days. At the fourth day the medium was changed with MEM for rabies plus 1% NCS. Subsequently, two cavities of an eight-well-microscope slide (Lab-Tek® Chamber slide, Nalge Nunc Int., Naperville) were filled with 0·5 ml liquid phase from the microflasks and 0·1 ml cell suspension BSR C1 13 ($1{\cdot}0 \times 10^6$ cells per ml) plus 10% NCS. The slides were put into an incubator (35 °C, 5% CO_2) for 48 h. After draining of medium the cells were fixed with 80% acetone for 30 min at room temperature. The well-slides were drained again and dried. FITC-labelled anti rabies IgG (Centocor Inc., Malvem) was added. The slides were stained for 30 min at 37 °C and afterwards analysed for rabies virus by the fluorescent antibody test (FAT). A single infected cell was regarded as positive; i.e. the specimen contained the rabies virus.

Also, the dissemination of the vaccine virus in

several animal species have been examined Nineteen silverfoxes, between 5–8 months old, and eight dogs were vaccinated p.o. with $1{\cdot}0 \times 10^8$ FFU and $2{\cdot}0 \times 10^8$ FFU SAD B19 vaccine virus, respectively. All animals did not have any detectable levels of rabies neutralizing antibodies before vaccination. Of six foxes, swabs of nasal and saliva secretion were collected 2 h post inoculation. On 1, 2, 3, 4, 8, 12, 15 and 20 days post inoculation two foxes were euthanized and the following tissue-samples were collected; mucosa of oral cavity (palatum durum), nasal mucosa (conchae nasalis sinistra/dextra), salivary glands (gl. mandubularis), regional lymph nodes (ln. mandibularis), tonsils, cerebellum, medulla oblongata, hippocampus and cerebral cortex. Of the eight dogs the same samples were collected, with the exception of the cerebellum, medulla oblongata and cerebral cortex. The following additional samples were taken only from the dogs: oesphagus, lungs, mucosal membrane of smaller intestine and the digastric muscle (M. masseter). The samples of the dogs were collected 1, 2, 4 and 8 days post inoculation. Between 1–2 g of tissue from each of the samples listed was removed aseptically and stored in sterile containers at −20 °C until further use. Between 0·5–1·0 g of this tissue-material was weighed and, using a mortar and pestle (with 10% sea sand added if required), a 10% trituration in MEM/SNT plus 2% NCS was prepared. Large particles were sedimented by short high-speed centrifugation. These samples were subsequently treated as described in the above section for saliva and nasal secretions.

Using a slightly different method for detection of SAD B19 in saliva, another 17 puppies and six adult dogs were vaccinated and examined; details of this test are listed in Table 4. Here, saliva was collected through chewing a cotton wool cylinder by the animal and by swabbing of the oral cavity for 1–1·5 min. Afterwards the cotton wool cylinder was placed in the inner holding tube of the Salivette® (Sardstedt AG, Nümbrecht). One ml of the following medium was added: 1 ml gentamicin and a mixture of 100 ml MEM/SNT – 10 ml NCS. Saliva was extracted by centrifugation (2000 r.p.m., 10 min) and collected. The samples were evaluated by the RFFIT and/or Mouse Inoculation Test (MIT). In case of MIT, for every saliva sample four mice were needed. The extracted saliva was administered to every mouse (0·5 ml s.c.). After 14 days the mice were bled. The blood samples from the mice inoculated with the same saliva sample were pooled. The blood samples were centrifugated at 3000 r.p.m. for 25 min. Serum was separated and stored at −20 °C until RFFIT.

In vivo stability of SAD B19

To test possible reversion to or increase in virulence of the attenuated virus vaccine, the initial vaccination should be carried out using the route of administration most likely to lead to reversion to virulence. Since passaging of SAD B19 by the oral route appeared unsuitable to ensure reliable results, the virus material was inoculated intracerebrally. The foxes were anaesthetized with 1·0–1·8 ml of a 1:1 mixture (v:v) of ketamine (100 mg/ml, Serumwerk Bernburg) and xylazine (2%, Serumwerk Bernburg), administered i.m. Subsequently, three groups of two animals (P1/P2, P3/P4, P7/P8) were shaved along a line between the outer canthuses in the region of the median cranial line and the area was disinfected with PPV-iodine solution. A strictly medical incision, approx. 1 cm in length, was then made in a craniocaudal direction up to the cranial bone. Small blood losses were stopped by a tamponade. The skin in the region of the wound was pushed slightly to the side and an opening of approx. 0·5 cm diameter bored through the cranium using a sterile trephine. Using a syringe needle, 1·0 ml of virus suspension ($1{\cdot}0 \times 10^8$ FFU) was injected approx. 1 cm deep into the left half of the cerebrum. The surgery wound was closed and then freed of blood coagulation. The foxes were euthanized after 6 days and tissue samples were collected from the vaccination site, medulla oblongata, hippocampus, cerebral cortex, salivary glands and regional lymph nodes. The salivary gland tissue sample of one group (P3/P4) from this first passage was questionable positive in the immunofluorescence test and was therefore inoculated into two foxes (P5/P6) in a second passage (10% suspension from the salivary gland). In a second study, the virus vaccine was passaged for 48 h. Two foxes (1A/1B) were injected i.c. with $1{\cdot}0 \times 10^8$ FFU SAD B19 as previously described. The animals were euthanized after 48 h and two other animals (3A/4A) were inoculated i.c. with a cell culture reisolate from the vaccination site of fox 1A. This procedure was repeated three times; the inoculation i.c. of the third passage in two foxes (7A/8A). Finally, four foxes (9A/10A/11A/12A), in which the fourth passage of the virus was inoculated i.c., were observed for 46 days. In addition, saliva swabs taken from these animals 1, 2, 3, 4, 5, 6, 8, 12 and 16 days post

Table 4. *Additional studies on possible vaccine virus excretion in dogs after inoculation with SAD B19*

Animal	Inoculation route	Number	Inoculation dose (FFU)	Saliva sampling*	Detection method	Institute
Puppy	p.o.	11	$4{\cdot}2 \times 10^8$	2, 24, 48, 72 h	RFFIT†	VCRI‡
Puppy	i.m.	6	$1{\cdot}0 \times 10^8$	4, 14, 19 d	MIT§	VCRI
Adult	p.o.	6	$4{\cdot}0 \times 10^7$	2, 24, 48, 72 h	MIT	VCRI

* Saliva sampling: time of sampling post inoculation: h, hours; d, days.
† Rapid Fluorescent Focus Inhibition Test.
‡ Etlik Central Veterinary Control and Research Institute, Ankara, Turkey.
§ Mouse Inoculation Test.

inoculation were examined for the presence of vaccine virus.

Furthermore, the cell-culture re-isolate from the vaccination site of fox 7A (fourth passage) with a titre of $2{\cdot}5 \times 10^7$ SAD B19 was administered i.c. as previously described in four juvenile dogs (approximately 6 months old). All dogs tested negatively for rabies neutralizing antibodies prior to inoculation. Forty days after inoculation the animals were euthanized. Also, saliva swabs were taken 1, 3, 5, 8 and 16 days after inoculation.

The genetic stability of SAD B19 was tested in rodents (NMRI-mice) by multiple passaging by the i.c. route at IDT. Four mice were inoculated with 0·025 ml vaccine virus ($1{\cdot}0 \times 10^8$ FFU/ml). Afterwards, a 10% brain suspension in MEM/SNT was centrifuged. The supernatant was removed and the virus vaccine was reisolated in BSR C113 and titrated. Subsequently, the reisolated vaccine virus of the succumbed mice was inoculated i.c. into the next group of four mice, up to 10 passages. Of all mice, with the exception of the first passage, the titre of the reisolated vaccine virus in the brain was determined (FFU/ml).

RESULTS

Safety tests in target and non-target species after a single administration of SAD B19

Carnivores

All carnivore species tested remained free of clinical signs of rabies during the entire study. Two animals died during the observation period, their death however was unrelated to the administration of SAD B19: One mink died of chronic nephritis and hepatitis, another ferret had to be euthanized because of an injury.

Table 5. *Neuropathogenicity of the tenth passage of SAD B19 in mice ($10^{5{\cdot}2}$ FFU/ml)*

Group	Number	Dose (ml)	Inoculation route	Mortality
1	20	0·05	p.o.	0/20
2	20	0·1	i.m.	1/20
3	20	0·025	i.c.	19/20

Rodents

Under experimental conditions, a low degree of residual pathogenicity of SAD B19 was observed. During the safety tests, a total of 5 of 87 wild rodents died of rabies (5·7%) within the average observation period of 34 days: $3 \times$ *M. musculus*, $1 \times$ *M. epiroticus* and $1 \times$ *A. sylvaticus*. A further three animals died during the observation period ($2 \times$ *M. musculus* and $1 \times$ *A. sylvaticus*), these however tested negative for the rabies virus (FAT). All the other vaccinated and control animals survived and tested negative for rabies, even though some of these animals were housed together with rabid animals. Also, the young of three litters of vaccinated dams survived and tested negative for the rabies virus. During the study with NMRI-mice at the experimental animal facility at IDT, also none of the control animals showed any clinical sign of disease throughout the study. All blood-samples of the control NMRI-mice housed together with the mice inoculated i.c. tested negative. The mortality rate of the NMRI-mice inoculated by the different routes is shown in Table 5.

Birds

Three of the 10 vaccinated magpies died between 18 and 22 days (FAT negative), probably due to the housing conditions and stress components. The other

Table 6. *Re-isolation of vaccine virus from organ samples from foxes vaccinated by the oral route*

Fox number	Days post inoculation	Tonsils	Lymph nodes	Oral mucosa	Nasal mucosa	*Gl. mandibularis*	*Med. oblongata*	Cerebellum	Cortex	Hippocampus
1	1	−*	−	−	−	−	−	−	−	−
2	1	+++§	−	−	−	−	−	−	−	−
3	2	+†	−	−	−	−	−	−	−	−
4	2	+	−	+	−	−	−	−	−	−
5	3	+	−	−	−	−	−	−	−	−
6	3	+	−	−	−	−	−	−	−	−
7	4	++‡	−	+	−	−	−	−	−	−
8	4	+++	−	+	−	−	−	−	−	−
9	8	−	−	−	−	−	−	−	−	−
10	8	−	−	−	−	−	−	−	−	−
11	12	−	−	−	−	−	−	−	−	−
12	12	−	−	−	−	−	−	−	−	−
13	15	−	−	−	−	−	−	−	−	−
14	15	−	−	−	−	−	−	−	−	−
15	20	−	−	−	−	−	−	−	−	−
16	20	−	−	−	−	−	−	−	−	−

* Negative.
† Weak presence in cell culture.
‡ Clear presence with numerous foci in cell culture.
§ Very intensive demonstration with complete fluorescence.

birds remained healthy. No rabies virus-neutralizing antibodies could be detected at the end of the study. All pigeons and chickens remained healthy during the entire observation period. Furthermore, these birds were also immunologically refractory to vaccine virus.

Primates

All animals remained healthy throughout the observation period of 90 days. No virus antigen was detected in brain samples of the animals (FAT); Ammon's horn, Medulla oblongata, cerebrum and cerebellum. Seven of 12 animals showed detectable levels of rabies neutralizing antibodies 90 days post vaccination.

Safety tests in young animals

The young foxes of all three age-groups remained clinically healthy throughout the observation period. Also, all dog puppies that died during the trials or were euthanized at the end of the observation period tested rabies negative (FAT). Although, hyperimmune sera against distemper, parvo and hepatitis was administered regularly, several puppies died as a result of parvo-enteritis. It can be concluded, that the SAD B19 vaccine virus was completely innocuous for young foxes and dogs less than 10 weeks of age.

Safety tests in immunodeficient animals

All SCID mice survived oral administration with SAD B19. Only two nude mice died from rabies 15 days after oral administration of the vaccine virus. Rabies was seen in all of the six nude mice and five of six SCID mice after administration by the i.m. route. All animals died between 9 and 12 days post inoculation. The survival rate for the 12 (control) NMRI mice was 100%. The vaccine virus was only demonstrable in the brain of the dead animals. All other investigated organs were FAT-negative; salivary gland, spleen, lung, oesphagus, oral cavity mucosa. No virus neutralizing antibodies were demonstrable in the sera of the surviving mice of all groups.

Dissemination and excretion of SAD B19

All saliva and, if collected, nasal swabs taken from the animals examined at different hourly intervals, irrespective of the route of vaccine virus administration, tested negative for the presence of the vaccine virus. In foxes, the SAD B19 virus was re-isolated from the tonsils and some regions of the oral mucosa up to 4 days post inoculation. All other tissue samples were rabies-negative (Table 6). Of the eight dogs tested, only in one sample (smaller intestine) one day post

Table 7. *Investigation of tissue samples from foxes after passaging of virus for 48 h*

Fox number	Passage number	Results											
		Vaccination site		Cerebellum		Hippocampus		*Med. oblongata*		Salivary glands		*Ln. mandibularis*	
		IFT*	CC†	IFT	CC	IFT	CC	IFT	CC	IFT	CC	IFT	CC
1A	1st	?§	+++††	n.s.‖	n.s.	−‡	++**	−	++	−	−	−	++
1B	1st	−	+++	n.s.	n.s.	−	+¶	−	+	−	−	−	−
2A	2nd	−	−	−	−	−	−	−	−	−	−	−	−
2B	2nd	−	−	−	−	−	−	−	−	−	−	−	−
3A	2nd	−	+++	−	+++	−	+++	−	+++	−	−	−	++
4A	2nd	−	+++	−	+++	−	+++	−	−	−	−	−	−
5A	3rd	−	++	−	+++	−	+	−	+++	−	−	−	+
6A	3rd	−	−	−	−	−	−	−	−	−	−	−	+++
7A	4th	−	−	−	−	−	−	−	−	−	−	−	−
8A	4th	−	+++	−	−	−	+	−	−	−	−	−	++

* Immunofluorescence test of impression smears.
† Cell culture.
‡ Negative.
§ Result questionable.
‖ Not studied.
¶ Weakly positive in the second cell culture passage/isolated fluorescent foci.
** Clearly positive in second cell culture passage/numerous foci.
†† Strongly positive in second cell culture passage/intensive infection of cells.

Table 8. *The titre of the reisolated vaccine virus (FFU/ml) after multiple passaging of SAD B19 in NMRI-mice by the i.c. route; the titre underscored was used for the next passage*

Mouse	2nd passage	3rd passage	4th passage	5th passage	6th passage	7th passage	8th passage	9th passage	10th passage
1	$3{\cdot}2\times10^{4}$	$1{\cdot}3\times10^{5}$	$5{\cdot}0\times10^{5}$	$\underline{2{\cdot}0\times10^{5}}$	$\underline{5{\cdot}0\times10^{4}}$	$3{\cdot}2\times10^{4}$	$\underline{1{\cdot}7\times10^{5}}$	$4{\cdot}0\times10^{4}$	$2{\cdot}5\times10^{4}$
2	$3{\cdot}2\times10^{3}$	$8{\cdot}0\times10^{5}$	$7{\cdot}9\times10^{4}$	$1{\cdot}6\times10^{4}$	$1{\cdot}0\times10^{5}$	$\underline{2{\cdot}0\times10^{5}}$	$1{\cdot}3\times10^{5}$	$\underline{2{\cdot}0\times10^{4}}$	$1{\cdot}2\times10^{4}$
3	$\underline{6{\cdot}3\times10^{4}}$	$\underline{4{\cdot}0\times10^{5}}$	$\underline{2{\cdot}0\times10^{5}}$	$4{\cdot}0\times10^{4}$	$2{\cdot}0\times10^{5}$	$4{\cdot}0\times10^{5}$	$1{\cdot}2\times10^{5}$	$1{\cdot}6\times10^{5}$	$1{\cdot}5\times10^{4}$
4	$2{\cdot}5\times10^{5}$	$4{\cdot}4\times10^{5}$	$3{\cdot}7\times10^{5}$	$4{\cdot}0\times10^{4}$	$1{\cdot}5\times10^{4}$	$8{\cdot}9\times10^{4}$	$1{\cdot}6\times10^{5}$	$1{\cdot}0\times10^{4}$	$2{\cdot}0\times10^{5}$

inoculation SAD B19 could be re-isolated, all other samples tested rabies-negative.

In vivo stability of SAD B19

Following intracerebral injection in dogs, the SAD B19 vaccine virus spread to numerous parts of the brain. Administration of $1{\cdot}0\times10^{8}$ FFU results in a virus concentration so low that a direct immuno-fluorescence test was too insensitive to demonstrate the virus. Re-isolation was possible after passaging twice in cell-culture. Intracerebral inoculation of the vaccine virus did not induce disease and the virus could no longer be demonstrated 6 days after inoculation (Table 7). A high titre of virus neutralizing antibodies at this time indicated that a possible intracerebral infection by the vaccine virus was thus immunologically interrupted. The pathogenicity of the virus was unchanged after five i.c. passages in foxes. The foxes used for the fifth passage similarly were free of clinical signs of disease and did not excrete the rabies virus in their saliva. They also had a high titre of virus neutralizing antibodies. None of the dogs vaccinated with the vaccine virus i.c. showed any clinical sign of rabies during the entire observation period.

All NMRI-mice inoculated i.c. with SAD B19 showed clinical signs of rabies. The titre of the reisolated vaccine virus is shown in Table 8.

DISCUSSION

Since 1978, many European countries participated in oral vaccination campaigns of wildlife against rabies using live attenuated SAD-derivates vaccines. The results obtained attest to the feasibility of oral

vaccination with these vaccines. However, the use of these virus vaccines continues to remain controversial, especially the potential residual pathogenicity [3, 8, 9]. Therefore, the vaccinia-rabies-glycoprotein recombinant (VRG) has been put forward as the most suitable candidate for oral vaccination of wildlife by several authors [10, 11]. However, no self-replicating system, either a modified live virus or recombinant-based, is without risk [12]. The use of VRG is also not without problems and risks to the health of humans and animals, which still partly have to be solved [13–16]. Especially, the risk of recombination between VRG and other orthopoxviruses circulating in many animal species, including man. For example, orthopoxviruses were found in the red fox populations in large areas of Europe; e.g. Norway, Great Britain, Belgium and Germany [17–22]. The cause of concern towards the use of the SAD-derivatives is mainly based on incidents with the SAD Berne vaccine virus. However, significant differences exist between the different SAD-derivatives concerning residual pathogenicity and stability [23, 24]. Shortly after the beginning of the field trials with SAD Berne in Switzerland three cases of vaccine induced rabies were reported [25]. Also, in the brains of two of four wild caught adult chacma baboons (*Papio c. ursinus*) the vaccine virus was isolated after oral administration of SAD Berne [8]. However, SAD B19 vaccine virus was completely innocuous for these primate species. Also, during a previous safety trial with chimpanzees (*Pan troglodytes*), all animals inoculated with SAD B19 remained healthy and developed protective antibody titres [26].

The results presented here have clearly showed the innocuity of SAD B19 for all mammal and bird species tested, except for certain rodent species. In the studies with rodents, no rabies virus was found in the unvaccinated control animals; although the Turkish control animals were kept in close contact with the vaccinated animals during the entire observation period, including animals that died of rabies. Also, none of the control mice died of rabies after being placed together with mice inoculated i.c. with SAD B19. Thus, indicating that excretion and horizontal transmission did not occur in these studies. Even in the immunocompromised mice that died from rabies no vaccine virus could be found in the salivary glands. The low residual pathogenicity for rodents observed under laboratory conditions could partially be explained by the procedure of inoculation; the forced administration of the vaccine virus. The virus did not only enter the mouth cavity but could also have entered the respiratory tract. For example, all three *M. musculus* that died from rabies in this study were anaesthetized with ether followed by ketamine-hydrochloride. Steck and colleagues [27] mentioned already that when the vaccine is administered under general anaesthetics, it may be refluxed into the nasal passage. Schneider and Cox [23] also reported a low residual pathogenicity of SAD B19 for rodents; two of 34 rats (5·8%) and 3 of 150 NMRI-mice (2%) succumbed to rabies. It is generally agreed, on the basis of laboratory and field studies, that there is no tendency for SAD-derivatives to spread and become established within the rodent populations [23, 28, 29]. Thousands of rodents have been collected in SAD B19 vaccination-areas (Germany, France, Belgium). No mortality by SAD B19 induced rabies in rodents during these field studies have been reported [29–31]. Also, no SAD B19 vaccine induced rabies has been reported in any other animal species examined in areas where SAD B19 vaccine baits had been distributed since 1983 [3, 24]. Only one laboratory study mentioned vaccine induced rabies in another mammal species, a skunk (*Mephitis mephitis*) which consumed a SAD B19 vaccine bait died from rabies ($4{\cdot}0 \times 10^5$ TCID 50/ml). Also all six skunks vaccinated by intranasal instillation succumbed to rabies [32]. However, the vaccine virus used in this study is not the same as the SAD B19 vaccine virus used presently for oral vaccination of wildlife in Europe.

Further extensive safety tests with SAD B19 have been carried out in other animals under supervision of the WHO Collaborating Centre for Rabies Surveillance and Research in Tübingen, Germany [26]; e.g. raccoon (*Procyon lotor*), raccoon dog (*Nyctereutes procyonoides*), jackals (*Canis aureus*), wolves (*Canis lupus*), wild boars (*Sus scrofa*). None of these animals succumbed from rabies after inoculation with SAD B19. Considering the differences between immunodeficient mice vaccinated by the oral and parenteral routes and immunocompetent standard mice, it is concluded that the immunodeficient mice model does not permit any conclusive statements on the risk of using SAD B19 vaccine virus. However, SAD B19 was not pathogenic for cats and foxes treated with immunosuppressant drugs, corticosteroids [33, 34].

The SAD B19 vaccine virus was not excreted via saliva in animal species examined, even just 2 h after administration (in dogs and foxes). In foxes the virus was only demonstrable in the tonsils up to 4 days post inoculation and in some parts of the oral mucosa after

repeated passaging. Since the lymph nodes, salivary glands and brain were negative, viraemia can be ruled out following primary virus replication in the tonsils and parts of the oral mucosa. In this study, also a possible change in the genetic properties of SAD B19 was studied through passaging in neural tissue of foxes and dogs. These studies permit the conclusion that the vaccine virus SAD B19 has no neurovirulent properties and that SAD B19 is genetically stable when passaged experimentally in neural tissue of these two animal species. Furthermore, ten consecutive passages of SAD B19 in mice after a lethal intracerebral inoculation was possible. The virus was isolated at a titre between $10^{3.5}$ and $10^{5.6}$ FFU/ml. From the results presented here and, most important, its performance during 15 years in the field without incidents, it can only be concluded that SAD B19 vaccine virus is as safe for use as any other presently available oral rabies vaccine. The critical attitude towards the use of this vaccine virus in comparison with other oral vaccine viruses is therefore unfounded.

REFERENCES

1. Müller T, Schlüter H. Oral immunization of red foxes (*Vulpes vulpes* L.) in Europe – A review. J Etlik Vet Microbiol 1998; **9**: 35–39.
2. Steck F, Wandeler A, Bichsel P, Capt S, Schneider L. Oral immunisation of foxes against rabies. Zbl Vet Med B 1982; **29**: 372–96.
3. Stöhr K, Stöhr P, Müller T. Ergebnisse und Erfahrungen aus den ostdeutchen Bundesländer. Tierärztl Umschau 1994; **49**: 203–11.
4. Blancou J, Meslin FX. Modified live-virus rabies vaccines for oral immunization of carnivores. In: Meslin FX, Kaplan MM, Koprowski H, eds. Laboratory techniques in rabies, 4th edn. Geneva: World Health Organization, 1996; 324–37.
5. Sato M, Maeda N, Yoshida H, et al. Plaque formation of herpes virus hominis Type 2 and rubella virus variants isolated from the colonies of BHK21/WI-2 cells formed in soft agar. Arch Virol 1977; **53**: 269–73.
6. Bachmann P, Bramswell RN, Fraser SJ, et al. Wild carnivore acceptance of baits for delivering of liquid rabies vaccine. J Wildl Dis 1990; **26**: 486–501.
7. Vos A, Sanli S. Evaluation of a bait delivery system for oral vaccination of dogs against rabies in Turkey. J Etlik Vet Microbiol 1998; **9**: 83–91.
8. Bingham J, Foggin CM, Gerber H, et al. Pathogenicity of SAD rabies vaccine given orally in chacma baboons (*Papio ursinus*). Vet Rec 1992; **131**: 55–6.
9. Brochier B, Blancou J, Thomas I, et al. Use of recombinant vaccinia-rabies glycoprotein virus for oral vaccination of wildlife against rabies: innocuity to several non-target bait consuming species. J Wildl Dis 1989; **25**; 540–7.
10. Pastoret PP, Brochier B, Lanquet B, et al. First field trial of fox vaccination against rabies using a vaccinia-rabies recombinant virus. Vet Rec 1988; **123**: 481–3.
11. Thomas I, Brochier B, Lanquet B, et al. Primary multiplication site of the vaccinia-rabies glycoprotein recombinant virus administered to foxes by the oral route. J Gen Virol 1990; **71**: 37–42.
12. Rupprecht CE, Hanlon CA, Koprowski H. General considerations in the use of recombinant rabies vaccines for oral immunization of wildlife. In: Meslin FX, Kaplan MM, Koprowski H, eds. Laboratory techniques in rabies, 4th edn. Geneva: World Health Organization, 1996: 341–6.
13. MacInnes CD. Control of wildlife rabies – the Americas. In: Campbell JB, Charlton KM, eds. Rabies. Boston: Kluwer Academic Publishers, 1988: 382–405.
14. Rupprecht CE, Kieny MP. Development of a vaccinia-rabies glycoprotein recombinant virus vaccine. In: Campbell JB, Charlton KM, eds. Rabies. Boston: Kluwer Academic Publishers, 1988: 335–64.
15. McNally R. Genetic madness – the European rabies eradication programme. Ecologist 1994; **24**: 207–12.
16. Boulanger D, Brochier B, Crouch A, et al. Comparison of the susceptibility of the red fox (*Vulpes vulpes*) to a vaccinia-rabies recombinant virus and to cowpox virus. Vaccine 1995; **13**: 215–9.
17. Pilaski J, Jacoby F. Die Kühpockenerkrankungen der Zootiere. Verhandlungsbericht des internationalen Symposiums über die Erkrankungen der Zoo- und Wildtiere 1993; **35**: 39–50.
18. Crouch AC, Baxby D, McCracken M, Gaskell RM, Benett M. Serological evidence for the reservoir hosts of cowpoxvirus in British wildlife. Epidemiol Infect 1995; **115**: 185–91.
19. Henning K, Czerny CP, Meyer H, Müller T, Kramer M. A seroepidemiological survey for orthopox virus in the red fox (*Vulpes vulpes*). Vet Microbiol 1995; **43**: 251–9.
21. Boulanger D, Crouch A, Brochier B, et al. Serological survey for orthopoxvirus infection of wild mammals in areas where a recombinant rabies virus is used to vaccinate foxes. Vet Rec 1996; **138**: 247–9.
22. Tryland M, Sandvik T, Mehl R, et al. Serosurvey for orthopoxviruses in rodents and shrews from Norway. J Wildl Dis 1998; **34**: 240–50.
23. Schneider LG, Cox JH. Ein Feldversuch zur oralen Immuniserung von Füchsen gegen Tollwut in der Bundesrepublik Deutschland. Tierärztl Umschau 1983; **5**: 315–24.
24. Masson E, Aubert MFA, Barrat J, Vuilaume P. Comparison of the efficacy of the antirabies vaccines used for foxes in France. Vet Res 1996; **27**: 255–66.
25. Wandeler AI. Control of wildlife rabies. In: Campbell JB, Charlton KM, eds. Rabies. Boston: Kluwer Academic Publishers, 1988: 365–80.
26. Müller W, Güzel T, Aylan O, et al. The feasibility of oral vaccination of dogs in Turkey – A European Union supported project. J Etlik Vet Microbiol 1998; **9**: 61–71.

27. Steck F, Wandeler AI, Bischel P, et al. Oral immunization of foxes against rabies. Laboratory and field studies. Comm Immun Microbiol Inf Dis 1982; **5**: 165–71.
28. Wandeler AI, Bauder W, Prochaska S, Steck F. Small mammal studies in a SAD baiting area. Comp Immun Microbiol Inf Dis 1982; **5**: 173–6.
29. Artois M, Guittré C, Thomas I, et al. Potential pathogenicity for rodents of vaccines intended for oral vaccination against rabies: a comparison. Vaccine 1992; **10**: 524–8.
30. Brochier B, Thomas I, Iokem A, et al. A field trial in Belgium to control fox rabies by oral immunisation. Vet Rec 1988; **123**: 618–21.
31. Artois M, Chillaud T, Maillot E, Rigal F, Blancou J. Première campagne de vaccination antirabique du renard par voie orale menée en France. Contrôles d'efficacité chez le renard et d'innocuité chez les micromammifères. Ann Med Vet 1987; **131**: 457–62.
32. Rupprecht CE, Charlton KM, Artois M, et al. Ineffectiveness and comparative pathogenicity of attenuated rabies virus vaccines for the striped skunk (*Mephitis mephitis*). J Wildl Dis 1990; **26**: 99–102.
33. Ciuchini F, Pestalozza AI, Buonavoglia C, et al. Effects of corticosteroids mediated immunosuppression on the distribution of rabies vaccine virus in red foxes orally immunized against rabies. J Vet Med B 1986; **33**: 628–31.
34. Ciuchini F, Bozzano AI, Pestalozza S, Tollis M. Research on susceptibility of cats to the oral administration of rabies vaccine SAD B19 Tü. J Vet Med B 1988; **35**: 677–80.