Studies of ERA/BHK-21 Rabies Vaccine in Skunks and Mice

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ABSTRACT

ERA® rabies vaccine virus grown in BHK-21 13S cells (ERA/BHK-21) and street rabies virus were titrated in mice by intracerebral, intranasal and intramuscular inoculation. Mice were also given undiluted ERA/BHK-21 in baits. Skunks were given undiluted ERA/BHK-21 in baits and by intramuscular, intranasal and intestinal inoculation. Virus neutralizing antibody titers against rabies virus were measured over a three month observation period. The surviving skunks were challenged by intramuscular inoculation with rabies street virus from a skunk salivary gland suspension.

When titrated in mice, ERA/BHK-21 had titers of $10^{7.0}$, $10^{5.2}$and $10^{3.9}$ median lethal doses per mL by the intracerebral, intranasal and intramuscular routes, respectively. All skunks (8/8) inoculated intranasally developed paralytic rabies by 12 days after exposure to ERA/BHK-21 virus. None of the skunks that developed vaccine-induced rabies had infectious virus in the submandibular salivary glands. Vaccine-induced rabies also occurred in 1/8 skunks in the intramuscularly inoculated group and in 1/8 in the intestinally inoculated group. The survival rates of challenged skunks in the various groups were as follows: intramuscular, 7/7; intestinal, 2/7; bait, 0/8; and control, 0/8. These results indicate that ERA/BHK-21 virus has a significant residual pathogenicity in mice and in skunks by some routes of inoculation. Skunks given vaccine intramuscularly were protected against challenge, while those skunks given the vaccine in baits were not.

RÉSUMÉ

Cette expérience consistait à titrer le virus rabique du vaccin ERA, cultivé dans des cellules BHK-21 13S (ERA/BHK-21), et le virus rabique de rue, chez des souris, par inoculation intracérébrale, intranasale et intramusculaire. On donna par ailleurs à des souris et à des mouffettes des appâts qui contenaient du virus ERA/BHK-21 non dilué; les dernières reçurent aussi ce virus par les voies intramusculaire, intranasale et intestinale. On mesura ensuite les titres d'anticorps neutralisants contre le virus rabique, au cours d'une période d'observation de trois mois. Les mouffettes survivantes subirent une infection de défi intramusculaire, avec du virus rabique de rue provenant d'une suspension des glandes salivaires de mouffettes enragées.

Le tirage du virus ERA/BHK-21, chez des souris, par les voies intracérébrale, intranasale et intramusculaire, en révéla les quantités de doses létales moyennes/mL suivantes: 10^{7}, $10^{5.2}$ et $10^{3.9}$. Les huit mouffettes vaccinées avec le virus ERA/BHK-21, par la voie intranasale, développèrent la rage paralytique, en 12 jours. Une des huit mouffettes vaccinées par la voie intramusculaire développa aussi la rage, tout comme une des huit vaccinées par la voie intestinale. Aucune d'entre elles n'excréta toutefois de virus dans ses glandes salivaires sous-maxillaires. À la suite d'une infection intramusculaire de défi, les sept mouffettes vaccinées par la voie intramusculaire survécurent, tout comme deux des sept vaccinées par la voie intestinale, mais aucune des huit vaccinées au moyen d'appâts et aucune des huit témoins ne survécurent.

Il semble donc que le virus ERA/BHK-21 possède une certaine pathogénicité résiduelle, chez la souris et la mouffette, par certaines voies d'inoculation. La vaccination de mouffettes, par la voie intramusculaire, les protégea contre une infection de défi, contrairement à celles qui reçurent le vaccin dans des appâts.

INTRODUCTION

Enzootic rabies in Canadian wildlife is a continual public health hazard and source of infection for domestic animals. In Ontario and Quebec, the main vectors are the fox and skunk, whereas the skunk is most frequently involved in transmission in Manitoba and Saskatchewan. Skunks comprised 23% of positive field specimens (all species) and 33% of positive skunk and fox specimens in Ontario and Quebec during the past decade (Data from Agriculture Canada, Animal Diseases Research Institute, NEPEAN).

Research on control of wildlife rabies has centered mainly on development of live oral vaccines. Field trials have been undertaken in Switzerland (1-4) and West Germany (5,6) using vaccines derived from the Street-Alabama-Dufferin (SAD) rabies virus. In Canada, ERA® vaccine and ERA® grown on BHK-21 cells (ERA/BHK-21) have been used. Results in foxes have been encouraging. Field trials in West Germany have shown that 50-75% of the fox population in the baiting area can be effectively immunized (5). However, except for laboratory trials with a vaccinia virus recombinant (7,8) no effective immun ization of skunks has been reported.

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Preliminary trials in foxes with ERA/BHK-21 at Connaught Laboratories Limited, resulted in high rabies antibody titers following oral immunization (unpublished data, K. Lawson). Previous reports on ERA/BHK-21 vaccines produced in other laboratories have demonstrated a residual pathogenicity by the oral route in rodents (2,4,9,10). Some of these vaccines are in use in field trials to control fox rabies in Europe (3,5).

Since oral administration of ERA® (produced by Connaught Research Institute) in baits has been shown to be nonpathogenic for skunks and other carnivores (11), ERA/BHK-21 was tested for pathogenicity by several routes in mice and skunks. The immunological response and resistance to challenge were tested in skunks given vaccine by intramuscular (IM), intranasal (IN), oral (bait) and intestinal (endoscopic deposition) routes. Our results indicate that the ERA/BHK-21 vaccine is only effective in skunks by the IM route of inoculation. The vaccine retains pathogenicity for white Swiss mice vaccinated by IC, IN and IM routes and for skunks given undiluted vaccine intranasally.

MATERIALS AND METHODS

VIRUSES

ERA/BHK-21 virus was prepared from the ERA® working seed stock used in commercial production of ERA® (12). BHK-21 13 S cells were grown in roller bottle cultures and were maintained in AV_2 medium (modified Earle's medium containing 2% fetal bovine serum, 10% tryptose broth, 3% of 7.5% sodium bicarbonate, 3% Hepes buffer, 1% glutamine and 25 units/mL potassium penicillin). The growth medium was poured off and the cultures were rinsed in Ca^{2+} and Mg^{2+} free phosphate buffered saline. ERA® WS III rabies seed virus (in Earle's balanced salt solution containing 1% gelatin and 100 μg/mL DEAE dextran) was added to the bottles and incubated for 45 min at 37°C on a roller apparatus at 1.0 rpm. The supernatant was poured off and 300 mL of AV_2 medium was added to each bottle. Cultures were incubated at 33°C on the roller machine at 1.0 rpm. After four days, the medium was harvested and had a virus titer of $10^{7.1}$ median mouse intracerebral lethal doses ($MICLD_{50}$)/mL by intracerebral inoculation in weanling mice.

Street virus was prepared as a 10% suspension (10^{-1} dilution) of submandibular salivary glands from naturally infected skunks from Ontario by homogenization with 0.01 M phosphate buffer (pH 7.4) containing 10% fetal bovine serum, 1000 IU penicillin and 2 mg streptomycin/mL and centrifuged at 600 *g* for 15 min (13). The virus titer was $10^{5.3}$ $MICLD_{50}$/0.03 g of tissue. Virus suspensions were stored at -70°C. Aliquots of stock solutions were rapidly thawed within 1 h prior to use and maintained at 4°C during inoculations.

Submandibular salivary glands were removed at necropsy from skunks that developed vaccine-induced rabies, which was confirmed by the rabies fluorescent antibody test on brain tissue (14). Ten percent suspensions of salivary gland tissue were prepared in Eagle's minimum essential medium (MEM) with 2% fetal bovine serum. The presence of rabies virus was determined by the mouse inoculation test (15) and by inoculation in BHK-21 cell cultures. In the tissue culture test 0.2 mL of salivary gland suspension was added to 0.1 mL of cells at a concentration of 5 x 10^5 cells/mL and incubated for four days at 36°C. The cultures were fixed in acetone and stained with fluorescein-labeled rabies antibody.

ANTIBODY TITERS

Blood samples were taken at the times specified (Table II). Sera were inactivated at 56°C for 30 min and the rabies virus neutralizing antibody titers determined by the fluorescence inhibition microtest (FIMT) (16). Titers were expressed in International Units (IU)/mL; titers less than 0.13 IU/mL were considered negative.

MICE

Three week old female CD 1 mice (Charles River, St. Constance, Quebec) were inoculated with tenfold serial dilutions of either ERA/BHK-21 or street rabies viruses by the following routes: intracerebral (IC) (0.03 mL), intranasal (IN) (0.02 mL), and intramuscular (IM) (0.02 mL). Five mice were used for each virus dilution. Mice to be given baits were starved for 24 h. The baits were polyurethane sponges coated three to four times with a beef fat and wax mixture (17). Each bait was loaded with 10 mL of undiluted ERA/BHK-21 virus by syringe. Mice were kept in individual cages and given one bait each. They were observed for 30 days. Brains of mice that died during the observation period were examined by the rabies fluorescent antibody staining test.

SKUNKS

Striped skunks (*Mephitis mephitis*) were obtained from Ruby's Fur Farm, New Sharon, Iowa and were approximately four months old at the beginning of the experiment. They were divided into four groups of eight skunks containing equal numbers of males and females. Each skunk was kept in a separate stainless steel cage and, except at the time of vaccination, was given food and water ad lib. Prevaccination blood samples were taken from all skunks just prior to or 24 h before vaccination.

Skunks in group 1 were starved for 24 h, and then each received one bait containing 10 mL of ERA/BHK-21 virus suspension. Eight skunks in group 2 were starved for 24 h prior to endoscopy. The skunks were anesthetized with ketamine hydrochloride (rogar/STB, London, Ontario) and acepromazine maleate (Ayerst Laboratory, Montreal, Quebec) (13) and given 0.5 mL of Tigan (Hoffman-LaRoche, Vaudreuil, Quebec) (18) following which 10 mL of vaccine were deposited into the duodenum by endoscope. For intramuscular inoculations, 1 mL of vaccine was injected into the right *biceps femoris* muscle of eight skunks in group 3. Eight skunks in group 4 were anesthetized and each received 0.5 mL of vaccine applied dropwise from a syringe into one nostril. Eight skunks were used as unvaccinated controls.

Serum samples were taken for rabies antibody titers on the days indicated (Table II). After a three month observation period, the surviving skunks were challenged by IM inoculation of 0.3 mL of the skunk salivary gland virus suspension. The route of inoculation was the *abductor*

digiti quinti muscle of the right pelvic limb. The animals were observed for a further 90 days. Animals that developed rabies were allowed to die or euthanized when complete paralysis developed. Rabies was confirmed by the rabies fluorescent antibody test on smears of brain or spinal cord.

RESULTS

MOUSE INOCULATIONS

By IC inoculation the titer of ERA/BHK-21 was $10^{7.0}$ $MICLD_{50}$/mL (Table I). This was identical to the value obtained with the street rabies virus prepared from skunk salivary glands. The level of pathogenicity by IN and IM inoculation of ERA/BHK-21 was less than the IC route with titers of $10^{5.2}$ and $10^{3.9}$ LD_{50}/mL, respectively. Similar results were obtained with street virus (Table I). In the group fed baits filled with ERA/BHK-21, 9/20 mice ate the baits. Four of the nine mice died of rabies.

TABLE I. Pathogenicity of ERA/BHK-21 Virus and Street Virus in Mice

Route of Exposure	Virus	Titer (LD_{50}mL)
Intracerebral	ERA/BHK-21	$10^{7.0}$
	STREET	$10^{7.0}$
Intramuscular	ERA/BHK-21	$10^{3.9}$
	STREET	$10^{4.2}$
Intranasal	ERA/BHK-21	$10^{5.2}$
	STREET	$10^{4.8}$
BAIT	ERA/BHK-21	4/9 died[a]

[a]FA positive

VACCINATION OF SKUNKS

ERA/BHK-21 virus was pathogenic by the IN route. All animals in this group developed paralytic rabies on day 12 and were killed *in extremis* on days 12, 13 or 14. Brain tissue from every skunk was positive by the rabies fluorescent antibody test. A 10% submandibular salivary gland suspension of each skunk that developed vaccine-induced rabies was inoculated IC into mice. The animals did not develop rabies during the 30 day observation period. Rabies virus was not detected in BHK-21 cell cultures inoculated with these suspensions.

In the group inoculated by the intestinal route, one animal died 17 days after inoculation. No clinical signs of rabies were observed prior to death. The hippocampus was positive by the rabies fluorescent antibody test; other regions of the brain were negative. Virus was not detected in a salivary gland suspension by mouse inoculation or tissue culture assays. The other skunks in this group remained healthy during the observation period.

One animal in the IM-inoculated group (#4, Table II) developed posterior ataxia 13 days after inoculation; this was quickly followed by paralysis of the pelvic limbs. After one week, motor function improved until the skunk became active and recovered almost full mobility. A residual weakness in the right rear leg was the only persistent clinical feature. This animal was excluded from the subsequent challenge study. Virus neutralizing antibody titers (FIMT) in serum and cerebrospinal fluid at six months were 7.4 IU/mL and 1.2 IU/mL respectively. The skunk was killed one year after vaccination. Histopathology revealed focal gliosis in the spinal cord at L-3. No other lesions were detected and virus was not isolated from brain or any other organ. It would appear that this animal recovered from rabies and had chronic residual disability.

One month after inoculation the highest levels of antibody were found in the IM-inoculated group (2-95 IU/mL) (Table II). All skunks in this group seroconverted. Moderate titers (0.13-11.0 IU/mL) occurred in 6/8 skunks in the intestinally inoculated group (Table II). Very low antibody titers, 0.22 and 0.24 IU/mL, were found in 2/8 skunks which were fed with baits (Table II).

It will be noted from the data in Table II that four out of 24 experimen-

TABLE II. Vaccination of Skunks with ERA/BHK-21 Rabies Vaccine

Vaccination Route and Skunk Number	Serum Neutralizing Antibody Titers (IU/mL)					Response to Challenge (Day of Death or Euthanasia)
	Day 0	Day 21	Day 33	Day 63	Day 105	
Oral (Bait-10 mL)						
1	0	0	0	0	0	R (24)
2	0	0	0	0	0	R (23)
3	0.13	0	0	0	0	R (23)
4	0	0	0	0	0	R (16)
5	0	0.15	0.22	0	0	R (17)
6	1.41	0.57	0.24	0	0	R (17)
7	0	0	0	0	0	R (18)
8	0	0	0	0	0	R (21)
Intramuscular (1 mL)						
1	0	13.70	4.91	12.2	4.96	S
2	0.20	3.17	2.05	7.61	1.76	S
3	0	9.28	13.3	13.8	7.22	S
4[a]	0.65	ND	72.3	161	ND	—
5	0	ND	95.4	251	236	S
6	0	7.46	14.5	49.2	29.6	S
7	0	ND	20.3	37.7	37.7	S
8	0	ND	19.50	18.7	30.1	S
Intestinal (10 mL)						
1	0	0.41	0.30	0.17	0	R (73)
2[b]	0	Died				
3	0	0.20	0.17	0.50	0.57	R (16)
4	0	1.57	0.87	0.67	1.87	S
5	0	0.20	0.13	0	0	R (17)
6	0	11.0	5.79	2.46	1.11	S
7	0	0.20	0.20	0	0	R (21)
8	0	0	0	0	0	R (17)

R =Succumbed to rabies on challenge; FA: positive (day euthanized/died)
S = Survived 90 day postchallenge observation period
ND = Not done
All animals in the oral groups consumed the bait
[a]Abortive rabies — not challenged
[b]Death from vaccine-induced rabies (FA positive)

tal skunks had positive serum antibody titers on day 0. Titers in two of these were very low (0.13 and 0.20 IU/mL) and were possibly false-positives. The other two were higher (0.65 and 1.41 IU/mL). The origin and significance of these titers is unclear at the present time, but it is apparent that they do not affect the overall conclusions.

CHALLENGE WITH STREET VIRUS

The survival rates in the various groups were: IM inoculation, 7/7; intestinal inoculation, 2/7; bait, 0/8; (Table II) and control 0/8. The incubation periods for the skunks that developed rabies were similar in the vaccinated groups and the controls (not shown).

DISCUSSION

Switzerland, West Germany and Canada have programs to control wildlife rabies using modified live rabies virus vaccines that have been derived from SAD virus (19). The vaccines used in the European programs have been adapted to cultures of BHK-21 cells (2,5,6). In the first Canadian field trial in 1985 a commercially prepared vaccine, ERA®, was used. It was grown in porcine kidney cells (12,20). This vaccine was selected for the Ontario program on the basis of its effectiveness in immunizing foxes (11,19) and its safety record when administered orally to potential contact species (11). The antigenic profile of the vaccine strain can be distinguished from street rabies virus by monoclonal antibodies (21). In the present investigation, we have examined the pathogenicity and immunogenicity of ERA/BHK-21 in mice and skunks when vaccine was administered by several different routes.

This study revealed that the pathogenicity of ERA/BHK-21 in CDI mice was similar to a standard preparation of street virus from skunk salivary glands when inoculated by IC, IN and IM routes. Undiluted vaccine in sponge baits induced rabies in 4/9 mice that ate the baits. It had previously been shown that ERA® retains a high level of pathogenicity in mice by the IC route but has a low level when administered orally in baits (11). A low level of pathogenicity was also reported with the Swiss SAD/BHK-21 vaccine in wild strains of mice (*Microtus arvalis, Apodemus silvaticus* and *Mus musculus*) after consuming vaccine-filled baits (1). No direct comparison of ERA/BHK-21 and SAD/BHK-21 vaccines has been made in mice.

ERA/BHK-21 given orally also had a low level of pathogenicity for wild rodents (unpublished data). The effect of rabies vaccines administered by the IN routes to mice has not been studied extensively. However, Steck *et al* (2) indicated that mice inoculated orally while under anesthesia had a sporadic mortality over a broad range of virus dilutions. They attributed this to intranasal uptake. In our investigation, the IN route was second to the IC route in sensitivity for measuring pathogenicity in mice.

In skunks, vaccine-induced rabies occurred in all animals in the IN inoculated group and in one of the intestinally inoculated group. One animal in the IM inoculated group developed clinical signs of rabies but recovered with slight residual paralysis. Other studies have shown that a small number of skunks developed rabies following the ingestion of bait containing high titers of another ERA/BHK-21 vaccine (Winkler, personal communication). These results demonstrate that the ERA/BHK-21 vaccine retains residual pathogenicity by all routes of inoculation when administered at high titers. As in mice, the intranasal route was highly sensitive for demonstrating pathogenicity.

Since the vaccines used in all field trials to date were efficacious and nonpathogenic in the main target species, the fox, a low level of infection by the oral route in nontarget species has been considered acceptable. Reports from Switzerland and West Germany have shown that vaccine-induced rabies has not become established in the wildlife population in the areas of the control programs (2,5). The results from the present investigation indicate that animals developing vaccine-induced rabies from ERA/BHK-21 would be very unlikely to transmit the disease since rabies virus was not isolated from the salivary glands of the skunks with vaccine-induced rabies.

While oral ERA® and ERA/BHK-21 vaccines were highly immunogenic in foxes (11; unpublished data, Lawson), skunks given ERA/BHK-21 vaccine orally had a low rate of seroconversion (1/8) and all succumbed to challenge. Similarly, intestinal deposition of ERA/BHK-21 was ineffective in skunks while ERA® administered by the same route to foxes was immunogenic (18). The ability of ERA/BHK-21 to immunize foxes but not skunks by the oral route (by sponge baits) cannot be explained at the present time.

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