Immunization of Foxes by the Intestinal Route Using an Inactivated Rabies Vaccine

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ABSTRACT

Approximately 30% of foxes given two doses of an inactivated rabies antigen delivered directly into the intestinal tract developed an immune response as measured by rabies serum neutralizing antibodies. Seven of ten previously immunized foxes showed an anamnestic response following a booster dose of inactivated rabies antigen delivered to the intestinal lumen.

Stomach and particularly intestinal contents were destructive to rabies antigen and virus. This effect could be partially neutralized *in vitro* by the addition of Questran and soybean trypsin inhibitor.

Small enteric coated tablets fed to foxes in a hamburger bolus remained in the stomach for up to 13 hours and therefore would provide a poor vehicle for the delivery of antigen to the intestinal tract.

RÉSUMÉ

Environ 30% des renards qui reçurent deux doses d'antigène rabique inactivé, directement dans la lumière intestinale, développèrent des anticorps, comme le démontra l'épreuve de séroneutralisation. Sept des dix renards déjà immunisés manifestèrent une réaction anamnestique, à la suite d'une dose de rappel d'un antigène rabique inactivé, directement dans la lumière intestinale.

Le contenu stomacal et surtout celui de l'intestin grêle se révélèrent destructifs pour l'antigène et le virus rabiques. On réussit à neutraliser partiellement cet effet, *in vitro*, par l'addition de questran et d'inhibiteur de la trypsine de la fève de soya.

Comme des petites tablettes entériques enrobées, données à des renards dans un hamburger bolus, demeurèrent dans l'estomac jusqu'à 13 heures, elles représentent un véhicule de piètre qualité pour l'acheminement d'antigène dans le tube intestinal.

INTRODUCTION

Wildlife, such as the fox, skunk or raccoon, are the main vectors of rabies in Canada, the United States and many countries in Europe. Control of rabies in wildlife by population reduction has not been particularly effective and has led to the concept of immunizing wildlife by distributing baits containing attenuated live virus rabies vaccines.

Trials conducted in Switzerland (1) and Germany (2) and more recently Canada (3), have shown this procedure to be successful. The vaccine used in these trials was the SAD strain of attenuated live virus. Since this strain retains some pathogenicity for non-target species (4,5,6), the use of inactivated vaccines (7,8) and immunogens prepared by genetic engineering (9,10) have been investigated.

Although immunization of foxes with attenuated live rabies virus occurs in the buccal cavity (11), vaccination of foxes by this route (*per os*) with inactivated rabies antigen has not been successful (Winkler, Black and Lawson — unpublished). As a result, immunization of foxes with an inactivated rabies antigen requires a delivery system different from that used with the live virus.

Lawson *et al* (7) reported on the selection of rabies antigen and delivery system for the evaluation of immunization of foxes with inactivated antigens. In those studies, it was shown that rabies serum neutralizing antibody could be produced after two doses of rabies antigen had been placed in the duodenum of foxes. This is a report on further studies carried out on the use of inactivated rabies antigen as a possible oral immunogen for foxes in the wild.

MATERIALS AND METHODS

ANIMALS

Foxes (*Vulpes vulpes*), red genotype were ranch bred and supplied as required. Foxes in the trials were eight weeks to five years of age. Laboratory mice, White Swiss strain, were obtained from Connaught Laboratories Limited and the CD-1 strain from a commercial supplier (Charles River Canada Inc., St. Constant, Quebec). The mice in the various trials were four to eight weeks of age and weighed 10-30 grams.

Connaught Laboratories Limited, 1755 Steeles Avenue West, Willowdale, Ontario M2R 3T4 (Lawson, Hertler), Ontario Ministry of Natural Resources (Johnston, Rhodes), Box 130, Rockwood, Ontario (Patterson) and University of Toronto, Toronto, Ontario (Campbell).

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TABLE I. Rabies Serum Neutralizing Antibody Response in Foxes Vaccinated with Inactivated Rabies Vaccine Delivered to The Intestinal Tract by Fiberscope

Total Number of Trials	Rabies Antigen[a]	Number of Animals	Antibody Results FIMT[b]					
			Postvaccination			Postbooster		
			Number Converting	Antibody Level < 0.35	Antibody Level > 0.35	Number Converting	Antibody Level < 0.35	Antibody Level > 0.35
6	2.1-34.2	79	6/79 (8%)	2/79 (3%)	4/79 (5%)	24/79 (30%)	7/79 (9%)	17/79 (22%)

[a]Recorded as International Units mL, as determined by the NIH test. Dose was 10 mL
[b]Recorded as International Units per mL. 0.35 IU/mL considered protective against challenge. Titers < 0.15 IU/mL are considered negative

VIRUSES

The vaccine viruses used in the trials were derived from a mouse passed strain of ERA (ERA H) and a plaque purified strain of the ERA H (ERA H pp), supplied by Dr. R.B. Stewart of Queen's University, Kingston, Ontario. The challenge virus was a standard challenge virus (CVS) originally obtained from the National Institutes of Health, Bethesda, Maryland.

The vaccine viruses were propagated in BHK-21 13S cell line grown in modified Earle's medium supplemented with 2% fetal calf serum, 10% tryptose phosphate broth, 1% L-glutamine, 3% HEPES buffer with pH adjusted to 7.0-7.3 with 7.5% sodium bicarbonate. Potency of viral harvests as determined by intracerebral inoculation of mice was $10^{7.0}$ to $10^{8.0}$ median mouse intracerebral lethal dose (MICLD50) per mL. Potencies of the inactivated viral fluids as determined by the NIH test were 2.1 to 34.2 international units per mL.

Virus was inactivated by the addition of betapropiolactone to a final dilution of 1:2500 and placed at room temperature for 4 h after which the virus was stored at 4-8° C. Tests to show that the virus had been inactivated consisted of inoculating ten mice intracerebrally with 0.03 mL of undiluted and 10^{-1} dilution of the vaccine. In addition, this procedure was repeated on fluids from the third tissue culture back passage of the inactivated vaccine. The back passage consisted of inoculating ten 150 cm^2 plastic flasks (AHS Canada, Mississauga, Ontario) of confluent BHK-21 cell cultures for each passage. The cells were grown to confluency at which time the medium was removed, cells washed with phosphate buffered saline (PBS) (12) pH 7.5 with the calcium and magnesium removed, after which the cell sheet was flooded with 10 mL of the inactivated vaccine or passage fluid containing 1% gelatin and 100 μg/mL of diethylaminoethyl (DEAE)-Dextran (Pharmacia Fine Chemicals, AB Uppsala, Sweden). The cells were incubated for 45 min at 34° C after which the seeding material was poured off, the cells washed with PBS without calcium and magnesium and replaced with maintenance media, and incubated at 34° C. Harvests of the back passages were made after three days incubation at which time the fluids from the ten flasks were pooled. This procedure was repeated until the three back passages had been made.

Rabies virus was concentrated by adding aluminum phosphate, 6 mg per mL, to inactivated rabies viral fluids, mixing and allowing to settle for 48 h at 4° C. The supernatant was removed and the sediment centrifuged for 30 min at 3000 g at 4° C. To the sediment an equal volume of 0.6 M sodium phosphate, pH 8.0, was added, shaken for 1 h at room temperature and then centrifuged at 3000 g at 4° C. The eluate was adjusted to pH 7.4 and 1% gelatin and 2% fructose were added as stabilizer. The fluids were further concentrated approximately threefold in an Amicon HIP-100 ultra filter. The concentrate was then centrifuged 1 h at 40,000 rpm in MSE A1 40 rotor at 30° C. The sediment was suspended in 0.05 M triethylamine hydrochloride, 0.001 M ethylenediaminetetraacetic acid, 0.15 M sodium chloride buffer pH 7.5 (TEN). The concentration achieved by volume reduction was 67.9 times and the NIH value of the concentrate was 34.2 IU/mL.

Mouse potency tests to determine live virus content of samples were performed by the method described by Koprowski (13). A rapid fluorescent antigen test (RFAT) for potency was

TABLE II. Anamnestic Response of Foxes Previously Immunized with Inactivated Rabies Antigen by Intramuscular Route to Intestinal Administration of Inactivated Rabies Vaccine

Fox No.	Antibody Results FIMT[a]						
	Primary Vaccination[b]			Booster[c]			
	Vaccine Strain	Days Postvacc. 0	Days Postvacc. 28-59	Vaccine Strain	NIH Value	Days Postbooster 0	Days Postbooster 7
237	ERA H	0	0.37	ERA H	8.1	0	0.98
238	ERA H	0	0.59	ERA H		0.20	0.71
239	ERA H	0	0.26	ERA H		0	6.55
240	ERA H	0	0.35	ERA H		0.17	0
241	ERA H	0	0.96	ERA H		0.44	0
242	ERA H	0	0.22	ERA H pp	3.2	0.22	11.75
243	ERA H	0	0.26	ERA H pp		0.17	0.41
244	ERA H	0	0.89	ERA H pp		0	1.51
245	ERA H	0	0.20	ERA H pp		0	0.20
246	ERA H	0	0.30	ERA H pp		1.7	2.84

[a]Recorded as International Units per mL. Titers < 0.17 IU are considered negative and < 0.35 IU nonprotective
[b]1 mL inactivated rabies vaccine, NIH value 4.5 IU/mL administered intramuscularly 8-16 weeks of age
[c]10 mL inactivated rabies vaccine by fiberscope, 192-225 days postvaccination

TABLE III. Rabies Serum Neutralizing Antibody Response in Foxes Vaccinated with Inactivated Rabies Vaccine by Fiberscope or Indwelling Catheter

Trial	Route	Antibody Response (FIMT)[a] Number Seroconverting/Number on Test Prevacc. Day 0	Prebooster Day 21	Postbooster Day 35
1[b]	Fiberscope	0/8	1/8	2/8 (25)
	Indwelling catheter	0/8	0/8	2/8 (25)
	Controls	0/8	0/8	0/8
2[c]	Fiberscope	0/6	0/6	2/6 (33)
	Indwelling catheter	0/6	0/6	2/6 (33)
	Controls	0/6	0/6	0/6 (0)

(percent)
[a]At least 0.35 IU/mL
[b]Dose 10 mL of antigen (3.7 IU/mL)
[c]Dose 10 mL of antigen (5.2 IU/mL)

performed according to the method described by Abreo (14), the results of which closely approximate those of the mouse test. The NIH Test was carried out according to the method described by Seligmann (15). The diluent used to make viral dilutions for live virus potency test was sterile saline containing 2.5% normal horse serum, 500 units of penicillin and 1 mg streptomycin/mL and for the inactivated NIH potency test, the diluent was phosphate buffered saline (PBS) pH 7.5.

Enteric coated tablets used in some of the studies were prepared using a hand operated pill press. The tablet consisted of 50% Hypaque (diatrizoate (de) sodium, Winthrop Laboratories, Aurora, Ontario), and 50% tablet mix (Novopharm, Toronto, Ontario). Tablets were prepared in a 3 mm and 4 mm size and were spray-coated with an air brush (Thayer and Chandler, Chicago, Illinois) in a specially designed cylinder. The coating consisted of 10% cellulose acetate phthalate (CAP) in acetone. Tablets produced by this method withstood dissolution in fox stomach contents *in vitro* for 4 h at 39° C.

PROCEDURES

The vaccine was introduced into the intestinal tract using a fiberscope by a method which has been previously described in detail (7). In brief, the inactivated virus was delivered to the duodenum by catheter threaded through a fiberscope which had been introduced into the stomach of a fox following appropriate anesthesia, antiemetic and antisecretory treatments.

Vaccine was also introduced into the lumen of the duodenum by an indwelling catheter (16). The introduction of the catheter AHS 2.7 mm feeding tube 38 cm long (McGraw Supply Ltd., Mississauga, Ontario) with plastic cap attached, was performed under methoxyflurane (Pitman-Moore Ltd., Mississauga, Ontario) and was securely fixed in the bowel. The delivery part of the tube was fixed under the skin with the plastic cap positioned in the inguinal region. Vaccine was introduced by syringe and hypodermic needle through the skin and into the tube through the plastic cap. After delivery of the vaccine, a 3 mL saline flush was used to clear the needle before withdrawal.

TEST SAMPLES

Blood samples were collected from the jugular vein of foxes after intramuscular administration of 0.5 to 1 mL Ketaset (rogar/STB, London, Ontario) containing 100 mg of ketamine HCl/mL. Samples were collected before and at various time periods after vaccination. Sera were stored at -20° C until tested for rabies serum neutralizing antibodies by the modified rapid fluorescent focus-forming inhibition test, referred to as the fluorescence inhibition microtest (FIMT) (17), and results recorded as international units (IU) per mL. A titer of 0.35 IU was considered as protective against a challenge with virulent virus (personal experience).

STATISTICAL METHODS

Fifty percent end-points for virus titrations and inactivated antigen values were calculated according to the method of Reed and Muench (18).

RESULTS

Table I shows the composite results obtained on six trials, carried out over a three year period, in which inactivated rabies antigen (ERA H or ERA Hpp) with a NIH titer of at least 2 IU per mL, was administered in two doses to the duodenum, with at least a 21 day interval. Six of 79 foxes (8%) in the trials developed antibody after the initial dose of vaccine. The number seroconverting increased to 24 of 79 foxes (30%) after a booster dose with the same vaccine. Twenty-two percent had titers greater than 0.35 IU. There was no correlation between potency of the vaccine used and degree of seroconversion.

The production of antibody after administering rabies antigen to the intestinal tract was shown in another experiment. In a trial to determine the age of immunological competence, ten fox pups (8 to 16 weeks of age) had each received 1 mL of inactivated rabies antigen by intramuscular

TABLE IV. Results Obtained on Challenge of Mice Previously Vaccinated With Inactivated Rabies Vaccine Diluted in Fox Stomach Contents, Intestinal Contents or Diluent

Antigen[a]	Number Dead/ Number Challenged[b]	% Protected
Rabies antigen + fox stomach contents	10/10	0
Rabies antigen + fox intestinal contents	9/10	10
Rabies antigen + rabies diluent	2/10	80
Controls	10/10	0

[a]Rabies antigen (NIH value 4.1 IU/mL), 1/10 dilution, 1 h at room temperature, 0.05 mL/mouse intramuscularly
[b]CVS challenge 0.05 mL intramuscularly

TABLE V. Results Obtained when Rabies Live Virus[a] was Diluted in Fox Stomach Contents, Intestinal Contents and Bile

Trial	Diluent	Potency Titers FAT/mL (log10) Dilutions of Diluent				
		Undil	1/5	1/25	1/125	1/625
1	Stomach contents	< 2.0	3.3	5.29	N.D.	
	Intestinal contents	< 2.0	< 2.0	< 2.0	< 2.0	
	Rabies diluent (control)	6.1				
2	Fox bile	< 2.0	< 2.0	5.8	6.3	6.4
	Rabies diluent (control)	6.5				

[a]Virus is diluted 1/5 and incubated 1 h at 39°C

inoculation. All animals responded although responses were low. At 192 to 225 days after inoculation, the pups were bled and a second dose of inactivated antigen was administered by fiberscope to the duodenum of each animal. The animals were bled seven days after the booster dose. The prebooster and postbooster sera were evaluated for antibody in the same test, the results of which are shown in Table II. Seven of the ten animals showed an anamnestic response after the intestinal instillation of rabies antigen. Two animals did not respond, one animal, number 241, showed no antibody, but did show a level of 0.54 IU on a preliminary screening test. One animal recorded a slightly higher value after the booster. These results show that inactivated rabies antigen placed in the duodenum of foxes produced an anamnestic response.

The administration of antigen by fiberscope required: (i) the animal be taken off food and water for 24 and 16 h, respectively, prior to treatment, (ii) be anesthetized and (iii) receive antiemetic and antisecretory drugs. It was postulated that this treatment may have favorably influenced the response of the animal to the rabies antigen in the trials described. To investigate this possibility, two trials were initiated in which animals received ERA Hpp antigen by fiberscope using the treatment described or by indwelling catheters under light sedation. The results obtained in these trials are recorded in Table III and show that 25-33% of the foxes receiving two doses of antigen in the duodenum either by fiberscope or indwelling catheter developed antibody. It was concluded that the pretreatment required for fiberscope administration did not unduly influence the immune response of foxes.

The activity of antigen in the intestinal tract is complicated by the presence of gastric and intestinal juices which contain substances such as acid, enzymes and bile which may be detrimental to the antigen. Trials were carried out to determine the destructive effect of fox gastrointestinal contents to rabies antigen. In this trial 1 mL of inactivated rabies antigen with an NIH value of 4.1 was diluted 1/10 in either fox stomach contents, intestinal contents or rabies diluent as a control. After 1 h at room temperature each preparation was inoculated at a dose of 0.05 mL intramuscularly into each of ten mice. Twenty-one days later the inoculated mice, along with uninoculated control animals, were challenged with 8 LD_{50} of CVS virus intramuscularly in a 0.05 mL dose. The results (Table IV) show that both stomach and intestinal contents are destructive to the rabies antigen.

In order to determine the destructive effect of stomach and intestinal content to live rabies virus, virus was added to make a final dilution of 1/5 to dilutions of fox bile, stomach contents, intestinal contents and rabies diluent. The mixtures were incubated for 1 h at 39°C, the body temperature of the fox, after which potency tests were carried out by RFAT. Table V shows that intestinal contents were the most destructive to the live rabies virus.

Trials to identify compounds which would neutralize the antiviral activity of fox intestinal contents *in vitro* identified Questran (Bristol Laboratories, Belleville, Ontario) containing 0.44 g of cholestyramine resin per gram and soybean trypsin inhibitor (Worthington Biochemical Corp. Freehold, New Jersey) as most promising. Table VI shows a checkerboard titration of these compounds with fox intestinal contents. Neither Questran nor soybean trypsin inhibitor alone neutralized the antiviral activity of fox intestinal contents, but when combined in the correct proportions, reduced this activity significantly. When DEAE Sephadex (Pharmacia, Uppsala, Sweden), DEAE cellulose (Sigma Chemical Company, St. Louis, Missouri) and Questran were used to neutralize the antiviral activity of fox bile, Questran proved to be the most promising (Table VII). DEAE cellulose in a concentration of 400 mg/mL reduced the antiviral activity of stomach contents (Table VIII).

In order to investigate an alternative system for delivery of antigen to the intestinal tract, enteric coated tablets, 3 mm and 4 mm in size, were prepared as described. The animals were taken off food for 19-23 h after which the pills were administered either in a 30 g hamburger bolus or by stomach tube under Ketaset anesthesia. Some animals were allowed to consume food after the administration of the tablets. The animals were examined by fluoroscopy at 3 to 11 h after administration of the tablets. Table IX shows the results obtained in this trial. Only those tablets 3 mm in size, administered by stomach tube and with food withheld before and after administra-

TABLE VI. Potency of Rabies Virus in Fox Intestinal Contents Containing Various Amounts of Questran and Soybean Trypsin Inhibitor

Questran mg/0.4 mL	Results RFAT/mL (log 10) Soybean Trypsin Inhibitor mg/0.4 mL				
	0	0.5	2.5	12.5	Control
0	< 2.0	< 2.0	< 2.0	< 2.0	6.4
5	< 2.0	3.3	4.1	< 2.0	
10	< 2.0	4.9	5.0	4.4	
20	< 2.0	5.2	5.2	5.1	

TABLE VII. Potency of Rabies Virus in Fox Bile Containing DEAE Sephadex, DEAE Cellulose and Questran

Additive 10 mg/0.4 mL	Results FAT/mL (log10) Fox Bile Dilution Undil	1/5	1/25	1/125	1/625	Control
None	< 2.0	< 2.0	4.8	5.3	5.4	5.5
DEAE Sephadex	< 2.0	< 2.0	< 2.0	5.3	4.9	
DEAE Cellulose	< 2.0	< 2.0	5.7	5.6	5.4	
Questran	< 2.0	4.6	5.0	5.6	5.4	

tion of tablets, moved freely into the intestinal tract. Tablets given with food remained in the stomach for at least 11 h. It was concluded that with food the delivery system of antigen would require preparation of microcapsules or tablets smaller than 3 mm in size in order to prove satisfactory.

DISCUSSION

From the work described, there is good evidence that inactivated rabies viral antigen placed directly into the duodenum of foxes can, under certain conditions, provoke an immune response as shown by the development of rabies serum neutralizing antibody. The fact that seven of ten animals showed an anamnestic response when a booster dose of inactivated rabies antigen was placed in the intestine is further evidence that the antigen can act as an immunogen in that location.

The stomach and intestinal contents were shown to be detrimental to rabies virus and antigen. This activity could be neutralized to some degree *in vitro* by the addition of chemicals such as Questran, DEAE cellulose and soybean trypsin inhibitor. Further studies are required to determine if antiviral activity can also be reduced or controlled *in vivo*.

Studies showed that small enteric coated tablets did not move freely into the intestine after being administered in a hamburger bolus. The preparation and administration of microcapsules should be considered. Many enteric coatings such as cellulose acetate phthalate (CAP), polyvinyl acetate phthalate (PVAP) and methylacrylate polymers (Eudragit) required solvents that are destructive to the rabies antigen, although some aqueous enteric coatings are now available (T. Reynolds, Novopharm, Toronto, Ontario).

Although these studies showed that it was possible to produce rabies serum neutralizing antibodies in foxes when an inactivated rabies antigen was placed directly into the duodenum, the number of animals seroconverting was low and two doses of antigen were required. This method of immunizing wildlife against rabies requires further study. Conjugation of antigen to other compounds to enhance absorption, neutralization of destructive elements of the gastrointestinal tract and development of a suitable vehicle to deliver antigen to the intestinal tract should be investigated.

A more promising approach to the control of rabies in the field, which we are currently pursuing, is the oral administration, in a bait, of the ERA® strain of attenuated live rabies virus propagated in primary porcine kidney tissue culture (5) or in a BHK-21 C13 cell line.

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TABLE VIII. Protective Action of DEAE Cellulose and Questran on Fox Stomach Contents to Live Rabies Virus

Fox Stomach Contents mL	Rabies Diluent Control mL	Additive Cellulose mg/mL	Additive Questran mg/mL	Incubation Period	Virus mL	Incubation Period	Results[a] # Dead/ # Inoculated
1	—	100	—	1 hour	0.5	1 hour	4/9
1	—	200	—	at	0.5	at	4/9
1	—	400	—	22-25°C	0.5	39°C	10/10
1	—	—	10	↓	0.5	↓	0/10
1	—	—	100		0.5		0/10
1	—	—	200		0.5		0/10
—	1	200	—		0.5		10/10
—	1	400	—		0.5		10/10
—	1	—	10		0.5		10/10
—	1	—	100		0.5		10/10
—	1	—	200	▽	0.5	▽	10/10

[a]Preparation was diluted to 10^{-2} for inoculation of 15-16 gram mice, 0.03 mL intracerebrally

TABLE IX. Passage of Enteric Coated Tablets into the Intestinal Tract of Foxes

Administration of Tablets[a]				Number of Tablets in Stomach Postadministration											
	Treatment		Fox	3 Hours		4 Hours		6 Hours		7 Hours		10 Hours		11 Hours	
Procedure	Pre	Post	Number	4 mm	3 mm	4 mm	3 mm	4 mm	3 mm	4 mm	3 mm	4 mm	3 mm	4 mm	3 mm
Stomach tube	off feed	not fed	666	3	2	3	2	0	0	0	0				
	23 h		800	3	2	3	0	3	0	3	0				
			736	3	2	2	2	2	0	2	0				
			Total	9	6	8	4	5	0	5	0				
Bait[b]	off feed	not fed	1012	ND	ND	ND	ND	ND	ND	3	3	3	3	3	3
	19 h		1010	ND	ND	ND	ND	ND	ND	3	3	3	3	3	3
			1009	ND	ND	ND	ND	ND	ND	3	3	3	3	3	3
			Total							9	9	9	9	9	9
Bait[b]	off feed	fed	1016	ND	ND	ND	ND	ND	ND	3	3	3	2	2	2
	19 h	pellets	1004	ND	ND	ND	ND	ND	ND	3	0	3	0	3	0
			792	ND	ND	ND	ND	ND	ND	3	3	3	3	3	3
			Total							9	6	9	5	8	5

[a]Each animal received 3 x 4 mm tablets and 3 x 3 mm tablets
[b]Bait was 30 gram hamburger bolus
ND = Not done — previous trials showed that 4 mm and 3 mm tablets did not move from stomach by seven hours

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