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SHORT COMMUNICATIONS

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Oral Efficacy of an Attenuated Rabies Virus Vaccine in Skunks and Raccoons

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ABSTRACT: Raccoons and skunks are major rabies reservoirs in North America. Oral vaccination is one method to consider for disease control in these carnivores. Under field conditions in the USA, only one oral rabies vaccine has been used. It is efficacious in wildlife such as raccoons (*Procyon lotor*), coyotes (*Canis latrans*), and foxes (*Vulpes vulpes*) but not in skunks (*Mephitis mephitis*). The objectives of this study were to evaluate an attenuated SAG-2 rabies virus vaccine for safety, immunogenicity, and efficacy by the oral route in skunks and raccoons. Two of five skunks and three of five raccoons developed virus neutralizing antibodies (VNA) by day 14 following oral administration of SAG-2 vaccine. All animals remained healthy. Upon challenge, naive controls succumbed to rabies. Among vaccinated animals, four of five skunks and all five raccoons had VNA on day 7 post-challenge and all survived. Given these results, SAG-2 is a promising candidate vaccine that may satisfy both safety and efficacy concerns for oral rabies immunization of major North American rabies reservoirs.

Key words: *Mephitis mephitis,* oral vaccination, *Procyon lotor,* rabies, rabies vaccine, raccoon, skunk.

Major reservoirs of rabies in the USA include raccoon (*Procyon lotor*) and striped skunk (*Mephitis mephitis*). Raccoon rabies affects all eastern seaboard states from Florida to Maine, and the epizootic has recently extended into Ontario and New Brunswick, Canada, and further inland within Alabama, West Virginia, Vermont, and Ohio (Krebs et al., 2000). In the late 1970s, the raccoon rabies epizootic was exacerbated because of animal translocation (Nettles et al., 1979). One million square km with a human population of over 90 million are now affected by raccoon rabies (Rupprecht and Smith, 1994; Rupprecht et al., 1995, 1996; Hanlon and Rupprecht, 1998). Rabies in skunks is widespread throughout the Americas (Charlton et al., 1991). In the USA, the total geographic area affected by skunk rabies is at least 3.5 million square km or nearly 40% of the entire contiguous lower 48 states (Krebs et al., 2000). In addition, skunks are the most commonly affected secondary species in the raccoon-rabies-enzootic area. The number of rabid skunks infected with raccoon rabies virus in this latter region sometimes surpasses the number of rabid raccoons. The observation of high numbers of rabid skunks in the eastern USA has led to speculation that this species may be capable of limited perpetuation of raccoon rabies virus, although neither field nor experimental data are as yet adequate for analysis.

Although oral vaccination of red foxes (*Vulpes vulpes*) has been practiced for several decades in Europe and Canada, oral vaccination of raccoons and skunks would extend this method of disease control to important North American rabies reservoir species. A vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine is licensed for oral rabies vaccination of raccoons and has been employed in an array of pilot programs at strategic epizootic fronts and in a few enzootic areas (Hanlon and Rupprecht, 1998). However, the V-RG vaccine is not effective by the oral route in skunks (Charlton et al., 1992). Moreover, at least one modified live rabies virus vaccine used for oral vaccination of red foxes (*Vulpes vulpes*), with demonstrated potential for immunization of raccoons (Rupprecht et al., 1989), resulted in vaccine-induced rabies in skunks (Rupprecht et al., 1990).

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In this study, a highly attenuated rabies virus vaccine, SAG-2, was evaluated by the oral route in skunks and raccoons. The SAG-2 rabies virus is a double mutant of the SAD-Bern fixed rabies virus strain that was derived under the neutralizing pressure of monoclonal antibodies resulting in two nucleotide changes at glycoprotein codon 333 (Flamand et al., 1993), a key site implicated in virulence (Dietzschold et al., 1983). In contrast to traditional modified-live rabies virus vaccines, the SAG-2 virus does not cause rabies when inoculated intramuscularly and intracerebrally in adult laboratory mice. The objectives of this study were to describe the clinical effects of the oral SAG-2 vaccine, measure the induction of rabies virus-neutralizing antibodies (VNA), and determine vaccine efficacy against lethal rabies virus challenge in skunks and raccoons.

Twenty adult striped skunks and 15 adult raccoons were obtained from a commercial source (Ruby's Fur Farm, New Sharon, Iowa, USA) and were quarantined for a minimum of 30 days for observation of general health. The animals were individually caged and offered cat chow (Lab Feline Diet #5003, PMI Nutrition International, Inc., Brentwood, Missouri, USA) and water ad libitum. When handled, skunks and raccoons were sedated with a mixture of 20 mg/kg ketamine hydrochloride (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA) and 0.4 mg/kg xylazine hydrochloride (Mobay Corporation, Animal Health Division, Shawnee, Kansas, USA). All animal care and experimental procedures were performed in compliance with the Centers for Disease Control and Prevention (CDC) Institutional Animal Care and Use Guidelines.

Levels of rabies VNA were determined by the rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1996). All raccoons and skunks were negative for detectable rabies VNA by the RFFIT at the beginning of the study. For initial titration of skunk rabies virus for rabies challenge, 10 adult skunks were sedated and inoculated in the right masseter muscle with 0.5 ml of dilutions of a salivary gland homogenate from a naturally infected skunk from California (virus identification R98-0100 AB; original titer $10^{6.3}$ mouse intracerebral lethal dose 50% [$MICLD_{50}$]/ml). With the formulation of three serial dilutions in 2% horse serum in phosphate buffered saline (PBS), three skunks received $10^{4.7}$ $MICLD_{50}$, four skunks received $10^{5.0}$ $MICLD_{50}$, and three skunks received $10^{6.0}$ $MICLD_{50}$ in a 0.5 ml volume.

The three skunks receiving $10^{4.7}$ $MICLD_{50}$ developed signs of rabies on days 16, 21, and 25 after inoculation and were euthanized. The four skunks receiving $10^{5.0}$ $MICLD_{50}$ developed signs of rabies and were euthanized on days 15, 16, 17, and 25. The three skunks receiving $10^{6.0}$ $MICLD_{50}$ developed signs of rabies and were euthanized on days 13, 15, and 16. Rabies was confirmed in all 10 skunks by the direct fluorescent antibody test (dFA) on fresh brain tissue samples (Velleca and Forrester, 1981). Similarly, a titration of a raccoon salivary gland pool from naturally infected animals (virus identification 6545; original titer $10^{6.2}$ $MICLD_{50}$/ml) in five raccoons indicated that 100% mortality could be achieved by the administration of 0.5 ml in the masseter muscle of naive raccoons at a dilution of 1:10 in 2% horse serum ($10^{4.9}$ $MICLD_{50}$ per raccoon).

The SAG2 vaccine (Batch number: RS2 007, VIRBAC Laboratories BP27-06511 Carros, Cedex, France) was frozen at −80 C until use. Immediately prior to administration, the preparation was thawed and diluted with minimal essential media to achieve a titer of $10^{9.0}$ tissue culture infectious doses (TCID)/ml.

Five skunks and five raccoons were sedated and given 1.0 ml of vaccine ($10^{9.0}$ TCID/animal) per os via a syringe without a needle. The animals were observed daily for adverse effects. Five naive skunks and five naive raccoons were included as control animals. On a routine schedule (Tables 1, 2), blood samples (2–3 ml) were ob-

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TABLE 1. Response of skunks to oral SAG-2 vaccination and rabies virus challenge.

		Rabies virus neutralizing antibody titers[a]					
		Days after oral vaccination			Days after rabies challenge[b]		
Skunk	Group	Day 7	Day 14	Day 21	Day 0	Day 7	Outcome
11	SAG-2	<5	9	10	60	1300	Survived
12	SAG-2	<5	<5	<5	<5	125	Survived
13	SAG-2	<5	50	50	50	440	Survived
14	SAG-2	<5	<5	<5	<5	19	Survived
15	SAG-2	<5	<5	<5	<5	<5	Survived
16	Control	<5	<5	<5	<5	<5	Rabid
17	Control	<5	<5	<5	<5	<5	Rabid
18	Control	<5	<5	<5	<5	<5	Died[c]
19	Control	<5	<5	<5	<5	<5	Rabid
20	Control	<5	<5	<5	<5	<5	Rabid

[a] Titers were determined using the rapid fluorescent focus inhibition test and reported in reciprocal values of the dilutions calculated for 50% reduction in fluorescent foci.

[b] Skunks received 0.5 ml of a salivary gland homogenate from a naturally-infected skunk from California (virus identification R98-0100 AB) diluted 1:10 with 2% horse serum in PBS and inoculated in the right masseter muscle ($10^{5.0}$ $MICDLD_{50}$/skunk) 30 days following vaccination.

[c] Skunk 18 died following sedation for a routine cage change on day 83 after rabies virus challenge. It was negative for rabies virus infection by the direct fluorescent antibody test.

tained from the jugular veins of all control and vaccinated skunks and raccoons while sedated.

At 30 days after oral vaccination, the five vaccinated and five control animals of each species were sedated and inoculated with 0.5 ml of virus in the right masseter muscle with either the skunk rabies virus isolate ($10^{5.0}$ $MICLD_{50}$/0.5 ml) from California for skunks or the raccoon salivary gland pool ($10^{4.9}$ $MICLD_{50}$/0.5 ml) for raccoons.

No adverse effects due to vaccination were observed among the vaccinated skunks and raccoons during the 2,775 total animal-days of observation (five raccoons and five skunks observed for 30 days after vaccination and prior to challenge and five skunks for 172 days post-challenge and

TABLE 2. Response of raccoons to oral SAG-2 vaccination and rabies virus challenge.

		Rabies virus neutralizing antibody titers[a]			
		Days after vaccination	Days after rabies challenge[b]		
Raccoon	Group	Day 14	Day 0	Day 7	Outcome
223	SAG-2	25	210	>1400	Survived
225	SAG-2	250	145	145	Survived
202	SAG-2	13	7	250	Survived
204	SAG-2	<5	<5	65	Survived
201	SAG-2	<5	<5	40	Survived
217	Control	<5	<5	5	Rabid
207	Control	<5	<5	<5	Rabid
215	Control	<5	<5	<5	Rabid
219	Control	<5	<5	<5	Rabid
221	Control	<5	<5	<5	Rabid

[a] Titers were determined using the rapid fluorescent focus inhibition test and reported in reciprocal values of the dilutions calculated for 50% reduction in fluorescent foci.

[b] Raccoons received 0.5 ml of a salivary gland homogenate pool from naturally-infected raccoons (virus identification 6545) diluted 1:10 with 2% horse serum in PBS and inoculated in the right masseter muscle ($10^{4.9}$ $MICLD_{50}$/raccoon) 30 days following vaccination.

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five raccoons for 323 days post-challenge) for both skunks and raccoons. Two of five vaccinated skunks developed rabies VNA by day 14 post-vaccination (Table 1). In raccoons, three of five vaccinated animals had rabies VNA by day 14 post-vaccination (Table 2).

Following rabies virus challenge, four of five vaccinated raccoons developed an apparent anamnestic response (i.e., greater than four fold rise in antibody titer) by 7 days post-challenge (Table 2) and all survived. Within 18 days of challenge, all five control raccoons succumbed to rabies. All vaccinated raccoons survived lethal rabies virus challenge and remained clinically normal for 323 days post-challenge (304 days past the last death due to rabies). Upon euthanasia, all survivors were confirmed to be negative for rabies virus infection by the dFA test.

Similarly, following rabies virus challenge, four of five control skunks succumbed to rabies on days 16 (two skunks), 25, and 78 post-challenge. The remaining animal died following sedation for a routine cage change on day 83 of the study; it was negative for rabies virus antigen. Four of five vaccinated skunks had detectable rabies VNA on day 7 post-challenge, suggestive of an anamnestic response (Table 1), and all vaccinated skunks survived lethal rabies virus inoculation. Vaccinated skunks were held for 172 days post-challenge or 94 days past the last death due to rabies among control skunks. Upon euthanasia, all survivors were confirmed to be negative for rabies virus infection by the dFA test.

Following the initial demonstration of the potential for oral immunization of foxes with modified live rabies vaccines (Baer et al., 1971), control of wildlife rabies through oral vaccination targeting red foxes has been successfully practiced in Europe since 1978 (Steck et al., 1982; Schneider et al., 1988) and more recently in Canada (Johnston et al., 1988). Initially, these trials used various modified live rabies virus vaccines. Subsequently, a V-RG recombinant vaccine was used in some European countries because of its oral efficacy, lack of genetic capacity to cause rabies (i.e., only codes for the rabies virus glycoprotein), and increased thermostability in comparison to modified live rabies virus vaccines (Kieny et al., 1984; Brochier et al., 1996). Concurrent with the initial field efforts targeting red foxes, raccoons were found to be refractory to oral vaccination using modified live virus vaccines (Rupprecht et al., 1989), but the V-RG vaccine induced protection against rabies (Rupprecht et al., 1986, 1992). Following the first North American field release (Hanlon et al., 1998), the V-RG vaccine was employed in a number of pilot programs for efficacy assessment at strategic raccoon rabies epizootic fronts and in enzootic areas (Hanlon and Rupprecht, 1998). Additional field trials using the V-RG vaccine have been under way in the USA for coyotes (*Canis latrans*) and foxes (Fearneyhough et al., 1998; Hanlon and Rupprecht, 1998) but not skunks. In an initial study with the V-RG vaccine in skunks where approximately 10^9 plaque forming units were offered in baits, six of seven skunks developed rabies VNA and five survived lethal rabies virus challenge (Tolson et al., 1987). In another study, a modified live SAD B19 virus in skunks demonstrated the potential for residual pathogenicity by causing rabies in some of the vaccinated animals, particularly when it was intentionally administered intranasally (Rupprecht et al., 1990). Moreover, the potential pathogenicity of SAD Bern administered orally to chacma baboons (*Papio ursinus*) was previously reported (Bingham et al., 1992). Similarly, vaccine-induced rabies cases have been documented in a number of species including a cat, stone marten (*Martes foina*), calf, and fox cub due to oral vaccination campaigns using modified live rabies virus vaccines targeting red foxes in Europe and Canada (Wandeler, 1991).

The evolution of vaccine development from modified live vaccine viruses to safer

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highly attenuated mutants has been possible due to technical advances involving the incubation of virus with neutralizing monoclonal antibodies to select for epitopic escape mutants. Two highly attenuated mutants have been derived from SAD Bern. The SAG-1 virus has a one-nucleotide substitution in the codon for amino acid position 333 of the rabies glycoprotein that renders it apathogenic for adult mice by the intracerebral route (Coulon et al., 1983; Seif et al., 1985; Flamand et al., 1993). The SAG-1 vaccine has been used extensively in the field in Europe for oral vaccination of foxes (Aubert et al., 1994). Further genetic stabilization of the epitopic escape mutant was accomplished with the production of a double mutation at amino acid 333 that resulted in replacement of arginine with glutamate through coding by GAA at position 333. This differs from codons for arginine by two nucleotides, thus further reducing the risk of potential epitopic reversion to the original parental (i.e., pathogenic) form (Flamand et al., 1993). To date, the SAG-2 vaccine has been evaluated for efficacy in various species, such as foxes, jackals (*Canis adustus* and *C. mesomelas*), dogs, and mongoose (*Cynictis penicillata*) and evaluated for safety in a number of non-target animals including rodents and primates (Flamand et al., 1993; Schumacher et al., 1993; Fekadu et al., 1996; Follman et al., 1996; Masson et al., 1996; Bingham et al., 1997, 1999; Rupprecht et al., 1998; Hammami et al., 1999).

As previously observed in dogs receiving SAG-2 by the oral route (Fekadu et al., 1996; Rupprecht et al., 1998), animals may be protected from rabies without having demonstrated antibodies prior to challenge. For example, two of 20 dogs receiving SAG-2 liquid deposited on the tongue and one of 20 dogs consuming a vaccine-laden bait did not demonstrate seroconversion prior to challenge. Yet these three dogs seroconverted by day 7 after challenge and survived, thus demonstrating an anamnestic response reflective of priming from vaccination (Fekadu et al., 1996). In the second study (Rupprecht et al., 1998), among five of 13 dogs that did not seroconvert after receiving SAG-2 orally, only four succumbed to rabies, and the remaining dog demonstrated an anamnestic response by day 7 after challenge and survived. In addition, among five of 13 dogs that did not seroconvert following consumption of a bait containing lyophilized SAG-2 vaccine, two demonstrated anamnestic responses by day 7 after challenge and survived. In the current study, three of five skunks and two of five raccoons did not have detectable antibodies after vaccination and before the rabies virus challenge and yet survived. Overall, the sensitivity of a positive antibody response at any time following vaccine administration to adequately predict survivors in these studies was 92% (37/40) (Fekadu et al., 1996), 84% (16/19) (Rupprecht et al., 1998), and 50% (5/10) (this study), recognizing that the days of blood sampling, dose of vaccine, method of delivery, and different species, as well as time of administration and variant of challenge viruses, may confound a direct comparison. Not unexpectedly, the sensitivity of detection of antibodies on the day of challenge to predict survivorship was lower (63%; 12/19) in the second study (Rupprecht et al., 1998) because four previously seropositive dogs had no detectable antibody by the time the animals were challenged 6 wk post-vaccination. Nonetheless, it is clear that a detectable antibody response at some time post-vaccination was 100% predictive of survivorship in these studies. Conversely, a lack of detectable antibodies among vaccinates was from 0 to 30% (0%, 0/3; 30%, 3/10; and 0%, 0/5, respectively) specific for susceptibility to challenge in these studies.

The kinetics and composition of an adequate immune response to vaccination, and in particular by the oral route, remain an enigma. Although measurement of antibodies is the most reliable test currently available, it is not possible to identify all

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animals capable of surviving challenge based upon VNA status alone. Potential measures of an adequate immune response may include evidence for priming of T helper cells, non-neutralizing antibodies to internal rabies virus proteins (Fu et al., 1994; Lafon et al., 1994), and the induction of cytokines, among others. It is unclear if the lack of detectable neutralizing antibodies and yet protection from rabies virus challenge following oral vaccination with SAG-2 is a phenomenon unique to this avirulent mutant or if this is a more generalized phenomenon perhaps characteristic of highly attenuated modified live rabies virus vaccines. Attenuated mutants may enhance apoptosis, as observed with recombinant rabies viruses (Pulmanausahakul et al., 2001). This may in turn result in a different presentation and composition of viral antigens than just glycoprotein expressed on the cell surface of intact neurons as may predominate with attenuated fixed and street rabies virus infections. As with the V-RG virus (Rupprecht et al., 1988; Thomas et al., 1990), the SAG-2 virus is cleared rapidly from the oral cavity after consumption (Fekadu et al., 1996) and the tonsils appear to be the primary site of uptake and replication of SAG-2 (Orciari et al., 2001). It is possible that in comparison to modified live rabies viruses, the attenuated mutant rabies viruses are arrested by the immune system before high levels of rabies VNA are induced, yet the memory, or some other as yet undetermined, component of the immune system has been adequately primed, thus yielding a safe, potent vaccine that may not be easily assessed by conventional immunologic measures.

Although there are many questions about the basic mechanisms underlying oral vaccination against rabies (Baer et al., 1975), our preliminary observations reveal a potential vaccine candidate that may satisfy both safety and efficacy concerns for oral rabies vaccination of free-ranging wildlife, including skunks. Effective dose or minimum potency may be a critical factor in the comparison and assessment of this potential candidate to previous and ongoing studies with either conventional, fixed, or recombinant viruses. Future studies should include determination of the minimum potency when offered in baits to skunks, as well as its efficacy against other skunk rabies virus variants of public health importance.

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