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IMMUNE RESPONSE AND PROTECTION IN RACCOONS (*PROCYON LOTOR*) FOLLOWING CONSUMPTION OF BAITS CONTAINING ONRAB®, A HUMAN ADENOVIRUS RABIES GLYCOPROTEIN RECOMBINANT VACCINE

L. J. Brown,[1,3] R. C. Rosatte,[1] C. Fehlner-Gardiner,[2] J. S. Taylor,[1] J. C. Davies,[1] and D. Donovan[1]

[1] Wildlife Research and Development Section, Ontario Ministry of Natural Resources, Trent University, DNA Building, 2140 East Bank Dr., Peterborough, Ontario K9J 7B8, Canada
[2] Centre of Expertise for Rabies, Canadian Food Inspection Agency, 3851 Fallowfield Rd., PO Box 11300, Station H, Ottawa, Ontario K2H 8P9, Canada
[3] Corresponding author (email: lucy.brown@ontario.ca)

ABSTRACT: We investigated the immune response and protection conferred in raccoons (*Procyon lotor*) following consumption of ONRAB® oral rabies vaccine baits. Forty-two wild-caught, captive raccoons were each offered an ONRAB vaccine bait; 21 controls received no vaccine baits. Blood samples collected from all raccoons before treatment, and each week posttreatment for 16 wk, were assessed for the presence of rabies virus antibody. In the bait group, an individual was considered to have responded to vaccination if serum samples from three or more consecutive weeks were antibody-positive. Using this criterion, 77% (20/26) of raccoons that consumed ONRAB baits with no observed vaccine spillage (full dose) demonstrated a humoral immune response. In the group that received a partial dose (0.05–0.90 mL vaccine recovered), 50% (8/16) of raccoons responded to vaccination. Regardless of the vaccine dose received, among the 28 raccoons that responded to vaccination 18 had antibody initially detectable at week 2 and 22 remained antibody-positive for at least 10 consecutive weeks. Kinetics of the humoral immune response suggest that the best time to conduct postbaiting surveillance for evidence of vaccination would be 6–13 wk following bait deployment, with the highest antibody prevalence expected between weeks 8–10. A sub-sample of 29 raccoons (20 ONRAB, 9 controls) was challenged with raccoon rabies virus variant 350 days posttreatment. Eight of nine controls (89%) developed rabies whereas 15/20 vaccinates (75%) survived. Survival following rabies challenge was significantly higher in raccoons presented ONRAB vaccine baits.

Key words: Human adenovirus type 5, immune response, ONRAB®, oral rabies vaccination, *Procyon lotor*, rabies virus challenge, raccoon, raccoon rabies.

INTRODUCTION

Rabies is an infectious and often fatal viral encephalitis that affects wild and domestic mammals and humans. Although rabies has been largely controlled in developed countries by parenteral vaccination of dogs (*Canis familiaris*), there is still a threat to humans, pets, and livestock due to rabies virus reservoirs in wild animals. In North America, raccoons (*Procyon lotor*) are considered to be the reservoir and main vector of raccoon rabies, which is enzootic in the eastern United States and, in the past, was epizootic in the Canadian provinces of Ontario, Quebec, and New Brunswick (Rosatte et al., 2006; Slate et al., 2009).

Control of rabies in wildlife has emphasized the development of safe and effective vaccines as well as practical and economical methods of administering the vaccine to important rabies vector species (Rosatte, 2011). Unfortunately, vaccines that have proved efficacious for canids such as coyotes (*Canis latrans*) and red foxes (*Vulpes vulpes*; e.g., ERA, V-RG, SAD_{B19}, and SAG_1) have not worked as well in raccoons (Baer, 1988; Rupprecht et al., 1989; Slate et al., 2009). Some of these vaccines also had residual pathogenicity in nontarget species such as rodents (Winkler et al., 1976; Artois et al., 1992) and, in Ontario, the ERA vaccine was responsible for nine cases of vaccine-induced rabies over 15 yr (four red foxes, two raccoons, two striped skunks [*Mephitis mephitis*], and one bovine calf (*Bos taurus*; Fehlner-Gardiner et al., 2008). During oral rabies

vaccination (ORV) campaigns in eastern Ontario to control raccoon rabies, a vaccinia-virus–rabies glycoprotein recombinant vaccine (V-RG) was deployed in Ontario "Slim" baits (Artemis Technologies, Inc., Guelph, Ontario, Canada) (Rosatte et al., 2008). Although recombinant rabies vaccines have considerable potential for overcoming some of the safety concerns regarding the use of attenuated live vaccines, serology results from field (Rosatte et al., 2008) and captive (Brown et al., 2011) studies suggested this particular vaccine-bait combination was probably not immunizing enough raccoons to control the disease.

The potential for re-emergence of raccoon rabies in Canada and the continuing spread of the disease in the United States, together with the ubiquitous and prolific nature of raccoons, has driven the search for new oral rabies vaccines that are safe, economical, and effective in raccoons. Human adenoviruses are particularly suitable as vectors in the development of rabies vaccines for wildlife because they are safe, stable, and effective by the oral route (Knowles et al., 2009a, b). Developed in Ontario, Canada, AdRG1.3 (ONRAB®) is a new live recombinant oral rabies vaccine that consists of the rabies virus (ERA strain) glycoprotein gene inserted within a human adenovirus type 5 virus vector (Rosatte et al., 2009a).

Our objectives were to 1) quantify the longitudinal humoral immune response of raccoons after ingesting ONRAB oral rabies vaccine baits, and 2) evaluate whether antibody development in response to ONRAB was protective by challenging animals with a field isolate of the raccoon rabies virus variant (RRVV) 350 days postvaccination.

MATERIALS AND METHODS

Study animals

Sixty-three wild raccoons were live-trapped in rural areas of Niagara, Ontario, Canada (43°05′N, 79°10′W) between 13 June and 25 July 2007. Although RRVV has been confirmed in raccoons and skunks in New York State, there has never been a reported case in adjacent areas on the Ontario side of the Niagara River. The health of all raccoons was assessed upon arrival at the Ontario Ministry of Natural Resources (OMNR) Wildlife Research Facility, Codrington, Ontario (44°10′N, 77°48′W), and each was dewormed (Strongid*T; 50 mg/mL; Pfizer Animal Health, Quebec, Canada) and vaccinated against canine distemper (Galaxy*D; Schering-Plough Animal Health, Nebraska, USA) and parvovirus enteritis (Fel-O-Vax PCT; Fort Dodge, Iowa, USA). Raccoons were housed in separate units within outdoor enclosures (Brown et al., 2011) and were provided a commercial feed once a day and natural foods (apples, carrots, corn, and frozen fish) when available; water was available ad libitum. Enrichment items were also provided throughout the study (wooden logs, small tires, and cardboard milk containers containing dog biscuits). All animals were maintained in accordance with national guidelines (Canadian Council on Animal Care Guidelines [CCAC, 2003] or Guide for the Care and Use of Laboratory Animals [ILAR, 2011]), and protocols were approved by Institutional Animal Care and Use Committees (OMNR or US Centers for Disease Control and Prevention).

Vaccine baits and vaccination protocol

Ultralite baits containing 1.8 mL (±0.1 mL) ONRAB oral rabies vaccine (10^{10} $TCID_{50}$/mL) were made by Artemis Technologies Inc., Guelph, Ontario, Canada (Rosatte et al., 2009a). Each raccoon was randomly assigned to either a control or vaccination group with minor adjustments to ensure similar age-sex representation among the groups. Forty-two raccoons (22 adults, 20 juveniles) were each offered an ONRAB vaccine bait. Plastic sheets were placed below each cage to collect bait debris and vaccine spillage. The amount of bait ingested, vaccine loss during ingestion, and timing of ingestion were recorded for each raccoon. Twenty-one controls (10 adults, 11 juveniles) received no vaccine baits.

Blood collection

A blood sample (3–5 mL) was collected from each raccoon in the control and vaccination groups before oral vaccination and then each week after treatment for 16 wk. Animals were anesthetized by intramuscular (IM) injection of ketamine hydrochloride (HCl; 100 mg/mL, Animal Health Canada Inc., Belleville, Ontario, Canada) and medetomidine HCl (1 mg/mL, Pfizer Canada Inc.) into

the hind leg. Blood samples were collected from the subclavian or brachiocephalic veins into 10-mL Vacutainer® tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) and a weight-dependant dose of atipamezole HCl (5 mg/mL, Novartis Animal Health Canada Inc., Mississauga, Ontario, Canada) was administered IM to reverse the sedation. Blood samples were refrigerated at 4 C for up to 48 hr and centrifuged at 1,000 × G for 12 min at 4 C. Serum (1–2 mL) was collected and frozen until testing. Prior to testing, serum was thawed and then heat-inactivated at 56 C for 30 min.

Serologic analyses

A competitive enzyme-linked immunosorbent assay (C-ELISA) as described by Elmgren and Wandeler (1996) was used to detect rabies virus antibody (RVA) in serum samples from the immune response study. The C-ELISA measures the ability of a test serum to inhibit the binding of a neutralizing, glycoprotein-specific, peroxidase-labeled monoclonal antibody (mAb) to immobilized ERA virus. Results are expressed as the percent inhibition of binding of the mAb. Serum samples were considered positive for RVA if inhibition was ≥25%. At a cut-off of 25%, the C-ELISA had a sensitivity of 75% and a specificity of 92% when compared to a rabies virus neutralization assay with a positive threshold value of 0.5 IU/mL (Knowles et al., 2009b; Fehlner-Gardiner et al., 2012).

Preparation of rabies challenge virus

The virus inoculum was prepared from submandibular salivary glands collected from naturally infected rabid raccoons from Pennsylvania in 1989–1990. Virus was characterized as the RRVV by mAb and genetic analysis. Salivary glands from infected raccoons were homogenized in sterile sand using a mortar and pestle in Eagle's minimum essential medium (MEM) supplemented with 10% heat inactivated fetal bovine serum (MEM-10), containing 2,000 IU penicillin G and 1 mg streptomycin sulfate/mL (Gibco/Life Technologies, Grand Island, New York, USA) for a 20% suspension. The homogenate was clarified by centrifugation (900 × G for 10 min) and the supernatant was stored at −80 C. A pooled suspension was prepared and a final titration was made by intracerebral inoculations into 4-wk-old mice, yielding a concentration of $10^{4.4}$ 50% mouse intracerebral lethal dose ($MICLD_{50}$)/0.03 mL ($10^{5.9}$ $MICLD_{50}$/mL). On the day of challenge, the pooled suspension was thawed and diluted 1:10 in 2% horse serum in phosphate buffered saline.

Challenge of raccoons with rabies virus

A sub-sample of 30 raccoons (20 ONRAB, 10 controls) from the longitudinal serology study was chosen for rabies challenge. Animals in the control group were selected to give an equal age-sex representation. To evaluate whether antibody development in response to ONRAB was protective, most raccoons (19/20) selected for challenge from the vaccination group were considered positive responders based on results from the longitudinal serology study. A greater number of female raccoons were included in the ONRAB group due to lower antibody prevalence in adult males.

On June 16, 2008, the 30 raccoons selected for challenge were transported to the US Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. During the 30-day quarantine and throughout the challenge, raccoons were housed in stainless steel squeeze cages. Animals were fed once per day and had free access to water. At 350 days posttreatment, raccoons were anesthetised by IM injection of telazol® (tiletamine HCl 50 mg/mL, zolazepam HCl 50 mg/mL, Fort Dodge) into a hind leg. A blood sample (3 mL) was collected from the jugular vein and then the raccoon was inoculated with 0.5 mL of the challenge virus preparation ($1\times10^{4.9}$ $MICLD_{50}$) into each of the left and right masseter muscles. A second blood sample was collected 7 days postinfection. Animals were observed at least twice daily by four independent observers for clinical signs of rabies (paralysis, ataxia, acute behavioural change, lack of food consumption, hyper-salivation, vocalization, agitation, tremors, convulsions, unprovoked aggression). A score was assigned to each clinical sign and raccoons were humanely euthanized by intravenous administration of 1.5 mL euthanasia solution (Beuthanasia®-D, Schering-Plough) when a score of 9 was reached. Surviving animals were euthanized 60 days postchallenge. Serum samples were assayed at CDC for RVA using a modification of the rapid fluorescent focus inhibition test (RFFIT; Smith et al., 1996). Brains of all animals were tested for the presence of rabies virus antigens by the direct fluorescent antibody test as described by Dean et al. (1996).

Statistical analyses

Fisher's exact tests (FET) were used to test whether serologic response differed among age-classes and between sexes and to test

whether survival following rabies challenge differed among the control and ONRAB groups. A Mann-Whitney *U*-test was used to test the null hypothesis that there was no difference in the number of days until death among the control and ONRAB groups after rabies virus infection (Zar, 1999). The software STATISTICA© was used for all analyses (StatSoft, Tulsa, Oklahoma, USA).

RESULTS

Vaccine consumption by raccoons

No spilled vaccine was recovered for 26 of 42 raccoons offered ONRAB vaccine baits. For 14 raccoons, varying amounts of spilled vaccine were recovered (0.05–0.90 mL). Vaccine recovery information was missing for two raccoons. An animal was assumed to have received a full dose of ONRAB if no vaccine was recovered from the plastic sheeting. If vaccine was recovered, or if vaccine recovery information was incomplete, the dose was considered partial.

Antibody production in response to ONRAB oral rabies vaccine

Throughout the study, no vaccine-induced morbidity or mortality was observed among raccoons. All serum samples collected from raccoons prior to the study were negative for RVA. Although serum samples from 17 of 21 controls were antibody-negative throughout the serology study, sporadic serum samples from four controls had C-ELISA values above the positive threshold of 25% (Fig. 1a). However, the pattern of C-ELISA values obtained over 16 wk for the controls contrasted greatly with that of raccoons fed ONRAB vaccine baits (Fig. 1b).

Because the C-ELISA is not 100% specific, and given that a small proportion of the serum samples from the nonvaccinated controls had positive C-ELISA values, a raccoon was considered a responder (to vaccination) only if C-ELISA values ≥25% were observed for three or more consecutive weeks. This criterion was used previously in a longitudinal study of raccoon antibody response to parenteral

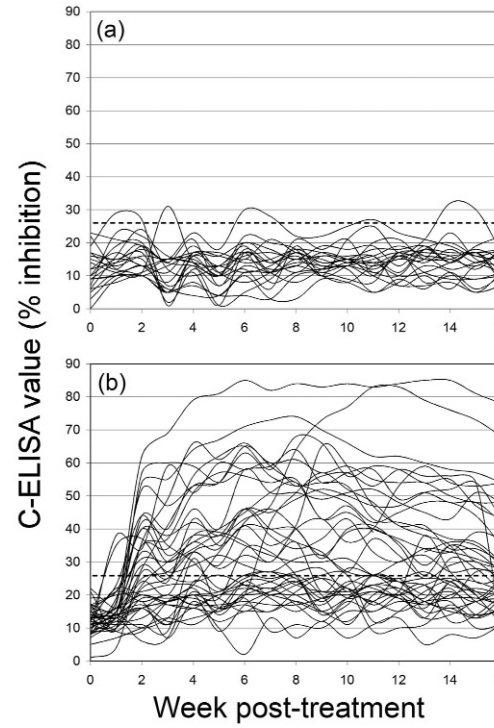

FIGURE 1. Longitudinal humoral immune response of raccoons in (a) control group and (b) group vaccinated orally via consumption of ONRAB oral rabies vaccine baits. Each line represents one raccoon. The hatched line at competitive enzyme-linked immunosorbent assay equal to 25% inhibition represents the cut-off value above which sera were considered positive for rabies virus antibodies.

administration of inactivated vaccine (Brown et al., 2011). Antibody development in the ONRAB group was assessed in two parts because some raccoons did not receive a full dose of vaccine during bait presentation. Seventy-seven percent (20/26) of raccoons that consumed ONRAB baits with no observed vaccine spillage (full dose) demonstrated a serologic response, and similar proportions of adults and juveniles responded positively to vaccination ($P=1.0$, FET; Table 1). Although females as a group responded slightly better than males, the difference was not significant ($P=0.6443$, FET). In the partial-dose group, eight of 16 raccoons (50%) showed serologic evidence of an immune response, with the response

TABLE 1. Number and proportion of raccoons with detectable antibody to rabies virus following consumption of ONRAB oral rabies vaccine baits.

Dose[a]	All raccoons	Adults	Juveniles	Females	Males
Full dose	20/26 (77%)	7/9 (78%)	13/17 (76%)	13/16 (81%)	7/10 (70%)
Partial dose	8/16 (50%)	6/13 (46%)	2/3 (67%)	7/9 (78%)	1/7 (14%)

[a] Full dose = no vaccine recovered during bait presentation; partial dose = any vaccine recovered or vaccine recovery information incomplete.

largely diminished by the absence of detectable RVA in adult males (0/6). The proportions of adults and juveniles that responded to vaccination in the partial-dose group were not significantly different ($P=1.0$, FET); however, the proportion of females responding to vaccination was significantly higher than for males ($P=0.0406$, FET; Table 1).

Combining results for both full- and partial-dose groups, most raccoons that responded to vaccination (18/28) had RVA initially detected at week 2 and, in 79% (22/28), detectable antibody was maintained for at least 10 consecutive weeks (Fig. 1b). At week 2, 58% (15/26) of raccoons in the full-dose group had detectable RVA compared to 31% in the partial-dose group (Fig. 2). Antibody prevalence in the full-dose group reached a peak of 77% (20/26) by week 6 postvaccination which was maintained through week 8. In the partial-dose group, antibody prevalence remained low through week 6 (31–38%) and increased to a peak of 50% (8/16) by week 8. Although antibody prevalence declined slightly in both vaccine groups after week 8, a higher proportion of positive responders was observed in the full-dose group (Fig. 2). In the last month of the study (weeks 13–16), antibody prevalence in the groups that received full and partial doses of ONRAB vaccine ranged from 60–69% and 38–44%, respectively. When data for all vaccinates were pooled, 55% of raccoons had RVA by weeks 4 and 5; antibody prevalence was highest at weeks 6 through 11 (57–67%) and declined only slightly in the last 5 wk of the study (51–57%; Fig. 2).

Challenge of study animals with RRVV

Eight control raccoons (89%) succumbed to rabies challenge as confirmed by FAT (Table 2). One adult male survived challenge despite the absence of detectable RVA throughout the longitudinal serology study. One raccoon in the control group was euthanized early in the challenge study because it bit a handler, and it is not considered in these analyses.

Fifteen of 20 raccoons (75%) in the ONRAB group survived rabies virus challenge (Table 2). Eleven of 16 raccoons (69%) in the group that received a full dose of ONRAB vaccine survived challenge. Ten of these animals responded to vaccination during the serology portion of the study

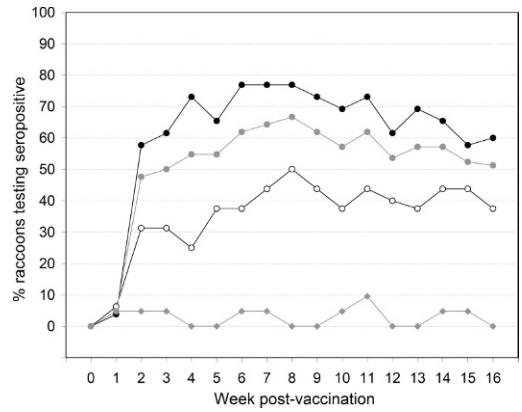

FIGURE 2. Kinetics of the humoral immune response for raccoons that received full (no vaccine recovered; $n=26$; closed circles) and partial (0.05–0.90 mL vaccine recovered or vaccine recovery information incomplete; $n=16$; open circles) doses of ONRAB oral rabies vaccine. Closed gray diamonds represent the proportion of controls antibody-positive ($n=21$); closed gray circles represent antibody prevalence for all vaccinates combined ($n=42$).

TABLE 2. Serologic response and survival of control and vaccinated raccoons following challenge with raccoon rabies virus variant. Vaccinates were each offered an ONRAB oral rabies vaccine bait. Vaccine dose was considered full (F) if no vaccine was recovered and partial (P) if vaccine was recovered or if vaccine recovery information was incomplete. Raccoons were challenged 350 days posttreatment.[a]

Control					ONRAB					
Rac	CWks ≥25%I	R0 (IU/mL)	R7 (IU/mL)	Challenge result[b]	Rac	Vaccine dose	CWks ≥25%I	R0 (IU/mL)	R7 (IU/mL)	Challenge result
1	0	0.04	0.05	D (14)	10	F	15	0.50	2.70	D (17)
2	0	0.04	0.05	D (17)	11	F	14	0.10	9.60	D (17)
3	0	0.04	0.05	D (17)	12	F	13	2.20	8.70	D (17)
4	0	0.04	0.05	D (17)	13	F	14	1.10	6.07	D (17)
5	0	0.04	0.05	D (20)	14	F	3	0.06	1.50	D (17)
6	0	0.04	0.05	D (20)	15	F	15	12.20	54.30	S
7	2	0.04	0.05	D (20)	16	F	15	2.20	20.90	S
8	0	0.04	0.05	D (25)	17	F	1	0.04	4.20	S
9	0	0.04	0.05	S	18	F	3	0.40	9.50	S
					19	F	15	2.40	15.20	S
					20	F	15	0.47	29.70	S
					21	F	15	2.80	11.40	S
					22	P	15	1.70	10.50	S
					23	F	7	0.04	3.40	S
					24	F	12	0.11	5.90	S
					25	F	15	2.40	15.20	S
					26	P	10	1.10	6.70	S
					27	P	15	0.04	1.70	S
					28	F	15	2.20	20.90	S
					29	P	3	0.05	1.20	S

[a] Abbreviations: Rac = raccoon identification; CWks ≥25%I = number of consecutive weeks with competitive enzyme-linked immunosorbent assay ≥25% inhibition; R0 = neutralizing antibody titer on day of infection; R7 = neutralizing antibody titer on day 7 postinfection; IU = International unit; D = died; S = survived.

[b] Day animal was humanely euthanized or found dead is shown in brackets.

and six had detectable neutralizing RVA on the day of challenge (range 2.2–12.2 IU/mL). All survivors, including the raccoon classed as a nonresponder, appear to have mounted an anamnestic response to rabies virus infection (range 3.4–54.3 IU/mL on day 7 postinfection). Five raccoons classed as responders to vaccination did not survive challenge, although three had detectable neutralizing RVA on the day of virus infection. All nonsurvivors also mounted an anamnestic response based on neutralizing antibody levels detected 7 days postinfection (range 1.5–9.6 IU/mL).

All four raccoons that received a partial dose of ONRAB were classed as vaccine responders and all survived rabies challenge. Two raccoons had detectable neutralizing RVA prior to virus infection (1.1 and 1.7 IU/mL) and all four mounted an anamnestic response based on serology results 7 days postinfection (range 1.2–10.5 IU/mL).

There was a significant association between treatment group and survival following challenge with RRVV ($P=0.0033$, FET), with higher survival in animals presented ONRAB vaccine baits. There was no significant difference between controls and vaccinates that succumbed to rabies in the number of days to death or humane euthanasia after rabies virus infection ($U=12.5$; $P=0.2073$). The median number of days to death, or exhibition of sufficient clinical signs to warrant humane euthanasia, was 17 (range 14–25).

DISCUSSION

Immunization of raccoons against rabies is an effective way to stop disease transmission among conspecifics and to other

wildlife species, livestock, pets, and humans and is most efficiently achieved over large geographic areas by deploying baits containing an oral rabies vaccine. Effective vaccination should stimulate production of antibodies that neutralize viral infectivity and ultimately provide protection from disease (Dietzschold et al., 1987; Bartlett et al., 2009). Our data support that ONRAB effectively stimulated the production of RVA in a high proportion of raccoons (67%) within the first 2 mo after vaccination, with RVA remaining detectable for an additional 2 mo. In those raccoons that responded to ONRAB, antibody was first detected between 1–7 wk, with the majority of responders first having antibody detectable at week 2. These response kinetics were similar to those observed in captive raccoons given IMRAB® 3 (inactivated whole-virus; Merial, Inc., Athens, Georgia, USA) intramuscularly or V-RG in Ontario "Slim" baits (Brown et al., 2011), two other vaccines that have been used extensively in North America for raccoon rabies control (Rosatte, 2011).

Not all raccoons consumed baits and vaccine similarly. This presented an opportunity to compare the immune response to vaccination under a best-case scenario (full dose) and where vaccine ingestion was considered to be less than optimal (partial dose). Raccoons that consumed ONRAB baits with no observed vaccine spillage demonstrated a rapid and sustained serologic response, with 77% of raccoons developing circulating RVA within 2 mo. In the group that received partial doses of vaccine, fewer raccoons (50%) developed detectable RVA in the same time period. Although the basic shape of the humoral immune response curve was similar for both groups, the immune response for the partial dose group was slower in the first 2 mo, and antibody prevalence remained much lower through week 16. Higher field antibody prevalence might be achieved by using a higher volume of vaccine or by increasing the vaccine titer. However, given the way raccoons manipulate food items as they eat, higher volumes of vaccine may still result in spillage. A higher vaccine titer may prove more effective; however, higher production costs may outweigh the benefits.

While it was not surprising to have fewer raccoons respond in the partial-dose group, several raccoons in the full-dose group also did not respond to ONRAB with RVA production. It is possible that factors such as age, general health status, diversity within immune response genes (Moore and Hanlon, 2010), and stress (Federoff, 2001) may affect immune response among animals. We did not detect differences in the proportion of adults and juveniles in the full-dose group that responded to vaccination, and all animals remained healthy throughout the study. However, it is possible there were underlying immunodeficiencies in some of the wild-caught raccoons that were not detected.

Antibody prevalence has been assessed in the field in Ontario and Quebec following large-scale aerial distribution of ONRAB vaccine baits and results were similar to those seen in our captive study. Although baits were distributed at different densities in the field studies (75–400 baits/km^2), our results closely matched field results for 150 baits/km^2. Using the same C-ELISA inhibition positive cut-off as in this study, 50% of raccoons captured in Ontario 5–6 wk after bait distribution (2006) and 56% and 51% of raccoons captured in Quebec (2007 and 2008, respectively) had detectable RVA (OMNR, unpubl. data; Mainguy et al., 2012). In our study, 50–55% of raccoons presented ONRAB baits were antibody-positive 3–5 wk postvaccination, which would equate to roughly 5–7 wk postbaiting, assuming most baits are consumed by raccoons within the first 2 wk of field distribution (Roscoe et al., 1998; Rosatte and Lawson, 2001; Blackwell et al., 2004). Based on the observed kinetics of the humoral immune response to ONRAB,

the best time to conduct post-ORV surveillance for evidence of vaccination would be 6–13 wk following bait distribution, with the highest antibody prevalence expected if blood samples are collected between weeks 8–10. In Ontario, baits are usually deployed during late August to mid-September to maximize bait uptake by both adult and juvenile raccoons. Although our results suggest the optimal timing of post-ORV assessment would be early to mid-October, surveillance results may be compromised at this time of year due to smaller sample sizes. In temperate regions, trapping success of raccoons usually declines in later months as more natural foods become available and raccoons prepare for winter denning. It may be necessary to adjust the timing of bait deployment and postbaiting surveillance in different regions depending on habitat and local raccoon behavior and ecology.

The presence of virus neutralizing antibodies is a good predictor of protection (Moore and Hanlon, 2010). However, the efficacy of a vaccine can only be assessed by virus challenge studies; such studies are recommended as part of the evaluation process for new vaccines (WHO, 1992). To determine to what extent antibody development in response to ONRAB conferred protection, 20 raccoons were challenged with RRVV 350 days postvaccination; this challenge dose killed eight of nine (89%) controls. One control animal and one ONRAB vaccinate that did not respond to vaccination survived challenge. Because all study animals were trapped in a raccoon-rabies–free area, and the surviving control did not show evidence of an anamnestic response following challenge, it is unlikely there was a previous exposure in the field that resulted in immunity. The nonresponding ONRAB vaccinate had a relatively high level of neutralizing RVA 7 days postinfection (4.2 IU/mL), suggesting a possible response to vaccination. Though the C-ELISA uses a neutralizing mAb as the competitor antibody, it is a binding assay that detects only the subset of antibodies in the test serum that binds to its particular epitope on the glycoprotein. Therefore, it is possible this animal developed an antibody repertoire in response to ONRAB for which the C-ELISA lacked sensitivity. Indeed, at the C-ELISA positive threshold selected to ensure high test specificity (minimize detection of false positives), the sensitivity of the assay as compared to a virus neutralization assay was 75% for raccoon sera.

Protection from virus infection is a complex process, part of which includes the ability to produce memory immune cells (Knowlton et al., 2001). All raccoons in the ONRAB challenge group had neutralizing RVA detectable 7 days postinfection, suggestive of an anamnestic response to the challenge virus. Of nine raccoons that had neutralizing RVA titers below 0.5 IU/mL before viral challenge, seven survived challenge. Together, these data suggest an effective memory immune response was generated in response to ONRAB and indicate that the bait densities and bait-drop frequency and timing (annual fall campaigns) currently used in Ontario and Quebec are adequate to achieve an immunity in individual raccoons that will persist until the next ORV campaign.

There were five rabies deaths among the vaccinates; however, one of these animals only met our minimum criterion for classification as a responder to vaccination. Nonetheless, it did have neutralizing RVA (1.5 IU/mL) at day 7 postchallenge, suggesting there was an anamnestic response. The death of the remaining four raccoons was more perplexing, given that each had serum samples positive for RVA for 13–15 consecutive weeks and three had detectable neutralizing RVA at challenge (0.5–2.2 IU/mL). In a similar longitudinal immune response and challenge study, all raccoons that exhibited a sustained antibody response survived challenge ($n=13$) regardless of the rabies vaccine (IMRAB 3 or V-RG) or method

of administration (IM or oral; Brown et al., 2011). However, in laboratory trials in which raccoons were immunized with V-RG by different routes, three raccoons succumbed to rabies despite having high levels of neutralizing RVA on the day of challenge (Rupprecht et al., 1988). Like V-RG, ONRAB is a recombinant vaccine encoding only the rabies virus glycoprotein. Our data support the conclusions of Rupprecht et al. (1988) that if additional components of the rabies virus also contribute to protection, an antiglycoprotein antibody response raised against a recombinant rabies vaccine will not necessarily confer protection.

While intramuscular vaccination effectively controls rabies in raccoons and other wildlife species (Rosatte et al., 1990; Rosatte et al., 2009b), its associated costs make ORV the only practiced method for wildlife rabies control over large areas. A marked reduction in the number of raccoon rabies cases in Quebec was observed following large-scale deployment of ONRAB vaccine baits (Rees et al., 2011), and encouraging antibody prevalence results were obtained from field trials in Ontario (Rosatte et al., 2009a; Rosatte, 2011), New Brunswick (Fehlner-Gardiner et al., 2012), and Quebec (Mainguy et al., 2012). ONRAB has been shown to be genetically stable and safe in target and nontarget species (Knowles et al., 2009a, b), and the extensive use of adenovirus vectors in human gene therapy research (Douglas, 2007) supports that ONRAB presents little risk to public health. These data, together with results of the present study, suggest ONRAB is a safe and effective alternative oral rabies vaccine for raccoons.

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