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Raccoon poxvirus rabies virus glycoprotein recombinant vaccine in sheep

Brief Report

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Summary. Twenty sheep were divided into groups and inoculated by various routes with recombinant raccoon poxvirus expressing the CVS rabies virus glycoprotein (rRCNV-G) or with raccoon poxvirus (RCNV). The apparent innocuous pathologic responses to each virus coupled with development of high levels of rabies virus neutralizing antibodies in animals vaccinated with rRCNV-G intradermally or intramuscularly suggested that the recombinant is effective and that RCNV would be a suitable substrate for further development of sheep vaccines. Poor antibody response to rRCNV-G given orally implied that it would be relatively harmless if inadvertently ingested by sheep. Virus transmission between vaccinated and sentinel sheep was not observed or detected serologically.

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Recombinant vaccines hold considerable promise for disease control [3, 4, 12], and it is probable that recombinant vaccines with the capacity to immunize against several diseases will soon be widely available [16]. Most of the recombinant virus vaccines developed to date, including a very effective vaccine for rinderpest in cattle [29], use vaccinia virus as a vector [12, 17, 18, 21–24, 27]. Fox rabies has been controlled in southern Belgium with the aid of a field-bait delivery system employing an apparently safe recombinant vaccinia-rabies vac-

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cine; the vaccinia-rabies vaccine has been used in 3 field trials in Virginia, Pennsylvania, and New Jersey with no complications reported [4, 23]. In contrast, during smallpox eradication, groups of dairy cattle in various countries became infected with vaccinia virus from smallpox vaccines and the virus was further transmitted to people from this non-target species [15]. Moreover, it appears that the vaccinia virus subspecies, buffalopox virus, which still causes infections in milking buffalo, dairy cattle, and persons that handle these animals and drink their milk, became established during the smallpox vaccination era in a yet unresolved animal reservoir in India and certain other countries [8, 15]. For this and other reasons, alternative poxviruses and other organisms continue to be researched for use as vaccine substrates.

Raccoon poxvirus (RCNV) was originally isolated from upper respiratory tissues of 2 raccoons trapped in 1962 in Maryland. RCNV appears to be a relatively attenuated, indigenous orthopoxvirus that infects North American raccoons (4% of 593 raccoons trapped in northern Maryland in 1988–89 were seropositive for RCNV neutralizing antibodies (NAbs)) [1, 11, 14, 26]. RCNV has been used to develop a recombinant rabies vaccine that expresses the rabies virus glycoprotein (rRCNV-G) [7, 9–12, 14, 19]. The purpose of the present study was twofold: (1) to begin evaluating RCNV as a potential substrate for development of sheep vaccines, and (2) to begin understanding the consequences of intradermal, intramuscular or oral rRCNV-G exposure in sheep, a non-target farm animal during a wildlife immunization campaign that delivers rRCNV orally via baits. Experiments were conducted to determine whether RCNV can be grown in sheep and goat cells useful in development of vaccines for these species, and to perform a limited evaluation of RCNV and rRCNV-G in sheep and sentinels in a containment facility to ascertain the immunogenicity, host response, and potential for horizontal transmission of these viruses. Taken together, the results of our limited studies, reported here, suggested that rRCNV-G is relatively safe, it appears to be highly immunogenic intradermally (ID) and intramuscularly (IM), and it appears likely that inadvertent ingestion of rRCNV-G by healthy sheep would be relatively innocuous.

The production of rRCNV-G and its effects in various other target and non-target animals, including raccoons and primates, has already been described [9, 11, 14]. RCNV and rRCNV-G were grown and plaque-forming units (pfu) were determined in BSC-1 African green monkey kidney cells by established techniques [13]. Ovine fetal lung (OFL) cells and goat synovial membrane (GSM) cells were grown from tissue explants of newborn animals and were verified to be free of mycoplasma and lentivirus [5]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, U.S.A.) supplemented with 5–10% fetal bovine serum (FBS; Irvine Scientific, Santa Anna, CA, U.S.A.), 29 μg/ml L-glutamine, 100 units/ml sodium penicillin G, 100 μg/ml streptomycin sulfate, and 2.5 μg/ml amphotericin B (Gibco, Grand Island, NY, U.S.A.) The capacity of rRCNV-G and RCNV to replicate in BSC-1, OFL, and GSM cells was compared by inoculating separate monolayers

(60 mm petri plates) of each cell line at an input multiplicity of 0.5 pfu per cell. Preliminary growth curves (not shown) indicated that both viruses replicated to slightly higher titers in BSC-1 cells (ca. 10^6 pfu/ml), that peak titers of each virus were obtained between 48 and 72 h post-infection (hpi) in all three cell lines, and that the kinetics of growth were slower in GSM cells than in BSC-1 or OFL cells.

To examine the effects of RCNV and rRCNV-G in sheep, 20 outwardly healthy adult Finn-Rambouillet ewes (age 1–2 years) were segregated into 5 groups in separate rooms of an isolation facility as shown in Table 1. Two or 3 sheep in each group were inoculated by 1 of 3 routes with rRCNV-G or RCNV, and 1 or 2 sentinel animals were included with each group to monitor horizontal transmission of virus. Intradermal (ID) and intramuscular (IM) inoculations were made near the midline at the base of the neck with 10^8 pfu of virus. Animals were test-bled periodically (see Tables 2–4); at 7 weeks post-inoculation (wk pi) each animal received a booster inoculation by the same route.

Rabies virus NAb titers were determined by the rapid immunofluorescent focus inhibition test (RFFIT) [25]. RCNV NAbs were determined by a 50% plaque-reduction test [20] in which sera from sheep immunized with RCNV or rRCNV-G and collected at 6 and 11 wk pi and were heat-inactivated (56 °C, 30 min). RCNV (100 pfu) in DMEM was mixed in equal amounts with serial four-fold dilutions of each test serum and incubated overnight (36 °C); the reaction samples were then applied to BSC-1 cell monolayers. Cultures were

Table 1. Experimental design for evaluation of sheep given recombinant raccoon poxvirus expressing the rabies virus glycoprotein (rRCNV-G) or raccoon poxvirus (RCNV)

Group	No. of sheep	Week 0		Week 7	
		inoculum	route	inoculum	route
A	2	RCNV	ID	rRCNV-G	ID
	1	sentinel		rRCNV-G	oral bait
B	3	rRCNV-G	ID	rRCNV-G	ID
	2	sentinel		rRCNV-G	oral bait
C	3	rRCNV-G	IM	rRCNV-G	IM
	2	sentinel		rRCNV-G	oral bait
D	3	rRCNV-G	oral drip	rRCNV-G	oral bait
	2	sentinel		rRCNV-G	oral bait
E	1	RCNV	ID titration	none	
	1	rRCNV-G	ID titration	none	

Virus doses: ID 10^8 pfu; IM 10^8 pfu; oral drip $10^{8.7}$ pfu; ampule baited 10^9 pfu; titration in group E was from 2×10^0 to 2×10^8 pfu

Table 2. Rabies and RCNV serum neutralizing antibodies (NAbs) in sheep immunized per os with rRCNV-G[a]

Animal number	Group D				Sentinel animals[b]						
	32	33	34		20	24	25	29	30	31	35
					rabies NAb (IU/ml)[d]						
Wk pi[c]	oral rRCNV-G primary vaccination			wk pi							
0	0	0	0		0	0	0	0	0	0	0
1	0	0.1	0		0	0	0	0	0	0	0
2	0	0.1	0		0	0	0	0	0	0	0
3	0	0.5	0		0	0	0	0	0	0	0
4	0	0.4	0		0	0	0	0	0	0	0
6											
7	oral booster with baited rRCNV-G			0	oral primary immunization with baited rRCNV-G						
8	0	0.2	0	1	0	0.07	0	0	0	0	0
9	0	0.1	0	2	0	0	0	0	0	0.06	0
10	0	0.1	0	3	0	0	0	0	0	0	0
11	0	0.2	0	4	0	0	0	0	0	0	0
					RCNV, Nab (titer)[e]						
11	< 10	< 10	< 10	4	< 10	< 10	< 10	< 10	< 10	< 10	< 10

[a] $10^{8.7}$ pfu of each virus inoculated per os by syringe drip (first inoculation) or with bait (second inoculation for sheep 32, 33, 34 or first inoculation for sentinel sheep at 7th week of the experiment)

[b] Sheep used as sentinels earlier in groups inoculated ID or IM (see Table 1)

[c] Week post-inoculation

[d] Rabies NAbs determined by the rapid fluorescent focus inhibition test [23], in which a rabies reference serum indicated that 1 International Unit (IU) equalled a 1:200 serum dilution

[e] Reciprocal dilution of serum by a 50% plaque-reduction RCNV NAb assay

incubated to 60 hpi and then stained with 0.13% crystal violet formalin solution to visualize plaques.

Individual animals of groups A–D (Table 1) were observed daily and monitored for behavioral differences, feed consumption, body temperature, and evidence of cutaneous lesions at sites of inoculation and elsewhere. Cutaneous lesions were scored using a semiquantitative scale (1–3+) by evaluating lesion color, diameter, surface roughness, and skin thickness. Periodically, the oropharynx of orally immunized sheep was examined with a mouth speculum. Euthanasia was performed at 11 wk pi by severing the carotid artery of sheep under heavy sedation with xylaxine. Tissue samples were collected into buffered formalin from each skin inoculation site of the two sheep in group A that received ID inoculations at different sites on the dorsum first with RCNV and then with rRCNV-G. At necropsy, the inoculation site (mouth, ID or IM site),

Table 3. Rabies and RCNV serum neutralizing antibodies (NAbs) in sheep immunized intradermally with rRCNV-G or RCNV[a]

	RCNV		rRCNV-G		
Animal number	18	19	21	22	23
Week post-inoculation					
	rabies NAb (IU/ml)[b]				
0	0	0	0	0	0
1	0	0	0.6	0.6	0.6
2	0	0	2.8	2.8	2.1
4	0	0	2.4	2.4	2.4
6	0	0	0.5	0.5	2.4
7	intradermal booster immunization with rRCNV-G				
8	0	0	> 14	> 14	13
9	0	0	> 14	> 14	6.3
10	0.08	0	> 14	> 14	6.3
11	0.09	0	ND[c]	ND	ND
	RNCV NAb (titer)[d]				
11	132	112	190	308	200

[a] 10^8 pfu virus given intradermally at the base of the neck with a booster at 7 weeks post-inoculation

[b] Rabies NAbs determined by the rapid fluorescent focus inhibition test (RFFIT) [23] in which the rabies reference serum showed 1 International Unit (IU) equalled a 1:200 serum dilution (14 IU = 1 628 400 dilution which was the routine final dilution used in screening the sera by RFFIT)

[c] Not determined

[d] Reciprocal dilution of serum by a 50% plaque-reduction RCNV NAb assay

regional lymph nodes, and thoracic and abdominal visceral organs of each animal were examined carefully. Samples of skin, lymph node, spleen, kidney, liver, lung, heart, and occasionally other tissues (tonsil, adrenal) were frozen for virus isolation or fixed in buffered formalin for histopathology. Specimens were trimmed, paraffin embedded, sectioned at 6 μm thickness, and stained with haematoxylin and eosin (H & E). Sections of various tissues were examined microscopically in a blinded fashion without knowledge of the immunization treatment that each animal received. H & E stained skin sections of ID inoculated animals were evaluated by a described system [7] that grades on a relative numeric scale the epidermal thickness, mitotic cell number, ulcer severity, focal hyperkeratosis, edema, haemorrhage, and the severity of dermal suppurative response.

The pathologic effects of different ID doses of RCNV or rRCNV-G were compared using 2 sheep (Table 1, group E) by outlining in ink, ten 2-cm squares on the shaved back, flanking the vertebral column. Ten-fold serial dilutions, containing 2×10^8 to 2×10^0 pfu of RCNV or rRCNV-G, were then inoculated ID in the center of each square on the left and right side, respectively (uninfected

Table 4. Rabies and RCNV NAbs in sheep inoculated intramuscularly with rRCNV-G[a]

	rRCNV-G		
Animal number	26	27	28
Week post-inoculation			
	rabies NAb (IU/ml)[b]		
0	0	0	0
1	0	0.6	0.1
2	0	2.8	0.6
4	0.1	0.6	0.1
6	0.1	0.5	0.1
7	intramuscular booster inoculation with rRCNV-G		
8	> 14	> 14	> 14
9	> 14	> 14	> 14
10	> 14	> 14	10
11	ND[c]	ND	ND
	RCNV NAb (titer)[d]		
11	113	294	144

[a] 10^8 PFU virus given in the muscle at base of neck with booster given at 7 weeks post-inoculation

[b] Rabies NAbs determined by the rapid fluorescent focus inhibition test (RFFIT) [23] in which the rabies reference serum showed 1 International Unit (IU) equalled a 1:200 serum dilution (14 IU = 1 628 400 dilution which was the routine final dilution used in screening the sera by RFFIT)

[c] Not determined

[d] Reciprocal dilution of serum by a 50% plaque-reduction RCNV NAb assay

cell culture fluid was also injected to serve as a control). The sheep were euthanized 4 days after inoculation to examine histological cross-sections of lesions that developed within the marked areas.

Virus isolation was attempted from spleen and prescapular lymph node specimens (1 cm^3 of tissue) of all animals at the termination of the experiment. Tissues were minced in DMEM, sonicated cold (3 cycles for 30 sec at output setting 1 of a Branson Model 450 Sonifier, Danbury, CT, U.S.A.), and clarified by centrifugation (10 min, 17 000 × **g**). Individual supernatant fluids were then screened for virus by inoculating duplicate BSC-1 monolayers, and at 5 days post-inoculation, staining for viral plaques [13, 20].

Three sheep (Table 1, group D; Table 2, animals nos. 32, 33, 34) each first received 2 ml containing $10^{8.7}$ pfu of rRCNV-G per os (PO) by drip from a syringe. All oral inoculations were preceded by visual examination of the oropharyngeal cavity to ensure that the animals had no apparent abnormalities. A prior feeding trial had shown that by withholding food for 1 day, the sheep would consume an alfalfa-coated wax ampule bait, which we subsequently filled with approximately 10^9 pfu rRCNV-G in 2 ml. Thus, animals nos. 32, 33, and 34 were given a second oral dose of rRCNV-G in bait at 7 wk pi, at a time

when all 7 sentinel animals of each group (Tables 1 and 2) were further used after screening for rabies and RCNV NAbs by administering an oral-baited dose of rRCNV-G (the 7 sentinel animals had failed to show seroconversion to rabies or RCNV Nabs by 6 wks pi; Tables 2–4). As suggested by Dr. S. Linhart, who kindly provided the bait materials, 0.5 cm diameter foam insulation caps were carefully placed over the mouth of 2 cc microcrystalline wax ampules filled with rRCNV-G and then melted wax was dripped around the cap to seal it. Sealed ampules were inserted into sleeves (urethane hair curler coverings) that were saturated with sorghum-molasses syrup and rolled in minced alfalfa.

There were no detectable symptomatic changes apparent between the virus-exposed and sentinel animals in body temperature, feed consumption, or behavior, nor were differences detected in these parameters before or after virus inoculation. In sheep given ID inoculations with RCNV or RCNV-G, there was a 2–4 day period of mild cutaneous erythema and edema followed by development of a 4–6 mm scab by 1 wk pi; scabs persisted for 3 to 6 wk pi. Lesions appeared similar following the second exposure to virus but were less severe with scabs reaching a size of 2–3 mm diameter. No cutaneous lesions were detected in sentinel sheep. There were no outward clinically apparent responses to IM inoculation, and the only oral lesion found during in vivo and necropsy examination of animals fed rRCNV-G was a circular rough area 15 mm diameter in the tonsil of sheep no. 32 which showed extensive tonsillar lymphoid follicular hyperplasia on histologic examination.

To assess and compare the local inflammatory response to RCNV and rRCNV-G, skin samples were taken at necropsy 4 days following inoculation of virus in 2 sheep (Table 1, group E). Both viruses at high titer (2×10^8 pfu) induced a pyogranulomatous inflammatory response (Fig. 1) that extended from the superficial dermis to the skeletal muscle underlying the deep dermis. This lesion was characterized by loosely scattered neutrophils and accumulations of lymphocytes and macrophages, particularly surrounding adnexal structures (hair follicles, sweat glands, and to a lesser extent, sebaceous glands). Edema was present, but there was no evidence of haemorrhage and minimal necrosis. Similar lesions, but without neutrophils and progressively less severe, were found in skin sites inoculated with 2×10^7 to 2×10^2 pfu of virus, but not in sites inoculated with less virus or no virus. Semiquantitative scoring of the lesions, using the criteria mentioned above, revealed progressive increase in severity of cutaneous lesions with virus dose in both sheep. The cumulative scores in both sheep ranged from 4 to 8 for control sites and low virus titers, 5 to 10 for intermediate titers, and 6 to 13 for high virus titers. However, there were no overall differences in severity of lesions induced by RCNV and rRCNV-G as the total score for all injection sites of both viruses did not differ significantly (two-tailed Student's t test at $P < 0.05$).

Changes at necropsy at 11 wk pi of the immunized sheep were limited to the site of virus inoculation. There was no gross or histopathologic evidence of systemic disease induced by poxvirus infection. Among sheep immunized ID

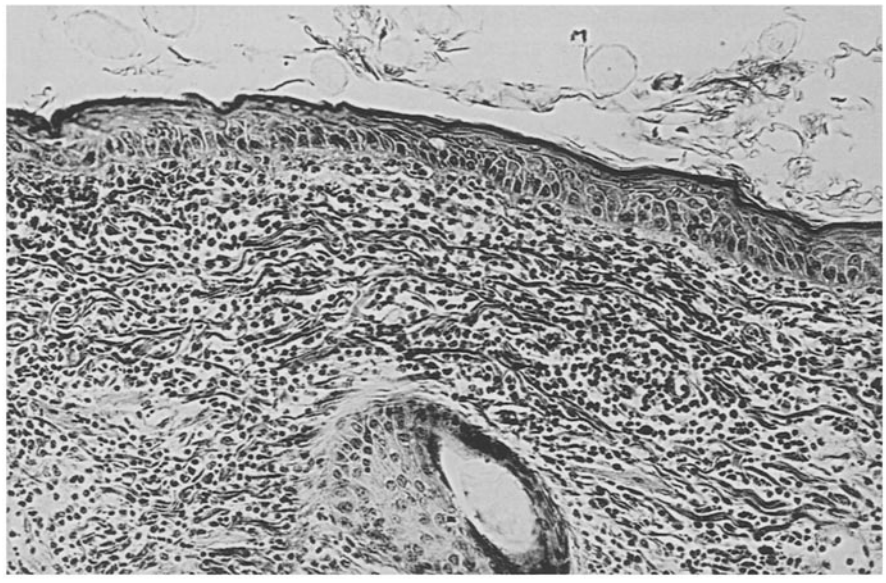

Fig. 1. Dermal inflammatory response consisting of lymphocytes, macrophages, and scattered neutrophils in the inoculation site of a sheep inoculated intradermally 4 days previously with 2×10^8 pfu RCNV. Bar: 100 μm

with rRCNV-G, (Table 1, groups A and B), at inoculation site, there was mild non-suppurative dermatitis histologically. All sheep, except one animal inoculated PO by syringe, had marked lymphoid hyperplasia in the prescapular lymph node draining the site of inoculation. This was manifested by enlarged prominent lymphoid follicles with active germinal centers, and increased numbers of lymphoblastoid cells and macrophages in the paracortical zone. The spleens of all sheep had varying degrees of follicular hyperplasia and increased thickness in the marginal zone. This change was less marked in 2 sheep inoculated with RCNV ID and in 3 sheep inoculated with rRCNV-G PO (Table 1). Pathologic change in other parenchymal organs of the sheep (liver, kidney, heart, lung) was limited to occasional incidental lesions that we associated with parasitism. Neither RNCV nor rRCNV-G virus could be isolated by duplicate attempts from prescapular lymph node or spleen specimens of any sheep, nor from the tonsil of sheep no. 32.

Of the three animals initially exposed to rRCNV-G PO by syringe drip (Table 2), sheep no. 33 developed a low titer and the two others were negative for rabies NAbs. There was no increase in rabies NAbs in any member of group D after boosting with baited rRCNV-G, which suggested to us that rRCNV-G does not infect sheep very well orally. Furthermore, of the 7 sentinel sheep exposed to baited rRCNV-G during the 7th week of the experiment (Table 2), only 2 developed very low rabies NAb levels when tested at 4 wk pi. No animal of this group (Table 2, nos. 20, 24, 25, 29, 30, 31, 35) developed a detectable

antibody response to RCNV, further suggesting to us that rRCNV-G takes poorly orally in sheep.

The capacity of ID rRCNV-G to induce rabies and RCNV serum NAbs was examined in 5 sheep (Table 3). No NAbs were detected in any animal prior to virus inoculation, in 2 animals inoculated with RCNV or in 3 sentinel sheep included with both groups. The three sheep first inoculated ID with rRCNV-G all developed NAb titers against rabies by 2 wk pi, and an approximate 5-fold further increase within 1 week of the booster ID inoculation at 7 wk pi. Of 2 ewes (Table 3, nos. 18, 19) inoculated ID with RCNV followed by an ID inoculation of rRCNV-G at 7 wk pi, one developed very low rabies NAbs when tested 3 weeks later, and the other remained seronegative to rabies, which suggested to us that prior exposure to RCNV may limit further responses via booster. However, these responses contrasted sharply with responses of animals nos. 21, 22, and 23 that had been given a primary ID rRCNV-G immunization (no prior exposure to RCNV). All the sheep in this group quickly developed rabies antibodies, and then after the booster inoculation, developed high levels of NAbs by 11 wk pi (Table 3).

As shown in Table 4, one of 3 sheep inoculated with rRCNV-G IM showed responses comparable to the ID group (Table 2), and the other 2 sheep developed much lower anti-rabies titers after the first immunization; however, all members of this group responded well to the second IM inoculation and all developed high antibody titers to rabies virus. All 3 animals also developed serum NAbs to RCNV by 11 wk pi. Considering that no potentially problematic skin lesions developed in the animals inoculated IM and good NAb responses were observed, the IM route may be considered a preferred method for vaccinating sheep in the field.

Thus, RCNV expressing the rabies virus glycoprotein was strongly immunogenic in sheep immunized by the ID and IM routes but not orally by syringe drip inoculation or by feeding the wax ampule baits. Relatively high levels of rabies NAbs were induced by a single ID dose of 10^8 rRCNV-G and these levels persisted for 6 weeks when the study was terminated. The World Health Organization level of 0.5 IU per ml is considered indicative of an adequate anti-rabies response to the immunogen in humans [3], and this level correlates with significant protection to rabies virus challenge in mice [28]. Booster immunizations by ID or IM routes led to marked rises in rabies NAbs, indicating an amnestic response and suggesting excellent priming by these vaccination routes. By oral immunization, rRCNV-G has been reported to induce high levels of rabies NAbs in raccoons, cats, dogs, cotton rats, foxes, rabbits, bobcats, but was poorly immunogenic in skunks, mice, primates and mongooses [9, 14]. Of the animals in the reported studies, only dogs, raccoons, and cotton rats had been challenged with no development of rabies. The percentage protection of skunks and mice generally correlated with presence of rabies Nabs; rabbits, foxes, bobcats, primates, and mongooses had not been challenged. A time-course study [14] (Esposito et al. unpubl. data), involving syringe feeding

rRCNV-G to raccoons, showed localization of the virus mainly in the tonsils with no apparent ill effects.

Although a tonsillar lesion developed in sheep no. 32 (Table 2) fed rRCNV-G, virus was not recovered from the lesion and neither this animal nor others in its group developed high rabies or RCNV NAbs. A low rate of seroconversion also was found in a study involving sheep exposed orally to a vaccinia rabies recombinant virus; only one of four animals developed protective levels of antibodies [2]. Interestingly, in the reported study [14] in which rRCNV-G or a vaccinia rabies recombinant virus was fed to patas or cynomolgus monkeys, the vaccinia recombinant was strongly reactive, producing a febrile response, visible oral lesions, and high levels of rabies NAbs, and the rRCNV-G was markedly less reactive.

RCNV NAbs developed in all sheep exposed ID and IM but not PO. Although the numbers of animals in groups A–D were relatively small, there seemed to be in group A (Table 3) an inhibition of the primary rabies NAb response to rRCNV-G in 2 animals that received prior ID inoculation of RCNV. In contrast, animals with prior exposure to rRCNV-G by ID or IM routes developed enhanced rather than inhibited NAb responses to rabies virus. These results, which may reflect significant differences in immune responses to wild type and recombinant viruses, are consistent with previous reports indicating interference with antibody response to recombinant vaccinia virus-expressed antigens in animals previously exposed to vaccinia virus [21], but not in those previously exposed to the expressed antigens [16]. The practical importance of these results in field immunization programs must await further studies, including data on the host range and seroprevalence of animals naturally exposed to RCNV.

As a candidate sheep vaccine substrate, RCNV must be evaluated in significant numbers of test animals for immunogenicity and safety after optimizing the route of inoculation and dose. Consistent with data obtained with raccoons [11, 14], (Esposito et al., unpubl. data) neither RCNV nor rRCNV-G induced severe local reaction or detectable systemic reactions in sheep in the present limited study, and transmission to in-contact sentinel animals was not detected. The relatively mild cutaneous lesions induced by ID inoculation of wild type or recombinant RCNV likely masked the evidence of attenuation of rRCNV-G because of insertional inactivation of its TK gene as reported previously [6] using more sensitive footpad or intranasal inoculations of rRCNV-G in neonatal mice. Additionally, sheep were relatively insusceptible to oral infection with rRCNV-G, hence, if used as a vaccine in domestic or wild carnivores rRCNV-G would appear to be innocuous for sheep which may be inadvertently exposed.

Taken together, these results suggested to us that rRCNV-G, which expresses the highly immunogenic rabies virus glycoprotein, is a potent, useful candidate vaccine for sheep using ID or IM routes, but not the oral route of immunization. On the other hand, this species of farm animal would not appear to be at risk if exposed to rRCNV-G if used as a bait for immunizing wildlife and domestic

animals. As a candidate vaccine, recombinant RCNV in sheep compares favorably with recombinant vaccinia virus in safety and immunogenicity studies performed in cattle [17, 27, 29].

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