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Raccoon Poxvirus Recombinants Expressing the Rabies Virus Nucleoprotein Protect Mice against Lethal Rabies Virus Infection

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Raccoon poxvirus (RCN) recombinants expressing the rabies virus internal structural nucleoprotein (RCN-N) protected A/WySnJ mice against a lethal challenge with street rabies virus (SRV). Maximum survival was achieved following vaccination by tail scratch and footpad (FP) SRV challenge. RCN-N-vaccinated mice inoculated in the FP with SRV were resistant to infection for at least 54 weeks postvaccination. Protection was also elicited by RCN recombinants expressing the rabies virus glycoprotein (RCN-G). Vaccination with RCN-G evoked rabies virus neutralizing antibody. Rabies virus neutralizing antibody was not detected in RCN-N-vaccinated mice prior to or following SRV infection. Radioimmunoprecipitation assays showed that sera from RCN-N-vaccinated mice which survived SRV infection did not contain antibody to SRV structural protein G, M, or NS. The mechanism(s) of N-induced resistance appears to correlate with the failure of peripherally inoculated SRV to enter the central nervous system (CNS). Support for this correlation with resistance was documented by the observations that SRV-inoculated RCN-N-vaccinated mice did not develop clinical signs of CNS rabies virus infection, infectious SRV was not detected in the spinal cord or brain following FP challenge, and all RCN-N-vaccinated mice died following direct intracranial infection of the CNS with SRV. These results suggest that factors other than anti-G neutralizing antibody are important in resistance to rabies virus and that the N protein should be considered for incorporation with the G protein in recombinant vaccines.

Rabies viruses cause a fatal disease that is associated with an acute infection of the central nervous system (CNS) of all warm-blooded species. At the onset of clinical symptoms, virus is widespread throughout the CNS, with highest concentrations present in the brain stem, basal ganglia, hippocampus, and cerebellum. After reaching the CNS, rabies virus spreads centrifugally from the CNS and is present in neurons throughout the body. There is no viremia.

The rabies virus RNA genome is linear, single stranded, nonsegmented, and of negative sense. Five proteins are encoded by the genome. Three of the proteins, nucleoprotein (N), phosphoprotein (P, MI, or NS), and large polymerase protein (L) associate with viral genomic RNA to form the ribonucleoprotein (RNP). The viral matrix protein (M) and transmembrane-spanning glycoprotein (G) are associated with the virion lipid envelope that surrounds the RNP (32).

The rabies virus G is generally regarded as the most relevant protein for eliciting protection, since only this protein has been shown to be responsible for the induction and binding of virus-neutralizing antibodies (7, 11, 20, 33). Furthermore, G is able to confer protection against lethal infection with rabies virus (34). It also has been demonstrated to be a target antigen of anti-rabies virus cytotoxic T-lymphocyte (CTL) clones (17), and it has been shown to elicit CTLs that may eliminate rabies virus-infected cells in vivo (8, 10, 21a). As a result of these and other studies, several vaccinia virus and adenovirus recombinant vaccines expressing rabies virus G have been constructed and used to successfully vaccinate different species of animals (5, 14, 18, 22, 29, 31). In contrast, we know of only one other study that has been done to determine the protective capabilities of the internal structural rabies virus N as expressed in a recombinant virus (27a).

In this report we analyze the protective properties of raccoon poxvirus (RCN) recombinants expressing either rabies virus G (RCN-G) or N (RCN-N) in street rabies virus (SRV)-susceptible A/WySnJ mice. As previously reported (14, 15), RCN-G induced high levels of anti-rabies virus neutralizing antibody and offered solid protection. More interestingly, RCN-N also induced long-lasting resistance against SRV. The RCN-N-induced resistance was highly dependent upon the route of vaccination and SRV challenge, as well as the concentration of virus used to vaccinate and challenge the mice. Furthermore, neither anti-rabies virus neutralizing antibody (anti-G) nor antibody to SRV protein NS or M was detected in RCN-N-vaccinated mice that survived a lethal SRV challenge.

RCN was plaque purified three times following isolation from the upper respiratory tissues of two apparently healthy raccoons (1, 30). Production of RCN recombinants with chimeric plasmids designed for inserting the challenge virus standard rabies virus G into the viral thymidine kinase (TK) locus has been described previously (14, 15). RCN recombinants expressing the rabies virus N open reading frame of the challenge virus standard rabies virus downstream of promoters for the vaccinia virus early/late 7.5-kDa protein or the late 11-kDa protein were made with the same chimeric plasmids described by Sumner et al. (27a) for TK insertional inactivation marker rescue of N open reading frame into vaccinia virus. Vaccinia virus recombinants expressing influenza virus hemagglutinin and Friend virus envelope protein were kindly provided by Bernard Moss through B. Chesebro. Human TK^- 143B cells (24) were used for propagation of RCN and RCN recombinants expressing rabies virus protein, which were then purified by sedimentation in

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TABLE 1. Protection of tail scratch-vaccinated mice against SRV[a]

SRV inoculation route and dose	No. of survivors/total no. of mice vaccinated with the indicated dose (PFU) of virus:								
	RCN-WT			RCN-G			RCN-N[b]		
	10^7	10^6	10^4	10^7	10^6	10^4	10^7	10^6	10^4
FP									
1×10^3 $MFPLD_{50}$	0/12	0/12	0/12	12/12	12/12	10/12	1/12	0/12	0/12
5×10^1 $MFPLD_{50}$	0/12	0/12	0/12	12/12	12/12	12/12	10/12[c]	8/12[d]	0/12
i.p.									
1×10^7 $MICLD_{50}$	0/12	0/12	0/12	12/12	12/12	12/12	2/12	3/12	0/12
6×10^6 $MICLD_{50}$	0/12	0/12	0/12	12/12	12/12	12/12	2/12	2/12	0/12
3×10^6 $MICLD_{50}$	0/12	0/12	0/12	12/12	12/12	12/12	10/12[c]	6/12[e]	1/12

[a] A/WySnJ mice were vaccinated via tail scratch with different concentrations of RCN-WT or RCN recombinants expressing rabies virus G or N. At 3 weeks postvaccination, mice were challenged in the FP or i.p. with SRV.
[b] The chi-square (χ^2) test was used to evaluate statistical significance. A value of $P < 0.05$ was considered significant.
[c] $P < 0.001$ compared with RCN-WT-vaccinated mice.
[d] $P < 0.01$ compared with RCN-WT-vaccinated mice.
[e] $P < 0.05$ compared with RCN-WT-vaccinated mice.

sucrose gradients as previously described by Esposito et al. (15). Mice were purchased from the Jackson Laboratory, Bar Harbor, Maine, and maintained as inbred stocks at the Rocky Mountain Laboratories. Three to six weeks after vaccination, they were challenged via the footpad (FP) or intraperitoneally (i.p.) with a 10% mouse brain suspension of a wild-type SRV which had been isolated from an adult bat (*Eptesicus fuscus*). In our initial experiments, SRV-susceptible A/WySnJ mice were vaccinated via tail scratch or i.p. with different concentrations of wild-type RCN (RCN-WT) or RCN recombinants expressing rabies virus G or N under the influence of the vaccinia virus 7.5-kDa early/late promoter. In subsequent experiments, only mice vaccinated via tail scratch with 10^7 PFU of virus in 10 μl of physiological saline were used. A FP SRV challenge of 5×10^1 mouse FP 50% lethal doses (LD_{50}) ($MFPLD_{50}$) was used in most of the experiments.

It was observed that >99% of the mice vaccinated via tail scratch with RCN-G survived. Furthermore, survival was not contingent upon the concentration of RCN-G that was used for vaccination, the route of SRV challenge, or the concentration of the SRV (Table 1). Interestingly, RCN-N also protected the A/WySnJ mice (Table 1). This protection was, however, highly dependent upon the concentration of the RCN-N used for vaccination and the concentration of SRV used to challenge the mice. Maximum protection (>83%) was observed in mice that had been vaccinated with the highest concentration of RCN-N (10^7 PFU) and subsequently received a FP SRV challenge of 5×10^1 $MFPLD_{50}$ or an i.p. challenge of 3×10^6 mouse intracranial (i.c.) LD_{50} ($MICLD_{50}$). Protection also occurred following vaccination with 10^6 PFU of RCN-N and a FP or i.p. challenge with similar amounts of SRV. In contrast, 10^4 PFU of RCN-N failed to protect mice, whereas 10^4 PFU of RCN-G protected mice following FP or i.p. challenge with the highest concentrations of SRV.

Somewhat different results were obtained with i.p.-vaccinated mice. Most importantly, 10^7 PFU of RCN-N did not protect mice against either FP or i.p. SRV challenge (Table 2). In contrast, mice vaccinated i.p. with either 10^7 or 10^6 PFU of RCN-G survived. The protection of the RCN-G i.p.-vaccinated mice was not as complete, however, as in tail scratch-vaccinated mice (Table 1), since 83 and 50% of the mice that had been vaccinated with the lowest concentration of RCN-G (10^4 PFU) died following FP challenge with 1×10^3 and 5×10^1 $MFPLD_{50}$ of SRV, respectively (Table 2). Because mice vaccinated with either RCN-G or RCN-N via tail scratch were protected, all subsequent studies were done with tail scratch-vaccinated mice.

Mice were challenged with SRV at intervals of 3 to 54 weeks postvaccination to test the longevity of the protection elicited following tail scratch vaccination with 10^7 PFU of either RCN-G or RCN-N. The data in Table 3 show that up to 54 weeks after vaccination, 100% of the mice vaccinated with RCN-G survived either FP or i.p. SRV challenge. Interestingly, vaccination with RCN-N protected 80 to 100% of the FP-challenged mice during this same length of time. Similar protection was noted at 3 and 10 weeks postvaccina-

TABLE 2. Protection of i.p.-vaccinated mice against SRV[a]

SRV inoculation and route	No. of survivors/total no. of mice vaccinated with the indicated dose (PFU) of virus:								
	RCN-WT			RCN-G			RCN-N		
	10^7	10^6	10^4	10^7	10^6	10^4	10^7	10^6	10^4
FP									
1×10^3 $MFPLD_{50}$	0/12	0/12	0/12	12/12	12/12	2/12	0/12	0/12	1/12
5×10^1 $MFPLD_{50}$	0/12	0/12	0/12	11/12	12/12	6/12	0/12	0/12	0/12
i.p.									
3×10^6 $MICLD_{50}$	1/12	2/12	0/12	12/12	12/12	12/12	3/12	1/12	1/12

[a] A/WySnJ mice were vaccinated i.p. with different concentrations of RCN-WT or RCN recombinants expressing rabies virus G or N. At 3 weeks postvaccination, mice were challenged in the FP or i.p. with SRV.

TABLE 3. Longevity of protection following tail scratch vaccination of mice[a]

No. of wks postvaccination	No. of survivors/total no. of mice vaccinated with the indicated RCN virus and challenged with SRV					
	RCN-WT		RCN-G		RCN-N	
	FP	i.p.	FP	i.p.	FP	i.p.
3	0/10	0/10	10/10	10/10	8/10[b]	8/10
10	0/10	0/10	10/10	10/10	10/10	8/10
20	0/10	1/10	10/10	10/10	10/10	3/10
30	1/10	0/10	10/10	10/10	10/10	4/10
54	0/10	0/9	9/9	10/10	8/10	4/10

[a] A/WySnJ mice were vaccinated by tail scratch with 10^7 PFU of virus. At various intervals postvaccination, mice were challenged in the FP with 50 $MFPLD_{50}$ or i.p. with 3×10^6 $MICLD_{50}$.
[b] $P < 0.01$ compared with RCN-WT-vaccinated mice.

tion with mice that had been challenged i.p., but the protection waned thereafter. We have also determined that 100% of A.SW/SnJ and SJL/J mice were susceptible to FP-inoculated SRV. Following tail scratch vaccination with either RCN-N or RCN-G, both strains were resistant to SRV for at least 6 months postvaccination (data not shown).

To measure serum antibody responses, vaccinated mice were bled 7 h before and 21 days after FP SRV challenge. Mouse neuroblastoma (MNB) cells infected with Evelyn-Rokitnicki-Abelseth (ERA) rabies virus were used to detect antibodies against rabies virus structural proteins G, N, NS, and M, using radioimmunoprecipitation assays with ^{35}S-labeled viral proteins. The MNB cells were infected with ERA rabies virus at a multiplicity of infection of 1. After 38 h, the cells were radiolabeled for 90 min in methionine-free medium containing 500 μCi [^{35}S]methionine. Five microliters of undiluted serum was reacted with the virus-infected MNB cell lysates containing [^{35}S]methionine-radiolabeled proteins or similar lysates of MNB cells persistently infected with SRV. Immunoprecipitates were bound to Pansorbin cells (Calbiochem Corporation, La Jolla, Calif.) alone or coated with rabbit anti-mouse immunoglobulin and centrifuged. The pelleted proteins were denatured and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9). Serum anti-rabies virus neutralization antibody titers (anti-G antibody) were also measured by the rapid fluorescent-focus inhibition test (26). It was observed that A/WySnJ mice vaccinated with RCN-G or RCN-N produced anti-rabies virus antibody against only the rabies virus protein expressed in the recombinants (Table 4). Furthermore, RCN-G-vaccinated mice that survived SRV infection did not produce antibody to SRV structural protein N, NS, or M, and RCN-N-vaccinated mice that survived SRV infection did not produce antibody to SRV structural protein G, NS, or M (Fig. 1; Table 4). These antibodies also were not detected when MNB cell lysates containing [^{35}S]methionine-radiolabeled SRV proteins were used in the radioimmunoprecipitation assays (data not shown). Furthermore, antibodies to the SRV structural proteins were not detected by radioimmunoprecipitation in the sera of unvaccinated A/WySnJ, A.SW/SnJ, or SJL/J mice 7, 10, or 12 days following FP SRV inoculation (data not shown).

Because mice vaccinated with RCN-G or RCN-N via tail scratch were resistant to either FP- or i.p.-inoculated SRV, similarly vaccinated mice were also challenged with SRV i.c., intramuscularly (i.m.), or subcutaneously (s.c.). RCN-G protected 100% of the mice following a direct i.c. SRV challenge of the CNS, as well as after SRV challenge by the four different peripheral routes (Table 5). As before, excellent protection was also observed in mice vaccinated with RCN-N following FP and i.p. SRV challenge, and 60% of the mice survived after either i.m. or s.c. SRV inoculation. However, in contrast to RCN-G-vaccinated mice, 100% of the RCN-N-vaccinated mice died following i.c. challenge (Table 5). The deaths of all RCN-N-vaccinated mice that had been challenged i.c. with SRV, and the 100% survival rate of similarly vaccinated mice that had been infected via the FP, suggested that the mechanism(s) of resistance elicited by RCN-N against FP-inoculated SRV was associated with the failure of the peripherally inoculated virus to enter the CNS. Support for this premise was obtained by examining the CNS of vaccinated mice for infectious SRV. Two to 21 days after FP SRV inoculation, the spinal cords and brains were removed from mice that had been vaccinated via tail scratch with RCN-WT, RCN-N, RCN-G, or a vaccinia virus expressing the Friend virus envelope protein (VAC-FR-ENV). Suspensions of these CNS tissues were tested for infectious SRV by i.c. inoculation of 21-day-old mice and by fluorescence staining for foci of SRV infection on MNB monolayers

TABLE 4. Serum antibody responses of A/WySnJ mice vaccinated with RCN recombinant viruses expressing either rabies virus G or N[a]

Vaccine	Antibody response[b]											
	10 wks postvaccination				30 wks postvaccination				54 wks postvaccination			
	Prior to SRV		21 days post-SRV		Prior to SRV		21 days post-SRV		Prior to SRV		21 days post-SRV	
	Anti-G[c]	Anti-N[d]	Anti-G	Anti-N	Anti-G	Anti-N	Anti-G	Anti-N	Anti-G	Anti-N	Anti-G	Anti-N
RCN-WT	–	–	ND	ND	–	–	ND	ND	–	–	ND	ND
RCN-G	2,220	–	4,370	–	550	–	1,660	–	53	–	251	–
RCN-N	–	+	–	+	–	+	–	+	–	+	–	+
VAC-FLU	–	–	ND	ND	–	–	ND	ND	–	–	ND	ND

[a] Ten mice per group were vaccinated via tail scratch with 10^7 PFU of wild-type or recombinant virus. At 10, 30, and 54 weeks postvaccination, animals were bled from the retro-orbital plexus and 7 h later were challenged in the FP with 50 $MFPLD_{50}$ of SRV.
[b] –, antibody not detected by radioimmunoprecipitation; +, antibody detected by radioimmunoprecipitation; ND, not done because all mice had died by the 21st day following SRV challenge.
[c] Neutralizing antibody (anti-G) titers were determined with the rapid fluorescent-focus inhibition test (26). Titers are expressed as reciprocal geometric mean titers. Sera of mice that survived SRV challenge and were negative for neutralizing antibody were additionally tested for anti-G antibody by radioimmunoprecipitation of ERA rabies virus-infected MNB cells or MNB cells persistently infected with SRV.
[d] Anti-N antibody was assayed by radioimmunoprecipitation of similarly infected MNB cells.

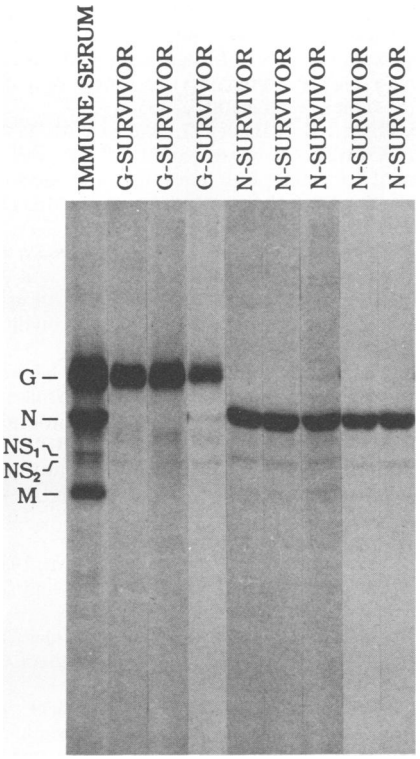

FIG. 1. SDS-PAGE analysis of sera with [^{35}S]methionine-labeled proteins of ERA rabies virus-infected MNB cells. Immune serum, immune serum pool from SJL/J mice that survived an i.p. inoculation of SRV. G-survivor, sera from three different RCN-G tail scratch-vaccinated A/WySnJ mice that survived a lethal FP challenge with SRV. N-survivor, sera from five different RCN-N tail scratch-vaccinated A/WySnJ mice that survived a lethal FP challenge with SRV. Unvaccinated A/WySnJ mice or A/WySnJ mice vaccinated with vaccinia virus expressing influenza virus hemagglutinin which are inoculated in the FP with a lethal dose of SRV die before serum antibody to the rabies virus structural proteins can be detected by radioimmunoprecipitation (results not shown). Thus, sera from these control mice were not included in Fig. 1.

that had been incubated with the tissues. Infectious SRV was never detected in the spinal cords or brains of mice vaccinated with RCN-G or RCN-N. In contrast, SRV was present in spinal cords by day 5 postinoculation and in both spinal cords and brains on days 7 to 21 postinoculation in the RCN-WT- and VAC-FR-ENV-vaccinated mice (data not shown).

TABLE 5. Resistance of RCN recombinant-vaccinated mice to SRV inoculated by different routes[a]

Route (challenge dose) of SRV inoculation[b]	No. of survivors/total no. of mice vaccinated with virus:		
	RCN-WT	RCN-G	RCN-N
FP (5×10^1 $MFPLD_{50}$)	2/10	10/10	10/10
i.p. (3×10^6 $MICLD_{50}$)	0/10	10/10	8/10[c]
i.m. (2.5×10^2 $MIMLD_{50}$)	0/10	10/10	6/10[d]
s.c. (5×10^1 $MSCLD_{50}$)	0/10	10/10	6/10
i.c. (2.7×10^4 $MICLD_{50}$)	0/10	10/10	0/10

[a] A/WySnJ mice were vaccinated via tail scratch with 10^7 PFU of wild-type or recombinant RCN virus. At 3 weeks after vaccination, mice were challenged via five different routes with SRV.

[b] $MIMLD_{50}$, mouse intramuscular LD_{50}; $MSCLD_{50}$, mouse subcutaneous LD_{50}.

[c] $P < 0.01$ compared with RCN-WT-vaccinated mice.

[d] $P < 0.05$ compared with RCN-WT-vaccinated mice.

We previously determined that neither RCN-WT alone nor recombinants expressing non-rabies virus proteins nonspecifically protected A/WySnJ mice against SRV or prevented SRV invasion of the CNS. Additional evidence for the specificity of protection elicited by RCN-G and RCN-N against rabies virus was shown by challenging vaccinated mice with encephalomyocarditis virus (23). It was determined that neither recombinant protected mice against an i.p. or FP challenge of encephalomyocarditis virus, whereas both recombinants protected mice against SRV (data not shown).

These studies demonstrated that RCN recombinants expressing the rabies virus internal structural protein N or the transmembrane-spanning structural protein G induce a long-lasting resistance against a lethal SRV infection. RCN-N-induced resistance was less substantive than that of RCN-G and was dependent upon the route of vaccination and SRV challenge, as well as the concentration of virus used to vaccinate and challenge the mice. These factors were less important in the resistance of RCN-G-vaccinated mice. Nonetheless, mice vaccinated via tail scratch with 10^7 PFU of RCN-N did not develop clinical signs of illness following a lethal FP inoculation of SRV and were highly resistant to infection for at least 54 weeks postvaccination.

The resistance of RCN-G-vaccinated mice was associated with the induction of rabies virus neutralizing antibody (Table 4) (7, 11, 20, 33) and possibly the activation of CTLs (8, 17). In contrast, neutralizing antibody was not detected in RCN-N-vaccinated mice prior to or following SRV infection. Radioimmunoprecipitation assays of sera from RCN-N-vaccinated mice that survived FP SRV challenge confirmed that these sera, as well as sera from unvaccinated mice that were destined to die following FP SRV inoculation, did not contain antibody to SRV structural protein G, M, or NS. Because these sera appeared to contain antibody only to N and were taken from mice that did not develop clinical illness or have detectable infectious virus in their CNS, we suggest that the mechanism(s) of resistance elicited by N occurred early after SRV challenge and was not related to neutralization of SRV by anti-G antibody. The susceptibility of the RCN-N-vaccinated mice to i.c. but not FP SRV challenge strengthens our impression that resistance involved the failure of peripherally inoculated SRV to enter the CNS. In a somewhat similar study, Dietzschold and colleagues (13) showed that vaccination of BALB/c mice and raccoons with liposomes containing the RNP of ERA rabies virus elicits protection against i.m.-inoculated challenge virus standard rabies virus and the rabies virus-related Duvenhage virus. The mechanism(s) by which RNP-immunized animals are protected was not clear, but the researchers proposed that RNP induces T helper cells that augment production of neutralizing antibody following a peripheral virus challenge. This proposed mechanism would not be applicable to our system, however, since the sera of RCN-N-vaccinated survivors did not contain neutralizing antibody. Interestingly, the RNP-liposome vaccines failed, as did our RCN-N recombinant, to confer protection against i.c. challenge. It has been suggested that viral N protein antigens which are not recognized by neutralizing antibodies may provide protection against lethal infection through their elicitation of CTL responses (2). It also has been shown that passively transferred N protein-specific T cells are very effective in protecting mice against influenza virus infection (2, 3) and in

stimulating CTLs in vitro (35). Germane to this observation, Dietzschold et al. (13) mention that rabies virus RNP is an excellent inducer of major histocompatibility complex class I-restricted CTLs in BALB/c mice. Nonetheless, to date, we have not been able to detect primary or in vitro-stimulated memory CTLs in A/WySnJ mice at several different intervals following RCN-N tail scratch vaccination. In contrast, strong secondary CTL responses were detected in RCN-G-vaccinated A/WySnJ mice before and after SRV challenge (21a).

The mechanism(s) by which RCN-N-vaccinated mice are protected is still unclear and is under study. Because RCN-N-vaccinated mice were not resistant to encephalomyocarditis virus and because neither RCN-WT alone nor recombinants expressing non-rabies virus proteins nonspecifically protected A/WySnJ mice against SRV or prevented SRV invasion of the CNS, we do not think the mechanism of resistance is a direct antiviral effect of interferon on target cells. We are considering, however, the possibility that resistance is associated with anti-N nonneutralizing antibodies. In this regard, Lafon and Lafage (19) have shown that artificially scrape-loaded (intracellularly loaded) monoclonal antibodies specific for the rabies virus protein N or NS block rabies virus replication. Protective nonneutralizing antibodies have also been detected in mice infected with Semliki Forest virus (4, 16) or Sindbis virus (27). It was suggested that these nonneutralizing antibodies trigger complement-mediated lysis of infected cells or are involved in an antibody-dependent cell cytotoxicity-type mechanism. Passive protection also has been demonstrated with nonneutralizing monoclonal antibodies directed against nonstructural proteins of dengue 1 virus (28), and nonneutralizing monoclonal antibodies have been shown to protect mice against vesicular stomatitis virus (21).

The question arises as to whether N should be included in recombinant rabies vaccines expressing G. There is no doubt that G alone provides for a powerful and highly efficacious vaccine. However, the antiviral effects of N in synergism with G may provide for a stronger and longer-lasting immunity through activation of additional pathways of the immune system. Furthermore, it is known that there is remarkable sequence conservation among N proteins of different rabies viruses (12, 25). Incorporation of N with G into live vectors such as RCN (14), a fowlpox virus (29), adenovirus (22), or an attenuated vaccinia virus (6) could provide for a vaccine that would be beneficial worldwide.

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