Sickness and Recovery of Dogs Challenged with a Street Rabies Virus after Vaccination with a Vaccinia Virus Recombinant Expressing Rabies Virus N Protein

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Dogs were vaccinated intradermally with vaccinia virus recombinants expressing the rabies virus glycoprotein (G protein) or nucleoprotein (N protein) or a combination of both proteins. The dogs vaccinated with either the G or G plus N proteins developed virus-neutralizing antibody titers, whereas those vaccinated with only the N protein did not. All dogs were then challenged with a lethal dose of a street rabies virus, which killed all control dogs. Dogs vaccinated with the G or G plus N proteins were protected. Five (71%) of seven dogs vaccinated with the N protein sickened, with incubation periods 3 to 7 days shorter than that of the control dogs; however, three (60%) of the five rabid dogs recovered without supportive treatment. Thus, five (71%) of seven vaccinated with the rabies N protein were protected against a street rabies challenge. Our data indicate that rabies virus N protein may be involved in reducing the incubation period in dogs primed with rabies virus N protein and then challenged with a street rabies virus and, of more importance, in subsequent sickness and recovery.

Rabies virus is a member of the family *Rhabdoviridae*, genus *Lyssavirus*, which possesses a negative-stranded RNA genome of about 12 kb. The rabies virus particles contain five virus-encoded proteins. Closely associated with the nucleoprotein (N protein) are the nonstructural (NS) protein and the virion transcriptase (L). The N protein represents the group-specific antigen of the genus. The matrix (M) protein and the glycoprotein (G protein) are located in a lipoprotein envelope, through which spikes of G protein project (5, 45, 52). Spike G proteins are amphophilic proteins, with at least one subunit of the oligomeric protein inserted into the lipid bilayer of the viral envelope anchoring the spike to the membrane (26). For rabies virus (and most other enveloped viruses), the spike G proteins are the antigens responsible for stimulating virus-neutralizing antibody (VNA) (6, 38, 46–49).

Various types of human and animal vaccines are used worldwide for the prevention of rabies. These vaccines are prepared from brain tissue of either adult or newborn animals (15, 19, 22, 37), avian tissues (18, 21, 31), or cell cultures (1, 10, 13). The virus strains used for vaccine production are usually derived from the Pasteur fixed rabies virus strain, with the virus inactivated by physical or chemical methods (1, 10, 13, 15, 16, 18, 19, 21, 22, 31, 37). Rabies vaccines are used for both pre- and postexposure vaccination in humans, whereas only preexposure vaccination is used for animals. Although the immunological basis of protection after vaccination is still not fully understood, it has been shown that both humoral and cellular immune responses are induced by rabies virus vaccines (7, 51).

The development of eukaryotic cloning vector systems has provided a novel alternative to vaccines derived from whole viruses and viral subunits. The role of individual virus proteins expressed in cloning vectors can now be evaluated

in protection and immunity experiments (23, 28, 53). Expression, immunization, and protection have been demonstrated with vaccinia virus recombinants containing foreign genes such as hepatitis B surface antigen, influenza virus hemagglutinin, and herpes simplex virus glycoprotein D (20, 27, 39). A vaccinia virus recombinant expressing the rabies G protein has also been shown to induce protective immunity in experimental animals (9, 36, 44, 50).

Furthermore, purified rabies virus virion N protein administered in complete Freund's adjuvant to mice and raccoons has been demonstrated to induce a protective immune response against rabies virus infection (8), and a vaccinia virus recombinant containing rabies virus N protein, administered by scarification, has been reported to protect mice against rabies virus challenge (43). In addition, rabies virus expressed in a baculovirus vector system and administered intraperitoneally to mice provides limited protection against rabies virus challenge (14, 34).

In previous studies, we reported that up to 20% of dogs experimentally infected with street rabies viruses recovered without supportive treatment; these recoveries were not dose or virus strain dependent (11). The mechanism of such recoveries, however, is still not well understood. We describe here studies with rabies virus N protein expressed in a vaccinia virus vector and used to protect dogs against peripheral challenge with a street rabies virus, and we discuss its involvement in sickness and recovery.

MATERIALS AND METHODS

Recombinant vaccinia virus (Copenhagen strain) expressing the rabies virus N protein. Recombinant vaccinia virus incorporating the N-protein gene coding region of the Challenge Virus Standard (CVS) strain of rabies virus used in this study was constructed as previously described (43).

Standardization of antigen. The amount of infectious recombinant vaccinia virus was standardized on the basis of

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the number of PFU per milliliter needed to protect mice vaccinated by scarification or by intramuscular, intraperitoneal, or subcutaneous inoculation before subsequent challenge with street rabies virus.

Animals. All animals used in this experiment were raised in closed colonies at the Centers for Disease Control animal breeding facility. None of the dogs were previously vaccinated against rabies, and none had rabies VNA at the time of vaccination.

Determination of VNA titers. VNA titers in sera from experimental animals were determined by the rapid fluorescent-focus inhibition test (41). Results were expressed in international units (IU), using an international standard reference serum (41). Antibody titers against N protein in dog sera were determined by an enzyme-linked immunosorbent assay (ELISA) in 96-well plates coated with a baculovirus recombinant expressing the rabies virus N protein as described elsewhere (33).

Immunofluorescence staining. All sera from dogs were tested for antibody to N protein titers by the indirect fluorescent-antibody technique, using fluorescein-conjugated rabbit anti-dog globulin.

Inoculation of dogs. Twenty-three laboratory-raised beagles 1 to 3 years of age and of either sex were divided into four groups: seven in group I and five in each of the other three groups. Each dog in group I was inoculated intradermally (i.d.), in the back, with $10^{8.5}$ PFU of the vaccinia virus-derived-recombinant virus expressing the rabies virus N protein (43). Other dogs were included for comparative purposes; group II was vaccinated i.d. with a vaccinia virus recombinant expressing the rabies G protein (acquired from J. J. Esposito, Centers for Disease Control), and group III was inoculated i.d. simultaneously with both G and N recombinants at two separate sites. Control dogs (group IV) were inoculated with only $10^{8.5}$ PFU of vaccinia virus. Serum specimens were collected at 7, 14, 30, and 90 days after vaccination to determine VNA titer by the rapid fluorescent-focus inhibition test against strain CVS or (in some cases) street rabies virus strains. At the end of the observation period, all dogs were bled and challenged in the masseter muscle with 1 ml of canine salivary gland suspension containing $10^{6.3}$ 50% mouse intracranial lethal doses of a street rabies virus obtained from a dog near the Texas-Mexico border (L-2596). Dogs were observed for signs of rabies: agitation, restlessness, dropped jaw, muscular incoordination, and paralysis. Serum specimens were then collected at days 95, 100, 120, and 130 to determine the possible booster effect of the challenge virus.

TABLE 1. Serum VNA titers of dogs vaccinated i.d. either with vaccinia virus recombinants expressing rabies virus N protein, G protein, or G plus N proteins or with vaccinia virus

Group	Serum VNA titer (IU/ml) at given wk postvaccination[a]						
	1	2	5	8[b]	9	10	12
I (N)	<0.1	<0.1	<0.1	<0.1	0.3[c]	1.5	1.5
II (G)	0.3	1.5	1.5	1.5	37.0	37.0	37.0
III (G + N)	<0.1	1.5	1.5	1.5	37.0	37.0	37.0
IV (vaccinia virus)	<0.1	<0.1	<0.1	<0.1	<0.1	0.3	NT[d]

[a] All titers were <0.1 at week 0.
[b] Challenge date.
[c] One of seven dogs in group I had VNA 1 week after challenge.
[d] NT, not tested.

TABLE 2. Rabies virus N antibody titers in dogs vaccinated with a vaccinia virus recombinant expressing the rabies virus N protein determined by ELISA against rabies virus N protein

Dog no.	Absorbancy determined at 1:100 serum dilution	
	Prechallenge	Postchallenge
27	0.639	2.391
84	0.573	2.141
366	0.585	1.436
24	0.490	1.891
36	0.660	1.586
40	0.651	1.875
65	0.490	0.761

RESULTS

No sign of disease was detected in any of the dogs prior to challenge. All dogs vaccinated with rabies virus G protein or G plus N proteins developed VNA titer (Table 1). Dogs vaccinated with N protein, however, had no detectable VNA titer prior to challenge, and the levels of antibody directed against N protein were low (Tables 1 and 2). Five of the seven dogs vaccinated with N protein developed clinical rabies 11 to 14 days after challenge. The incubation period in these dogs was 3 to 7 days shorter than that of the control dogs (Table 2). The difference in incubation periods between control dogs and those vaccinated with N protein was statistically significant ($P < 0.02$). All control dogs died. Of the seven dogs vaccinated with N protein, only two died of rabies (although five had sickened). Viral antigen was demonstrated by fluorescent antibody in the brain tissue of all dogs that died. The remaining three dogs in group I gradually recovered without supportive treatment, after a morbidity period ranging from 7 to 12 days (Table 3). The recovery was confirmed by the presence of VNA titer in the cerebrospinal fluid (CSF) (Table 4) collected after the disappearance of clinical signs. None of the dogs vaccinated with the G protein (group II) or with the G and N proteins (group III) and none of the control dogs (group IV) developed any detectable amounts of VNA titer in the CSF. All dogs in groups II and III had a high booster response 5 days after challenge, but only one of the dogs in group I developed a VNA titer 8 days after challenge; a similar VNA titer was also detected in one dog in group IV (Table 1). Protection, sickness, or survival of dogs vaccinated with the N protein was not dependent on the titer of VNA or antibody against N protein prior to challenge, as dogs with detectable amounts of antibody died whereas others that had no VNA survived challenge.

DISCUSSION

Recovery from rabies has been reported for only a limited number of humans and animals since Pasteur's time (2, 12,

TABLE 3. Sickness and recovery in dogs vaccinated i.d. with vaccinia virus recombinants expressing either rabies virus N protein, G protein, or G and N proteins simultaneously or with vaccinia virus

Group	Incubation period (days)	Sickness	Death/ survival	Recovery
I (N)	11–14	5/7	2/5	3/5
II (G)		0/5	0/5	0
III (G + N)		0/5	0/5	0
IV (vaccinia virus)	14–21	6/6	6/6	0

TABLE 4. VNA titers of dogs vaccinated with a vaccinia virus recombinant expressing rabies virus N protein and then challenged with street rabies virus

Dog no.	VNA titer (IU/ml)[a]		
	Postchallenge, serum	Postrecovery	
		Serum	CSF
27	8.2	14.1	12.9
84	0.1	7.3	1.4
366	0.6	12.9	10.1
24	<0.1	<0.1	<0.1
36	<0.1	<0.1	<0.1
40	0.6[b]	NT[c]	NT
65	<0.1[b]	NT	NT

[a] Pre- and postvaccination serum titers were <0.1 in all cases.
[b] Dogs that died.
[c] NT, not tested.

17, 29, 30, 35, 42). Pasteur was the first to report that dogs occasionally recovered from rabies, and he considered subsequent resistance of these dogs to reinfection as a strong indication of previous abortive infection (29). The most commonly used criterion for detecting nonfatal rabies virus infection is the isolation of virus from saliva, brain, or other tissues of animals that recover after sickness. Brain biopsy specimens have even been taken from humans for virus isolation tests to confirm a diagnosis of rabies when apparent recovery from rabies had occurred (12, 17, 30). However, a high VNA titer in the brain or the CSF has been shown to be the only definitive diagnostic test for demonstrating recovery from central nervous system rabies virus infection in humans and animals (3, 12).

In previous studies, we reported that up to 20% of dogs experimentally infected with street rabies viruses recovered without supportive treatment; these recoveries were not dose or virus strain dependent (11). The mechanism of such recoveries, however, is still not well understood. Dogs that survived street rabies challenge without signs of disease and failed to develop VNA titer were challenged after a 2-year observation period; they developed a rather high anamnestic serum VNA response and resisted challenge, indicating that factors other than VNA induced by rabies virus G protein play a role in protecting dogs from rabies virus infection. It has also been reported that animals with VNA titers succumbed when challenged, whereas others that had no detectable amounts of antibody resisted (4, 32). In addition, the role of cell-mediated immunity has been noted in recovery from attenuated rabies virus infection in mice, with T cells stimulated by both rabies virus G and N proteins (24, 25, 40). VNA, therefore, may not be the only factor involved in recovery from rabies.

Nevertheless, the role of individual rabies virus proteins in resistance to challenge is still not well understood. Recent reports have shown that the N protein can induce a protective immune response to rabies virus infection in mice (8, 43). It has also been reported that mice primed with ribonucleoprotein and given a booster vaccination with homologous rabies virus G protein developed only low levels of VNA and were not protected against lethal challenge (8). Our present study shows that all dogs vaccinated with G protein were fully protected and developed a high booster response after challenge. Five (71%) of the seven dogs vaccinated with the N protein survived challenge with or without clinical signs of rabies. Two (28.5%) of the seven

N-protein-vaccinated dogs that did not sicken failed to develop VNA after challenge, unlike dogs vaccinated with G protein, indicating that priming dogs with N protein may not induce higher VNA response, contrary to previous reports (8). Three of five N-protein-vaccinated dogs (60%) sickened and recovered without supportive treatment and developed high VNA titers in both the serum and CSF, indicating that N protein may be involved not only in induction of the T-cell response (24, 25, 40) but also in sickness and recovery. The specific role of N protein in sickness and recovery in dogs is not clear. The mechanism of protection against rabies virus challenge in the absence of VNA could be attributed to the induction of cytolytic T cells as well as T helper cells that support the activity of VNA-producing B cells, or it could act by promoting the attachment of anti-N antibody via the Fc receptor to phagocytic cells, which are then stimulated by the infecting (challenge) virus to produce cytokines that inhibit viral replication. Because of the unavailability of specific dog T-cell epitope markers, it is difficult to explain the role of T cells in sickness and recovery of dogs vaccinated with N protein. Further studies should determine the role of other rabies virus proteins in sickness and recovery or abortion of rabies virus infection.

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