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Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein

(rabies vaccine/antigen priming/intrastructural antigen recognition/virus-neutralizing antibody/cytolytic T cells)

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ABSTRACT **We have studied the ability of rabies virus ribonucleoprotein (RNP) to induce a protective immune response in animals against lethal challenge with rabies and rabies-related lyssa viruses. Liposomes containing either RNP or the glycoprotein (G protein) of a variant virus with multiple alterations in the G antigenic structure conferred no or poor protection, respectively, against lethal intracerebral challenge with rabies virus. By contrast, liposomes containing RNP and the variant G protein induced a good protective response, comparable to that achieved with inactivated virus vaccine against intracerebral challenge. Moreover, mice or raccoons immunized with RNP alone resisted lethal peripheral challenge with homologous or heterologous virus strains. These results indicate that the RNP of rabies virus plays a crucial role in induction of protective immunity.**

Rabies virus particles represent complex antigens consisting of five different structural proteins (1). Attention concerning the induction of protective antiviral immunity has focused primarily on the response to the viral surface glycoprotein (G protein) based on the observation that neutralizing antibody to rabies virus, considered so far as the main source of protection against a rabies virus infection, shows exclusive specificity for the G protein (2). Purified G protein and a vaccinia-rabies G gene-recombinant virus expressing the rabies virus glycoprotein have been shown to be effective vaccines against lethal rabies infection (2, 3). However, antigenic analysis of a wide variety of fixed (vaccine strains), street, and rabies-related lyssa viruses with G-specific monoclonal antibodies has revealed considerable antigenic variation among the G proteins of viruses isolated from different host species or geographical locations (4). These marked antigenic variations have potentially serious implications for rabies control strategies (4, 5). To circumvent the problem of antigenic variability of rabies virus G, we turned to the rabies virus nucleoprotein, which is more conserved antigenically among rabies virus strains (4). Based on observations in other pathogenic virus systems that internal viral antigens can contribute significantly to the induction of protective immunity (6, 7), we analyzed the role of rabies virus ribonucleoprotein (RNP) in induction of protective immune response. We have shown (5) that an inactivated rabies vaccine prepared from a variant virus having multiple amino acid substitutions in the G protein can confer protection against a lethal infection with a virus strain that has an identical nucleoprotein but that differs considerably in the antigenic composition of the G protein from the vaccine strain. This observation, together with the finding that the virus-neutralizing antibody (VNA) titer against rabies challenge virus induced by the variant virus vaccine was lower by a factor of 30 than the VNA titer induced by the parent virus vaccine, suggests that other structural proteins such as the nucleoprotein might contribute to the induction of protective immune responses against rabies.

We demonstrate here that immunization with rabies RNP can effectively prime animals for the production of VNA. Furthermore, administration of rabies RNP resulted in protection of mice or raccoons from a lethal peripheral challenge with rabies or rabies-related lyssa viruses.

MATERIALS AND METHODS

Viruses and Antigens. The fixed rabies strains ERA and CVS-11 and the rabies-related strains Mokola 3 (MOK) and Duvenhage 6 (DUV6) were propagated on BHK-21 cell monolayers as described (8). Challenge virus strains CVS-11, CVS-24, DUV6, and MD5951 were prepared from suckling mouse brain as described (9). Rabies or rabies-related viruses were purified as described (9). The purified virus was suspended in phosphate-buffered saline, inactivated with β-propiolactone, and adjusted to a protein concentration of 100 μg/ml. Viral RNP was isolated and purified from rabies virus-infected BHK-21 cells as described (10). G protein was purified from virions by preparative isoelectrofocusing as described (11). Liposomes were prepared according to the method of Thibodeau *et al.* (12).

Selection of Multiple Variant Virus CVS-V7. The method used for selection of the multiple variant virus CVS-V7 has been described (5). The sequentially selected neutralization-resistant variant CVS-V7 was not neutralized by any of 41 neutralizing antibodies.

Protection Experiments with Inactivated CVS-V7 Virus and Liposomes Containing G Protein or RNP. Four-week-old female ICR mice (Harlan Sprague–Dawley) in groups of seven were immunized intraperitoneally (i.p.) on days 0 and 7 with 0.1 ml of one of five serial dilutions (16–10,000 ng) of either inactivated CVS-V7 virus or liposomes containing CVS-V7 G protein, CVS-V7 RNP, or both CVS-V7 RNP and CVS-V7 G protein. On day 14 blood was collected from vaccinated mice. They were then infected intracerebrally (i.c.) with 0.02 ml (50 $MICLD_{50}$, where $MICLD_{50}$ represents mouse i.c. lethal dose) of CVS-11 virus. Mice were observed for 3 weeks and deaths were recorded daily. The effective doses protecting 50% of mice (ED_{50}) were calculated as described (5).

Abbreviations: VNA, virus-neutralizing activity(ies); RNP, ribonucleoprotein; G protein, glycoprotein; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; RFFIT, rapid fluorescence focus-inhibition test; i.c., intracerebral(ly); i.p., intraperitoneal(ly); i.m., intramuscular(ly); s.c., subcutaneous(ly); $MIMLD_{50}$, mouse i.m. lethal dose; $MICLD_{50}$, mouse i.c. lethal dose; MOK, Mokola 3; DUV6, Duvenhage 6.

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Determination of VNA. The neutralizing activity of mouse or raccoon immune sera to CVS-11 or MOK virus was determined as described (13).

Priming of Mice for VNA with RNP. Six-week-old female ICR mice (Harlan Sprague–Dawley) were immunized i.p. with 5 μg of ERA RNP plus complete Freund's adjuvant (CFA) or CFA alone. Four weeks after priming, groups of seven mice primed with either RNP plus CFA or CFA alone were immunized i.p. with 0.1 ml of five serial dilutions (8–5000 ng) of either inactivated ERA virus or ERA G protein. Blood was collected 10 days after booster immunization and mice were challenged i.c. with 50 $MICLD_{50}$ of CVS-11 virus. The ED_{50} of the vaccines was calculated as described (5).

Protection Experiments with RNP. Six-week-old female BALB/c mice (Harlan Sprague–Dawley) were immunized i.p. with 10 μg of ERA RNP or MOK RNP in CFA or CFA alone with 10 μg of ERA RNP without CFA. Four weeks after immunization, mice were challenged intramuscularly (i.m.) in the hind leg with 10^6 $MICLD_{50}$ (10 $MIMLD_{50}$, where $MIMLD_{50}$ represents mouse i.m. lethal dose) of CVS-24 virus or with 10^5 $MICLD_{50}$ of DUV6 virus. Animals were observed for 3 weeks and deaths were recorded daily.

Five adult raccoons received 100 μg of ERA RNP in CFA i.p. and five controls received only CFA i.p. Thirty days after the primary immunization, the RNP-primed raccoons again received 100 mg of ERA RNP in incomplete Freund's adjuvant (IFA) subcutaneously (s.c.) and the control animals received IFA alone. Sixty days after primary immunization, RNP-treated and sham-immunized animals were challenged with $10^{5.5}$ $MICLD_{50}$ of the street rabies virus strain MD5951 as described (14). Blood was collected for all raccoons on days 0, 14, 21, 60, and 17 and VNA were determined as described (13).

Protection Experiments with Synthetic Peptides. The amino acid sequence and synthesis of peptides N-V12b and N-V10c have been described (15). To facilitate the incorporation of the peptides into liposomes, palmitic acid was linked to the amino-terminal end of the peptide as described (16) and peptide liposomes were formed (12). Groups of 6-week-old BALB/c mice (Harlan Sprague–Dawley) were immunized i.p. with either 15 μg of N-V12b liposomes in CFA, 15 μg of N-V10c liposomes in CFA, or liposomes in CFA. Four weeks after immunization, mice were challenged i.m. with either 2, 4, or 8 $MIMLD_{50}$ of CVS-24 virus.

RESULTS

Effect of RNP on Induction of Immunity Against i.c. Virus Challenge. VNA as well as protection were provided by the inactivated CVS-V7 virus vaccine; only 33 ng of CVS-V7 vaccine was necessary to protect 50% of mice against an i.c. challenge with CVS-11 virus in which 100% of control animals succumbed (Table 1). In contrast, the protective activity of liposomes prepared with the CVS-V7 G protein was low; 588 ng of CVS-V7 G protein (inserted into liposomes) was necessary to protect 50% of mice. Whereas the RNP-liposome vaccine failed to confer protective immunity against a lethal i.c. challenge, a preparation of liposomes containing the CVS-V7 G protein and the CVS-V7 RNP resulted in a 13-fold increase of the protective activity over the CVS-V7 G protein liposome vaccine. The effective dose of the G protein-RNP liposome vaccine was similar to that of the inactivated CVS-V7 virus vaccine.

Production of VNA and Protection Against i.c. Virus Challenge in RNP-Primed Mice. VNA titers in mice primed with RNP in CFA and given booster immunizations with inactivated rabies virus vaccine were 10–20 times higher than those in mice primed only with CFA and then given booster immunizations with inactivated virus vaccine (Table 2). In addition, the ED_{50} of rabies virus vaccine was found to be lower by a factor of ≈10 in the mice that had been primed with RNP in CFA. Mice that received booster vaccinations of ERA virus G developed only low levels of VNA and were not protected against lethal i.c. challenge with CVS-11 virus. No significant differences in VNA titers were found among the groups of mice primed with RNP in CFA or CFA alone. Mice primed with ERA RNP in CFA and then given booster immunizations with inactivated MOK virus vaccine developed MOK-specific VNA titers that were 6-fold higher than VNA titers in mice primed with CFA only (Table 3). Furthermore, no VNA against CVS virus were detected in mice primed with ERA RNP and then given booster immunizations with MOK virus, indicating that the increased MOK-specific VNA in ERA RNP-primed mice are not due to a contamination of the ERA RNP with ERA G protein.

Table 1. Protective activities of an inactivated CVS-V7 virus vaccine and CVS-V7 subunit vaccines against an i.c. challenge infection with CVS-11 virus

	CVS-V7 virus		CVS-V7/G protein liposome		CVS-V7/RNP liposome		CVS-V7/RNP-G liposome	
Vaccine, ng	VNA against CVS-11 GMT (range)*	Mortality rate†	VNA against CVS-11 GMT (range)*	Mortality rate†	VNA against CVS-11 GMT (range)*	Mortality rate†	VNA against CVS-11 GMT (range)*	Mortality rate†
10,000	1000 (270–1620)	0/7	109 (<10–810)	1/5	0	7/7	588 (90–4860)	1/6
2000	223 (60–540)	2/7	275 (90–540)	2/7	0	7/7	223 (60–810)	1/7
400	177 (60–540)	3/7	37 (<10–180)	4/6	0	7/7	323 (90–1620)	1/7
80	100 (<10–540)	3/7	21 (10–60)	3/6	0	7/7	53 (<10–540)	1/7
16	25 (<10–540)	3/7	<10 (<10–30)	5/6	0	7/7	<10 (<10–90)	5/7
ED_{50},‡ ng	33.3		588		>10,000		45	

Groups of mice were immunized i.p. on days 0 and 7 with 0.5 ml of the listed dose of vaccine, bled for VNA on day 14, and then challenged i.c. with 0.03 ml (50 $MICLD_{50}$) of CVS-11 virus.

*Titers are listed as the reciprocal of the highest dilution of serum yielding a 50% reduction in a modified rapid fluorescence focus-inhibition test (RFFIT). GMT, geometric mean titer.

†100% of nonimmunized control animals succumbed to challenge infection.

‡Nanograms of vaccine protecting 50% of mice.

Table 2. Effect of RNP priming on VNA titers and mortality rates

	Booster immunization with ERA virus				Booster immunization with ERA G protein			
	Priming with ERA RNP + CFA		Priming with CFA		Priming with ERA RNP + CFA		Priming with CFA	
Vaccine, ng	VNA against CVS-11 GMT (range)*	Mortality rate†	VNA against CVS-11 GMT (range)*	Mortality rate†	VNA against CVS-11 GMT (range)*	Mortality rate†	VNA against CVS-11 GMT (range)*	Mortality rate†
5000	1000 (180–2430)	3/7	44 (<10–180)	5/5	169 (30–540)	7/7	117 (20–540)	6/7
1000	489 (60–1620)	4/7	39 (10–180)	6/7	32 (20–180)	6/7	19 (<10–90)	7/7
200	138 (20–1620)	3/7	10 (<10–60)	6/7	1.3 (<10–10)	6/7	1.5 (<10–20)	7/7
40	34 (<10–270)	5/6	10 (20–60)	7/7	0	7/7	0	7/7
8	26 (<10–540)	6/7	10 (<10–30)	7/7	0	7/7	0	6/6
ED_{50},‡ ng	625		>5000		>5000		>5000	

Groups of mice were immunized i.p. on day 0 with 5 μg of ERA RNP + CFA or CFA alone. Four weeks later the mice received booster immunizations with different concentrations of either inactivated ERA virus or ERA G protein, and 10 days after immunizations the mice were bled for VNA and challenged i.c. with CVS-11 virus (50 MICLD).
*Titers are listed as the reciprocal of the highest dilution of serum yielding a 50% reduction in a modified RFFIT. GMT, geometric mean titer.
†100% of nonimmunized control animals succumbed to challenge infection.
‡Nanograms of vaccine protecting 50% of mice.

Induction of Protective Immunity to Peripheral Rabies Virus Challenge by Rabies RNP. To circumvent the variability of results caused by heterogeneity in the genetic background of outbred mice, peripheral virus challenge experiments were carried out in BALB/c inbred mice. Groups of mice immunized i.p. with ERA or MOK RNP in CFA or CFA alone or immunized s.c. with ERA RNP without CFA were challenged 4 weeks later i.m. with 8 $MIMLD_{50}$ ($\approx 10^6$ $MICLD_{50}$) of CVS-24. Though only 10% of the sham-immunized control animals survived the rabies virus challenge, 80% of mice immunized with ERA RNP survived (Table 4). Interestingly, i.p. immunization with MOK RNP in CFA resulted in 90% protection against a heterologous CVS-24 challenge. In addition, i.p. administration of MOK or ERA RNP in CFA also resulted in substantial protection (90–100%) of mice challenged with the rabies-related virus DUV6 (Table 4).

To determine whether immunization with RNP confers protection in species other than mice, we performed protection experiments in captive raccoons (data not shown). Four of five raccoons immunized parenterally with RNP in CFA survived challenge with the street rabies virus strain MD-5951, whereas four of five sham-immunized animals succumbed to rabies. None of the RNP immunized raccoons had VNA titers at the time of challenge.

Table 3. Priming with ERA RNP for MOK VNA

Experimental method		Geometric mean VNA titer*	
Antigen priming (day 0)	Booster immunization (day 28)	Against MOK virus (day 39)	Against CVS-11 virus (day 39)
ERA RNP (10 μg) + CFA	BPL MOK (10 μg)	646 (270–2430)	0
CFA	BPL MOK (10 μg)	112 (30–270)	0

Groups of mice were immunized i.p. with 10 μg of ERA RNP + CFA or CFA alone. On day 28, the primed mice received a booster immunization of 10 μg of inactivated MOK virus. BPL, β-propiolactone.
*Titers are listed as the reciprocal of the highest dilution of serum yielding a 50% reduction in test virus in a modified RFFIT.

Protective Immunity Induced by a Synthetic Rabies RNP Fragment. We have recently described two synthetic peptides covering amino acids 369–383 (N-V10c) and 313–337 (N-V12b) of the N-protein sequence. Both peptides were recognized by different monoclonal antibodies and by human T cells (16). These were used to immunize BALB/c mice against an i.m. challenge with the CVS-24 strain of rabies virus. Depending on the dose of the challenge virus, immunization with N-V12b peptide liposomes resulted in partial but significant protection (62–82%) (Table 5). In contrast, immunization with N-V10c peptide liposomes in CFA or liposomes alone in CFA failed to protect mice against a lethal i.m. challenge with CVS-24.

DISCUSSION

We have described the induction of protective immune responses by rabies virus RNP against infection with rabies and rabies-related lyssa viruses. The efficacy of the RNP vaccine was dependent upon the route of the virus challenge. Liposomes containing RNP isolated from CVS-V7 virus induced no protection against i.c. challenge with CVS-11 virus. Furthermore, liposomes containing the CVS-V7-derived G

Table 4. Immunization of BALB/c mice with rabies RNP and MOK RNP against an i.m. challenge with CVS-24

	Mortality rate† (%)	
Experimental treatment*	CVS-24 challenge	DUV6 challenge
ERA RNP (10 μg) + CFA i.p.	2/10 (20)	0/10 (0)
MOK RNP (10 μg) + CFA i.p.	1/10 (10)	1/10 (10)
CFA i.p.	18/20 (90)	6/10 (60)

ND, not done.
*Groups of BALB/c mice were immunized i.p. with either ERA RNP + CFA, MOK RNP + CFA, or CFA alone.
†Four weeks after immunization, all mice were challenged i.m. in the hind leg with 10^6 MICLD (≈ 10 $MIMLD_{50}$) of CVS-24 virus or with 10^6 $MICLD_{50}$ of DUV6 virus.

Table 5. Protective activities of N-V12b peptide liposomes in BALB/c mice to i.m. challenge with CVS-24

Vaccine, μg	Vaccine	Mortality rate
	Experiment 1*	
15	N-V10c liposome + CFA	5/5
15	N-V12b liposome + CFA	1/8
—	None	7/10
	Experiment 2†	
15	N-V12b liposome + CFA	3/8
—	Liposome + CFA	7/8
	Experiment 3‡	
15	N-V12b liposome + CFA	4/10
15	N-V10c liposome + CFA	9/10
—	Liposome + CFA	8/10

Groups of BALB/c mice were immunized i.p. with either N-V10c peptide in CFA, N-V12b peptide liposomes in CFA, or liposomes in CFA alone. Four weeks after immunization, mice were challenged i.m. with 2–8 $MIMLD_{50}$ of CVS-24 virus.
*i.m. challenge with 2 $MIMLD_{50}$.
†i.m. challenge with 4 $MIMLD_{50}$.
‡i.m. challenge with 8 $MIMLD_{50}$.

protein induced only poor protection. However, when RNP was combined with the G protein in liposomes, the protection was much better and comparable to that obtained with whole virus vaccine. Differences in survivorship to CVS-11 challenge of the RNP liposome, G liposome, and the G-RNP liposome-immunized groups appeared consistent with the levels of VNA against CVS-11 virus induced by these vaccines—i.e., RNP induced no detectable VNA, G protein induced low VNA, and RNP combined with G protein stimulated a VNA response of the same magnitude as whole virus.

Whereas RNP alone did not confer protection against a lethal i.c. challenge with CVS-11 virus, mice or raccoons receiving RNP plus CFA i.p. were protected against a peripheral i.m. challenge with CVS-24 virus. Similar findings were obtained with raccoons (data not shown). Importantly, immunization with MOK RNP resulted in protection against heterologous CVS-24 virus. Because neutralizing antibodies raised against MOK virus only minimally neutralize rabies virus (13), this experiment indicates that it is unlikely that the protection conferred by RNP is due to the presence of VNA induced by undetectable amounts of G protein. In addition, ERA RNP and MOK RNP induced protective immunity against the rabies-related European bat strain DUV6. Together, these results demonstrate that RNP purified from rabies and rabies-related viruses can induce protective immunity against heterologous viruses.

The mechanism(s) by which RNP-immunized animals are protected is not yet clear. Possibly, RNP induces T-helper cells that augment the activity of VNA-producing B cells; the G protein and the RNP have been shown to stimulate proliferation of rabies-specific T-helper cells, and most T-helper cell lines tested are highly responsive to RNP and less so to G protein (17). Furthermore, internal influenza virion antigens are known to generate murine T-helper cells able to help anti-hemagglutinin antibody responses (18), results confirmed *in vitro* and *in vivo* (19). Like mice primed with internal influenza proteins and given booster immunizations with whole influenza virus, mice primed with rabies RNP in CFA and then given booster immunizations with inactivated rabies virus developed significantly higher VNA than did control mice. The helper effect mediated by RNP occurred only when mice were given booster immunizations with inactivated whole virus and no effect was observed when G protein was used for booster immunization. For animals that were protected after RNP immunization against peripheral (i.m.) virus challenge, the challenge itself would act as a booster immunization. In fact, RNP-immunized animals have higher VNA titers 5 days after challenge when sham-immunized control animals (1:20 vs. 1:5); however, VNA titers in the protected animals 5 days after challenge may be too low to block the spread of virus to the central nervous system. Cytolytic T cells might play a major role in the elimination of infectious virus, since rabies RNP is an excellent inducer of major histocompatibility complex class I-restricted cytolytic T cells in BALB/c mice (H.E., unpublished). It has also been suggested that protection of mice against a lethal challenge with influenza virus can be attributed to priming of influenza nucleoprotein-specific cytolytic T cells (6).

Results of the protection experiments with the synthetic peptides are encouraging with respect to the development of a synthetic vaccine against rabies. However, since the recognition of peptide antigens is controlled by immune response genes and is often restricted to a given major histocompatibility complex haplotype (20), it is unclear whether a single peptide fragment can be applied as a vaccine in an outbred population. We have previously shown that mice immunized with liposomes containing herpes simplex virus I (HSV-1) or HSV-2 peptides are protected against a lethal HSV-2 challenge and that $Lyt\text{-}2^+$ cells, and not antibody, are responsible for this protection (21). In the case of rabies, the protective mechanism remains unclear, although the possibility that $Lyt\text{-}2^+$ cells are involved is consistent with the data generated with whole RNP.

The immunization experiments with RNP from rabies and rabies-related lyssa viruses indicate that RNP plays a significant role in inducing protective immunity, especially against infection with heterologous lyssa viruses. Since VNA alone are known to be ineffective in rabies post-exposure situations (22), the use of RNP might be considered in light of its ability to induce immune effectors such as cytolytic T cells, which could be effective in the post-exposure rabies treatment.

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