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Immunology

Rabies virus nucleoprotein expressed in and purified from insect cells is efficacious as a vaccine

(baculovirus/helper T cells/antigen priming/virus-neutralizing antibody)

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ABSTRACT **A cDNA copy of the RNA gene that encodes the nucleoprotein N of rabies virus Evelyn–Rokitnicki–Abelseth strain was cloned into baculovirus. The recombinant baculovirus expressed the N protein abundantly in *Spodoptera frugiperda* cells. The N protein was extracted from infected *Spodoptera frugiperda* cells and purified to near homogeneity by affinity chromatography. The purified N protein reacted with 31 of 32 monoclonal antibodies that recognize native rabies virus ribonucleoprotein. Like the ribonucleoprotein, the purified N protein was a major antigen capable of inducing virus-specific helper T cells. Priming of mice with the purified N protein prior to a booster inoculation with inactivated Evelyn–Rokitnicki–Abelseth virus vaccine resulted in a 20-fold increase in the production of virus-neutralizing antibodies. After immunization with the purified N protein, mice developed a strong anti-ribonucleoprotein antibody response and were protected against a lethal challenge of rabies virus. These data indicate that the N protein expressed in insect cells is antigenically and immunogenically comparable to the authentic rabies virus ribonucleoprotein and therefore represents a potential source of an effective and economical vaccine for large-scale immunization of humans and animals against rabies.**

Rabies virus contains a single negative-strand RNA genome that codes for five proteins, a major nucleoprotein designated N, a nominal phosphoprotein designated NS, a RNA polymerase designated L, a matrix protein, and the surface glycoprotein G (1). The N, NS, and L proteins together with the viral RNA form the ribonucleoprotein (RNP) complex. Rabies virus glycoprotein G has been recognized (2) as the only antigen capable of inducing virus-neutralizing antibodies (VNA) and conferring protective immunity against rabies. Recent studies have demonstrated that, in addition to glycoprotein G, the internal RNP can also induce protective immunity against a subsequent peripheral challenge with rabies virus in mice, raccoons, and monkeys (ref. 3; M. Tollis, B.D., C. Buonavoglia, and H.K., unpublished data). The RNP-induced protection against rabies is apparently mediated by a complex interaction of various immune effector mechanisms, including B and T lymphocytes and possibly macrophages (4, 5).

Since RNP plays an important role in the induction of protective immunity, it represents a logical focus in the development of a new vaccine. Therefore, we have utilized DNA recombinant technology to design an effective economical vaccine that could provide a low-cost alternative for primary rabies vaccination. As the majority of RNP-induced B cells and helper T cells (T_H cells) are directed against the N protein, which is the major component of the RNP complex, we cloned the N protein gene of the rabies virus Evelyn–Rokitnicki–Abelseth (ERA) strain into a baculovirus that can express foreign proteins to high levels in insect cells (6). The recombinant baculovirus expressed the N protein abundantly in insect cells, and this protein was purified to homogeneity by affinity chromatography and tested for its antigenicity and immunogenicity in a mouse model.

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MATERIALS AND METHODS

Virus and Antigens. The baculovirus used in this study is *Autographa californica* nuclear polyhedrosis virus (AcNPV). The virus was propagated in *Spodoptera frugiperda* (Sf9) cells in the presence of Grace's medium (7). The fixed rabies virus ERA strain and challenge virus standard (CVS-24 and CVS-11) strains were propagated in BHK-21 cells and purified as described (8). Rabies virus vaccine was prepared with purified ERA virus inactivated with β-propriolactone (ERA-βPL) (3). ERA RNP was extracted from rabies virus-infected BHK-21 cells as described (9).

Construction of Transfer Vectors Containing the Rabies N Protein Gene and Selection of Recombinant Baculovirus. The transfer vector pVL1393 (10) containing the baculovirus polyhedrin promoter was obtained from M. D. Summers (Texas A & M University). The cDNA containing the complete coding sequence of the N protein gene of rabies virus was derived from plasmid pN1R2 (W.H.W., D. Marchadier, and P. J. Curtis, unpublished data) by digestion with *Xba* I and *Pst* I and then force-cloned into pVL1393 previously digested with *Xba* I and *Pst* I. A recombinant plasmid with the rabies virus N protein gene sequence in the correct orientation with respect to the polyhedrin promoter was selected and confirmed by restriction enzyme digestion and dideoxynucleotide sequencing. The recombinant plasmid DNA was cotransfected with purified AcNPV DNA into Sf9 cells as described (7). The recombinant baculovirus containing the N protein gene of rabies virus was selected by two cycles of limited dilution in combination with dot-blot hybridization (11) and then was plaque-purified (7).

Purification and Analysis of N Protein Expressed in Sf9 Cells. For purification, monolayers of Sf9 cells in 10 150-cm^2 tissue culture flasks (Nunc) were infected with the recombinant baculovirus at a multiplicity of infection of ≈10 plaque-forming units per cell. Infected cells were harvested 50–60 hr after infection, washed with phosphate-buffered saline, and disrupted with distilled water and sonication. The cell lysates

Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; VNA, virus-neutralizing antibodies; RNP, ribonucleoprotein complex; CFA, complete Freund's adjuvant; BRN, recombinant baculovirus containing rabies nucleoprotein gene; $MICLD_{50}$ and $MIMLD_{50}$, mouse i.c. and i.m. median lethal dose (kills 50% of mice); mAb, monoclonal antibody; CVS, challenge virus standard; ERA, Evelyn–Rokitnicki–Abelseth; T_H cell, helper T cell; Sf9, *Spodoptera frugiperda*; βPL, β-propriolactone; GMT, geometric mean titers.
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were clarified by centrifugation at 20,000 rpm for 30 min in an SW 27 rotor. After adjustment of pH to 7.2 and NaCl to 0.1 M, the supernatant was loaded onto an Affi-Gel 10 (Bio-Rad) column coupled with rabbit anti-RNP antibody, and the N protein was eluted with 0.15 M ethanolamine, pH 11/10% (vol/vol) glycerol. The amount of N protein eluted from the affinity column was determined by using a known amount of bovine serum albumin as a standard. For protein analysis, recombinant and wild-type virus and mock-infected Sf9 cell lysates, as well as the purified N protein, were subjected to electrophoresis in a sodium dodecyl sulfate (SDS)/10% polyacrylamide gel (12). The gels were either stained with Coomassie blue or transferred onto nitrocellulose membrane and immunoblotted (Western blotted) with monoclonal antibody (mAb) 377-7, specific to rabies virus RNP (13). The mAb probe was detected with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Boehringer Mannheim).

ELISA. The antibody-binding specificity of the N protein purified from Sf9 cells was compared with that of intact virus by an ELISA using microtiter ELISA plates coated with either purified ERA-βPL or the N protein purified from Sf9 cells at 0.2 μg per well.

Stimulation of Rabies Virus-Specific T-Cell Hybridomas. The two T-cell hybridomas used in this study, 9C5.D8-H and 3E6.E11-H, were specific for rabies virus N protein. They were shown to be phenotypically Thy-1.2$^+$, CD4$^+$, and CD8$^-$ and thus belonged to the T_H-cell subset. The development, selection, and specificity of these T-cell hybridomas will be described elsewhere (H.C.J.E., B.D., and L. Otvos, unpublished data). T-cell hybridomas were tested for lymphokine release in response to antigen as described (5).

Stimulation of Lymph Node Lymphocytes. Various concentrations of antigen diluted in complete Freund's adjuvant (CFA) were injected into the footpads of groups of female C3H/He mice from The Jackson Laboratories. Six days later, mice were sacrificed, and draining lymph nodes (i.e., popliteal and para-aortal) were tested for lymphokine release in response to antigen as described (5).

Protection Experiments with ERA RNP and N Protein Purified from Sf9 Cells. ICR mice (Harlan–Sprague–Dawley; 6–10 wk old) divided into groups of 10 mice were immunized i.p. twice at 10-day intervals with different amounts of ERA RNP in CFA or N protein purified from Sf9 cells in CFA. One group was sham-immunized with CFA alone. Two weeks after the last immunization, the mice were challenged i.m. in the hind leg with 10 MIMLD_{50} [mouse i.m. median lethal dose (50% of animals died)] of CVS-24 virus. Blood was withdrawn from these animals prior to challenge for the determination of anti-RNP antibodies as described (14). Animals were observed daily for 3 wk after challenge for development of symptoms and deaths.

Priming Effect of ERA RNP and N Protein Purified from Sf9 Cells on VNA Production and Vaccine Potency. To test the priming effect of ERA RNP and the N protein purified from Sf9 cells, 6-wk-old female ICR mice were immunized (10 animals per group) i.p. with 5, 1, or 0.2 μg of ERA RNP in CFA or N protein purified from Sf9 cells in CFA or with CFA alone. Ten days after priming, all of these mice were immunized i.p. with 5 μg of ERA-βPL. Blood was collected 5 and 10 days after booster immunization, and VNA titers were determined as described (15). To measure the effect of the N protein purified from Sf9 cells on the potency of a rabies virus vaccine, groups of ICR mice were immunized with 1 μg of the N protein purified from Sf9 cells in CFA or with CFA alone. Ten and 20 days after the priming, these mice were immunized twice with different amounts (0.04–5 μg) of ERA-βPL. Ten days after the last immunization, the mice were challenged intracerebrally with 10 MICLD_{50} (mouse i.c. median lethal dose) of CVS-11 virus.

RESULTS

Construction of Recombinant Plasmids and Expression of N Protein in Sf9 Cells. To express the N protein of rabies virus to high levels in the baculovirus expression system, the complete coding sequence of rabies virus N protein gene excised from pN1R2 was force-cloned into the transfer vector pVL1393. One recombinant plasmid, named pVL-RN, was confirmed by restriction enzyme digestion and dideoxynucleotide sequence analysis as having the rabies virus N protein gene in the correct orientation in relation to the polyhedrin promoter. After cotransfection of pVL-RN DNA and AcNPV DNA in Sf9 cells, a recombinant baculovirus was selected by limiting dilution in combination with dot-blot hybridization. This recombinant was subsequently plaque-purified and designated recombinant baculovirus containing the rabies N protein gene (BRN).

Purification of the Baculovirus-Expressed N Protein by Affinity Chromatography and Analysis of the Protein by SDS/PAGE and Western Blotting. Sf9 cells infected with BRN recombinant virus at a multiplicity of infection $\approx$10 plaque-forming units per cell were harvested 50–60 hr after infection, lysed with distilled water, and sonicated briefly. After clarifying by centrifugation, the supernatant was loaded onto an Affi-Gel column coupled with rabbit anti-RNP IgG, and the bound N protein was eluted with 0.15 M ethanolamine (pH 11). The lysates of BRN-, AcNPV-, and mock-infected Sf9 cells as well as the purified N protein were subjected to SDS/PAGE analysis with purified rabies virus proteins as standard markers. The resolving gel was either stained with Coomassie blue (Fig. 1 *Left*) or transferred to nitrocellulose paper for immunoblotting (Fig. 1 *Right*). The N protein expressed in BRN-infected Sf9 cells was found to have the same molecular weight as authentic rabies virus N protein by SDS/PAGE, and both reacted specifically with an anti-RNP antibody. The N protein was purified by affinity chromatography to near homogeneity with only one band visible in the gel (Fig. 1 *Left*); 5 mg of purified N protein was obtained from 2 $\times$ 10^8 infected cells. The purified N protein expressed in the baculovirus expression system is designated "B-N."

Antigenic Comparison of B-N and RNP. A panel of 32 anti-rabies RNP mAbs, two anti-NS protein mAbs, and one mAb specific for the N protein of a rabies-related virus were used in an ELISA test to compare the antigenicity of B-N in comparison with authentic N protein present in rabies virus. While ERA reacted with all of the 32 mAbs specific to rabies virus RNP, B-N reacted with 31 of them (Fig. 2). mAb 590-2, that recognizes rabies RNP, failed to react with B-N. B-N

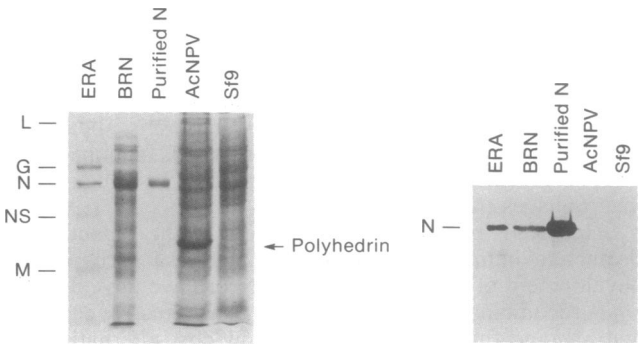

FIG. 1. Analysis of the rabies virus N protein synthesized in and purified from insect cells. Lysates of Sf9 cells mock-infected or infected with AcNPV or the BRN recombinant virus as well as the affinity-purified N protein from BRN-infected Sf9 cells were analyzed by SDS/PAGE and then either stained with 0.25% Coomassie blue (*Left*) or electrophoretically transferred to nitrocellulose membrane for Western blot analysis (*Right*).

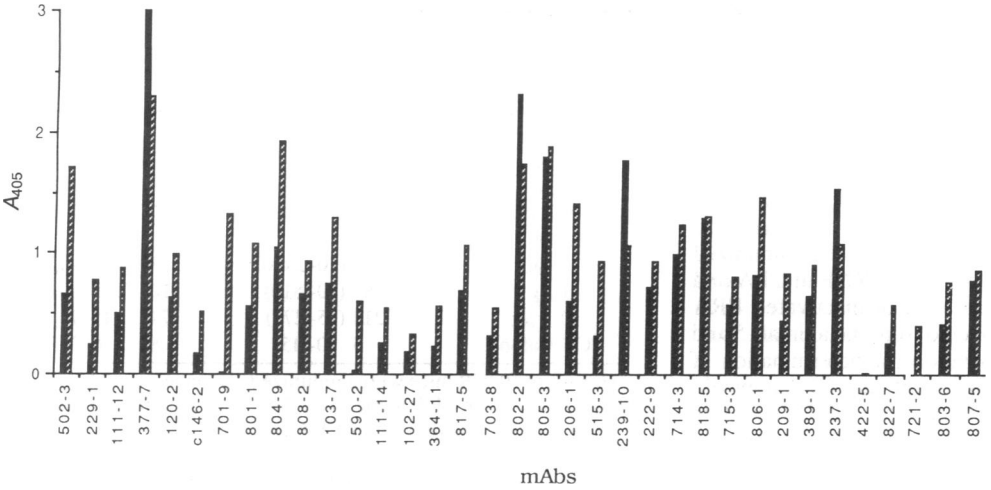

FIG. 2. Comparison of the antibody-binding specificity of ERA strain (□) and B-N (■). ERA strain of rabies virus and B-N were used to coat the wells of ELISA microtiter plates (0.2 μg per well). Antigens were blotted with a panel of 32 anti-rabies N protein mAbs, two anti-rabies NS protein mAbs (701-9, 721-2), and one mAb to a rabies-related virus (422-5). The conjugate used was an anti-mouse IgG antibody coupled to alkaline phosphatase. The absorbance value was read at 405 nm using a micro-ELISA reader. Data are means of triplicate measurements.

was not recognized by two NS protein-specific mAbs (i.e., 701-9 and 721-2), whereas ERA reacted with both. Neither ERA nor B-N reacted with mAb 422-5, which is specific for a rabies virus-related virus. To test for the T_H-cell antigenicity of B-N, two T_H-cell hybridomas with a known specificity for the ERA N protein were tested for lymphokine release in response to various amounts of ERA-βPL, RNP, or B-N presented on γ-irradiated syngeneic splenocytes. T-cell hybridomas responded to all three antigens (Fig. 3). The RNP was, on a weight basis, ≈5 times more efficacious than B-N. As a control, the hybridomas were tested for response to lysates of BRN- and BRNS-infected Sf9 cells. (BRNS represents recombinant baculovirus containing rabies virus NS protein gene.) While the hybridomas responded well to the unpurified N protein (2896 cpm for 9C5.D8-H and 2988 cpm for the 3E6.E11-H line at a dilution of 1:700), no response to the lysate of BRNS-infected cells was observed (92 cpm for 9C5.D8-H and 87 cpm for the 3E6.E11-H line at a dilution of 1:700), thus indicating that the response was elicited by the N protein and not by residual baculovirus or Sf9 cell proteins.

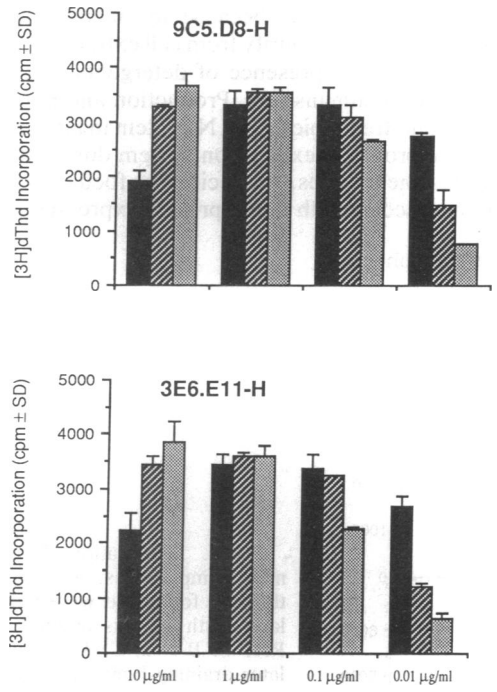

FIG. 3. Stimulation of T-cell hybridomas specific for rabies virus N protein. Irradiated C3H/He splenocytes were incubated with 25 μl of ERA-βPL (■), RNP (▨), and B-N (▩) diluted to 0.01–10 μg/ml in DMEM in wells of the microtiter plate. T-cell hybridomas 9C5.D8-H or 3E6.E11-H were then added in 50 μl of DMEM per well. After an incubation period of 24 hr, supernatants were transferred onto HT-2 cells. Proliferation of HT-2 cells (a T-cell line that depends on interleukin 2 or 4 for its growth) was measured 48 hr later by incorporation of [^{3}H]dThd. Data are means of triplicate experiments ± SD. The medium control for the hybridoma cells was as follows: 9C5.D8-H, 179 ± 49 cpm; 3E6.E11-H, 80 ± 19 cpm.

Comparison of the Immunogenicity of B-N and RNP. To test the potential for B-N to induce anti-RNP antibody as well as protective immunity, groups of ICR mice were immunized i.p. with different amounts of B-N or RNP in CFA and challenged 3 wk later i.m. with 10 $MIMLD_{50}$ of CVS-24. The geometric mean titers (GMT) of anti-RNP antibody induced by B-N were slightly lower than those induced by RNP (Table 1). While only 10% of the sham-immunized control animals survived the rabies virus challenge, 90%, 66%, and 35% of the mice immunized with 5 μg, 1 μg, or 0.2 μg of B-N, respectively, were protected. In comparison, 80% of mice immunized with 0.2 μg of RNP survived, indicating that the protective activity of RNP is better than that of B-N.

B-N, which was shown to stimulate a T_H-cell response of rabies virus-specific lymphocytes *in vitro*, was also tested *in vivo* for the induction of rabies virus-specific T_H cells. To compare the T_H-cell response to B-N and RNP, groups of C3H mice were immunized with various amounts of either B-N or RNP in CFA, and draining lymph node lymphocytes were tested 6 days later for lymphokine release (indicative for T_H-cell activity) in response to ERA-βPL. Both RNP and B-N induced lymphocytes that, upon *in vitro* stimulation with

Table 1. Induction of RNP-specific antibody and protective immunity by ERA, RNP, and B-N

Vaccine*	Anti-RNP antibody GMT† (range)	Mortality
RNP in CFA		
5 μg	10,660 (8100–24,300)	2/10
1 μg	7257 (2700–24,300)	3/10
0.2 μg	2389 (900–8100)	2/10
B-N in CFA		
5 μg	9041 (2700–24,300)	1/10
1 μg	2387 (1000–8100)	3/9
0.2 μg	1496 (900–2700)	7/10
CFA alone	0	9/10

*Groups of 10 ICR mice were immunized twice i.p. with RNP in CFA, B-N in CFA, or CFA alone.

†Two weeks after the last immunization, all mice were bled and challenged i.m. in the hind leg with 10 $MICLD_{50}$ of CVS-24 virus. Anti-RNP antibody titers were determined by the indirect fluorescent antibody staining technique as described (14).

ERA virus, released lymphokines (Fig. 4). RNP was more efficacious when compared with B-N, since 10–100 ng of RNP induced optimal stimulation of ERA virus-specific T cells, whereas 10 ng of B-N protein gave an intermediate response and 1 ng gave an insignificant response. RNP on the other hand, still induced a measurable rabies virus-specific T_H-cell response at 1 ng.

Since N protein-specific T_H cells can augment the production of VNA, we studied the priming effect of B-N in comparison to the priming effect of RNP. Groups of ICR mice were immunized with different amounts (0.2–5 μg) of B-N or RNP in CFA and given a booster immunization with 5 μg of inactivated ERA virus 10 days later. Blood was collected from the animals 5 and 10 days after the booster immunization for determination of VNA titers. In the absence of priming antigen (CFA control), only low VNA titers (day 5 = 1:5, day 20 = 1:81) could be detected (Table 2). In contrast, VNA titers 10 days after booster immunization in mice primed with either B-N or RNP were (depending on the concentration of the priming antigen) 6–19 times higher than those in mice primed with CFA alone. Mice that were primed with B-N responded as well as or better than mice primed with RNP.

To investigate the effect of B-N on vaccine potency, groups of ICR mice were immunized with 1 μg of B-N in CFA or CFA alone on day 0, followed by two booster immunizations with various amounts of ERA-βPL on day 10 and day 20. Ten days after the last immunization, the mice were challenged intracerebrally with 10 $MICLD_{50}$ of CVS-11. The VNA titers in the B-N-primed mice were (depending on vaccine concentration) 2 times (5 μg of ERA-βPL) to 95 times (0.04 μg of ERA-βPL) higher than the corresponding VNA titers in CFA-primed mice (Table 3). The higher VNA titers in the B-N-primed mice were reflected by an increased potency of the vaccine. The ED_{50} (median effective dose) of the vaccine in B-N-primed mice was 0.171 μg per mouse and the ED_{50} of the same vaccine in CFA-primed mice was 0.872 μg per mouse, indicating a 5-fold increase in vaccine potency after priming with B-N.

Table 2. Priming with ERA RNP and B-N for VNA

Priming antigen* (day 0)	VNA GMT† (range)	
	Day 15	Day 20
RNP in CFA		
5 μg	139 (10–3645)	716 (30–10,935)
1 μg	286 (30–2430)	829 (90–10,935)
0.2 μg	97 (30–810)	504 (30–10,325)
B-N in CFA		
5 μg	117 (10–2430)	1539 (135–10,935)
1 μg	187 (30–810)	1107 (135–10,935)
0.2 μg	214 (15–270)	743 (45–10,535)
CFA alone	5 (0–45)	81 (0–2430)

*Groups of 10 ICR mice were immunized i.p. once with RNP in CFA, B-N in CFA, or CFA alone. Ten days after priming all mice were immunized i.p. with 5 μg of ERA-βPL.

†VNA titers are listed as the reciprocal at the highest dilution of serum yielding a 50% reduction of virus-infected cells in the rapid fluorescence focus-inhibition test (15).

DISCUSSION

A rabies virus subunit vaccine consisting of the viral N protein was prepared from a baculovirus expression system. Data are presented that show that this subunit vaccine elicits T- and B-cell responses in mice and induces protection against a subsequent lethal challenge with rabies virus. It was shown previously that the viral surface glycoprotein G and the internal RNP are each capable of inducing protective immunity in a variety of species (2, 3, 16).

Expression systems that generate large amounts of individual proteins potentially provide an economic source of a viral subunit vaccine devoid of the risks associated with live recombinant subunit vaccines. A practical problem with production of such protein vaccines, however, is the need for extensive purification before the vaccine can be accepted for use in humans. It is not practical to produce the rabies virus glycoprotein G in this way because the membrane-bound glycoprotein is difficult to purify from cell extracts and is kept in solution only in the presence of detergents that are not suitable for use in humans (17). Production and purification of the membrane-free rabies virus N protein in large amounts by using an appropriate expression system does not require detergent treatment. Thus, we decided to focus on developing a subunit vaccine with the N protein expressed in insect

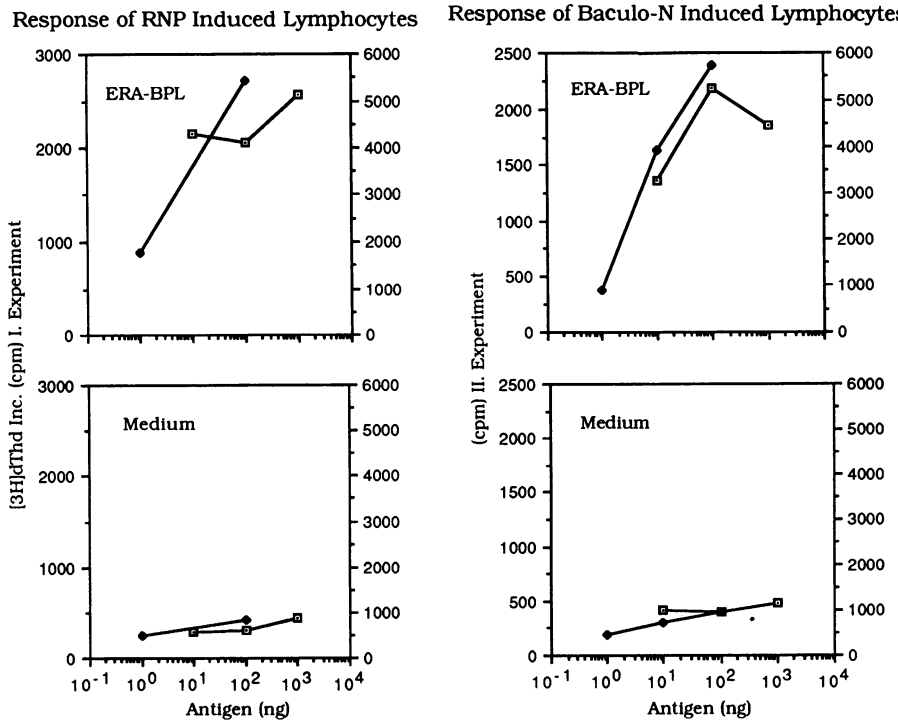

FIG. 4. Stimulation of lymph node lymphocytes. Groups of three or four mice were inoculated with various amounts of RNP or B-N in CFA. Six days later draining lymph node lymphocytes were tested for lymphokine release in response to ERA-βPL (250 ng per well) (*Upper*) or as the negative control in response to medium without antigen (*Lower*). Data are means of quadruplicate sets of values of two experiments: solid symbols relate to experiment I (left *y* axis); open symbols relate to experiment II (right *y* axis). Inc., incorporated.

Table 3. Effect of B-N priming on vaccine potency

Vaccine,† μg	Priming antigen* B-N VNA GMT‡ (range)	B-N Mortality	CFA VNA GMT‡ (range)	CFA Mortality
5.00	7467 (240–32,805)	2/10	3602 (405–10,935)	2/10
1.00	3361 (270–10,935)	3/9	479 (15–7290)	4/10
0.20	939 (45–21,870)	4/10	63 (5–1215)	10/10
0.04	666 (45–7290)	5/9	7 (5–90)	8/9
0.00	7 (5–10)	10/10	0 —	8/9

*Ten days after priming, mice were immunized twice with different concentrations of ERA-βPL. Ten days after the last immunization, mice were bled and challenged intracerebrally with 10 $MICLD_{50}$ of CVS-11 virus.

†Groups of 10 ICR mice were primed with 1 μg of B-N in CFA or with CFA alone.

‡VNA titers are listed as the reciprocal of the highest dilution of serum yielding a 50% reduction of virus-infected cells in the rapid fluorescence focus-inhibition test (15).

cells infected with a recombinant baculovirus containing the N protein gene of rabies virus. The N protein not only represents the major protein component of the viral RNP complex but also is an excellent inducer of functional B and T_H cells (4, 5). Furthermore, the N protein is highly conserved, both antigenically and genetically, among different rabies and rabies-related viruses (1, 3) and, therefore, is expected to induce cross-protection against a variety of virus strains circulating in various parts of the world.

The N protein from the CVS-11 strain of rabies virus has been expressed in baculovirus-infected insect cells (18, 19), but in these studies, no attempts were made to study its immunogenicity in any quantitative way. From the results presented in this paper, it is clear that ERA B-N protein expressed in insect cells and purified to near homogeneity is antigenically and immunogenically comparable to that of authentic rabies virus RNP. By using a panel of mAbs with known binding specificity to internal proteins of rabies virus, we demonstrated that the B-N protein not only possessed the three antibody binding sites but also contained 31 of 32 B-cell epitopes present in the native protein.

To evaluate the cellular immune response to B-N, two T_H-cell hybridomas, which are derived from ERA virus-immune C3H/He mice and are specific for an immunodominant epitope of the N protein, were tested for lymphokine release in response to purified B-N, ERA-βPL, or RNP. Slightly higher amounts of B-N as compared to RNP were required to induce optimal activation of the T-cell hybridomas. Similar results were obtained *in vivo*, where stimulation of rabies virus-specific T_H cells (defined by lymphokine release upon *in vitro* restimulation with ERA-βPL virus) by RNP was somewhat superior to stimulation by B-N. The superiority of RNP was also demonstrated in the protection experiment. The superior efficacy of the RNP complex over B-N protein may be explained by two possibilities. First, trace amounts of NS protein in the RNP complex, which can be demonstrated by Western blot analysis with anti-NS mAbs (data not shown), might enhance induction of T_H cells or anti-viral antibodies, although the contribution of NS is expected to be small based on the amounts of NS protein present in the RNP, and NS protein is a poor immunogen for both B and T_H cells (J. K. Larson, W.H.W., and H.C.J.E., unpublished data). The second possibility is that greater stability of the N protein in the RNP complex makes it a better immunogen.

In this study, we have demonstrated that immunization with B-N can effectively prime animals for the production of VNA. The priming for VNA of a subunit vaccine could have more practical implications for rabies immunoprophylaxis of humans than animals. In areas where urban animal rabies is endemic, the entire human population could be primed by immunization with B-N. In the case of an exposure to rabies virus, a single inoculation of the B-N-primed individuals with inactivated rabies virus vaccine would probably be sufficient to protect them from developing rabies or rabies virus infection. Furthermore, the time lapse between exposure to rabies virus and postexposure treatment, determined to be crucial in successful prevention of lethal rabies virus infection, might be less critical in individuals primed with B-N prior to rabies virus exposure.

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