Preparation and Testing of Vaccines Prepared from the Envelopes of Venezuelan, Eastern, and Western Equine Encephalomyelitis Viruses

CARL E. PEDERSEN, JR.

United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

Received for publication 6 October 1975

Envelope components were separated from Venezuelan, Eastern, and Western equine encephalomyelitis viruses after treatment of the virions with detergent. Vaccines prepared from the envelope component were capable of stimulating mice to produce humoral antibodies. Protective efficacy studies were performed using mono-, di-, and trivalent vaccine combinations. These elicited varying degrees of homologous protection, and Eastern and Venezuelan equine encephalomyelitis envelope products appeared to confer protection to mice challenged with Western equine encephalomyelitis virus.

Casals first introduced the concept that the arboviruses (togaviruses) could be clustered into complexes that were serologically related (3). It has since been suggested that antigenic determinants located in the envelope of the group A arboviruses (togaviruses) may be responsible for serological specificity of these virions (8). Further, Karabatsos (14) has recently presented evidence that the specificity of plaque neutralization is due to these type-specific determinants. We previously reported that the isolated envelope component from the attenuated TC-83 strain of Venezuelan equine encephalomyelitis (VEE) virus elicited neutralizing antibodies in mice and that test animals thus immunized were protected from challenge with the virulent Trinidad strain of VEE virus (19). To further assess the feasibility of vaccines prepared from virion components, we studied the immunogenicity and protective efficacy of the envelope products derived from VEE, Eastern equine encephalomyelitis (EEE), and Western equine encephalomyelitis (WEE) viruses.

MATERIALS AND METHODS

Attenuated virus strains used. The TC-83 strain of VEE virus (16), the Arth 167-SP strain of EEE virus (a gift from P. H. Coleman of the Medical College of Virginia, Richmond, Va.), and the B628, clone 15, strain of WEE virus (13) were used for the preparation of virion envelope subunit components. Plaque assays of virus infectivity were performed on primary chicken embryo monolayers in flasks as previously described (20).

Virus concentration and purification. The concentration and purification procedures used in this study have been described (19). Briefly, after propagation of virus for 18 to 24 h in roller bottles contain-

ing chicken embryo cell monolayers, cell debris was removed, and the virus was pelleted through a cushion of 10% (wt/wt) sucrose in NTE buffer [0.1 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.001 M ethylenediaminetetraacetate, pH 7.6] at $59,000 \times g$ for 2.5 h. After suspension in NTE buffer, the virus was applied to a 10 to 30% (wt/wt) linear sucrose gradient and centrifuged at $90,000 \times g$ for 2 h. The opalescent band was collected, pelleted at $90,000 \times g$ for 90 min, and suspended in 1.5 ml of NTE buffer. The resulting preparations contained approximately 10^{12} plaque-forming units/ml and were stored at -70°C until used.

Dissociation of the virus and recovery of envelope component. Concentrated, purified viruses were mixed with the nonionic surfactant Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) at a final concentration of 0.5% (vol/vol) surfactant. After constant shaking for 60 min at room temperature, the virion envelope material was separated from the core component by application of 1 ml of the reaction product on the top of a 10 to 30% linear sucrose gradient in NTE buffer (19). The top 2.0-ml fraction was collected after centrifugation for 60 min at $286,000 \times g$ (SW41 rotor) and monitored for infectious virus and serological activity.

Immunization and challenge schemes. Studies were conducted using adult, CD-1 strain, Swiss white mice (Charles River, Wilmington, Mass.). All vaccinations were made via the subcutaneous route using 0.2 ml of vaccine. Secondary immunizations were given 14 days after the primary vaccinations. Mice were challenged 21 days after the second vaccine dose by intracerebral inoculation of 10^3 suckling mouse median lethal doses of the challenge virus (21).

Challenge virus strains. Trinidad strain VEE virus (20), California strain WEE virus (1), and Cambridge strain EEE virus (1) were used to challenge laboratory animals.

Serological tests. Tests were performed on sera

collected at the time of challenge. Complement-fixation (CF) tests were performed in microtiter plates according to the method of Casey (5). Antigens were provided by the Center for Disease Control, Atlanta, Ga. Gel diffusion tests (7) were performed by using 0.75% agarose buffered with sodium veronal (pH 8.6), ionic strength 0.075, in 0.1% Triton X-100. Plaque reduction neutralization tests (PRNT) were performed essentially as described by Dulbecco et al. (9). Constant virus (approximately 10^2 plaque-forming units) and diluted antisera were warmed and mixed. After overnight incubation at 4 C, samples were titrated for residual virus by plaque assay on Vero cells. End-point titers were reported as 80% reduction in test plaques over control values.

Mouse hyperimmune antisera were produced by inoculating animals once every 7 days for 4 weeks intramuscularly with approximately 200 μ g of isolated envelope components. Globulin fractions were prepared from these sera using ammonium sulfate precipitation as described by Hebert et al. (12).

Polyacrylamide gel electrophoresis. High pH, discontinuous gel electrophoresis was performed essentially as previously described (18) with 7.5% acrylamide gels in glass tubes (125 by 3 mm). Briefly, purified, concentrated virus or antigen preparations were disrupted with 1% (wt/vol) sodium dodecyl sulfate and 1% (vol/vol) β -2-mercaptoethanol, heated for 2 min at 100 C, mixed with a solution of 60% sucrose and bromophenol blue tracking dye, and layered under the electrode buffer (6.0 g of tris(hydroxymethyl)aminomethane-hydrochloride, 28.8 g of glycine, 10 ml of 10% sodium dodecyl sulfate, 990 ml of distilled water) directly on top of

the gel columns. Proteins were separated by electrophoresis at 2 mA/gel until the tracking dye reached the bottom of the gel.

Protein determination. Protein was determined by the method of Lowry et al. (15) using bovine serum albumin fraction V (Armour Pharmaceutical Co., Kankakee, Ill.) as a standard.

RESULTS

Figure 1 depicts the electrophoretic profiles obtained from the concentrated, purified virus strains and the isolated envelope components after polyacrylamide gel electrophoresis. Envelope preparations used as vaccines in this study were free from infectious virus (determined by plaque assay and suckling mouse inoculation) and contaminating core components.

After separation from the concentrated, attenuated virus pools, envelope components were adjusted with NTE buffer to contain approximately the same amount of protein (TC-83 VEE, 1,400 μ g/ml; Arth 167-SP EEE, 1,350 μ g/ml; clone 15 WEE, 1,250 μ g/ml). For the preparation of vaccines the subunit concentrations were adjusted as shown in Table 1 so that groups of mice received the same amount of specific virion envelope antigen regardless of the type of vaccine (mono-, di-, or trivalent), although the total amount of antigen varied with each vaccine injected. Freeze-dried antigens were not used in this study.

FIG. 1. Profile of virions and envelope components after electrophoresis in polyacrylamide gels. CL, clone; Env, envelope.

We found that with a single injection of antigen mice responded poorly, if at all, serologically, and, as expected, direct intracerebral challenge resulted in a very high mortality rate (unpublished data). However, when given two injections at 14-day intervals, mice showed antibody responses indicative of serological conversion (Table 2) and were protected from challenge (Table 3).

Due to its relative simplicity, we used the gel diffusion technique as a screening procedure for sera from animals inoculated with envelope

components. Whereas globulin prepared from hyperimmune mouse sera against monovalent envelope vaccines did not cross-react in the gel diffusion test against heterologous envelope preparations (Fig. 2), the serum from one mouse inoculated with EEE envelope gave a positive reaction with WEE antigen. In addition, two sera from mice inoculated with divalent envelope vaccines showed a reaction against the third component.

Sera from mice immunized with envelope vaccines contained CF and neutralizing anti-

TABLE 1. *Envelope component vaccines administered to mice*

Vaccine	Original concn ^a (μ g/ml)	Dilution (calculated concn, μ g/ml)	Used as vaccine (ml)	Calculated pro- tein/injection (μ g)
Monovalent				
VEE	1,400	1:3 (466)	0.2	93.2
EEE	1,350	1:3 (450)	0.2	90.0
WEE	1,250	1:3 (416)	0.2	84.0
Divalent, combined				
VEE	1,400	1:1.5 (933)	0.1	93.3
EEE	1,350	1:1.5 (900)	0.1	90.0
				183.3
VEE	1,400	1:1.5 (933)	0.1	93.3
WEE	1,250	1:1.5 (833)	0.1	83.3
				176.6
WEE	1,250	1:1.5 (833)	0.1	83.3
EEE	1,350	1:1.5 (900)	0.1	90.0
				173.3
Trivalent, com- bined				
VEE	1,400	None (1,400)	0.07	98.0
EEE	1,350	None (1,350)	0.07	94.5
WEE	1,250	None (1,250)	0.07	87.5
				280.0

^a Determined by the method of Lowry et al. (16).

TABLE 2. *Serological responses of mice after administration of envelope component vaccines*

Vaccine received ^a	Gel diffusion (positive/total)			CF (geometric $\bar{X}$)			80% PRNT (geometric $\bar{X}$)		
	VEE	EEE	WEE	VEE	EEE	WEE	VEE	EEE	WEE
Monovalent									
VEE	8/8	0/8	0/8	27	— ^b	—	73	—	—
EEE	0/8	8/8	1/8	—	18	4	—	16	4
WEE	0/8	0/8	8/8	—	—	26	—	—	90
Divalent									
VEE/EEE	10/10	10/10	1/10	19	18	—	12	13	—
VEE/WEE	9/10	1/10	10/10	14	—	24	21	—	36
WEE/EEE	0/10	10/10	9/9	—	16	16	—	16	38
Trivalent									
VEE/EEE/WEE	10/10	10/10	10/10	18	14	16	16	14	26

^a Viruses used for the preparation of envelope vaccines were: VEE strain TC-83; EEE strain Arth 167-SP; WEE strain B628 clone 15. Mice were injected subcutaneously with 0.2 ml of envelope antigen and boosted 14 days later with an additional 0.2 ml.

^b No titer.

TABLE 3. *Protective efficacy of envelope component vaccines in mice^a*

Vaccine ^b	Survivors (%) after i.c. challenge with 10 ⁵ SMICLD ₅₀ (no./total)		
	VEE	EEE	WEE
Monovalent			
VEE	88 (15/17)	12 (2/17)	53 (8/15)
EEE	5 (1/18)	63 (12/19)	59 (10/17)
WEE	6 (1/16)	6 (1/16)	95 (19/20)
Divalent			
VEE/EEE	67 (12/18)	58 (11/19)	73 (11/15)
VEE/WEE	71 (12/17)	6 (1/17)	100 (19/19)
WEE/EEE	6 (1/16)	47 (8/17)	100 (18/18)
Trivalent			
VEE/EEE/WEE	63 (12/19)	50 (11/22)	100 (23/23)

^a Mice were injected subcutaneously with 0.2 ml of envelope antigen and boosted 14 days later with an additional 0.2 ml. Mice were challenged 21 days after the booster injection. Challenge viruses (10⁵ suckling mouse median lethal doses [SMICLD₅₀]) injected intracerebrally (i.c.) (0.03 ml) were: VEE strain Trinidad Donkey; EEE strain Cambridge; WEE strain California.

^b Viruses used for the preparation of envelope vaccines were: VEE strain TC-83; EEE strain Arth 167-SP; WEE strain B628 clone 15.

bodies directed against homologous virions. Only one serum from a mouse immunized with EEE envelope showed a spurious cross-reaction, and this was the sample that reacted with WEE antigen in the gel diffusion test. None of the other test sera gave reactions that indicated that *in vitro* serological responses could be elicited to antigens other than the one inoculated.

Protective efficacy studies (Table 3) showed that the envelope components elicited homologous protection when administered singly. Administration of envelope antigens as divalent or trivalent vaccines showed that component products were capable of eliciting protection against the homologous virus. In addition, both EEE and VEE envelope products appeared to confer an unusually high degree of protection to animals against challenge with WEE virus. In contrast, WEE envelope did not confer protection against either VEE or EEE virus challenges. It did not appear that VEE or EEE envelope products offered any reciprocal cross-protection.

In a separate experiment an adult male burro

was inoculated subcutaneously with 1.0 ml of the trivalent envelope vaccine. Three weeks later the animal was given a booster immunization. At this time the 80% PRNT for VEE virus was <1:10, for EEE virus 1:10, and for WEE virus 1:10. Four weeks later the 80% PRNT for VEE virus was 1:20, for EEE virus 1:160, and for WEE virus 1:80.

DISCUSSION

Equines in this country have been routinely protected against WEE and EEE by immunization with formalin-inactivated vaccines. Recently, the U.S. Department of Agriculture licensed a three-component vaccine, composed of inactivated VEE, EEE, and WEE viruses, for use in equines (22). Whereas inactivated, intact virus vaccines protect against the diseases, we felt that studies should be performed to assess the immune response proffered by purified antigenic components free from non-essential virion subunits for possible use in humans. We used attenuated viruses for the preparation of vaccine components to minimize the constraints required for propagation and purification of highly virulent togaviruses.

Serological assays for the presence of humoral antibodies gave results similar to those described by other arbovirologists (2, 4, 8, 10, 11, 14). Casals' concepts of clusters of group A arboviruses (togaviruses), more closely related serologically than others, places the VEE, EEE, and WEE viruses in separate subgroups (3). These viruses have previously been studied by gel diffusion (2, 10), and, although the sensitivity of this technique is relatively low, it has a narrow specificity and cross-reactions infrequently occur. We utilized this test for an initial assay for the presence of precipitating antibodies directed against the immunizing envelope components. The test was positive only when two or more injections of vaccine were administered, however, and in a few instances heterologous positive reactions were observed. The CF test, used extensively for detection of antibodies against group A arboviruses (togaviruses), exhibits some degree of cross-reactivity, notably among the WEE and EEE viruses (4). The PRNT has the greatest degree of specificity (14) and shows that the group A arboviruses (togaviruses) are less closely related than indicated by the hemagglutination inhibition of CF tests (4). In our studies, both the CF and PRNT tests showed (with the exception of one serum) that antibodies were produced that reacted only with the homologous virus strains.

Whereas *in vitro* serological tests are a valid index for the assay of circulating antibodies in

FIG. 2. Gel diffusion reactions of virion envelopes (V, E, W = VEE, EEE, and WEE virus envelopes, respectively) and globulin fractions from rabbit anti-envelope sera (V_γ, E_γ, W_γ = anti-VEE, -EEE, and -WEE envelopes, respectively).

an immunized animal, the ultimate criterion for protection can only be measured by challenge studies. Since the WEE virus used in this study will uniformly kill adult mice only when inoculated intracerebrally (21), we used this route for challenge with all three viruses. Although we found that protection was not always absolute, we believe that this study demonstrates the feasibility of using immunogenic components of group A togaviruses either alone or in combination. Several factors may have been responsible for the suboptimal protection observed. Possibly the virus challenge load was excessive for the route used. Alternatively, one or two additional booster immunizations might be required for complete homologous and perhaps heterologous protection; however, in studies using Semliki forest virus, Mussgay and Weiland (17) reported that subunit vaccines prepared by detergent treatment were less protective than vaccines containing nondisrupted inactivated virus. It is therefore possible that protective efficacy is dependent on total antigenic mass or on the size of the components carrying immunogenic sites. Admittedly, additional studies are warranted in this area to adequately define the optimum immunization schedule and challenge regimen.

Cole and McKinney (6) found that formalin-

inactivated VEE, EEE, and WEE viruses injected singly would not induce any cross-protection against challenge strains. However, Cole and McKinney (6), using hamsters, and Byrne et al. (1), using burros, have described partial protection using live VEE or EEE as vaccines followed by challenge with WEE. In the latter studies VEE-immune burros were also found to be relatively resistant to EEE. Hearn (11) has also described cross-protection in mice with VEE and EEE.

We found that the envelope component from these viruses elicits homologous antibody responses and will induce protection against the homologous virus. In addition, it appears from challenge studies that a cumulative protective effect might be produced, in that mice immunized with VEE and EEE, either alone or in combination, were protected against WEE challenge. Cole and McKinney (6) found that in the level of circulating heterologous antibody at the time of challenge did not play a role in their studies, since inactivated and live viruses induced only homologous neutralizing antibody. Casals (3) has suggested that pre-experienced animals might produce cross-reacting antibodies at an accelerated rate after challenge. Why this might be true with WEE virus but not VEE virus or EEE virus is not clear. Perhaps se-

quential immunization schedules using envelopes from group A viruses would clarify this point.

It would appear that preparation of purified envelope subunits from group A togaviruses represents one avenue of approach for the development of noninfectious vaccines. These products contain the antigenic determinants that protect mice from the equine encephalomyelitis viruses studied. In addition, in the burro study the envelope component vaccine was capable of inducing humoral antibodies. Envelope vaccines represent purified components freed from undesirable cell culture material and unnecessary virion antigens. In light of the intriguing proposal for immunization with combined vaccines for specific geographical areas (6), further investigations on the immunogenicity of subunit products from other group A togaviruses should be performed.

ACKNOWLEDGMENT

I am grateful to Everett Lucas for his technical assistance in this study.

LITERATURE CITED

- Byrne, R. J., G. R. French, F. S. Yancey, W. S. Gochenour, P. K. Russell, H. H. Ramsberg, O. A. Brand, F. G. Scheider, and E. L. Buescher. 1964. Clinical and immunologic interrelationship among Venezuelan, Eastern and Western equine encephalomyelitis viruses in burros. *Am. J. Vet. Res.* 25:24-31.
- Calisher, C. H., and K. S. C. Maness. 1970. Arbovirus identification by an agar-gel diffusion technique. *Appl. Microbiol.* 19:557-596.
- Casals, J. 1963. Relationships among arthropod-borne animal viruses determined by cross-challenge tests. *Am. J. Trop. Med. Hyg.* 12:587-596.
- Casals, J. 1972. Antigenic characteristics of Venezuelan equine encephalitis (VEE) virus: relation to other viruses, p. 77-83. *In* Venezuelan encephalitis. Scientific Publ. no. 243, Pan American Health Organization, Washington, D.C.
- Casey, H. L. 1965. Adaptation of LBCF method to micro techniques, p. 31-34. *In* Standardized diagnostic complement fixation method and adaptation to micro test. Public Health Service, Monogr. no. 74, Washington, D.C.
- Cole, F. E., Jr., and R. W. McKinney. 1971. Cross-protection in hamsters immunized with group A arbovirus vaccines. *Infect. Immun.* 4:37-43.
- Crowle, A. J. 1958. A simplified micro double-diffusion agar precipitin technique. *J. Lab. Clin. Med.* 52:784-787.
- Dalrymple, J. M., S. N. Vogel, A. Y. Teramoto, and P. K. Russell. 1973. Antigenic components of group A arbovirus virions. *J. Virol.* 12:1034-1042.
- Dulbecco, R., M. Vogt, and A. G. R. Strickland. 1956. A study of the basic aspects of neutralization of two animal viruses, Western equine encephalitis virus and poliomyelitis virus. *Virology* 2:162-205.
- Hawkes, R. A., and I. D. Marshall. 1967. Studies of arboviruses by agar gel diffusion. *Am. J. Epidemiol.* 86:28-44.
- Hearn, H. J., Jr. 1961. Cross protection between Venezuelan equine encephalomyelitis virus and Eastern equine encephalomyelitis virus. *Proc. Soc. Exp. Biol. Med.* 107:607-610.
- Hebert, G. A., P. L. Pelham, and B. Pittman. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse, and goat antisera. *Appl. Microbiol.* 25:26-36.
- Johnson, H. N. 1963. Selection of a variant of Western encephalitis virus of low pathogenicity for study as a live virus vaccine. *Am. J. Trop. Med. Hyg.* 12:604-610.
- Karabatsos, N. 1975. Antigenic relationships of group A arboviruses by plaque reduction neutralization testing. *Am. J. Trop. Med. Hyg.* 24:527-532.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- McKinney, R. W., T. O. Berge, W. D. Sawyer, W. D. Tigertt, and D. Crozier. 1963. Use of an attenuated strain of Venezuelan equine encephalomyelitis virus for immunization in man. *Am. J. Trop. Med. Hyg.* 12:597-603.
- Musgay, M., and E. Weiland. 1973. Preparation of inactivated vaccines against alphaviruses using Semliki Forest virus-white mouse as a model. I. Inactivation experiments and evaluation of double inactivated subunit vaccines. *Intervirology* 1:259-268.
- Pedersen, C. E., Jr., S. C. Marker, and G. A. Eddy. 1974. Comparative electrophoretic studies on the structural proteins of selected group A arboviruses. *Virology* 60:312-314.
- Pedersen, C. E., Jr., D. R. Slocum, and G. A. Eddy. 1973. Immunological studies on the envelope component of Venezuelan equine encephalomyelitis virus. *Infect. Immun.* 8:901-906.
- Randall, R., and J. W. Mills. 1944. Fatal encephalitis in man due to the Venezuelan virus of equine encephalomyelitis in Trinidad. *Science* 99:225-226.
- Robinson, D. M., S. Berman, and J. P. Lowenthal. 1972. Mouse potency assay for Western equine encephalomyelitis vaccines. *Appl. Microbiol.* 23:104-107.
- U.S. Department of Agriculture. 1975. USDA licenses triple vaccine against equine encephalomyelitis. News Release 812-75 (17 March 1975), U.S. Department of Agriculture, Washington, D.C.