Serological Characterization of the Three Major Proteins of Vesicular Stomatitis Virus

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The three major proteins of vesicular stomatitis virus-Indiana, glycoprotein (G), nucleoprotein (N), and membrane protein (M), were isolated and characterized by means of specific monocomponent antisera. G, N, and M proteins are distinct, nonrelated antigens with specific serological properties. The G protein is the only antigen inducing the formation of virus-neutralizing antibodies and was shown to confer immunity to mice. Specific complement-fixing and precipitating activity was demonstrated for each of the three antisera. The future use of isolated rhabdovirus components and of monospecific antisera is considered for therapeutic and diagnostic purposes as well as for virus strain differentiation and classification work.

By polyacrylamide gel electrophoresis five polypeptides have repeatedly been demonstrated in vesicular stomatitis virus (VSV) (8, 18). The so-called three major proteins are the glycoprotein (G), nucleoprotein (N), and membrane protein (M). Recently, G and N proteins were isolated in a biologically active form (6, 12); however, to date only the N protein was obtained sufficiently pure to allow the production of a specific monocomponent antiserum which did not cross-react with the other virus components.

The present work deals with the isolation and further separation of biologically active G and M proteins. The three major proteins of VSV-Indiana and the specific monocomponent antisera were compared and evaluated by serological techniques. The G protein was potency-tested for immunizing capacity by *in vivo* and *in vitro* systems.

MATERIALS AND METHODS

Virus strain. VSV serotype Indiana was obtained from R. E. Dierks, Ames, Iowa.

Virus production and purification. Virus was propagated in roller cultures of BHK-21 C 13 cells (17). Culture conditions and media were described before (11). Infectious cell culture fluids and infected cells were harvested after general cytopathological effect (CPE) was evident. For labeling of virus proteins the infected cells were incubated with a 1:10 mixture of Eagle medium and Earle salt solution containing 5 μ Ci of [35 S]methionine per ml of medium. The virus from clarified supernatant fluids was precipitated with polyethylene glycol 6000 (PEG) according to McSharpy and Benzinger (15). The

precipitate was eluted three times with 0.15 M sodium chloride, 0.01 M Tris-hydrochloride, 0.001 M ethylenediaminetetraacetic acid (STE) buffer, pH 7.4, by using 10 ml of buffer per 1,000 ml of original cell culture fluid. The suspension was clarified by centrifuging 10 min at $5,000 \times g$, layered on a preformed potassium tartrate and glycerol gradient 0 to 50% (wt/vol) potassium tartrate and 30 to 0% (wt/vol) glycerol, and was centrifuged in a Spinco SW 27 rotor at 25,000 rpm for 3 h at 4 C.

The virus band was collected and dialyzed against 500 volumes of 0.01 M Tris-hydrochloride, pH 7.8, 0.15 M NaCl (Tris) buffer.

Infectivity assay. The infectivity of virus preparations was titrated by the plaque assay by using the cell suspension technique (13). Virus dilutions were made in Eagle medium containing 0.3% BSA. Two petri dishes were inoculated each with 0.1 ml of the virus. Plaques were counted after 48 h of incubation at 35 C and the titer was calculated.

Isolation of glyco- (G) and membrane- (M) proteins. The G protein was liberated from purified virus by treatment with Triton N 101 (0.5% wt/wt). By means of the Albertson two phase technique, as described by Bishop and Roy (2), the G protein was separated from the remaining virus components. To remove the detergent and PEG the G protein contained within the PEG phase was intensively dialyzed against 0.01 M Tris buffer, pH 7.8. After dialysis the G protein was adsorbed to DEAE cellulose equilibrated with 0.01 M Tris buffer and was washed with 10 times the void volume of the column. The G protein was then eluted with 0.5 M NaCl.

The M protein was recovered from the dextran phase of the Albertson technique. The dextran phase was treated 6 $\times$ with three times its volume of 6% PEG in Tris buffer. NaCl and Nonidet P-40 (NP-40) was added to the dextran phase at a final concentration of 1 M and 1% (wt/wt), respectively. The mixture was

incubated for 30 min at 20 C and then was extracted twice with an equal volume of 6% PEG in Tris buffer. The PEG phases were pooled and the dextran phase was discarded.

Isolation of nucleoprotein (N). The N protein was isolated from VSV-infected cells as described for rabies virus nucleoprotein (12).

Isoelectric focusing. The G protein was intensively dialyzed against 1% (wt/vol) glycine and 1% (wt/vol) glycerol in double distilled water. After clarification at $5,000 \times g$ for 10 min the preparation was electrofocused in a 0 to 40% (wt/vol) sucrose gradient containing 1% carrier ampholytes (pH 3 to 10) and 0.5% (wt/vol) Triton X 100 as described by Friesen et al. (5).

Sedimentation experiments. Potassium chloride (0.5 M) was added to the electrofocused G protein, and the mixture was layered on a 5 to 20% (wt/vol) sucrose gradient in Tris buffer. The gradient was centrifuged in a Spinco SW 65 rotor at 57,000 rpm for 16 h at 4 C. ^3H -methylated BSA served as marker protein.

Polyacrylamide gel electrophoresis. Acrylamide gel electrophoresis was carried out as described by Bolognesi and Bauer (3) with the following modifications. The protein samples were treated with sodium dodecylsulfate (SDS) and dithiothreitol (DTT) to give a final concentration of 1.0% and 0.1 M, respectively, and were heated for 1 min at 100 C. Samples of 50 μl containing approximately 100 μg of protein were applied to the gels.

Neuraminidase digestion. A preparation containing 200 μg of [^{35}S]methionine-labeled G protein was dialyzed intensively against 0.05 M sodium acetate buffer pH 5.5, 0.15 M NaCl, 0.0078 M CaCl_2 . Neuraminidase (*Clostridium perfringens*, A-grade) was then added to a final concentration of 2 $\mu\text{g}/\text{ml}$ and the solution was incubated at 37 C for 1 h.

Protein determination. Proteins were determined by the Lowry method (7) with BSA as reference.

Production of antisera. Anti-G, -N, -M, and -virion sera were prepared by hyperimmunization of rabbits with purified virus or isolated components. Immunization schedule: three intramuscular injections during the first week (the first injection with complete Freund adjuvant) were followed by one booster inoculation given 4 weeks later. Blood was obtained by heart puncture and tested after thermal inactivation (three times 30 min, 56 C) for specific activities.

Agar gel precipitation. The test procedure has been described before (12).

Immunoelectrophoresis. The formerly described technique (12) was used which includes 1% (wt/vol) Agarose (SERVA, Heidelberg) in Veronal buffer, pH 8.6, at an ionic strength of 0.05. The sample was electrophoresed at 250 V for 75 min; the diffusion time was 24 h at 4 C. Proteins were stained with amido black.

CF test. The microplate method as described before (12) was used for block titrations of sera and antigens. The reciprocal of the highest dilution of antiserum giving 25 to 30% hemolysis for a volume of 0.025 ml was taken as the complement fixation (CF) activity of the sample.

Serum neutralization (SN) test. Serial fivefold dilutions of sera were prepared. To each dilution an equal volume of virus containing 1,000 PFU per ml was added. The mixture was incubated for 90 min at 35 C after which 0.1 ml of each serum-virus mixture was inoculated on at least three plates. After 48 h of incubation plaques were counted, and the 50% plaque reduction end point was determined (19).

Vaccination of mice. Virus was inactivated by overnight incubation (4 C) with beta-propiolacton (BPL) at a final dilution of 1:4,000. Vaccination and challenge of mice were performed according to the National Institute of Health potency test for rabies vaccines (14). Serial fivefold dilutions of the vaccine were inoculated intraperitoneally on days 1 and 8 (0.5 ml each time) into 6-week-old mice followed by an intracerebral challenge infection on day 14 by using 10 to 15 mouse LD_{50} . The median effective dose (ED_{50}) of each vaccine was calculated from the dilution of vaccine which protected 50% of the mice by using the formula of Reed and Muench (10).

Antibody-binding test. The procedure (1) was used to determine the antigenicity and potency of the vaccines in vitro. Principle: Antibody from a standard immune serum is adsorbed by serial dilutions of the vaccine; the presence of nonadsorbed antibody is then determined by the plaque reduction test. Procedure: Serial twofold dilutions of the vaccine are mixed with an equal volume of the standard immune serum in a dilution containing 25 to 100 antibody units (that is, 25 to 100 times the limiting concentration of the specific virus-neutralizing activity). After incubation of the vaccine-serum mixture for 1 h at 35 C, VSV containing 1,000 PFU/ml is added in equal volume portions to each vaccine-serum dilution. The vaccine-serum-virus mixtures were incubated for 90 min at 35 C and inoculated in 0.1-ml portions on at least two petri dishes. Determination of the 50% plaque-reduction end point was carried out as described under SN test.

RESULTS

Isoelectric focusing. The crude preparation of VSV-Indiana protein obtained by two-phase separation by using dextran and PEG still contained traces of M protein as revealed by electrophoresis and serology. Therefore, the G protein was subjected to isoelectric focusing. Figure 1 shows the distribution of ^{35}S -labeled G protein after electrofocusing. The G protein is found in two peaks with isoelectric points (IP) of 5.4 and 5.8, as shown by distribution of radioactivity and CF. A better separation by isoelectric focusing in ampholine gradients of pH 3 to 6 was not obtained. The material from the two peaks was separately isolated and refocused in an ampholine gradient, pH 3 to 10. Again, the material of the first peak appeared at pH 5.4 and the second at pH 5.8 (Fig. 2 and 3). When the material with IP 5.4 and 5.8 was subjected to SDS acrylamide gel electrophoresis no differences in the molecular weights were observed.

FIG. 1. Isoelectric focusing of [^{35}S]methionine-labeled VSV-Indiana glycoprotein in an ampholine gradient, pH 3 to 10. The glycoprotein preparation was electrofocused in an isoelectrofocusing column (LKB 8100-10, volume 110 ml) at 300 V for 48 h, after which time the current dropped from 10 to 1 mA. Fractions were collected and assayed for radioactivity ($\bullet$), complement fixation activity ($\circ$), and pH (.....).

FIG. 2. Re-electrofocusing of separately isolated glycoprotein peak with isoelectric point of 5.4. Electrofocusing was carried out as described in Fig. 1. Symbols: see legend to Fig. 1.

FIG. 3. Re-electrofocusing of separately isolated glycoprotein peak with isoelectric point of 5.8. Symbols: see legend to Fig. 1.

After treatment with neuraminidase followed by isoelectric focusing (pH 3 to 10) the two G protein peaks were shifted to the anode and appeared at pH 6.1 and 6.4 (Fig. 4).

Sucrose gradient sedimentation. The profiles of the refocused [^{35}S] methionine-labeled G protein (peaks 1 and 2) after cosedimentation with ^3H -methylated BSA are shown in Fig. 5. VSV-Indiana G protein sedimented in a distinct peak, its sedimentation constant being somewhat lower than BSA.

Electrophoresis of the isolated proteins. The VSV-Indiana G protein purified by the Albertson technique and by isoelectric focusing was subjected to SDS polyacrylamide gel electrophoresis as was the membrane (M) protein and nucleoprotein (N) which were isolated, as described in Materials and Methods (Fig. 6B to D). Figure 6A represents the protein pattern of the purified VSV-Indiana. It may be seen from Fig. 6B to D that each structural protein prepa-

FIG. 4. Isoelectric focusing of ^{35}S -labeled VSV-Indiana glycoprotein after treatment with neuraminidase. Symbols: see legend to Fig. 1.

FIG. 5. Sedimentation profiles of [^{35}S]methionine-labeled VSV-Indiana glycoprotein ($\circ$) and ^3H -methylated BSA ($\bullet$) in a 5 to 20% (wt/vol) sucrose gradient after centrifugation at 57,000 rpm for 16 h.

FIG. 6. Polypeptide patterns of purified VSV-Indiana (A) and the three isolated major proteins (B, C, D). Virus and isolated proteins were denatured with 1% SDS and electrophoresed in 7.5% polyacrylamide gel. Drawings represent optical density of the Coomassie brilliant blue-stained gels measured by using a Beckman Analytrol photometer.

ration was not contaminated with other polypeptides. Even a fivefold increase of the protein concentration did not give evidence of contamination.

Serological properties of the three major proteins of VSV-Indiana: G, N, and M. CF test. Monocomponent antisera against the three major proteins were prepared in rabbits. Each antiserum was reacted with each of the purified antigens in complement fixation "checker board" experiments. Table 1 shows that all antigens except BHK cell extract react with anti-virion serum. In addition, G, N, and M proteins react only in the homologous system, indicating serologically distinct antigens.

Agar-gel precipitation. Results obtained in the double diffusion test (Fig. 7) correspond to those of the CF test. G, N, and M antigens formed single, individual precipitation lines which did not join but crossed when reacted in neighboring positions. This clearly indicates that besides G and N, the M protein is the third major virion-derived protein of VSV-Indiana which represents a distinct antigen.

Immunoelectrophoresis. Immunoelectrophoresis of the two G-protein fractions obtained after isoelectric focusing showed a single precipitation line when reacted with G antiserum (Fig. 8). This indicates that these two fractions represent one antigen.

SN test. Antisera against the virus and the

TABLE 1. CF tests with monocomponent sera against VSV-Indiana structural proteins and anti-BHK serum

Antigens	Antisera				
	Virion	G protein	N protein	M protein	BHK
Virion	2560/128 ^a	640/128	640/32	320/64	Neg. ^b
G protein	320/128	640/64	Neg.	Neg.	Neg.
RNP	1280/128	Neg.	640/128	Neg.	Neg.
M protein	40/8	Neg.	Neg.	320/16	Neg.
BHK extract	Neg.	Neg.	Neg.	Neg.	640/320

^a Results of checker-board experiments: reciprocal of antiserum titer over antigen titer at 75 to 100% inhibition of hemolysis.

^b Neg. = <10/2 (antiserum 1:10 over antigen 1:2).

FIG. 7. Agar-gel double diffusion test employing purified virus (3 and 6), isolated glycoprotein (1 and 4), nucleoprotein (2), and membrane protein (5). The center well contains antiviral serum.

FIG. 8. Immunoelectrophoresis of the isolated glycoprotein peaks from Fig. 1 against homologous antiserum. A, G protein; B, G antiserum; ▼, start. (Fig. 8 was kindly supplied by W. Matthaeus)

three major proteins were tested for virus neutralizing activity employing the 50% plaque reduction test. The antiserum directed against the virus had a neutralizing titer of 1:20,000. The virus neutralizing activity of the anti-G serum was 1:60,000. Anti-N and anti-M sera did not neutralize virus even when used undiluted.

Vaccination experiments. Since only antiviral and anti-G sera showed virus-neutralizing activity, it was of interest to compare the protective activity of the two preparations in vaccination experiments and vaccine potency tests. BPL-inactivated vaccines were prepared from VSV-infected cell culture supernatant, from concentrated virus, and from purified G

protein. The potency of the vaccine was tested in the antibody-binding test; the protective activity was measured in mice. Further, the CF activity of the three vaccines was tested, and the protein content of concentrated virus and G protein was determined. The results are summarized in Table 2.

The vaccination experiments show that the isolated G protein is highly immunogenic and offers good protection of mice even against intracerebral challenge. The protective activity of the G protein compares well to that of the vaccine prepared from concentrated virus. It is also seen that the antibody-binding test, a simple and quick in vitro method for potency testing of vaccines, corresponds well with the in vivo system.

DISCUSSION

In a previous communication (12) we described for VSV and other rhabdoviruses the isolation of N protein obtained by disruption of purified virus or of virus-infected cells by NP-40. The present report describes the isolation of two further structural proteins—G and M—of VSV-Indiana in a biologically active form. The liberation of the G protein by Triton N 101 and its separation from other virus components by a two-phase separation method was reported before (2). In our hands this method yielded G-protein preparations of high specific activity but which still contained traces of contaminating M protein. The use of isoelectric focusing led to the removal of the latter protein. After isoelectric focusing, two separate G-protein peaks were observed which reappeared after refocusing. Neuraminidase treatment altered the isoelectric position of both peaks indicating that neuraminic acid is con-

tained in the sugar molecules of each peak fraction. If subjected to acrylamide gel electrophoresis the material from the two G-protein peaks proved to be of the same molecular weight. The preparation contained no other protein. CF, agar-gel precipitation, and immunoelectrophoresis employing homologous and heterologous virus-component antisera proved the electrofocused G protein to be one single antigen without contaminants.

The uniformity of the G-protein preparation, leads us to the conclusion that the observed heterogeneity after electrofocusing is the result of a heterogeneity in charge of the sugars contained in the preparation, as has been observed with fetuin (9). To prove whether the heterogeneity in charge is a result of micro- or central heterogeneity (9, 16), treatment with other glycolytic enzymes or analysis of the sugars in both fractions, or both, must be carried out.

Cartwright et al. (4) first proposed that the G protein of VSV could be responsible for the induction of virus-neutralizing antibodies. Kelley et al. (6) demonstrated the specific, neutralizing antibody-adsorbing activity of VSV-Indiana G protein. The present report demonstrates that of the three isolated major VSV-proteins only G protein induced the formation of virus-neutralizing antibody (Table 3). In addition, we have shown by in vivo and in vitro experiments that VSV-Indiana G protein is the immunity-inducing protein of the virus. Mice immunized with BPL-inactivated vaccines prepared either from virus or from G protein were protected against a subsequent challenge infection. The protective activity of G protein is surprisingly high and compares well to that of concentrated virus. The extreme purity of such preparations leads us to believe that future vaccines made, for example, from G protein of another rhabdovirus, rabies virus, will be much safer and more efficient than vaccines presently in use for humans.

This report describes for the first time the isolation of M protein, the third major protein of VSV. Anti-M serum has CF and precipitating but no virus-neutralizing activity (Table 3). M protein is the third biologically active antigen of VSV and does not cross-react serologically with G- and N-protein preparations.

As shown in Table 3, each of the three virus proteins represents a distinct, serologically active antigen. Each antigen has specific biological properties such as induction of virus-neutralizing antibody and immunity, immunofluorescence, etc. We see the further potential use of isolated virus components in the following:

TABLE 2. Vaccination experiments with BPL-inactivated VSV-Indiana and a G protein preparation

Vaccine	Protective values		CF test (CFU/ml)	Protein (mg %)
	Mouse	Cell culture		
	ED ₅₀ ^a	AD ₅₀ ^b		
Infectious cell culture supernatant	20 ^c	32 ^c	2.5 × 10 ³	
Virus, concentrated	175	515	2 × 10 ⁴	111.0
G protein	271	650	1.6 × 10 ⁵	79.2

^a ED₅₀ = 50% end point of vaccine dilution (highest vaccine dilution protecting 50% of mice).

^b AD₅₀ = effective 50% adsorbing dose (highest vaccine dilution at which the number of plaques is reduced by half).

^c Reciprocal of vaccine dilution.

TABLE 3. *Properties of the isolated three major proteins of VSV-Indiana and monospecific antisera*

Properties of antigens	G protein	N protein	M protein
Molecular weight (dalton)	67,000	52,000	25,000
Origin	Envelope	Core	Envelope
Properties	Induces virus-neutralizing antibody and immunity	Group-antigen	Unknown
Properties of antisera	Anti-G	Anti-N	Anti-M
Neutralizing activity	+	Negative	Negative
Immunofluorescence	+	+	Negative
CF activity	+	+	+
Precipitating activity	+	+	+

preparation of safe vaccines, of diagnostic sera, and of monocomponent sera for virus strain differentiation and for serological classification work.

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