

## Rabies Virus Glycoprotein

### II. Biological and Serological Characterization

JAMES H. COX,\* BERNHARD DIETZSCHOLD, AND LOTHAR G. SCHNEIDER

*Federal Research Institute for Animal Virus Diseases, Tübingen, Germany*

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Purified rabies virus glycoprotein (G) was shown by complement fixation and immunodiffusion tests to be a second distinct antigen of the virus. It is the only structural protein of the virus that induces the formation of virus-neutralizing antibodies and which confers immunity to animals. When the G protein is taken as antigen, the complement fixation test can be used for the assay of virus-neutralizing antibodies. The total protective activity of the virus was recovered in the purified G protein preparation. The protective activity of G protein increased with purification: 9 ng of G protein was required to protect 50% of the mice as compared to 1.63  $\mu$ g of the virus. Selective immunofluorescent membrane staining and immunocytolysis of rabies virus-infected cells were shown to be G protein specific. Due to its purity and potency, the G protein preparation can be considered the ideal human antirabies vaccine.

Attempts aimed at the isolation of a biologically active component of the rabies virus responsible for inducing immunity in animals resulted in preparations either low in activity (23) or still containing several proteins (1, 6). In a separate communication (B. Dietzschold, J. H. Cox, and L. G. Schneider, manuscript in preparation) we describe the isolation of glycoprotein (G) by isoelectric focusing (IEF) from Triton X-100-disrupted rabies virus. The G protein focusing at pH 7.0 was shown to be the "spike" protein of the virus, free from lipids and other proteins of the viral envelope. The biological and serological properties of IEF-purified G protein are described in the present report.

#### MATERIALS AND METHODS

**Virus.** Viruses used were the cloned ERA strain of rabies virus, obtained from T. J. Wiktor, Philadelphia, Mokola virus (16) plaque cloned in BHK-21 C13 (19) cells and the vesicular stomatitis virus (VSV) Indiana strain (5).

**Virus production and purification.** Virus was propagated in roller cultures of BHK-21 cells. Culture conditions and media have been described before (12). Infectious cell culture fluids were harvested after 96 h postinfection. The clarified cell culture supernatant was centrifuged in a Beckman R19 rotor at 19,000 rpm for 120 min at 4°C. The supernatant was discarded and the virus pellet was resuspended in a small volume of 0.15 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, 0.001 M ethylenediaminetetraacetic acid (STE) buffer overnight. The resuspended virus was clarified by low-speed centrifugation and layered on a preformed 10 to 50% (wt/vol) sucrose gra-

dient, and was centrifuged in a Beckman SW27 rotor at 25,000 rpm for 90 min at 4°C. The virus band was collected and dialyzed extensively against STE buffer.

**Isolation of G protein.** Dissociation of rabies virus was carried out as follows. To a suspension of 1 mg of purified rabies virus per ml, Triton X-100 in STE was added to a final concentration of 1% (protein-Triton ratio, 1:10). The mixture was kept for 20 min at room temperature, followed by centrifugation in a Beckman SW65 rotor at 45,000 rpm for 60 min at 4°C. The resulting 120,000  $\times$  g supernatant was subjected to IEF as previously described (5).

**Isolation of N.** The nucleocapsid protein (N) was isolated from rabies virus-infected BHK cells as described by Schneider et al. (13).

**Removal of ampholine.** For separation of ampholine from IEF material, gel filtration was used. Sephadex G-50 (Pharmacia, Uppsala, Sweden) was equilibrated with borate buffer (0.2 M sodium borate, 0.1 N HCl, and 0.1 M NaCl, pH 7.3). Column size was 1 by 15 cm. Assay material was eluted by ascending flow with borate buffer, and fractions were screened at optical density of 1 cm at 280 nm using a LKB constant-flow ultraviolet recorder (Uppsala, Sweden), followed by CF testing.

**Protein determination.** Proteins were determined either by the method of Lowry et al. (11), with bovine serum albumin as a reference, or with samples containing Triton X-100, by the method of Wang and Smith (21).

**Production of antisera.** Antirabies-G, -N, and -virion sera were prepared by hyperimmunization of rabbits with purified virus or isolated components. One intraperitoneal injection with complete Freund adjuvant and two intradermal injections during the first week were followed by one booster inoculation given 4 weeks later. One week after the booster,

blood was obtained by heart puncture and was tested after thermal inactivation (three times, 30 min, 56°C) for specific activities.

**Vaccination of mice.** NMRI mice were taken from the breeding station of our institute. BALB/c mice were obtained from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. The *in vivo* protection test (National Institutes of Health test) was performed as described (15). The median effective dose ( $ED_{50}$ ) of each vaccine was calculated from the dilution of the vaccine that protected 50% of the mice, using the Spearman-Kärber formula (10). The antigenic value of each vaccine was obtained by dividing the  $ED_{50}$  of the test vaccine by that of the reference vaccine. The rabbit brain vaccine A 18 (Behringwerke, Marburg, Germany) served as a reference vaccine, which by previous comparison proved to be equal to the World Health Organization International Antirabies Reference Vaccine.

**Human antirabies sera.** Human antisera obtained at different times after prophylactic vaccination with an antirabies vaccine of human diploid cell culture origin (3) were assayed in parallel for neutralizing and complement-fixing activity. Purified rabies virus G protein was used as antigen in the complement fixation (CF) test.

**Serum neutralization SN test.** The rapid-fluorescent-focus-inhibition test was performed as described by Smith et al. (17) with modifications (3).

**Antibody-binding test.** The antibody-binding test was performed as previously reported (3).

**CF test.** The microplate method as described before (13) was used for block titrations of sera and antigens.

**ID test.** The immunodiffusion (ID) method has been described before (5).

**IF test.** In the indirect immunofluorescence (IF) test (20), fluorescein-labeled goat anti-rabbit serum (Behringwerke, Marburg, Germany) was used at a 1:200 dilution in M/90 phosphate-buffered saline (PBS). Infected BHK cells were stained after fixation on glass slides or in suspension. Infected mouse brains were minced with scissors and treated three times for 10 min each with 0.125% (wt/vol) trypsin in PBS. Trypsinized cells were carefully poured off, chilled, sedimented ( $1,000 \times g$  for 5 min, 4°C), and washed twice in PBS, followed by fixation and serum treatments. Direct IF staining using fluorescein-labeled anti-N serum has been described before (13).

**Assay for lytic antibodies.** The original procedure (22) was modified. The test was performed on Lab-Tek chamber slides (Lab-Tek Products, Division of Miles Laboratories, Inc., Westmont, Ill.). The cells were chicken embryo fibroblasts (4) grown in medium T199 mixed 1:1 with Hanks balanced salts solution, supplemented with 10% calf serum, and were used either infected with ERA virus (72 h) or uninfected. For the test  $1.5 \times 10^5$  infected cells per ml were mixed with  $3.5 \times 10^5$  uninfected cells per ml. To 0.2 ml of the cell mixture was added 0.1 ml from serum dilutions and 0.1 ml of complement. Guinea pig serum, absorbed with agarose (Serva, Heidelberg, Germany) (2), served as complement. As a control for each serum dilution, complement

was replaced by medium. After incubation for 2 h at 37°C, the slides were fixed and stained by the direct IF method. A 50% reduction of infected cells was considered as the end point for lytic activity.

**HA test.** The hemagglutination (HA) test has been described before (7). HA inhibition by virus components was performed according to Sokol et al. (18).

## RESULTS

In a separate communication (Dietzschold et al., in preparation) we have characterized rabies virus G protein. Purified virus was solubilized by Triton X-100 and centrifuged at  $120,000 \times g$ , and the supernatant was subjected to IEF. Of the three IEF peaks demonstrated by CF, one (pH 7.0) was shown to be purified G protein, and the second peak (pH 4.5) represented a complex of membrane proteins ( $M_1$ ,  $M_2$ ) associated with lipids and traces of G protein. The third peak (pH 8.0) consisted mainly of lipids.

**Protection studies.** When injected into animals, IEF-purified G protein induced virus-neutralizing (VN) antibodies with high titers, whereas the response to the IEF-pH 4.5 material was low. In order to evaluate the protective activity of the two preparations, immunization experiments were conducted in NMRI mice. The two vaccines were compared, *in vivo* and *in vitro*, to a beta-propiolactone-inactivated, concentrated virus preparation and to a standard reference vaccine. Before dilutions, the IEF vaccines were adjusted to a volume corresponding to the initial volume of the concentrated virus before Triton treatment. The antigenicity of each vaccine was tested in the antibody-binding test, and the antigenicity of three vaccines was tested in the CF test. The results are summarized in Table 1.

The vaccination experiments show that only beta-propiolactone virus and the G protein vaccine offered protection against the intracerebral infection of mice. Moreover, the *in vivo* test clearly indicated that the total protective capacity of the initial virus preparation was quantitatively recovered in the G protein peak, which corresponded to the pH 7.0 material of IEF. In contrast, it was seen that no protection was given by the M proteins of the viral envelope contained in the IEF pH 4.5 vaccine.

The capacity to bind VN antibodies was associated with the IEF-G protein fraction of pH 7.0 but not with the IEF-pH 4.5 vaccine.

Despite excessive dialysis, IEF-purified G protein still contained ampholytes that interfered with protein determinations. Sephadex G-50 filtration was used to remove excessive ampholyte. The separation of IEF G protein from ampholyte is shown in Fig. 1. All of the CF

TABLE 1. Protection experiments with beta-propiolactone-inactivated ERA strain of rabies virus and with peak material from IEF (IEF, pH 4.5, pH 7.0) of a 120,000 × g supernatant of Triton-treated ERA virus<sup>a</sup>

Vaccine	Protective value		CF test (CFU <sup>d</sup> /ml)	Antigenic value	
	Mouse test (ED <sub>50</sub> ) <sup>b</sup>	Antibody-binding test (AD <sub>50</sub> ) <sup>c</sup>		Mouse test (AV) <sup>e</sup>	Antibody-binding test (AV)
Virus, concentrated	3,458 <sup>f</sup>	6,400 <sup>f</sup>	8.2 × 10 <sup>4</sup>	56.7	5
IEF peak, pH 7	3,311	3,200	4.1 × 10 <sup>4</sup>	54.3	2.5
IEF peak, pH 4.5	<3	<100	2.6 × 10 <sup>3</sup>	<0.05	<0.08
Reference	61	1280	NT <sup>g</sup>	1	1

<sup>a</sup> The initial vaccine dilution of the IEF materials was adjusted to the original virus volume of 5 ml with 0.15 M NaCl.

<sup>b</sup> ED<sub>50</sub>, 50% end point of vaccine dilution (reciprocal of highest vaccine dilution protecting 50% of mice). Virus titer of the intracerebrally inoculated CVS challenge virus was 80 LD<sub>50</sub>/0.03 ml.

<sup>c</sup> AD<sub>50</sub>, Effective 50% adsorbing dose (reciprocal of highest vaccine dilution at which the neutralizing capacity of a standard immune serum preparation is reduced by half).

<sup>d</sup> CFU, Complement-fixing units.

<sup>e</sup> The antigenic value of a test vaccine is obtained by dividing the 50% protective or adsorbing end point by that of the reference vaccine.

<sup>f</sup> Reciprocal of vaccine dilution.

<sup>g</sup> NT, Not tested.

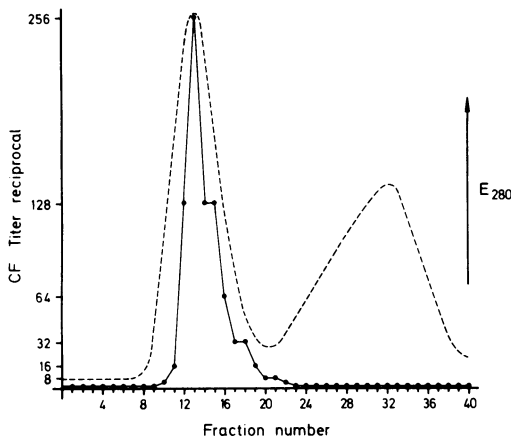


FIG. 1. Elution profile of IEF material after Sephadex G-50 gel filtration. Elution buffer was 0.2 M sodium borate, 0.1 N HCl, and 0.1 M NaCl, pH 7.3. Symbols: CF activity, —; E<sub>280</sub>, ---.

activity and 6% of the total proteins were associated with the first peak, whereas the second peak contained mainly ampholine.

The removal of ampholine allowed quantitation of the G protein that was recovered during the purification and correlation of the protein contents of vaccines with their protective activity (ED<sub>50</sub>) and with their capacity to bind VN antibodies (ABU). The results of this immunization experiment carried out in BALB/c mice are shown in Table 2.

Approximately 20% of the total protein was recovered in the 120,000 × g supernatant (vac-

cine no. 2), with 6% remaining after ampholine removal (vaccine no. 4). The protective activity of vaccine no. 2 equaled that of the starting material (vaccine no. 1) and increased after IEF (vaccine no. 3) and ampholine removal (vaccine no. 4). The ratio of ABU-ED<sub>50</sub> decreased during purification (vaccines no. 2 to 4). The amount of protein needed to protect 50% of the mice decreased from 1.63 μg to 9 ng.

A repetition of this experiment comparing virus produced at 37°C with that at 32°C showed a similar protein-ABU-ED<sub>50</sub> correlation, the only difference being a fivefold-higher virus and G protein yield from virus grown at 32°C.

CF. Rabbit anti-G serum had a VN antibody titer of 1:10,800. The CF titer against G protein and whole virus was 1:640. CF cross-reactions were not observed with either N protein, with complete or disrupted Mokola virus, or with BHK cells, demonstrating that the G protein is a distinct and serotype-specific antigen of the rabies virus.

Our results proved that the G protein is the only antigen responsible for the induction of VN antibodies and for immunity. Therefore, an effort was made to link VN activity and CF activity against G protein in human postvaccinal antirabies sera. The results (Fig. 2) show a direct correlation between VN activity and CF antibody titer.

ID. Results obtained in the double-diffusion test (Fig. 3) correspond to those of the CF test. G and N antigens formed single, individual precipitation lines which joined the analogous lines of Triton-disrupted virus.

IF. In the indirect IF test using infected

TABLE 2. Recovery of protein, antibody binding (ABU), and protective activities ( $ED_{50}$ ) during preparation of G-protein

Vaccine	Total vol (ml)	Protein ( $\mu$ g/ml)	ABU/ml	$ED_{50}$ /ml	Correlation of:
					$ED_{50} = ABU = \mu$ g of protein
1. Concentrated virus	10	2,000	36,000	1,230	1 = 30 = 1.63
2. 120,000 $\times$ g	20	200	48,600	759	1 = 64 = 0.26
3. G-protein plus ampholine	6	3,500	18,000	5,012	1 = 4
4. G-protein after Sephadex	12	100	16,200	10,715	1 = 1.5 = 0.009
5. Ampholine after Sephadex	16	1,300	200	<10	
6. Reference		100,000	8,538	64	1 = 133 = 1,562

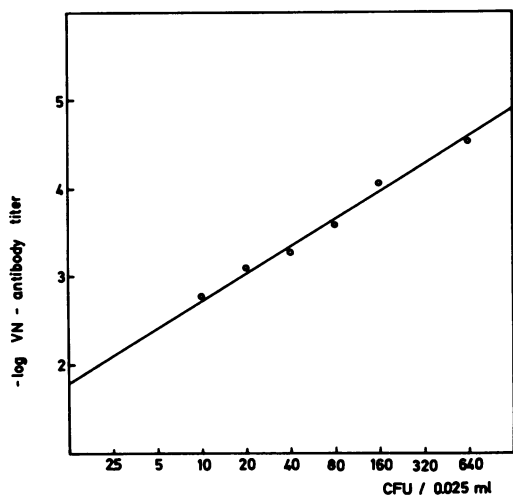


FIG. 2. Evaluation of VN versus complement-fixing antibodies from human antirabies sera. Purified G protein served as antigen in the CF test.

BHK cells, treatment with anti-G serum resulted in a selective fluorescence of the cell membrane, whereas the fluorescence induced by anti-N serum was restricted to the cytoplasm. The selective membrane staining of infected brain neurons was only possible when the brain tissue was trypsinized and when the cells were kept in suspension.

**Lytic antibody.** The assay is based on the immunolysis of cells by antibody in the presence of complement. The sera were tested, and the results obtained are given in Table 3. Sera induced by whole virus, G protein, and antirabies vaccine showed lytic antibody activity, which correlated with the respective VN antibody titer. Sera directed against the nucleocapsid and the membrane proteins (IEF-pH 4.5) of rabies virus and against VSV Indiana did not show such activity, indicating that immunolysis in rabies is G protein specific.

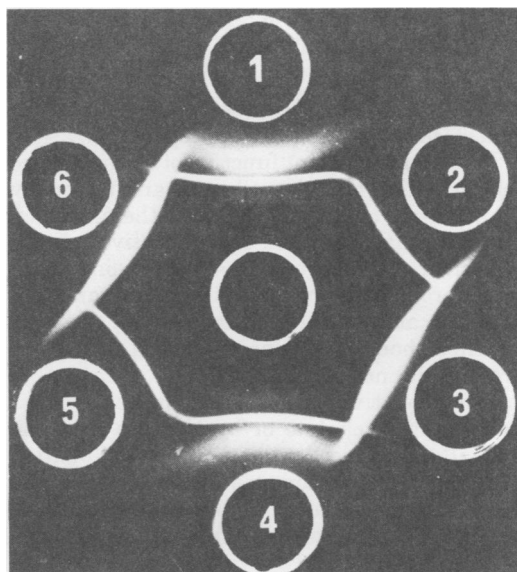


FIG. 3. Agar gel double-diffusion test employing purified Triton-disrupted virus (1 and 4), glycoprotein (2 and 5), and nucleoprotein (3 and 6). The center well contains antiviral serum.

**HA.** Isolated and purified G protein did not hemagglutinate goose erythrocytes in a pH range of 6.0 to 7.0. When mixed with the virus before the addition of erythrocytes, G protein inhibited the HA reaction of 3 to 4 HAU of intact rabies virus. The technique may be used for antigen titrations; however, the sensitivity is low as compared with CF and antibody-binding tests.

### DISCUSSION

By the use of IEF-purified G protein and anti-G monocomponent sera, known biological and serological activities of rabies virus could be associated with this structural component,

TABLE 3. Comparison of lytic and VN antibody titers as demonstrated by antisera of different origin

Species	Antiserum directed against <sup>a</sup>	Antibody titer	
		Lytic	VN
Rabbit	ERA virus, complete	640	75,000
Rabbit	ERA virus, glycoprotein <sup>a</sup>	160	10,800
Rabbit	ERA virus, membrane proteins <sup>b</sup>	<5	640
Rabbit	ERA virus, nucleocapsid protein	<5	<5
Humans	HDGS vaccine <sup>c</sup>	160	9,000
Rabbit	VSV Indiana complete <sup>c</sup>	<5	15,000

<sup>a</sup> IEF-pH 7.0 material.

<sup>b</sup> IEF-pH 4.5 material.

<sup>c</sup> HDGS, human diploid cell culture strain; VSV, vesicular stomatitis virus.

shown to represent the "spike" protein of the virus (Dietzschold et al., in preparation).

The most important function of G is to induce the formation of VN antibodies and to convey immunity to animals, protecting them from lethal challenge infection. The protective activity was clearly shown to be associated only with the G protein and not with the membrane proteins M<sub>1</sub> and M<sub>2</sub> (IEF peak, pH 4.5; Table 1) which, although containing residual traces of G protein, did not protect mice.

The protective activity of G protein solubilized by deoxycholate or by Nonidet P-40 and partially purified by gradient centrifugation was found by Wiktor et al. (23) to be 9 to 24 times lower as that of undissociated virus. In several experiments using two different strains of mice (Tables 1 and 2), we found that the total protective activity of the virus was recovered in the G protein fraction of IEF. It is evident from protein determinations during purification that the protective activity of G increases with purity (Table 2). In the final G preparation, 9 ng of protein protected 50% of the mice, compared with 1.63  $\mu$ g of undissociated virus. Since it is not known whether high-molecular-weight ampholine is completely removed by Sephadex filtration, it is possible that the value of 9 ng represents an even lower amount of G protein. These results indicate that IEF-purified G protein 3S monomers (Dietzschold et al., in preparation) immunize mice at least as well as the complete virus does.

The above findings apply equally to virus grown at 32°C; however, five times more G was recovered from 32°C virus as compared with virus grown at 37°C. The higher virus yield at temperatures below 37°C has been noted before (24) and may be important when manufacturing antirabies vaccines.

In analogy to previous results obtained with VSV (5), CF and ID tests proved the G protein of rabies virus to be a second major antigen distinct from the rabies group-specific N antigen which has been isolated before (13). Its lack of cross-reaction with Mokola virus, another member of the rabies subgroup of rhabdoviruses (13), indicates that G is a serotype-specific antigen.

The correlation found between CF and VN antibody titers of human antirabies postvaccinal sera encourages the use of the CF test as a specific VN-antibody assay provided that G is used as antigen.

The selective IF staining of cell membranes differing from the N-specific cytoplasmic fluorescence is another characteristic of anti-G serum. It indicates that viral G protein accumulates at the budding sites of the virus and not in the cytoplasm. Membrane staining of neurons was not demonstrable in sections or smears of infected brains but only in suspended cells. This may explain the failure of membrane staining by antiviral sera in spite of high VN antibody titers. This type of serum is used almost worldwide for the routine IF diagnosis of rabies but exclusively exhibits N-specific cytoplasmic staining.

Immunocytolysis is another function that we could associate with anti-G antibodies. Only sera directed against the virus surface (anti-G, antiviral) show cytolytic activity, whereas anti-N and anti-M sera do not cause cytolysis (Table 3). This and the results of external labeling of rabies virus by pyridoxal phosphate (Dietzschold et al., in preparation) indicate that G protein is identical to the "spike" protein of the virus envelope. The original assay for lytic antibody (22) was modified for the use in Lab-Tek plates, which at present are widely used for various rabies seroassays (3, 18) since only minimal amounts of reagents are required.

HA activity as shown by intact virus in the presence of goose erythrocytes (7) is not associated with purified G preparations. Lipid solvents (9) and detergents (18), with the exception of saponin (12), destroy the HA activity of the virus, indicating that this reaction is dependent on the integrity of the viral unit membrane (see also 14). In contrast, envelope proteins solubilized by deoxycholate show an inhibition of the HA reaction (18). IEF-purified G protein also inhibits HA when mixed with intact virus particles, probably as a result of competition for erythrocyte receptor sites.

The biological and serological functions associated with the two major antigens of rabies virus as described here for G protein and in a

previous report for N protein (13) give an almost complete account of the antigenic composition of the virus. Most important is the fact that the isolated G protein devoid of lipids and other proteins has the same immunizing capacity as the intact virus. This seems to justify the earnest consideration of the production and application of such a preparation as a vaccine for human use. A water-soluble preparation consisting of only one viral protein has been regarded as the ideal human antirabies vaccine (8).

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