

Enzyme-Linked Immunosorbent Assay for Determination of Antibodies to the Envelope Glycoprotein of Rabies Virus

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The envelope glycoprotein G of rabies virus induces neutralizing antibodies, which are important in protection against rabies. This protein was solubilized from purified virus and isolated by differential and sucrose density gradient centrifugation followed by high-performance liquid chromatography. Conditions for solubilization and purification of G were optimized by using immunoblotting and enzyme-linked immunosorbent assay techniques. The reaction with conventional antisera and monoclonal antibodies indicated that purified G protein was essentially devoid of internal viral proteins. Microdilution plates were coated with purified G protein, and sera from humans vaccinated against rabies were tested for the presence of antibodies. Results were compared with those of the rapid fluorescent focus inhibition assay, which is the standard neutralization assay for antibodies to rabies virus. The results of this comparison indicate that the enzyme-linked immunosorbent assay for G is a reliable and simple alternative to the neutralization test.

Current rabies vaccines induce the formation of antibodies directed to all viral proteins. Neutralization of viral infectivity, however, is due to antibodies to the envelope glycoprotein G (2, 5, 27). Although virus-neutralizing antibodies are not the only factor responsible for protection against rabies (3, 6), the presence of neutralizing antibodies in serum is taken as a reliable indicator for the success of active immunization. According to current World Health Organization (WHO) guidelines, neutralizing antibodies should be assayed in mice or in cell culture. In the latter case, the rapid fluorescent focus inhibition assay (RFFIT) is the method of choice. Enzyme-linked immunosorbent assay (ELISA) techniques with either whole virus or purified G protein have been described in the literature (1, 17, 21, 22). Assays based on whole virus as the antigen also detect antibodies against proteins other than G and consequently may lead to erroneous estimates of protection. Economic reasons may preclude the widespread use of currently available ELISAs for antibodies to G protein. We have therefore designed a simple and inexpensive procedure for the purification of G. The results presented in this communication indicate that the ELISA based on this protein preparation detects neutralizing antibodies with high sensitivity and specificity.

MATERIALS AND METHODS

Production of virus. The SAD strain of rabies virus was grown in BHK-21-C13 cells as described previously (10). After precipitation with ammonium sulfate (final concentration, 50% [wt/vol]), the virus was purified by centrifugation at 4°C on a 20 to 45% (wt/vol) discontinuous sucrose gradient at 25,000 rpm in a Beckman SW28 rotor for 5 h. Virus was collected at the interphase, diluted with phosphate-buffered saline, and pelleted in an SW41 rotor at 38,000 rpm for 90 min. The virus was then suspended in TEN buffer (10 mM Tris hydrochloride, 10 mM EDTA, 150 mM NaCl [pH 7.4]), and the protein concentration was estimated by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Solubilization of G protein. Virus suspended in 300 mM NaCl-50 mM Tris hydrochloride (pH 7.4) was incubated

with octyl- β -glucopyranoside (Sigma, St. Louis, Mo.; final concentration, 68 mM) (18). After incubation at room temperature for 1 h, viral cores were sedimented in a Beckman SW55 rotor at 50,000 rpm for 1 h. The supernatant containing G protein was then subjected to high-performance liquid chromatography (HPLC).

HPLC. Size-exclusion HPLC was carried out with LKB Spherogel TSK 3000SW or Beckman TSK 4000SW columns. In some experiments, the material was passed through an LKB TSK precolumn before application to the separation column. The equipment consisted of a high-pressure pump (LC414) and a Uvikon 720 LC photometer connected with an Anacomp microcomputer (Kontron, Zurich, Switzerland). Conditions for HPLC were as follows: running buffer, 20 mM Tris hydrochloride-0.6% sodium deoxycholate (pH 7.4); flow rate, 0.2 ml/min; collection of fractions at intervals of 2 min.

Monoclonal antibodies. Mice were immunized with either SAD strain rabies virus ribonucleoprotein (M. Bruns, Ph.D. thesis, University of Giessen, Giessen, Federal Republic of Germany) or with intact infectious virus. Ribonucleoprotein was injected intraperitoneally in complete Freund adjuvant, and infectious virus was injected intramuscularly. Spleen cells were fused with P3U1 myeloma cells by established methods (9). Four antibodies (10AF12, 11AH6, 7AG8, 5DF12) were specific for the nucleoprotein (N), three (10ED8, 10EC9, 10AA9) were directed against G protein, and one (16AD8) was specific for the NS protein.

Analysis of purity of G protein. Fractions obtained by HPLC were diluted 10-fold in phosphate-buffered saline (pH 7.4), and 100- μ l samples were coated onto 96-well microdilution plates (Dynatech Laboratories, Billingham, Sussex, England). After incubation in a moist chamber at 20°C for 16 h, the plates were washed three times with 500 mM NaCl containing 0.5% (vol/vol) Tween 20. Monoclonal antibodies to G or N were then added (100- μ l samples). After incubation for 3 h, the plates were washed. Bound antibody was detected with goat antibody to mouse immunoglobulin G conjugated to alkaline phosphatase (Tago, Burlingame, Calif.). The enzyme was allowed to react with *p*-nitrophenyl phosphate disodium, and optical density was measured in a Titertek Multiskan spectrophotometer. Sodium dodecyl sul-

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fate-polyacrylamide gel electrophoresis was performed in a 12% polyacrylamide gel as described by Laemmli (13), and protein bands were visualized by the silver staining method of Morrissey (16). Immunoblotting was performed as described by Towbin et al. (23).

G ELISA. G protein purified and analyzed as described above was diluted in phosphate-buffered saline (pH 7.4) and was used for coating of 96-well microdilution plates (16 h of incubation). Optimal dilutions of G were determined in checkerboard titrations against positive and negative control sera. The approximate concentration of G used for coating was 1 to 2 $\mu\text{g/ml}$. Plates were stored with the coating fluid in the wells at -20°C without apparent loss of antigenic activity. Sera to be assayed were diluted 1:50 in milk-Tris buffer (20 mM Tris hydrochloride, 0.1% [vol/vol] Tween 20, 0.05% [wt/vol] sodium azide, 500 mM NaCl, 5% [wt/vol] skim milk powder). Samples of 100 μl were added to the wells and incubated for 3 h at 20°C in a moist chamber. Plates were then washed, and bound antibody was detected by means of rabbit antibody to human immunoglobulin conjugated to alkaline phosphatase as described by Fey et al. (7). After incubation for 2 h, plates were again washed, and the enzyme reaction was initiated by the addition of *p*-nitrophenyl phosphate disodium (2 mg/ml). The enzyme reaction was not stopped.

RFFIT. Neutralizing antibodies were measured by using the RFFIT protocol of Smith et al. (20) modified for 96-well flat-bottom microdilution plates (28). Titers are expressed as the reciprocal of 50% endpoint dilution calculated by the method of Spearman and Kärber as described by Lorenz and Bögel (14). A positive reference serum (3.4 IU/ml, obtained from the WHO, Copenhagen, Denmark) was used to standardize the test.

Human sera. Serum samples were obtained from persons vaccinated with a commercial human diploid cell culture vaccine. Negative control sera were obtained from unvaccinated persons. All sera were assayed in parallel with the RFFIT and with the G ELISA.

RESULTS

To monitor the purification, samples were taken at various stages and analyzed by immunoblotting. The results of a representative experiment are shown in Fig. 1. Mouse serum obtained by immunization with intact rabies virus recognized proteins in the range of 30 to 80 kilodaltons, corresponding to proteins M, NS1, NS2, N, and G (Fig. 1A, lane 1). Solubilization of purified rabies virus followed by centrifugation only incompletely removed internal viral proteins from G (Fig. 1A, lanes 2 and 3). However, these proteins were essentially removed from this material by HPLC (Fig. 1A, lane 4). Importantly, HPLC removed the M and NS proteins to a level beyond the detection limit of our assay. To assess more specifically the presence of N, which accounts for approximately 30 to 34% of total protein in the intact viral particle (4), we used a monoclonal antibody directed to N (Fig. 1B). As can be seen from the more dense staining compared with that in Fig. 1A, this antibody detects N with higher sensitivity than does the polyclonal mouse serum. Only trace amounts of N remained in the preparation of G protein after HPLC (Fig. 1B, lane 4). A monoclonal antibody specific for G was used to monitor the purification of this protein (Fig. 1C). To investigate whether the preparation of G obtained by HPLC was suitable for the detection of antibodies to this protein in the ELISA, we carried out a series of experiments similar to those shown in Fig. 2.

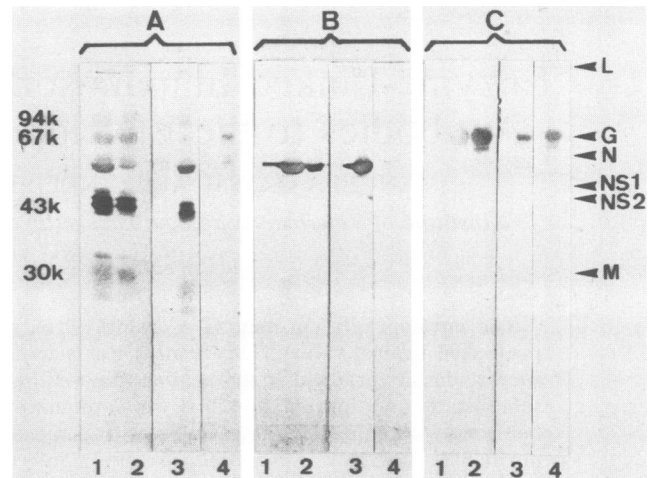


FIG. 1. Analysis of rabies G protein purification by immunoblotting. Proteins were transferred to a nitrocellulose membrane after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by incubation with mouse immune serum to rabies virus (A) or monoclonal antibodies 5DF12 to N (B) or 10AA9 to G (C) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse serum and subsequent reaction with aminoethyl carbazole as the substrate. Molecular weights (in thousands) were calculated from standards (not shown) run along with the samples and visualized by the silver staining method. Lanes: 1, interphase-purified virus; 2, supernatant after solubilization with octylglucopyranoside; 3, pellet after solubilization with octylglucopyranoside; 4, G protein purified by HPLC.

Material eluting from the HPLC column was coated to ELISA plates and subsequently reacted with monoclonal antibodies to G or N. G eluted between 30 and 54 min after the start of the HPLC run in a total volume of 40 ml (Fig. 2). The optical density observed with the monoclonal antibody to N never exceeded a value of 0.056 and remained constant throughout the peak of G, showing that the trace of N observed in immunoblotting (Fig. 1) was not detected in the ELISA. Additional experiments with a monoclonal antibody specific for NS revealed no evidence for the presence of this protein in the preparation of G protein (data not shown).

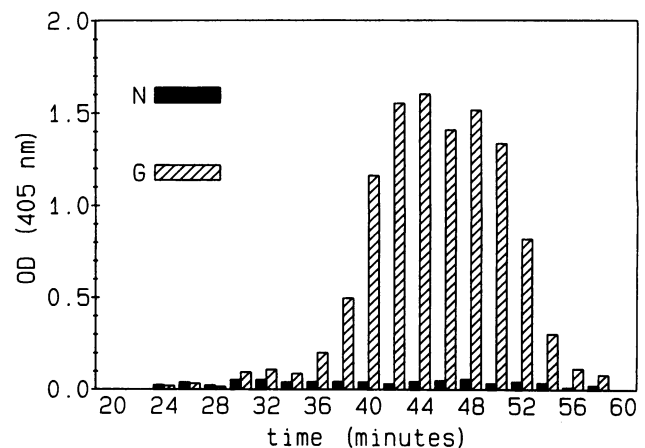


FIG. 2. Elution profiles of rabies G and N proteins in size exclusion HPLC. Purification of rabies G protein was as described in Materials and Methods. After HPLC, samples of the fractions were diluted and coated to ELISA plates. Monoclonal antibodies were used to detect G (10EC9) and N (5DF12). OD, Optical density.

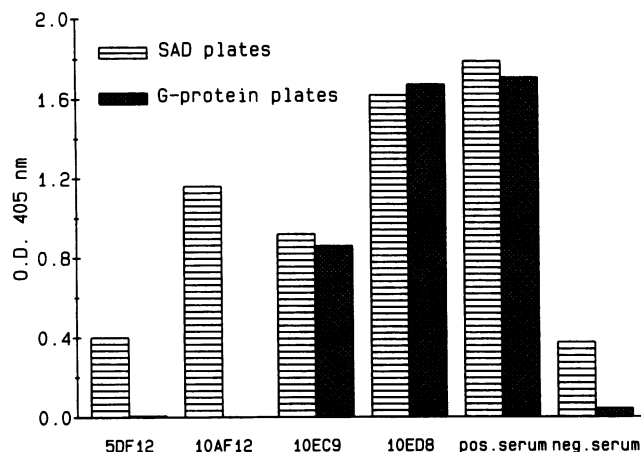


FIG. 3. Comparison of ELISA with G protein versus whole virus as the antigen. Plates were coated with G protein or with rabies virus purified by differential and sucrose density gradient centrifugations. Binding of human sera and mouse monoclonal antibodies to the plates was assayed as described in Materials and Methods. Antibodies 5DF12 and 10AF12 are specific for N, and antibodies 10EC9 and 10ED8 are specific for G. O.D., Optical density.

We next compared ELISA plates coated with virus purified by differential and sucrose gradient centrifugations with plates coated with G protein. The monoclonal antibodies to G and the positive control serum bound to both types of ELISA plates (Fig. 3). In contrast, monoclonal antibodies to N bound only to plates coated with whole virus. Interestingly, also nonimmune human serum bound to this antigen to some extent. It is unclear whether this is due to the presence in the nonimmune serum of antibodies to contaminating cellular antigens or bovine serum albumin in the interphase-purified virus preparation or whether it reflects nonspecific binding.

To assess more specifically the performance of the G ELISA in detecting antibodies to G protein and to evaluate ways of standardization, we carried out two series of experiments.

In the first, 44 serum samples of vaccinated persons and 75 serum samples of unvaccinated persons were included. A serum pool with a RFFIT titer of 56 was used as a standard at various dilutions on every plate. The plates were read when the 1:10 diluted standard had reached a value of approximately 1.2. At this point the 75 serum samples of the unvaccinated persons reached an optical density of 0.040 ± 0.032 . The arithmetic mean plus 3 standard deviations was used to define the negative range. Only 1 of the 44 serum samples of vaccinated persons was in the negative range. The serum of this person had also no neutralizing activity in the RFFIT.

A WHO working group recommended that a minimum value of 0.5 IU should be attained to demonstrate seroconversion (19) indicative of successful vaccination. In the second series of experiments we used a serum pool containing this concentration of antibody (as measured by the RFFIT) to define the border between positive and negative in both the RFFIT and the ELISA. Seventy serum samples from persons vaccinated with a diploid cell culture vaccine were compared in the RFFIT and the ELISA in this way (Table 1). Of the 70 serum samples, 15 were in the negative range in the RFFIT as compared with 13 in the ELISA. Divergent results were obtained with six sera. Two serum samples that were positive in the RFFIT were in the negative

TABLE 1. Comparison of RFFIT with ELISA

ELISA result	No. of RFFIT results			
	Negative		Positive ^a	
	<0.2 IU	0.2-0.49 IU	0.5-0.99 IU	>1 IU
Negative	7	4	2	0
Positive ^b	0	4	4	49

^a Positive is defined as seroconversion (19) with WHO standard serum.

^b Positive is defined as serum specimens with an optical density greater than that of standard serum with RFFIT titer (0.5 IU).

range in the ELISA, and four serum samples that were negative in the RFFIT were positive in the ELISA. Serum samples of unvaccinated persons were negative in both assays (data not shown).

DISCUSSION

The aim of determining antibodies directed to rabies virus differs somewhat from that of most other viruses because information on protection rather than immunologic reactivity as such is sought. Although internal viral proteins have been demonstrated to play a role in the protection conferred by cellular immunity (3, 6), neutralizing antibodies are held to be the most reliable indicator of protection against rabies. According to current WHO guidelines (25), the sera of vaccinated persons should be examined for the presence of neutralizing antibodies, preferably by the RFFIT or the mouse neutralization test. Since these tests involve cell culture techniques or require facilities for keeping mice, they are beyond the possibilities of many laboratories, particularly those in developing countries.

Any assay to replace the neutralization test should be specific for the envelope G protein, which induces neutralizing antibodies (24), and ideally the test should be specific for the epitopes in this protein that are involved in neutralization. Specificity for the G protein is particularly important because certain vaccines, particularly those of nervous tissue origin (8), contain a high concentration of nucleoprotein. ELISAs based on antigen prepared from intact viral particles detected antibodies to both G and N (Fig. 3). Western blots (immunoblots) of sera from vaccinated persons were bound to all viral proteins, both internal and external (results not shown). This situation, i.e., detection of antibodies that confer protection together with antibodies that fail to protect against infection, would be of no consequence with sera of persons vaccinated with potent cell culture vaccines containing a high concentration of G or with recombinant vaccines containing or expressing G (11, 26). However, the use of whole virus as an antigen in the ELISA could lead to the erroneous and potentially dangerous interpretation that persons vaccinated with inefficient vaccines are protected against rabies because such vaccines induce predominantly antibodies to internal viral proteins. As verified by immunoblotting and by ELISA, this problem is solved by using purified G protein as the antigen.

For the standardization of the ELISA we chose two approaches. In the first we tried to find criteria allowing the differentiation between antibody-positive and -negative sera. This was achieved by using sera of unvaccinated persons to define the negative range. With one exception, which was also found to lack antibodies in RFFIT, sera of all vaccinated persons could be determined to be in the positive range. However, as recommended by a WHO Working Group,

demonstration of seroconversion after vaccination should be based on a certain minimum concentration of antibody rather than on the calculation of positive and negative based on the determination of the negative range with sera from nonimmune persons. Preexposure vaccination against rabies is considered successful when a concentration of 0.5 IU of neutralizing antibody is present in serum (19). We therefore attempted to define a cutoff point for positive by using a serum pool with an antibody concentration of 0.5 IU in the RFFIT. Using this approach for the standardization of the ELISA, we found that most sera gave concordant results in the RFFIT and the G ELISA, with only slight discrepancies with sera close to the cutoff points in both assays. Similar discrepancies have been noted between the current standard assays, i.e., the RFFIT and mouse neutralization tests (12, 15).

The proposed procedure for the purification of G protein is relatively inexpensive. In our hands, 1 liter of supernatant of a cell culture infected with rabies virus yielded enough G protein for coating 120 to 240 ELISA plates, with which 5,000 to 10,000 sera can be tested. The antigen coat on the ELISA plates remained stable for several months when stored at -20°C . Since the plates contain no infectious virus, the G ELISA can be carried out also in laboratories lacking the safety facilities required for working with rabies virus. Furthermore, the ELISA is technically more simple and allows more samples to be assayed in a shorter time than the neutralization assay in mice and the RFFIT in cell culture.

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