Enzyme Immunoassay for Rabies Antibody in Hybridoma Culture Fluids and Its Application to Differentiation of Street and Laboratory Strains of Rabies Virus

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A rapid and sensitive enzyme immunoassay is described for detecting rabies antibody in hybridoma culture fluids. Glass fiber filter disks were used to immobilize gamma-irradiated mouse neuroblastoma cells infected with street or laboratory strains of rabies virus. Bound rabies-specific antibody was detected by reaction with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G. The assay was performed in a 96-well filtration device developed by Cleveland et al. (J. Clin. Microbiol. 15:402–407, 1982) for the typing of herpes simplex viruses. When partially disrupted cells were used, both internal and external viral antigens were available for reaction. The procedure is rapid (<4 h for completion) and requires only small amounts of fluid, and the gamma-irradiated antigen is noninfectious. When the procedure was used to screen 145 fluids from rabies-immune spleen-myceloma cell fusions, 132 were positive for rabies antibody. Other commonly used assays for the detection of rabies-specific antibody were less sensitive. Simultaneous analyses of many hybridoma fluids against a battery of street and laboratory strains of rabies virus are possible and allow rapid selection of useful monoclonal hybridoma lines.

For successful hybridoma production, it is essential to have an assay system of high sensitivity and specificity in which the results of large-scale screenings can be obtained within 24 h. The aim of our monoclonal antibody (Ab) studies was the production of Abs capable of detecting antigenic differences among rabies virus strains used as vaccine seed stock and those rabies “street” isolates currently producing the disease in domestic and wild animals worldwide. Vaccine or laboratory-adapted rabies virus can easily be grown to high titers in cell culture; however, street rabies virus strains grow poorly in cell culture, and only small amounts of complete virus particles are found in cell-free supernatants (1, 15). It is, therefore, difficult to produce the purified virus necessary in the assay systems suitable for large-scale screening for rabies antibody (2, 11, 21). The enzyme immunoassay (EIA) assay (EIA) described by Cleveland et al. (4) was readily applicable to our work. Gamma-irradiated rabies virus-infected cells rather than purified virus were used as target antigens, thus reducing the risk of laboratory exposure to infectious material and overcoming the difficulty of producing large quantities of purified street rabies virus.

MATERIALS AND METHODS

Production of hybrid cells. All hybrid cells were derived from the spleen of one 6-month-old male BALB/c mouse (Center for Disease Control breeding facility, Lawrenceville, Ga.) which had been injected in the left and right footpads with 0.03 ml and intraperitoneally with 0.8 ml of a 20% suckling mouse brain suspension of the ERA vaccine strain of rabies virus (10^9.9 50% mouse intracerebral lethal doses per ml). An intravenous booster injection of gamma-irradiated, ERA-infected mouse neuroblastoma (MNA) cell culture supernatant (crudely purified by centrifugation at 25,000 rpm for 2.5 h through a glycerol cushion, followed by suspension in phosphate-buffered saline [PBS] at a concentration of 30×1) was given 8 weeks later (ca. 1 × 10^5 50% tissue culture infectious dose of virus). The spleen was removed 2 days later, and cell fusion with an Sp2/0-Ag14 derivative of the BALB/c myeloma line P3-X63-Ag8 was performed by a modification of the procedure described by Kohler and Milstein (13). Selected hybridoma cultures were cloned by limiting dilution with BALB/c peritoneal macrophages as feeder cells. When necessary, ascitic fluid containing large amounts of monoclonal Ab was produced by injecting cloned hybrid cells into BALB/c mice pretreated with pristane.

Cell culture. An MNA cell line of A/J origin (H-2a) (supplied by T. J. Wiktor, Wistar Institute, Philadelphia, Pa.) was used for virus isolation and target antigen preparation. The cells were grown in Eagle minimal essential medium containing 10% fetal bovine serum.

The mouse myeloma cell line, Sp2/0-Ag14 (Salk Institute, San Diego, Calif.), was propagated in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) medium with 20% fetal calf serum containing 2 mM glutamine, 100 U of penicillin per ml, 50 mg of streptomycin per ml, and 5 × 10^-5 M 2-mercaptoethanol. Hybrid cells resulting from fusion of Sp2/0 cells and spleen cells from rabies virus-immunized mice were propagated in RPMI 1640—hypoxanthine—aminopterin—thymidine medium supplemented with 20% fetal calf serum containing 2 mM glutamine, 100 U of penicillin per ml, 50 mg of streptomycin per ml, 5 × 10^-5 M 2-mercaptoethanol, 0.1 mM hypoxanthine, 1 μM aminopterin, and 40 μM thymidine.

EIA. Laboratory and street strains of rabies virus were incubated with MNA cells in Falcon plastic tissue culture flasks. The progress of the infection was monitored by fluorescent antibody staining of parallel cultures grown on Lab-Tek slides (Lab-Tek Products, Westmont, Ill.). When cell infectivity reached 100%, cell monolayers were collected by scraping, suspended in Eagle minimal essential medium, frozen on dry ice, and irradiated for 3 h in a model 220 gamma cell containing 400 C with an activity of 1.96 × 10^8 rads/h in October, 1977. The absence of infectious rabies virus was confirmed by intracerebral inoculation of 3-week-old Institute of Cancer Research mice.

The EIA was performed in disposable 96-well microtiter
plates (V & P Enterprises, San Diego, Calif.). The bottom of each well contained a glass fiber filter and a capillary tube-like drain hole small enough to prevent liquid from draining through unless vacuum was applied.

Suspensions of normal or ERA-infected MNA cells in PBS containing 10% fetal bovine serum, 1% bovine serum albumin, and 0.3% gelatin were added to each well (10,000 to 200,000 cells per 50 μl per well). Liquid was removed by vacuum, leaving particulate matter larger than 1.5 μm trapped on the glass filter.

Fifty microliters of serum or hybridoma fluid was added and incubated with the filters for 1 h at 37°C. Vacuum was then applied, and the glass filters were washed with gelatin buffer (PBS plus 0.3% gelatin and 0.001% thimerosal). Bound mouse immunoglobulin G was then detected by adding 50 μl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Tago, Inc., Burlingame, Calif.). After 60 min of incubation at 37°C, excess conjugate was removed by vacuum, and the filters were again washed with gelatin buffer. The drain holes were then sealed with stop-cock grease and 200 μl of substrate (O-phenylenediamine [Sigma Chemical Co., St. Louis, Mo.] and H2O2) was added per well. After 30 min at room temperature, 6 N H2SO4 was added, and the reaction mixtures were transferred to a clean 96-well plate. Absorbance at 490 nm (A490) was measured on a Dynatech Micro-ELISA reader. Specific absorbance at 490 nm was calculated by the following formula: [A490 (virus-infected cells + antirabies antibody) − A490 (normal cells + antirabies antibody)] − [A490 (virus-infected cells + anti-monkey pox antibody) − A490 (normal cells + anti-monkey pox antibody)] = specific absorbance at 490 nm. A specific absorbance at 490 nm equal to or greater than 0.1 was considered positive for antirabies antibody.

**Virus-neutralizing antibody assay.** As virus-infected monolayers were collected for EIA target antigen, cell culture supernates were collected for use as challenge virus in a virus neutralization test.

Neutralizing antibody titers were measured against 10 to 50 fluorescent foci of virus per 0.1 ml of inoculum in MNA cells by a modification of a previously described procedure (20). Residual virus was detected after a 48 h incubation by staining acetone-fixed monolayers with fluorescein-conjugated antirabies serum (BBL Microbiology Systems, Cockeysville, Md.). A 50% reduction in challenge virus infectivity was considered to be evidence of virus neutralization.

**Immunofluorescent antibody staining for intracytoplasmic antigen.** Virus-infected MNA monolayers grown on eight-chamber Lab-Tek slides or slides made from virus-infected mouse brains were fixed with acetone, treated with hybridoma fluids or control sera for 30 min at 37°C, washed to remove unbound Ab, and then restained with fluorescein-conjugated goat anti-mouse immunoglobulin G (Tago). Slides were examined at a 200 to 400× magnification with a Zeiss standard universal microscope with vertical illumination. The light source was an XBO-150-W xenon bulb with KP-490 and LP-510 filters.

**IFA staining for membrane-associated viral antigen.** Immunofluorescent antibody (IFA) staining was performed using the procedure of Lodmell et al. (17). Unfixed ERA rables virus-infected cell suspensions were washed with PBS and stained at 4°C for 30 min with hybridoma fluid diluted in PBS supplemented with 2% fetal bovine serum and 0.01 M NaCl (PBSS). After being washed in PBSS, the cells were incubated for 30 min at 4°C with fluorescein-conjugated goat antimouse serum diluted in PBSS. The cells were then washed again in PBSS and suspended in PBS containing 1% Formalin and 50% glycerol. Fluorescent microscopy was performed as described above.

**Determination of hybridoma Ab specificity.** Only antibodies directed to membrane-associated viral antigens (glycoprotein and possibly the membrane protein) are capable of rabies virus neutralization (15, 24). Hybridoma supernates which neutralized one or more strains of rabies virus and stained the membranes of live ERA-infected MNA cells but not the intracytoplasmic inclusions of acetone-fixed infected MNA cells were considered to contain Ab directed toward membrane antigens (Abmem). Hybridoma fluids which neither neutralized rabies virus nor stained the membranes of live infected cells but did stain the intracytoplasmic inclusions characteristic of rabies-infected acetone-fixed cells (18, 23) were considered to contain Ab directed toward the nucleocapsid antigens of rabies virus (Abnc).

**Rabies virus strains (laboratory strains).** (i) The ERA vaccine strain (1) was obtained from the American Type Culture Collection, plaque-purified once (19), and grown on BHK-21 13s cells. Supernate and cells from the first passage in MNA cells were used as antigen for IFA staining, EIA, and neutralization. Brain material from a first intracerebral passage in suckling mice was used as antigen for immunization of mice for hybrid-cell production.

(ii) The CVS-11 fixed virus strain (12) repeatedly passaged on BHK-21 13s cells was maintained as a laboratory challenge virus at the Centers for Disease Control (20). Supernate and cells from the first passage in MNA cells were used as antigen for IFA staining, EIA, and neutralization.

(iii) The official production vaccine strain (8) was obtained from the American Type Culture Collection and had 10 passages on BHK-21 13s cells. Supernate and cells from the first passage in MNA cells were used as antigen for IFA staining, EIA, and neutralization.

(iv) The Flurys Hybrid Egg Passage vaccine rabies strain (14, 16) was obtained from the American Type Culture Collection. Supernate and cells from the first passage in MNA cells were used as antigen for IFA staining, EIA, and neutralization.

**Rabies virus strains (street strains).** (i) Strain MD9591 was obtained as dog salivary gland material collected by Mario Martell, Mexico City, Mexico, from a naturally infected rabid dog. Supernate and infected cells at the fourth passage level in MNA cells were used as target antigens for neutralization, EIA, and IFA staining.

(ii) Strain TBM-L4 was obtained as bat salivary gland material collected by Lois Leffingwell, Texas State Health Department, Austin, Texas, from a rabid Mexican free tail bat, *Tadarida brasiliensis mexicana*. A clarified 20% suspension of the brains of mice dying after injection with this isolate was used to infect MNA cells. Supernate and infected cells at the third passage level in MNA cells were used as target antigens for neutralization, EIA, and IFA staining.

(iii) HB virus was obtained as hoary bat (*Lasius cinereus*) salivary gland material collected by Dora F. Woodall, Arizona State Health Department, Phoenix, Arizona. A clarified 20% suspension of the brains of mice dying after injection with this isolate was used to infect MNA cells. Supernate and infected cells at the third passage level in MNA cells were used as target antigens for neutralization, EIA, and IFA staining.

**RESULTS**

Normal mouse sera and rabies-immune mouse sera of known titer (Table 1) were used to determine the number of ERA-infected MNA cells to be added to each well of the EIA
reaction plate. Checkerboard titrations were performed to assess the optimal reaction times and dilutions of anti-mouse immunoglobulin G and substrate (data not shown). The number of infected cells per well for each different virus isolate was then adjusted so that absorbance values after the addition of control sera were approximately those obtained by using ERA-infected target cells (Fig. 1).

**Screening of hybridoma supernatants for Ab activity.** Results of the initial screening for Ab reacting with ERA in 145 hybrid cultures produced in the spleen fusion experiment are shown in Table 2. It is apparent from these findings that ERA rabies virus is a very effective antigen for hybridoma production and that the EIA system is the most sensitive of the three assay methods tested. Before being cloned by limiting dilution, all 132 antibody-producing hybrid cultures were expanded to larger cultures and then stored in the frozen state. Supernates from these cultures were tested against a battery of laboratory and street strains in the EIA (data not shown), and those hybrid cultures which reacted with some (but not all) of the test virus strains were then retrieved from frozen storage and cloned by limiting dilution.

Individual clones were then reselected by their reaction pattern against a battery of virus strains, expanded to larger cultures and stored in the frozen state. Twenty-six Abmc and 49 Abnc were selected by this procedure. Examples of the reaction patterns displayed by representative clones against five selected rabies virus strains are shown in Fig. 2 and 3 and Tables 3 and 4.

Clone 62-15-2 is an Abnc which reacts equally well with all five laboratory and street strains. Table 3 shows the results of IFA and neutralization testing and Fig. 2A gives the EIA reaction of this Ab.

Clone 62-73-1 is an Abnc which consistently reacts much more strongly in EIA with ERA-infected target cells than with CVS and MD5951 and reacts poorly, or not at all, with TbmLc4 and HB (Fig. 2B). The values shown in Fig. 2B reflect the results of testing by three other assay methods (Table 3).

Clone 62-24-10 is also an Abnc. The data in Fig. 2C and Table 3 show that it reacts strongly with four of the five virus strains tested but fails to react with HB rabies virus.

Table 4 shows the IFA and neutralization results of testing clone 62-61-2, an Abmb which reacts with all five virus strains. Figure 3A gives the EIA reaction of this Ab.

Figure 3B and Table 4 illustrate the results of testing clone 62-105-2, an Abmb which reacts strongly with four of the five virus strains tested but fails to react with CVS rabies virus.

Figure 3 and Table 4 illustrate that membrane-associated viral antigens are available for reaction with Ab in the EIA. It should be noted that IFA staining of acetone-fixed cell cultures with Ab directed to membrane antigens gives positive results with some viruses (primarily the laboratory strains of rabies), but the staining pattern is a fine granular one and can be easily distinguished from nucleocapsid staining (15).

**Comparison of EIA with neutralization assay for detection and classification of Abmb.** As we proceeded in comparative testing of many rabies virus strains, it became apparent that neutralization was not always a satisfactory method for determining the reaction pattern of the Abmb. For example, although 24 of 26 Abmb reacted strongly with CVS in the EIA, only 16 of 26 were capable of neutralizing CVS, although these same Abmb could neutralize other rabies virus strains in the same assay system. Ascitic fluids produced by these hybridoma clones were, however, capable of neutralizing CVS, although their titer against CVS was lower than that noted against other challenge viruses. This remained true even if 10-fold less CVS than other virus strains was used in the challenge inoculum (data not shown).

Although less commonly observed, other rabies virus strains would occasionally resist neutralization by an Abmb.
with which they had reacted quite strongly in EIA. Figure 4 shows the reaction pattern for culture fluid from hybridoma clone 62-80-6, which reacted very strongly in EIA with ERA, CVS, and MD5951, neutralized both CVS and MD591 (neutralization titer against CVS, 1:20; against MD5951, 1:200), but failed to neutralize ERA even when undiluted. Ascites fluid prepared from this clone was capable of neutralizing all three viruses (neutralization titer against ERA, 1:8,000; against CVS, 1:1,000; against MD5951, 1:100,000).

DISCUSSION

In the present study, we describe an EIA suitable for screening large numbers of hybridoma supernates for their Ab reaction to a variety of laboratory and street rabies virus strains. Although both laboratory and street rabies virus strains readily infect some cell culture systems, street rabies strains do not grow well in vitro, and repeated passage in cell culture is necessary for the adaptation of street virus to efficient growth in this system. However, cell cultures infected with these viruses accumulate sufficient cellular-associated antigen for immune detection even when only small amounts of infectious virions are released into the culture fluid (22; J. Smith, unpublished observations). This allows use of cell culture-grown street rabies virus as target antigen after only a limited number of passages in vitro. The infectious nature of rabies virus isolates is further reason to

**TABLE 3.** Reciprocal titer of hybridoma culture fluid tested by three different assay methods against five different virus strains  

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<tr>
<th>Rabies virus strain</th>
<th>Clone and assay method</th>
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<td>62-15-2</td>
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<td>IFA&lt;sub&gt;amb&lt;/sub&gt;</td>
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<tr>
<td>CVS</td>
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<td>MD5951</td>
<td>3.0</td>
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<td>Neg</td>
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<td>HB</td>
<td>2.0</td>
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<sup>a</sup> For definitions of the assay methods, see footnote <sup>a</sup>, Table 1. Neg, negative; —, test not performed; +/−, only slight reactivity even when undiluted fluid was used.

<sup>a</sup> Reciprocal of maximum serum dilution still promoting fluorescence.
antigen destruction or change with this method is minimal (5).

In the EIA described here, the disruption of virus-infected cells by a single freezing and thawing process allows both internal and external cell-associated viral antigens to react with Ab. This is important if street rabies virus is used as an antigen. Whereas most laboratory rabies strains have been shown to exhibit extensive cytoplasmic membrane budding (10), street rabies virus has been shown to bud primarily from the endoplasmic reticulum, and even there, virus production is sparse in comparison with the growth of laboratory-adapted rabies strains (9).

The ability of the EIA to detect Ab_{snr} reaction with street rabies virus is probably its most important advantage over other assay systems. As demonstrated in the work presented here and by others (3, 6), the neutralization reaction is not a satisfactory means of differentiating rabies virus strains. The challenge virus dose used in the neutralization assay is critical, and some strains of rabies virus are less easily neutralized than others, even when minimal amounts of challenge virus are used. This is not a new observation but is a most interesting confirmation by modern technology of an old observation. For example, in 1948 Wright and Habel (25) used cross-immunity tests to compare the antigenicity of the six substrains of Pasteur’s fixed rabies virus in use for vaccine production at that time. Ordinarily in such a test between two similar but not identical viruses, the degree of neutralization afforded by homologous serum is higher than that of heterologous serum. However, one of the virus strains tested (virus I) gave uniformly low Ab titer results against immune sera induced by all six viruses, whereas another virus strain (virus III) gave uniformly high Ab titers for the same sera. Similarly, when vaccines made from each of the six viruses were used in a cross-immunity test, vaccine made from virus I gave better protection against infection with virus III than it did against itself. Furthermore, vaccine made from virus III, which gave good protection against itself and four other virus strains, gave little or no protection against infection with virus I.

In summary, the EIA developed by Cleveland et al. (4) is readily applicable to the selection of useful anti-rabies monoclonal Abs and allows the evaluation of strain differences associated with both nucleocapsid and membrane viral antigens.

ACKNOWLEDGMENTS

The authors acknowledge with gratitude the advice and assistance of Luanne H. Elliott (Special Pathogens Branch, Center for Infec-
LITERATURE CITED


