Presence of Cross-Reactions with Other Viral Encephalitides in the Indirect Fluorescent-Antibody Test for Diagnosis of Rabies

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The antemortem diagnosis of rabies in humans employs techniques that require accuracy, speed, and sensitivity. A combination of histochemical analysis, in vitro virus isolation, immunological methods, and molecular amplification procedures are utilized in efforts to diagnose the disease. Modern medicine now offers potentially life-saving treatment for a disease that was considered invariably fatal once clinical signs develop. However, medical intervention efforts require a rapid and accurate diagnosis as early in the course of clinical disease as possible. Indirect fluorescent-antibody (IFA) testing on cerebrospinal fluid and serum specimens provides rapid results, but the specificity of the assay has not been well studied. Because false-positive IFA results could significantly affect patient treatment and outcomes, it is critical to understand the specificity of this assay. In this study, IFA testing was performed on 135 cerebrospinal fluid and serum specimens taken from patients with viral encephalitis or a presumed viral infection involving an agent other than rabies virus. Results indicate that false-positive results can occur in interpreting the rabies IFA test. Staining patterns morphologically similar to antirabies staining were observed in 7 of the 135 cerebrospinal fluid specimens examined. In addition, a majority of the cerebrospinal fluid specimens tested from patients with encephalitis presented immunoglobulin that bound to antigens present in the cell culture substrate. Of marked concern was the frequent presence of cross-reactive antibodies in encephalitis cases associated with West Nile and Powassan flaviviruses. Because IFA testing for rabies on human specimens may result in false-positive results, it should not be used as the sole basis for initiating antirabies treatment.

MATERIALS AND METHODS

Cell culture. BHK-21 cells (C-13; ATCC CCL10) (American Type Culture Collection, Rockville, MD) were used at passages 70 to 95. Mouse neuroblastoma cells (2) were used at passages 700 to 750. Both cell lines were cultured and maintained as previously reported (3).

Virus inoculum. The ERA strain of rabies virus (4) was utilized as the rabies antigen source in the IFA test procedure. The virus inoculum used to infect cells was obtained from a commercially available veterinary vaccine vial (5). Prior to use in the preparation of the IFA antigen slides, the stock virus was passaged twice in BHK-21 cells using the medium previously reported (3). At the second passage of cell confluence, the flask were placed at −80°C overnight. Cells were thawed to a frozen slurry, agitated, and refrozen at −80°C. Upon thawing, lysed cellular debris was removed by centrifugation at 1,000 × g, and aliquots were prepared from the supernatant for storage at −80°C.

Antigen slide preparation. Stored virus inocula had previously been titrated to identify endpoint values and infectivity profiles in both neuroblastoma and BHK-21 cell cultures. Virus inocula were added to trypsinized cells at a multiplicity of infection and cell count suitable to produce 40 to 50% cell infection with 3 days of growth at 34°C with a 5%
CO₂ atmosphere in a moist chamber incubator. Cells were grown on multiwell Teflon-coated slides (Cel-Line/Thermo Fisher Scientific catalog no. 30-225H). After 3 days of cell growth, the medium was removed, and cells were washed once (2 min) in 0.01 M phosphate-buffered saline (PBS) (pH 7.6) and air dried before storage at −80°C. Upon use, antigen slides were thawed, air dried, fixed in −20°C acetone overnight, and air dried prior to the addition of serum or CSF.

**Anti-human IgG and IgM antibodies.** Goat anti-human IgG-FITC (catalog no. IF0001) was obtained from Focus Diagnostics (Cypress, CA). This secondary antibody was used directly from the vial, with no further dilutions or additions. FITC-conjugated goat anti-human IgM (μ) was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD), reconstituted as directed by the manufacturer, and then diluted 1:40 in 0.01 M PBS (pH 7.6) containing Evans blue counterstain at 0.00125%.

**Clinical specimens.** A total of 135 CSF samples from viral encephalitis patients were tested with the rabbits IFA assay. The sample set included 10 cases of Epstein-Barr virus (EBV), one of eastern equine encephalitis virus (EEEV), one of human herpesvirus 6 (HHV-6), one of HHV-6 plus enterovirus, four of enterovirus six of herpes simplex virus 2 (HSV-2), and one of EBV plus varicella-zoster virus (VZZ) infection, all confirmed by real-time PCR assays with CSF samples (6). Thirty CSF samples were from patients with serologically diagnosed West Nile virus infections and five CSF samples were from patients with serologically diagnosed Powassan virus encephalitis. The IgG and IgM assays were performed on rabbits virus-infected murine neuroblastoma cells (i.e., rabies virus-infected cells [RICs]) and non-rabies virus-infected neuroblastoma cells (i.e., non-rabies virus-infected cells [NRICs]). With a smaller subset of specimens, rabies virus-infected and noninfected BHK-21 cells were employed to compare results with those of the neuroblastoma cell assay. The IgG IFA test was performed on 135 cerebrospinal fluid specimens and 17 serum specimens. IgM IFA testing was performed for 115 of the cerebrospinal fluid specimens. CSF samples were tested undiluted. Serum samples were available from two types of encephalitis cases, i.e., West Nile virus and Powassan virus, which tested positive in cross-species plaque reduction neutralization tests (7). These serum samples were tested in the rabbits IFA test at a screening dilution of 1:20. For the IgG assay, serum samples were depleted of IgG by an initial 1:8 dilution with goat anti-human IgG (Gull-Sorb; Meridian Bioscience, Inc., Cincinnati, OH). Serum samples for the IgM assay were tested at a 1:20 dilution. Rabies-positive control serum was from a vaccine recipient with a titer of 2.0 IU/ml and serum and CSF samples were obtained from a human rabies case. Negative-control sera were from healthy individuals with no rabies virus neutralization antibodies detected. The positive-control serum was diluted in serial 2-fold dilutions in PBS. A serum dilution of 1:32 or 1:64 was used as a control for each IFA assay.

**Indirect fluorescent-antibody assay.** Fifteen microliters of CSF or diluted serum was applied to each well of multiwell antigen slides. All sera were tested on RICs and NRICs for comparison. Slides were incubated at 37°C for 30 min in a moist chamber. The unbound antibody was eluted by a gentle wash with 0.01 M PBS (pH 7.6) from a wash bottle, and the slides were then soaked for 15 min at room temperature in a PBS-filled Coplin jar. Fifteen microliters of anti-human IgG was applied to each well, and the slides were incubated at 37°C for 30 min. After the second incubation, the conjugate was gently washed with PBS from a wash bottle before the slides were soaked in PBS for 15 min at room temperature. Slides were air dried, and coverslips were mounted with a mountant consisting of 0.05 M Tris, 0.15 M NaCl (pH 9.0), with 20% glycerol. IgM testing was performed like the IgG assay, with the substitution of goat anti-human IgM-FITC conjugate. All slides were evaluated by R. J. Rudd, and pertinent images were simultaneously viewed on a computer monitor by S. J. Wong for discussion and grading of the reaction intensity.

**Microscopy and imaging.** Photomicroscopy was performed using a Zeiss AxiosImager A1 microscope equipped for fluorescence microscopy. A Zeiss AxioCam MRc camera captured images using Zeiss AxioVision 3.1 software. Images were optimized for brightness and contrast using Adobe Photoshop Elements 2.0 software.

**Rabies virus neutralization assay.** CSF and serum samples that presented structural patterns of staining similar to staining patterns observed for known rabies-positive cases were further examined using an in vitro rabies virus neutralization assay (8).

**RESULTS**

The attachment of antibodies, as reflected by the attachment of FITC-labeled anti-human IgG conjugate, produced either structurally specific patterns or generalized background staining. Staining patterns appearing similar to specific anti-rabies staining patterns were observed for 7 of the 135 cerebrospinal fluid specimens examined for IgG specific for rabies antigen. This staining pattern was present on RICs and absent on NRICs. The IFA procedure examining IgM antibodies identified 2 of 115 cerebrospinal fluid specimens with rabies-like immunoreactivity patterns. Specific staining on RICs is identified as either intracytoplasmic inclusions consisting of rabies virus ribonucleoprotein or membrane-associated rabies glycoprotein (Fig. 1A and B). All of the CSF or serum samples that presented IFA staining similar to rabies-specific staining were negative for rabies virus-neutralizing antibodies. Comparison of reactivity patterns between RICs and NRICs identified patterns that ranged from antibody attachment seen only in rabies virus-infected cells (Fig. 1A; see also Fig. S1 to S5 in the supplemental material) to strong reactivity in both RICs and NRICs (see Fig. S6 to S8 in the supplemental material). In certain clinical samples, the pattern of the staining in the RICs, although strong, was not typical of a specific RIC staining pattern (see Fig. S6 and S9 in the supplemental material). Samples were also identified in which there was a reduced reaction pattern in the NRICs when there was a very strong reaction in the RICs (see Fig. S10 in the supplemental material). Of the 135 samples examined for IgG reactions, 70 (51.5%) reacted to RICs and 58 (42.6%) reacted to NRICs. Of the 115 samples examined for IgM reactions, 30 (26%) reacted to RICs and 28 (24%) reacted to NRICs. Evidence of reactivity to RICs and NRICs with respect to the etiological agent responsible for the encephalitis is presented in Table 1. Reaction patterns of Powassan virus encephalitis-positive CSF specimens with BHK-21 RICs and NRICs (see Fig. S6 in the supplemental material) were particularly striking, revealing antibodies with strong avidity for these cells. Neither anti-human IgG-FITC nor anti-human IgM-FITC produced nonspecific binding to antigen slides containing RICs or NRICs. Nonspecific attachment of immunoglobulins from encephalitis patients to RICs and NRICs was frequently observed (Table 1), often presenting a staining pattern that targeted the cytoskeleton or specific organelles and was distinguishable from a specific rabies reaction.

**DISCUSSION**

We examined CSF and serum samples from encephalitic humans patients and identified a subset that presented a positive reaction in the indirect fluorescent-antibody test designed for the demonstration of antit rabies antibodies. When these positive samples were tested with the standard rabies virus neutralization test, the samples were negative for antirabies antibody. In most cases, an alternative etiological agent was identified by either PCR testing of CSF and/or identification of serum antibodies to other pathogens. The potential for false-positive results in a test designed to diagnose rabies in a human is disconcerting, as rabbits is noted to be an
invariably fatal disease. Additionally, if rabies is diagnosed in a patient, then it likely will initiate the events associated with the Milwaukee protocol (1).

The presence of cross-reactive antibodies induced by viral infections has been well documented (9–12). Srinivasappa et al. (9) demonstrated molecular mimicry when monoclonal antibodies developed against numerous viral pathogens reacted with normal tissues from mice. Antibodies directed against measles virus cross-reacted with cellular stress proteins of mammalian cells infected with heterologous viruses (10). Rabies virus-infected cell cultures could produce similar stress proteins that would be recognized by antibodies directed against a heterologous encephalitic agent. Solid-phase assays such as the IFA test measure all antibodies that bind to the antigen source. The antigen sources in the rabies IFA test are rabies virus-infected and noninfected cell cultures. The microscopist evaluating the fluorescence reaction pattern is tasked with discerning the proper staining pattern associated with a positive reaction due to attachment of antibody to rabies antigen in the infected cell culture. Specific staining patterns of RICs may show both intracytoplasmic inclusions containing rabies ribonucleoprotein and rabies virus surface antigen containing glycoprotein (13). The staining patterns for these two antigens may be differentiated by examining RICs stained with monoclonal antibodies directed against these two antigens (14). The recognition of staining patterns that are specific only to rabies infections should become problematic only when similar staining patterns are in-

![Image of IFA test results](https://journals.asm.org/journal/jcm)
TABLE 1 Evidence of antibody attachment in CSF samples examined with the indirect fluorescent-antibody test

<table>
<thead>
<tr>
<th>Causative agent of encephalitis</th>
<th>No. with antibody reactivity/no. tested (%)</th>
<th>RICs</th>
<th>NRICs</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Nile virus</td>
<td>20/30 (66.6)</td>
<td>18/30 (60)</td>
<td></td>
</tr>
<tr>
<td>Powassan virus</td>
<td>4/5 (80)</td>
<td>2/5 (40)</td>
<td></td>
</tr>
<tr>
<td>EBV</td>
<td>8/10 (80)</td>
<td>6/10 (60)</td>
<td></td>
</tr>
<tr>
<td>HSV-2</td>
<td>3/6 (50)</td>
<td>3/6 (50)</td>
<td></td>
</tr>
<tr>
<td>HHV-6 and enterovirus</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>Enterovirus</td>
<td>1/3 (33)</td>
<td>0/3 (0)</td>
<td></td>
</tr>
<tr>
<td>HHV-6</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>EBV and VZV</td>
<td>1/1 (100)</td>
<td>1/1 (100)</td>
<td></td>
</tr>
<tr>
<td>EEEV</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>45/78 (57.6)</td>
<td>35/78 (44.8)</td>
<td></td>
</tr>
</tbody>
</table>

a West Nile virus and Powassan virus cases were diagnosed by serology; all others were diagnosed by real-time PCR and real-time reverse transcription-PCR with CSF samples (6).

b Rabies virus-infected cells (RICs) and non-rabies virus-infected cells (NRICs) were examined by the rabies indirect fluorescent-antibody (IFA) testing procedure.

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R.J.R. and S.J.W. conceived and designed the experiments; R.J.R., K.A.A., and S.J.W. performed the experiments; and R.J.R. and S.J.W. wrote the paper.

REFERENCES