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.

Rapid antemortem rabies diagnosis in humans has been imperative for palliative patient care and for treatment of individuals potentially exposed to the patient. The Milwaukee protocol (1) was introduced as a potentially life-saving treatment for human rabies, and the sooner the protocol is initiated the greater the chances of success. This paradigm demands speed and accuracy from the rabies diagnostician. The test most likely to provide a quick rabies diagnosis is the direct fluorescent-antibody (DFA) test (see Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing [www.cdc.gov/rabies/pdf/RabiesDFASPv2.pdf]) performed on a nuchal skin biopsy specimen from the patient. However, since the results of this test may be negative in earlier stages of the disease, other procedures are relied upon and are carried out concurrently with the DFA test. The indirect fluorescent-antibody (IFA) test performed with cerebrospinal fluid (CSF) and serum specimens from rabies-suspect patients can yield results within a few hours. To perform an IFA test, serial dilutions of serum or CSF samples are placed on fixed, rabies virus-infected, cultured cells. If the serum or CSF contains antibodies to rabies, then these antibodies attach to rabies antigens present in the infected cell substrate. A fluorescein isothiocyanate (FITC)-labeled secondary antibody specific for human immunoglobulins is applied, and the slides are then examined by fluorescence microscopy. An experienced microscopist can recognize fluorescent staining patterns indicating the presence of an immune response to rabies virus.

The IFA test is a quick and sensitive procedure. However, the specificity of the assay has not been studied in detail. This study analyzed the specificity of the rabies IFA test through the examination of specimens from rabies-negative patients who presented with encephalitis of known or unknown origin. The results indicate that the specificity of the rabies IFA test is not 100%, and thus this test should not be the sole basis for initiating antirabies therapy.

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 was 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. IP0001) 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 rabies 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, six of herpes simplex virus 2 (HSV-2), and one of EBV plus varicella-zoster virus (VZV) 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 rabies 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 reaction neutralization tests (7). These serum samples were tested in the rabies IFA test at a screening dilution of 1:20. For the IgM 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 screening dilution of 1:20. Rabies-positive control serum was Sorb; Meridian Bioscience, Inc., Cincinnati, OH). Serum samples for the IgM assay were tested at a screening dilution of 1:20. Rabies-positive control serum was

**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. 1; 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 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.

**DISCUSSION**

We examined CSF and serum samples from encephalitic human patients and identified a subset that presented a positive reaction in the indirect fluorescent-antibody test designed for the demonstration of antirabies 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 IFA 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 rabies 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-

FIG 1  Indirect fluorescent-antibody test results for serum and CSF specimens from rabies virus- and non-rabies virus-infected patients with encephalitis. (A) Serum IgG (1:40) staining of RICs (BHK-21 cells) in a human rabies case, showing numerous punctate intracytoplasmic inclusions. Magnification, ×200. (B) IgG staining on RICs (neuroblastoma cells) with a 1:100 dilution of serum with a neutralizing antirabies titer, showing rabies-specific intracytoplasmic inclusions and coalescent fluorescence produced by membrane-associated rabies glycoprotein staining. Magnification, ×200. (C) IgG staining pattern on RICs (neuroblastoma cells) similar to that in panel B with neat CSF from an Epstein-Barr virus-infected patient (CSF-39). Magnification, ×400. (D and E) Neat CSF sample from a Powassan virus-infected patient (POW-7), exhibiting a strong reaction on RICs (D) and a weak reaction on NRICs (neuroblastoma cells) (E). Magnification, ×200.
CSF samples were diagnosed by real-time PCR and real-time reverse transcription-PCR with validation by interlaboratory proficiency testing. Such tests as the IFA test are used for the diagnosis of rabies and to determine the prevalence of cross-reactivity when solid-phase approaches to antirabies treatment. Further work is warranted to rabies in humans and the implementation of experimental approaches to antirabies treatment. However, this definition allows for the diagnosis of rabies in a human solely on the basis of the results of IFA testing with CSF and serum samples from encephalitic patients. Because the IFA test for rabies may result in false-positive results in human specimens, it should not be used as the sole basis for the diagnosis of rabies in humans and the implementation of experimental approaches to antirabies treatment. Further work is warranted to determine the prevalence of cross-reactivity when solid-phase assays such as the IFA test are used for the diagnosis of rabies and to validate any such test by interlaboratory proficiency testing.

### REFERENCES


