The antemortem diagnosis of rabies in humans employs techniques that require accuracy, speed and sensitivity. A combination of histochemical analysis, \textit{in vitro} virus isolation, immunologic methods and molecular amplification procedures are utilized in an effort 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 signs as possible.

The indirect fluorescent antibody (IFA) procedure on cerebral spinal fluid and serum provides a rapid result but the specificity of the assay has not been well studied. Because a false positive IFA could significantly affect patient treatment and outcome, it is critical to understand the specificity of this assay. In this study, IFA was performed on 135 cerebral spinal fluid and serum specimens taken from patients with viral encephalitis or a presumed viral infection from an agent...
Results indicate that false-positive results can occur when interpreting the rabies IFA procedure. A staining pattern morphologically similar to anti-rabies staining was observed in 7 of the 135 spinal fluids examined. In addition, a majority of the spinal fluids tested, from patients with encephalitis, presented immunoglobulin that bound to antigens present in 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 the IFA test for rabies on human specimens may result in false-positive results, it should not be used as the sole basis for initiating anti-rabies treatment.

Introduction

Rapid and accurate ante mortem rabies diagnosis in humans has been an imperative for palliative patient care and for treatment of individuals potentially exposed to the patient. Since the Milwaukee protocol (1) was introduced as a potential life-saving treatment for human rabies, the sooner the protocol is initiated the greater the chance 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 test (DFA) (2) performed on a nuchal skin biopsy from the patient. However, since this test may be negative in earlier stages of the disease, other procedures are relied upon and are carried out concurrently with the DFA. The indirect fluorescent antibody test (IFA) used on cerebral spinal fluid (CSF) and serum from the rabies suspect patient can yield results within a few hours. To perform an IFA, serial dilutions of serum or CSF are placed onto fixed rabies-infected cultured cells. If the serum or CSF contains antibodies to rabies, these antibodies will 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 will recognize fluorescent staining patterns indicating the presence of an immune response to rabies virus.

The IFA 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 by examination of specimens from rabies-negative patients who present with an encephalitis of known or unknown origin. Results indicate that the specificity of the rabies IFA is not 100%, and thus should not be the sole basis for initiating rabies therapy.

Materials and Methods

Cell Culture. BHK-21 cells (C-13) (ATTC CCL10) (American Type Culture Collection, Rockville, MD.) were used at passages 70 to 95. Mouse neuroblastoma cells (NA), (10) were used at passages 700 to 750. Both cell lines were cultured and maintained as previously reported (11). Viro inoculum. The ERA strain of rabies virus (12) was utilized as the rabies antigen source in the IFA procedure. The virus inoculum used to infect cells was obtained from a commercially available veterinary vaccine vial (13). Prior to use in the preparation of the IFA antigen slides the stock virus was passaged twice in BHK-21 cells using the media previously reported (11). At second passage cell confluency the flasks were placed at -80°C overnight. Cells were thawed to frozen slurry, agitated and refrozen at -80°C. Upon thawing lysed cell debris was removed by centrifugation at 1000G and aliquots were prepared from the supernatant for storage at -80°C.
Antigen slide preparation. Stored virus inoculum had previously been titrated to identify end-point values and infectivity profiles in both neuroblastoma and BHK-21 cell cultures. Virus inoculum was added to trypsinized cells at a MOI and cell-count suitable to produce 40 to 50% cell infection with three days growth at 34°C with 5% CO₂ atmosphere in a moist chamber incubator. Cells were grown on multi-well teflon coated slides (Cel-Line/Thermo Fisher Scientific, Cat no. 30-225H). After 3 days of cell growth medium was removed, cells washed once (2 min.) in phosphate buffered saline, 0.01M, pH 7.6 (PBS) 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 sera/CSF.

Anti-human IgG and IgM antibodies. Goat anti-human IgG-FITC (Catalog # IF0001) was obtained from Focus Diagnostics (Cypress, CA 90630). This secondary antibody is used directly from the vial, with no further dilutions or additions. Goat anti-human IgM(µ)- FITC was obtained from Kirkegaard & Perry Laboratories, (Gaithersburg, MD 20879), reconstituted as directed by the manufacturer, and then diluted 1:40 in .01M PBS, Ph 7.6 containing Evans Blue counterstain at 0.00125%.

Clinical Material. A total of 135 CSF samples from viral encephalitis patients were tested by the rabies IFA procedure. The sample set included ten cases of Epstein Barr virus(EBV), one eastern equine encephalitis(EEE), one human herpesvirus 6 (HHV6) , one dual HHV6+enterovirus , four enterovirus, six herpes simplex virus(HSV2), and one dual EBV/varicella zoster virus(VZV), all confirmed by real-time PCR on CSF (16). Thirty CSF samples
were from patients serologically diagnosed with West Nile infections and five CSF samples were serologically diagnosed as Powassan encephalitis cases. The IgG and IgM assays were performed on rabies infected murine neuroblastoma cells (RIC) and non-infected neuroblastoma cells (NRIC). On a smaller subset of specimens rabies infected and non-infected BHK-21 cells were employed to compare results with the neuroblastoma cell assay. The IgG IFA was performed on 135 spinal fluids and 17 sera. IgM IFA assay was performed on 115 of the spinal fluids. CSF samples were tested undiluted. Sera were available from two types of encephalitis cases: West Nile and Powassan which were tested as positive in cross-species plaque reduction neutralization tests(14). These sera were tested in the rabies IFA at a screening dilution of 1:20. For the IgM assay sera samples were IgG depleted by an initial 1:8 dilution in goat-anti human IgG (GullSorb, Meridian Bioscience, Inc., Cincinnati, OH 45244). Sera for IgM assay were tested at a 1:20 dilution. Rabies positive control sera was from a vaccine recipient with a titer of 2.0 IU and serum and CSF 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 1/32 or 1/64 serum dilution was used as a control for each IFA assay.

Indirect Fluorescent Antibody Assay. Fifteen µl of CSF or diluted sera was applied per well of multiwell antigen slides. All sera were tested on RIC and NRIC for comparison. Slides are incubated at 37°C for 30 min in a moist chamber. The non-bound antibody was eluted by a gentle wash with PBS (.01M, 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 µl of anti-human IgG was applied to each well and the slide incubated at 37°C for 30 min. After the second incubation 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 mounted with a mountant consisting of 0.05M Tris buffered 0.15 saline, pH 9.0 with 20% glycerol. IgM testing was performed as the IgG assay with the substitution of goat anti-human IgM-FITC conjugate. All slides were evaluated by RJR, and pertinent images were simultaneously viewed on a computer monitor by SJW for discussion and grading of the reaction intensity.

**Microscopy and Imaging.** Photomicroscopy was performed using a Zeiss AxioImager A1 microscope equipped for fluorescence microscopy. A Zeiss AxioCam MRe 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 a structural pattern of staining similar to staining pattern observed with known rabies positive cases were further examined using an in-vitro rabies virus neutralization assay. (15)

**Results**

**Staining patterns.** The attachment of antibodies, as reflected by the attachment of anti-human IgG FITC labeled conjugate, produced either structurally specific patterns or a generalized background staining. A staining pattern appearing similar to specific anti-rabies staining was observed in 7 of the 135 spinal fluids examined for IgG specific for rabies antigen. This staining pattern was present on RIC and absent on NRIC. The IFA procedure examining IgM antibodies identified 2 out of 115 spinal fluids with a rabies-like immunoreactive pattern.
Specific staining on RIC is identified as either intracytoplasmic inclusions consisting of rabies virus ribonucleoprotein or membrane associated rabies glycoprotein (Fig. 1A,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 RIC and NRIC identified patterns that ranged from antibody attachment seen only in rabies infected cells (Fig. 1 & S1-S5) to strong reactivity in both RIC and NRIC (Fig.S6 - S8). In certain clinical samples the morphology of the staining in the RIC, although strong, was atypical of a specific RIC staining morphology (Fig. S6, S9). Samples were also identified where there was a reduced reaction pattern in the NRIC when there was a very strong reaction in the RIC (Fig. S10). Of the 135 samples examined for IgG reactions, 70 (51.5%) reacted to RIC and 58 (42.6%) reacted to NRIC. Of the 115 samples examined for IgM reactions, 30 (26%) reacted to RIC and 28 (24%) reacted to NRIC. Evidence of reactivity to RIC and NRIC as compared to the etiologic agent responsible for the encephalitis is presented in Table 1. Reaction patterns with Powassan positive CSF in RIC and NRIC (Fig.S6) in BHK-21 cells were particularly striking, revealing antibodies with a strong avidity to these cells. Neither anti-human IgG-FITC or anti-human IgM-FITC produced non-specific binding to antigen slides containing RIC or NRIC. Non-specific attachment of immunoglobulins from encephalitic patients to RIC and NRIC was frequently observed(Table 1), often presenting a staining pattern that was targeting cytoskeleton or specific organelles and was discernable from a specific rabies reaction.

**Discussion**
We examined CSF and sera from encephalitic human patients and identified a subset that presented a positive reaction in the indirect fluorescent antibody test designed for the demonstration of anti-rabies antibody. When these positive samples were tested on the gold-standard rabies virus neutralization test the samples were negative for anti-rabies antibody. In most cases an alternate etiologic agent was identified by either PCR testing of the CSF and/or the 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 as an invariably fatal disease. Additionally, if rabies is diagnosed in a patient it likely will initiate the events associated with the Milwaukee protocol.

The presence of cross reactive antibodies induced by viral infections has been well documented (3-6). Srinivasappa et al (3) demonstrated molecular mimicry when monoclonal antibodies, developed against numerous viral pathogens, reacted with normal tissues from mice. Antibodies directed against measles virus cross react with cellular stress proteins of mammalian cells infected with heterologous viruses (4). Rabies infected cell culture could produce similar stress proteins that would be recognized by antibodies directed against a heterologous encephalitic agent. Solid phase assays such as the IFA measure any antibody that binds to the antigen source. The antigen sources in the rabies IFA are rabies infected and non-infected 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 RIC may show both intracytoplasmic inclusions containing rabies ribonucleoprotein and rabies virus surface antigen containing glycoprotein (7). The morphology of the staining pattern for these two antigens may be differentiated by examining RIC stained with monoclonal antibodies directed against these.
two antigens (8). The recognition of staining patterns that are specific only to rabies infections should become problematic only when there are morphologically similar staining patterns induced by sources other than a rabies infection. We present five instances (Fig. S1-S5) in which there was a positive reaction in RIC and the NRIC presented little to no reaction. In three of these cases (Fig. S1, S4 & S5) the staining pattern in the RIC could, in our opinion, be interpreted as similar to staining due to a rabies positive antibody source.

The 2011 case definition for the diagnosis of human rabies includes the identification of Lyssavirus specific antibody by indirect fluorescent antibody test or complete rabies virus neutralization at 1:5 dilution in the CSF (9). This case definition strongly recommends that laboratory confirmation is accompanied by a positive result with the other diagnostic techniques presently in use for antemortem human rabies diagnosis. This definition nevertheless allows for the diagnosis of rabies in a human based solely on the results of the IFA procedure on CSF and sera samples from encephalitic patients. Because the IFA test for rabies on human specimens may result in false-positive results, it should not be used as the sole basis for diagnosis of rabies in humans and implementation of experimental approaches to anti-rabies treatment. Further work is warranted to determine the prevalence of cross reactivity when employing solid phase assays such as the IFA for the diagnosis of rabies and to validate by inter-laboratory proficiency testing any such test.

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in the IFA test. We are grateful for the editing provided by Drs. Harry Taber, Ron Limberger and Kirsten St. George. The Photography and Illustrations core at the Wadsworth Center was instrumental in preparing the Table and Figures used in this work. We are indebted to the Viral Encephalitis Laboratory and Diagnostic Immunology Laboratories at the Wadsworth Center for the sharing of samples that made this study possible.

Author Contributions

RJR and SJW conceived and designed the experiments. RJR, SJW and KAA performed the experiments. RJR and SJW wrote the paper.

References


TABLE 1  Evidence of antibody attachment in CSF samples examined with the Indirect Fluorescent Antibody Test.

<table>
<thead>
<tr>
<th>Causative Agent of Encephalitis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Evidence of antibody reactivity on rabies infected and non-infected cell culture&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>RIC</strong></td>
</tr>
<tr>
<td>West Nile</td>
<td>20/30 (66.6%)</td>
</tr>
<tr>
<td>Powassan</td>
<td>4/5 (80%)</td>
</tr>
<tr>
<td>E BV</td>
<td>8/10 (80%)</td>
</tr>
<tr>
<td>HSV2</td>
<td>3/6 (50%)</td>
</tr>
<tr>
<td>HHV6 &amp; Entero</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>Entero</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>HHV6</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>E BV &amp; VZV</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>EEE</td>
<td>0/1 (0%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>45/78 (57.6%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>West Nile and Powassan cases diagnosed by serology, all others diagnosed by real-time PCR and real-time RT-PCR on CSF (16).

<sup>b</sup>Rabies infected cells (RIC) and non rabies infected cells (NRIC) were examined by the rabies indirect fluorescent antibody (IFA) procedure.
Fig. 1  Indirect fluorescent antibody results from serum and CSF of rabies and non-rabies infected encephalitis patients.

Serum IgG, 1:40 (human rabies case) staining of RIC (BHK-21 cells) showing numerous punctate intra-cytoplasmic inclusions, (A) 200X. IgG staining on RIC neuroblastoma cells (NA), 1:100 dilution of sera with a neutralizing anti-rabies titer showing rabies specific intracytoplasmic inclusions and coalescent fluorescence produced by membrane associated rabies glycoprotein staining (B) 200X. A IgG staining pattern similar to (B) from the neat CSF of an Epstein-Barr virus patient (CSF-39) on RIC (NA) (C) 400X. Neat CSF from Powassan virus infected patient (POW-7) exhibiting a strong reaction on RIC (D); weak reaction on NRIC (E), (NA) 200X.