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Rapid Diagnosis of LaCrosse Encephalitis: Detection of Specific Immunoglobulin M in Cerebrospinal Fluid

T. I. DYKERS,[1] K. L. BROWN,[2] C. B. GUNDERSEN,[2] AND B. J. BEATY[1]*

Department of Microbiology and Environmental Health, Colorado State University, Fort Collins, Colorado, 80523,[1] and Gundersen Clinic-LaCrosse Lutheran Hospital, LaCrosse, Wisconsin 54601[2]

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An immunoglobulin M (IgM) antibody capture enzyme immunoassay (MAC-EIA) was developed for the rapid and early diagnosis of LaCrosse (LAC) virus infections. The MAC-EIA was a sensitive and specific technique for the detection of IgM antibodies to LAC virus in cerebrospinal fluid specimens and in acute-phase serum specimens. In a retrospective study, cerebrospinal fluid and acute-phase serum paired samples from 108 patients were tested by the MAC-EIA and by an IgM immunofluorescence assay. The results were compared with the original diagnosis, which was made by using a variety of classical serological tests including serum neutralization, hemagglutination inhibition, and complement fixation. Thirty patients were confirmed as having LAC virus infections; of these, 30 (100%) were diagnosed as positive by serum MAC-EIA, and 27 (90%) were positive by cerebrospinal fluid MAC-EIA. The MAC-EIA was more sensitive than the IgM immunofluorescence assay. Two patients who were not previously confirmed as positive cases were diagnosed as having LAC virus infections by the MAC-EIA. One patient who was subsequently diagnosed as having a Jamestown Canyon virus infection and two patients who were previously infected with Jamestown Canyon virus were not falsely identified as having LAC virus infections by the MAC-EIA.

LaCrosse (LAC) virus remains a major cause of arboviral encephalitis in the United States (15). Rapid diagnosis of LAC virus infections is of value to physicians because of similarities in clinical presentation with other encephalitides (1). Rapid diagnosis of an LAC virus infection would preclude other unnecessary and perhaps invasive diagnostic procedures (for example, brain biopsy in suspected herpesvirus encephalitis) and would assist the physician in prescribing patient care (1, 7, 12).

Conventional diagnostic tests such as complement fixation, hemagglutination inhibition, and virus neutralization (NT) typically require acute- and convalescent-phase serum specimens to obtain a diagnosis (5). In addition, these tests usually are performed in centralized facilities such as state laboratories of hygiene, which further delays provision of diagnostic results to physicians.

Recent development of an immunoglobulin M (IgM) immunofluorescence assay (M-IF) and an IgM antibody capture enzyme immunoassay (MAC-EIA) (2, 3, 14) for the detection of specific IgM in acute-phase serum specimens provides rapid and clinically relevant diagnostic capability for LAC virus infections. However, even stronger serological evidence of etiology is provided by detection of specific IgM in cerebrospinal fluid (CSF). Since specific IgM does not normally cross the blood-brain barrier, the presence of specific IgM in CSF is indicative of virus replication in the central nervous system (CNS) (4, 8, 9, 13, 19). In terms of rapid diagnosis of CNS infections, examination of CSF offers other advantages. CSF is a less complex clinical specimen than serum, reducing problems with extraneous proteins, rheumatoid factor (6), and IgM specific for other agents which may not have caused CNS infection. Thus, CSF can be assayed in more concentrated form than serum. In addition, CSF specimens are frequently obtained immediately upon admission from patients with CNS dysfunction. In some instances, this may be up to 24 h before blood samples are obtained.

Because of these considerations, we investigated the potential of an MAC-EIA for diagnosis of LAC virus infections by examination of CSF specimens.

MATERIALS AND METHODS

Antigen preparation. The procedure for preparation of a whole virion antigen was similar to that previously published (10), with certain modifications suggested by Vance Vorndam (Centers for Disease Control, Fort Collins, Colo.). The procedure was as follows.

Monolayers of BHK-21 cells were infected with plaque-purified LAC virus (multiplicity of infection, 0.01). After adsorption for 1 h, unattached virus was washed from the flasks, maintenance medium was added, and the cells were allowed to incubate at 37°C. When approximately 75% of the infected cells demonstrated cytopathic effects, the supernatants [infected (+) and mock-infected (−) controls] were harvested. Both (+) and (−) antigens were treated identically throughout the following purification procedure.

The supernatants were centrifuged at 12,000 × *g* for 30 min at 4°C in a Beckman J2-21 centrifuge to remove the cellular debris. Proteins were then precipitated from the clarified supernatants by the addition of 10% polyethylene glycol (molecular weight, 6,000) and 2.3% NaCl with slow mixing for 3 to 4 h at 4°C. Precipitated proteins were pelleted in 250-ml polypropylene centrifuge bottles by centrifugation at 12,000 × *g* for 40 min. The precipitates were then suspended in several milliliters of STE buffer (0.145 M saline, 0.043 M Tris, 0.1 mM EDTA, pH 7.8) and were then stored at 4°C for 12 h to enhance suspension. The next day the suspensions were cleared of undissolved debris with low-speed centrifugation at 5,000 × *g* for 15 min. The supernatant was saved, and the pellet was suspended, lightly mixed on a vortex apparatus, and centrifuged again. This procedure was repeated to enhance virus recovery from the polyethylene glycol pellet. The supernatants were then

* Corresponding author.

pooled and further purified by pelleting through a 20% sucrose cushion by ultracentrifugation at 111,000 × *g* for 2 h. This pellet was then suspended in 1.0 ml of STE buffer and stored overnight at 4°C to enhance the suspension. The virus and control samples were then carefully layered onto 45% potassium tartrate–30% glycerol gradients. The samples were centrifuged at 82,500 × *g* for 4 h at 4°C. The resulting visible virus band and a "pseudoband" [(−) antigen sample from a tube location with a density corresponding to the position of the (+) virus band] were removed. The bands were then diluted to a concentration of less than 10% potassium tartrate and pelleted at 111,000 × *g* for 3 h at 4°C. The resulting pellets were suspended in approximately 0.5 ml of STE buffer, dispensed in 0.1- and 0.2-ml volumes, and stored at −70°C. The mean virus titer of the (+) antigens was 7.7 $\log_{10}$ 50% tissue culture infectious doses per ml; no infectious virus was detected in the (−) antigen. The protein concentration of the (+) antigen was estimated with a Bio-Rad protein assay to be 0.17 mg of protein per ml; the (−) antigen was 0.05 mg of protein per ml.

Antibody preparation. Antibodies to LAC virus were prepared by immunization of a rabbit. The rabbit was inoculated intramuscularly with a suspension of approximately 4.5 $\log_{10}$ 50% tissue culture infectious doses of stock (plaque-purified) LAC virus in 1.0 ml. On days 35 and 55 the rabbit was inoculated intramuscularly (1.0 ml) with approximately 5.5 $\log_{10}$ 50% tissue culture infectious doses of gradient-purified LAC virus. The rabbit was then bled 8 days later, and the serum was found to have an anti-LAC virus titer of ≥1:2,560 by indirect fluorescent antibody staining (2).

The antibodies were then separated from the serum by ammonium sulfate precipitation (17). After two successive 50% fractionations at 4°C, antibodies were suspended in phosphate-buffered saline (PBS) and dialyzed with PBS buffer; both precipitations were done at pH 7.3. The protein concentration of this suspension, as determined with the Bio-Rad protein assay, was approximately 15 mg/ml.

Test specimen selection. The paired acute serum and CSF specimens were selected from stored (−70°C) samples obtained from patients who had symptoms compatible with encephalitis at the Gundersen Clinic LaCrosse-Lutheran Hospital (LLH) during the arboviral seasons of 1979 through 1983. In general, both CSF and acute-phase serum specimens were obtained upon the day or within 1 day of admission. The samples ranged from 0 to 14 days after onset of symptoms, with a mean of 3.9 days. Exclusion of one sample obtained 14 days after onset of symptoms resulted in a mean of 3.4 days.

A total of 108 paired acute-phase serum and CSF samples were studied. Serum specimens from certain of these patients had previously been examined by the Immunology Section of LLH (by counterimmunoelectrophoresis and M-IF), the Virology Section of the Wisconsin State Laboratory of Hygiene (complement fixation and hemagglutination inhibition), or the Zoonosis Research Unit of the University of Wisconsin, Madison, Wis. (by NT); 28 of the patients were diagnosed as having had LAC virus infections on the basis of one or more of these tests (2). One patient had apparently been previously infected with LAC virus; there was no detectable IgM, nor were there changes in specific IgG titers in acute and convalescent specimens obtained during the current disease episode. Investigation revealed that the patient had been hospitalized 4 years previously and was serologically confirmed as having had an LAC virus infection at that time. One patient was diagnosed as having Jamestown Canyon (JC) virus infection because the serum specimens reacted to higher titer with JC virus than with LAC virus in the NT test. Of the remaining 78 patients with negative tests, 18 were confirmed as having enterovirus infections, and two had serological evidence of previous JC virus infections. Of the remaining 58 patients with negative tests, 5 had little or no patient history or test results available. Typically, these latter patients were discharged from the hospital within 1 or 2 days of admission and did not return for further treatment. Thus, convalescent-phase serum specimens were not available for analysis.

MAC-EIA. (i) Optimizing test conditions. Optimal working dilutions of all reagents were determined separately by checkerboard titration. Incubation times and temperatures were similar to those determined previously (14). To minimize nonspecific reactions, appropriate blocking agents were determined by trial and error. Immulon II 96-well flat-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were used throughout. Only the inner 60 wells of the plates were used to minimize inconsistent results often detected in the perimeter wells of a microtiter plate. Unless otherwise stated, all well volumes were 100 μl, and washes consisted of five replicates of approximately 200 μl of 0.1 M PBS (pH 7.3)–0.05% Tween 20 (PBS-TW20).

(ii) Test protocol. The microtiter plates were coated with goat anti-human IgM (Hyclone, Logan, Utah) at least 1 day before (at most 7 days before) the day of testing. This was accomplished by using a 1:100 dilution of the anti-human IgM antibody in carbonate buffer (pH 9.6). The plates were incubated at 37°C for 2 h and then stored at 4°C until the day of the test.

On the day of the test, plates were warmed, washed five times with PBS, and then blocked for 30 min with 5% normal goat serum diluted in PBS–TW20. After washing, patient specimens (CSF, 1:10; serum, 1:100) were diluted in diluting buffer (PBS, 0.1% gelatin, 0.05% TW20, 1% normal goat serum). Specimens were added to the plates in replicates of six and incubated for 2 h at 37°C. After another wash, the (+) and (−) antigens were diluted 1:300 in PBS-TW20 and added in replicates of four and two, respectively, to each set of specimen wells. The plates were then incubated for 1 h at 37°C and washed before the addition of the detector antibody. Rabbit anti-LAC virus detector antibody was diluted 1:2,000 in diluting buffer (PBS, 0.1% gelatin, 0.05% TW20, 5% normal goat serum) and added to each of the 60 wells. The antibody was incubated for 1 h at 37°C, and then the plates were thoroughly washed. The indicator antibody, goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.), was diluted in diluting buffer (PBS, 0.1% gelatin, 0.05% TW20, 1% normal goat serum) and was added to each well. The plates were incubated for 1 h at 37°C, washed with Tris buffer (pH 9.0), and incubated with the substrate *p*-nitrophenyl phosphate (2 mg/ml) in substrate buffer for 30 to 40 min. The color development was stopped by the addition of 3 N NaOH (50 μl per well), and the plates were read immediately for absorbancy at 410 nm with a Dynatech Multiskan Plate Reader (model MR600).

(iii) Diagnostic criteria. Each patient specimen was tested in six wells of the microtiter plate; four wells contained the LAC virus (+) antigen, and two wells contained the (−) antigen. The specific activity of each test specimen was calculated as the mean absorbance of (+) antigen wells minus the mean absorbance of (−) antigen wells. The negative cutoff value or diagnostic criterion for both serum and CSF was established by testing 10 pairs of serum and CSF which were known to be seronegative. The mean of the

10 samples plus three standard deviations was the value used as the highest possible specific activity for a negative sample [mean (−) specific activity + 3 standard deviations = the diagnostic cutoff line]. Specific activities above this value indicated that the patient specimen contained IgM antibodies to LAC virus. Different diagnostic (cutoff) criteria were determined for sera (0.045) and CSFs (0.056). All 108 pairs of CSF and serum were tested within 2 days to permit the use of the same criterion for all samples tested. In addition, known positive and negative samples were included on each plate as internal controls.

M-IF. Each specimen pair was examined for specific IgM by M-IF and MAC-EIA. The two tests of a given sample were conducted on the same day at Colorado State University. In addition, many specimens had been examined by M-IF upon admission of the patient at LLH.

Methods for preparation of spot slides and test protocols for the M-IF were detailed previously (2). In brief, LAC virus-infected cells were affixed to 8 spots of 12-spot microscope slides. Uninfected cells were affixed to the other 4 spots as controls.

Sera (diluted 1:10) and CSF (diluted 1:2) in PBS were then applied to two of the spots containing LAC virus-infected cells and one spot containing noninfected cells. After incubation for 40 min at 37°C, the slides were washed twice in PBS for 10 min, rinsed in distilled H_2O, and air dried. Goat anti-human IgM antibody conjugated with fluorescein isothiocyanate was diluted 1:100 in 0.1% Evans blue (Cappel Laboratories, Cochranville, Pa.) in PBS and was applied to each spot. Slides were incubated for an additional 40 min, and the washes were repeated. The slides were then dried, and cover slips were mounted with PBS-glycerol (1:1). Slides were stored at 4°C until examined with an Olympus BH-2 epifluorescence microscope. Cells were examined at 200× or 400× for specific fluorescence with blue light excitation. The specimens were scored as either positive, weakly positive, +/− (equivocal), or negative when compared with the level of fluorescence seen in positive and negative control spots.

Sensitivity and specificity of MAC-EIA. The sensitivity of the MAC-EIA was compared with the diagnostic results from LLH, the Virology Section of the State Laboratory of Hygiene, and the Zoonoses Research Unit, as described above. The diagnostic efficacy of the MAC-EIA and M-IF tests using serum and CSF specimens was compared. In addition, the sensitivity and specificity of MAC-EIA diagnosis of LAC infections by examination of CSF were determined by using the results of MAC-EIA examination of serum as the referent standard (11). The false-negative rate was calculated as 1 minus sensitivity; the false-positive rate was calculated as 1 minus specificity.

RESULTS

Detection of LAC virus IgM in serum. Of the 108 patient serum specimens tested, 28 were from patients who had been serologically confirmed as having LAC virus infection. All 28 specimens were positive by the MAC-EIA. In addition, the MAC-EIA detected anti-LAC IgM antibodies in the serum specimens of two patients whose cases had not been serologically confirmed previously. Both of these patients had been scored as questionable in the M-IF test conducted upon admission at LLH. Since both were discharged within several days after admission, convalescent sera were not obtained, and samples had not been sent to the State Laboratory of Hygiene for testing by hemagglutination inhibition or NT. Thus neither patient had been confirmed as having an LAC virus infection. These two specimens were subsequently demonstrated to contain NT antibody to LAC virus and were thus considered to be confirmed cases for these analyses. Therefore, of the 108 patient CSF and serum specimen pairs, 30 were considered to be from patients with confirmed LAC virus infections.

TABLE 1. Comparison of the MAC-EIA and M-IF for the clinically relevant diagnosis of LAC virus infections

Samples	No. positive/total[a] (% positive)		
	MAC-EIA	M-IF	
		LLH[b]	CSU[c]
Sera	30/30 (100)	21/30 (70)	17/30 (57)
CSF	27/30 (90)	3/11 (27)	3/30 (10)

[a] Number of specimens scored as positive by either MAC-EIA or M-IF/number of samples examined from serologically confirmed cases of LAC virus infection.
[b] M-IF test conducted upon presentation at LLH.
[c] M-IF test conducted in parallel with MAC-EIA at Colorado State University (CSU).

The serum specimen from the patient who experienced an LAC virus infection 4 years previously was negative by MAC-EIA. One patient, who was diagnosed as having a JC virus infection by NT, was tested twice in the LAC MAC-EIA; negative results were obtained on both occasions. There were two additional patients in the study who had detectable serum NT antibody to JC virus; both were negative in the LAC virus MAC-EIA.

The MAC-EIA detected IgM antibody to LAC virus in the acute-phase serum specimen of 100% (30 of 30) of the confirmed LAC virus infections (Table 1). When the same serum specimens were examined by M-IF upon presentation at LLH, 70% (21 of 30) had contained detectable anti-LAC virus IgM (Table 1). In contrast, when the M-IF test was conducted at Colorado State University up to several years postinfection 57% (17 of 30) were positive. Apparently long-term storage, manipulation of samples, or other variables resulted in less efficient detection of IgM by M-IF. Regardless, the MAC-EIA was more sensitive than the M-IF for detecting IgM in serum specimens.

Detection of LAC virus IgM in CSF. The MAC-EIA detected specific IgM in 90% (27 of 30) of the CSF specimens from confirmed LAC virus infections (Table 1). The two patients who had not been confirmed as having LAC virus infections because convalescent-phase specimens were not obtained had anti-LAC virus IgM in the CSF. Only a few CSF samples had been tested at LLH by M-IF upon presentation of the patient; thus results cannot be directly compared. However, of the 11 positive patients whose CSF specimens were examined by M-IF upon admission, only 27% (3 of 11) were diagnosed as having LAC virus infections (Table 1). The test result for one CSF specimen was considered to be equivocal. Only 10% (3 of 30) of the CSF samples were positive when tested at Colorado State University; the results for two samples were considered equivocal. The MAC-EIA (90%) was clearly more sensitive than the M-IF for detection of specific IgM in CSF.

Comparison of IgM detection in CSF and serum for the diagnosis of LAC virus infections. The sensitivity and specificity of IgM detection in CSF and serum by MAC-EIA were calculated by using the results of the serum MAC-EIA as the referent standard (Table 2); 30 specimens were positive by serum MAC-EIA, and 27 were positive by CSF MAC-EIA.

TABLE 2. Comparison of IgM detection in CSF and serum by MAC-EIA for the diagnosis of LAC virus infections[a]

CSF MAC-EIA results	Serum MAC-EIA results		
	Positive	Negative	Total
Positive	27	0	27
Negative	3	30	33
Total	30	30	60

[a] Sensitivity, 0.90; specificity, 1.0; false-negative, 0.10; false-positive, 0.0.

The sensitivity rate of the CSF MAC-EIA was 0.90; the specificity rate was 1.0 (Table 2).

DISCUSSION

The sensitivity of the MAC-EIA for diagnosis of LAC virus infections in these specimens was surprising; 100% (30 of 30) of the patients with confirmed LAC infections were diagnosed with the acute-phase serum specimen. In a previous study, a similar MAC-EIA diagnosed 83% (24 of 29) of the cases with the acute phase specimens (14). Whether the increased sensitivity is due to fortuitous selection of patient specimens or to alterations in the MAC-EIA protocol has not been addressed. In regard to the latter possibility, major differences in the EIA test protocol used herein were removal of the *N*-acetylcysteine treatment and the use of normal goat serum instead of bovine serum in the blocking step and in certain diluents. The use of goat serum (the anti-human IgM was prepared in goats) proved most effective in reducing background noise (data not shown).

The MAC-EIA proved to be a sensitive technique for detection of IgM in CSF; IgM was detected in 27 of 30 (90%) confirmed positive cases. In contrast IgM was only detected in 3 of 30 (10%) samples by M-IF even though a fivefold more concentrated sample was examined. A number of the patients had detectable IgM in the serum and the CSF on the day of presentation (data not shown).

In general, CSF results were concordant (27 of 30) with those obtained from serum samples. Thus anti-LAC IgM is detectable in the CSF of most children who are hospitalized as a consequence of infection with LAC virus. Of the three discordant CSF specimens, two were scored as equivocal, and one was scored as negative. The patients with equivocal cases did not have unusual case histories, and in the absence of other serological information they would not have been diagnosed. However, both had detectable IgM in their serum specimens; thus these patients were diagnosed as having LAC virus infections. If the diagnostic criteria had been less stringent (i.e., the mean plus two standard deviations), both would have been diagnosed as positive. In the third case, the serum MAC-EIA was scored as a weak positive, and the CSF MAC-EIA was negative.

JC virus, a California group virus related to LAC virus, has recently been recognized to cause significant morbidity in humans (18). Three patients examined in these studies had antibodies to JC virus. In one case, there was a demonstrable increase in NT antibody titer to JC virus between acute and convalescent serum specimens. In the other two cases, NT antibody titers did not change, suggesting that these two patients had been infected with JC virus before the current disease episode. None of these three cases was falsely identified as LAC virus infection by the MAC-EIA. Of course, the previous infections would be unlikely to be diagnosed.

In approximately 30% (11 of 30) of the cases, the IgM specific activity in the CSF was somewhat greater than that of the respective serum (data not shown). This phenomenon has been noted in the diagnosis of other encephalitides (4, 9). Detection of LAC virus-specific CSF IgM may be aided by the reduced competition of other nonspecific IgM molecules for the capture antibody. A CSF specimen would be less likely than a serum specimen to contain a wide variety of IgM antibodies. Quantitative information may also be provided by MAC-EIA. For example, MAC-EIA endpoint titers for anti-St. Louis encephalitis virus IgM correlated strongly with absorbance values obtained from 1:100 dilutions of patient serum (16). If a similar correlation can be demonstrated for LAC IgM, then the attending physician could be provided with both qualitative and quantitative information on the nature or extent of the immune response based upon one acute-phase specimen.

Testing of CSF samples by MAC-EIA proved to be a promising method for diagnosing LAC virus infections. In most CSF and serum sample pairs, IgM test results were in agreement (Table 2). CSF samples are presumably not as prone to nonspecific reactions (including rheumatoid factor and other immunologic cross-reactions) and are frequently obtained upon presentation of patients with CNS dysfunction. Thus, detection of IgM in CSF may be a valuable addition to the techniques for diagnosis of LAC virus infections.

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