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Serodiagnosis of La Crosse Virus Infections in Humans by Detection of Immunoglobulin M Class Antibodies

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Sera from 92 humans with illnesses clinically compatible with those caused by California serogroup virus infections were tested for antibody to La Crosse (LAC) virus by using the immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent assay (MAC ELISA), the IgG ELISA, and the hemagglutination inhibition (HI), complement fixation and serum dilution-plaque reduction neutralization tests. On the reported day of onset of illness in 18 individuals, 94% had IgM antibody, 50% had neutralization antibody, 33% had HI antibody, and 11% had IgG antibody. Neutralization, HI, and IgG antibody prevalence rates increased thereafter, whereas IgM antibody prevalence remained high (92% 2 or more weeks after the onset of illness). It was concluded that the MAC ELISA is a sensitive test for the presence of antibody to LAC virus. The sensitivity of the MAC ELISA and the rapidity with which it can be performed appear to provide a powerful tool for the clinically relevant serodiagnosis of LAC virus infections in humans.

La Crosse (LAC) virus, a member of the California serogroup, family *Bunyaviridae*, causes more pediatric encephalitis than does any other arbovirus in the United States (2). Human infections caused by LAC virus have been documented in 24 states, most of them east of or contiguous with the Mississippi River (2). At present, most state and local health agencies use insensitive or otherwise inadequate methods for the serodiagnosis of LAC virus infections (3). Others do not test for the presence of antibodies to this or other California serogroup viruses, including the related snowshoe hare and Jamestown Canyon (JC) viruses which have been shown to cause encephalitis in humans (8, 9). When the mild-to-severe clinical picture with frequent and severe sequelae is considered (10), gross underreporting and overtreatment of LAC virus infections are possible. Specific serodiagnosis of LAC virus infections is required to (i) prevent unnecessary and invasive diagnostic procedures, (ii) provide information necessary for judicious treatment, (iii) provide data regarding indicator cases in potential epidemic situations, and (iv) provide background information for understanding subsequent seizure disorders (7, 10).

In an attempt to provide an antibody assay that could be performed rapidly enough to have an impact on the treatment of patients with LAC virus infections, Jamnback et al. (11) and Beaty et al. (1), testing a limited number of specimens, compared the sensitivities of enzyme immunoassays for immunoglobulin M (IgM) antibody with those of hemagglutination inhibition (HI), complement fixation (CF), neutralization, counterimmunoelectrophoresis, and indirect fluorescent antibody tests. We attempted to confirm and extend their results by including IgG antibody assays. The results of our tests suggest that the IgM antibody-capture enzyme-linked immunosorbent assay (MAC ELISA), as performed, is a highly sensitive tool for use in the rapid diagnosis of California serogroup virus infections, including those caused by LAC virus.

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MATERIALS AND METHODS

Sera. Serum specimens were obtained from patients with illnesses clinically diagnosed as California encephalitis, encephalitis, aseptic meningitis, central nervous system infection, or fever of unknown origin. Paired serum samples were collected by personnel of the Ohio Department of Health from people in Ohio or were submitted for serodiagnostic testing to the Centers for Disease Control, Fort Collins, Colo., from persons in Ohio, Indiana, North Carolina, Mississippi, Tennessee, Arkansas, Missouri, Maryland, Georgia, Oklahoma, Michigan, and West Virginia. Of the 92 individuals studied, 62 were males with an age range of <1 to 14 years (mean, 8.3 years), 27 were females with an age range of <1 to 12 years (mean, 6.3 years), and 3 were males of unknown age.

All sera had been tested previously with LAC virus by one or more of the following tests: HI, CF, serum dilution-plaque reduction neutralization, or a serum dilution neutralization test using as an indicator inhibition of cytopathic effects in Vero cell tube cultures. Many of these sera had also been tested for heterologous antibody to the California serogroup viruses snowshoe hare, JC, Trivittatus, and Keystone by the accepted method of determining the etiologic agent in infections caused by California serogroup viruses, the neutralization test, using a comprehensive battery of member serotypes (5). The sera had been stored at −20°C for as long as 6 years before being tested. Twenty sera from children with illnesses similar to those of the test patients but who did not have HI antibody to LAC virus were included as controls.

Antigens. For preliminary testing, standardization of reagents, and screening sera, we used continuous sucrose gradient-purified LAC virus, prototype strain (4). In later tests, we used a pretitrated dilution of supernatant fluid from cultures of Vero cells infected with LAC (prototype strain) virus. Supernatant fluid from cultures of uninfected Vero cells was included as a negative control.

MAC ELISA. The procedures used in the MAC ELISA

TABLE 1. Comparative tests for antibody to LAC virus and neutralization tests for antibody to four other California serogroup viruses in 10 individuals with fourfold and greater HI titer rises between paired acute- and convalescent-phase serum samples

Patient no.	Day after onset of illness	LAC virus					Titer of N antibody to[a]:			
		Antibody titer			ELISA OD ratio[b]					
		HI	CF	N	MAC	IgG	SSH	JC	TVT	KEY
1	2	10	<8	<10	4.23	1.45	<10	<10	<10	<10
	9	80	8	640[c]	3.78	1.72	640	80	80	80
2	0	20	<8	320	3.66	1.74	640	20	40	<10
	20	640	8	640	3.50	3.02	640	640	640	20
3	1	40	<8	640	3.02	1.92	320	40	20	80
	15	320	<8	640	2.29	1.68	640	320	160	320
4	0	<10	<8	40	2.67	1.08	<10	<10	<10	<10
	26	40	8	640	4.01	2.39	640	160	80	40
5	6	20	<8	640	3.36	1.58	640	NT[d]	NT	NT
	17	640	32	640	3.48	3.07	640	NT	NT	NT
6	5	<10	<8	<10	2.49	1.22	20	<10	<10	<10
	30	320	16	640	2.92	3.09	640	640	320	640
7	3	40	<8	<10	2.89	1.11	40	<10	<10	<10
	21	5,120	32	640	2.66	2.70	640	320	80	20
8	14	10	<8	640	2.37	1.61	640	20	80	<10
	32	40	128	640	3.44	2.53	640	320	320	320
9	0	<10	<8	<10	4.24	1.88	<10	<10	NT	<10
	23	80	64	640	3.66	2.38	640	320	NT	80
10	0	10	<8	640	2.99	1.29	320	160	NT	<10
	18	80	16	640	2.52	2.30	640	640	NT	160

[a] SSH, Snowshoe hare virus; JC, Jamestown Canyon virus; TVT, Trivittatus virus; KEY, Keystone virus; N, serum dilution-plaque reduction neutralization.
[b] Ratio of OD of test serum with antigen to that of serum without antigen; ≥2.0 was considered positive.
[c] 640 indicates ≥640.
[d] NT, Not tested.

were essentially those reported previously for alphaviruses (4). Briefly, flat-bottomed polystyrene plates were coated with goat IgG anti-human IgM antibody diluted in carbonate-bicarbonate buffer (pH 9.6). Patient serum (a 1:100 dilution), antigen, hyperimmune mouse ascitic fluid, horseradish peroxidase-conjugated goat IgG anti-mouse IgG, and substrate were then added sequentially. The diluent for all but the coating (capture antibody) step was phosphate-buffered saline with 5% fetal bovine serum (pH 7.4). Diluted patient serum, antigen, detecting (secondary) antibody, and conjugated antibody were added in 50-μl volumes and incubated for 1 h at 37°C. Coating antibody and substrate were added in 75-μl volumes and incubated overnight and for 10 to 20 min, respectively, at room temperature. After the addition and incubation of all reagents except the substrate, the plates were washed mechanically 10 times with phosphate-buffered saline containing 0.05% Tween 20. Optical densities (OD) of individual wells were read mechanically at 405 nm and recorded by the same instrument. Controls included the assay without the patient serum, without antigen, and a plate with substrate only, for determination of the background reading owing to the plate itself. When the OD of the test serum was ≥0.300 and that of the serum without antigen was ≤0.150, results were considered acceptable. Ratios of the OD of test serum with antigen to that of serum without antigen of ≥2.0 were considered positive. Sera considered positive in preliminary MAC ELISA screenings were titrated at fourfold dilutions. Positive controls included sera from individuals with documented LAC or JC virus infections, as determined by both HI and neutralization tests. Sera from JC virus-infected individuals were generously provided by R. Deibel, New York State Department of Health, Albany, and sera from snowshoe hare virus-infected individuals were provided by H. Artsob, University of Toronto, Toronto, Ontario, Canada.

IgG ELISA. For the IgG ELISA, individual wells were coated with 500 ng of purified virus in 75 μl of carbonate-bicarbonate buffer and incubated overnight at room temperature. Then, 50 μl of patient serum diluted 1:100, 50 μl of horseradish peroxidase-conjugated goat IgG anti-human IgG, and 75 μl of substrate were added sequentially to each well with 10 washes, as described above, after the addition of each reagent. Plates were read, recorded, and interpreted as described above for IgM.

RESULTS

All individuals for whom serologic results are presented had illnesses compatible with California serogroup virus infections. None of the 20 HI-negative controls had IgM antibody to LAC virus.

The results of comparative tests with paired serum samples from 10 of 42 persons with fourfold and greater HI antibody titer changes between acute- and convalescent-phase serum samples are given in Table 1. HI antibody titer

changes of fourfold and greater were always accompanied by either fourfold or greater changes (usually rises) in neutralization titers or high, stable neutralization titers; CF antibody was either undetectable, minimally positive, or relatively low, with one or two exceptions. IgM antibody was detected in 87 (99%) and IgG antibody was detected in 36 (41%) of 88 sera from the 42 patients. An additional 38 sera from 19 individuals with fourfold and greater HI titer changes were tested for IgM antibody but not IgG antibody. Thirty-six (95%) of these sera and all 19 individuals had IgM antibody.

Another group of 27 individuals with stable (twofold or lower) changes in HI titers between acute- and convalescent-phase serum samples or with no HI antibody were tested by CF and neutralization for antibody to LAC virus and for IgM and IgG antibodies to LAC virus. The results of tests with sera from 10 of these patients are presented in Table 2. As with sera from patients with fourfold and greater HI titer changes, CF antibody in these sera was undetectable, minimal, or in the rare exception, positive at diagnostically useful levels. Neutralizing antibody titer rises were detected between acute- and convalescent-phase serum samples, but more often the titers were at high, stable levels. IgM antibody was detected in 50 of 54 (93%) specimens and in at least 1 specimen from 26 of 27 (96%) individuals.

Single serum specimens from four other individuals with HI or CF antibody titer rises were tested for IgM and IgG antibodies. All had IgM antibody, whereas none had IgG antibody.

TABLE 2. Comparative tests for antibody to LAC virus in 10 individuals with twofold or lower HI titer changes between paired acute- and convalescent-phase serum samples

Patient no.	Day after onset of illness	Antibody titer			ELISA OD ratio[a]	
		HI	CF	N[b]	MAC	IgG
11	0	20	<8	640[c]	2.92	1.62
	14	40	<8	640	3.23	2.33
12	0	<10	<8	<10	2.74	1.39
	10	20	<8	320	3.38	2.03
13	4	160	<8	320	2.74	1.49
	39	320	<8	640	3.07	2.20
14	5	80	<8	640	2.85	1.33
	19	40	<8	640	2.97	2.26
15	0	<10	<8	<10	1.39	1.01
	13	20	<8	640	2.08	1.69
16	0	80	8	640	3.06	2.54
	14	160	8	640	2.18	2.44
17	8	<10	<8	<10	2.50	1.11
	104	<10	<8	640	4.11	2.12
18	14	40	<8	160	2.16	1.03
	58	40	16	320	3.53	2.38
19	17	2,560	8	640	2.23	2.11
	39	1,280	64	640	3.15	2.68
20	31	40	<8	640	1.17	1.19
	37	20	<8	640	1.13	1.13

[a] Ratio of OD of test serum with antigen to that of serum without antigen; ≥2.0 was considered positive.
[b] N, Serum dilution-plaque reduction neutralization.
[c] 640 indicates ≥640.

TABLE 3. Relationships among HI, neutralizing, IgM, and IgG antibodies to LAC virus at different times after reported onset of illness[a]

Day of illness	Presence or absence of antibody	No. of sera with antibody:		
		IgM+ IgG+	IgM+ IgG−	IgM− IgG−
0–7	NI+ N+	4	9	0
	HI+ N−	1	4	0
	HI− N+	0	6	0
	HI− N−	0	11	1
8–14	HI+ N+	4	5	0
	HI+ N−	0	0	0
	HI− N+	0	0	0
	HI− N−	0	0	0
15–120	HI+ N+	23	7	3
	HI+ N−	0	0	0
	HI− N+	2	1	0
	HI− N−	0	0	0

[a] N, Serum dilution-plaque reduction neutralization; +, antibody present; −, antibody absent.

The relationships among HI, neutralizing, IgM, and IgG antibodies at different times after the reported onset of illness are shown in Table 3. None of the 81 sera examined in this study was IgG antibody positive and IgM antibody negative, irrespective of the presence or absence of HI, CF, and neutralizing antibodies. Within week 1 after onset, 35 of 36 (97%) sera had detectable levels of IgM antibody, 19 (53%) had neutralizing antibody, 18 (50%) had HI antibody, and 5 (14%) had IgG antibody. Only nine sera were available from individuals bled 8 to 14 days after onset; all had IgM, HI, and neutralizing antibodies; four (44%) had IgG antibody.

Of 36 sera from individuals bled more than 2 weeks after the onset of illness, all had neutralizing antibody, 33 (92%) had IgM antibody, 33 (92%) had HI antibody, and 35 (69%) had IgG antibody. Overall, 77 of 81 (95%) had IgM antibody, 64 (79%) had neutralizing antibody, 60 (74%) had HI antibody, and 34 (42%) had IgG antibody.

Of sera collected from 18 individuals on the reported day of onset of illness, 17 (94%) had IgM antibody, 9 (50%) had neutralizing antibody, 6 (33%) had HI antibody, and 2 (11%) had IgG antibody.

We noted no correlation of IgM, IgG, HI, CF, or neutralizing antibody presence or titer with the severity of illness, the available clinical description of the illness, age, or sex.

Cerebrospinal fluids from two individuals were tested by the MAC ELISA. Fluid was collected from one individual 162 days after the onset of illness confirmed to be caused by LAC virus; there were HI and neutralizing antibodies but no IgM antibody in serum collected at the same time, and there was no IgM antibody in the cerebrospinal fluid. Fluid was collected from the other individual 7 days after the onset of illness. This patient had neither HI nor neutralizing antibody in a serum sample collected at the same time but did have IgM antibody in both the serum sample and the cerebrospinal fluid.

DISCUSSION

IgM antibody was detected in 177 of 184 (96%) serum specimens and in 91 of 92 (99%) patients with LAC virus

infections serologically confirmed by one or more tests (HI, CF, or neutralization) or identified as presumptive recent infections by these tests. These results are indicative of the sensitivity of the MAC ELISA in tests for antibody to LAC virus. Although reported in Tables 1 and 2 as OD ratios, all MAC ELISA-positive sera were titrated. Irrespective of day of illness, titers ranged from 500 to >100,000 (data not shown). The heightened sensitivities of the tests described here, compared with those reported by Jamnback et al. (11), may be attributable to differences in the techniques used. Although Jamnback et al. used essentially the same methods and reagents for capturing antibody, wash solution, and diluting serum, the antigens we used, particularly in the final tests, were diluted supernatant fluids from infected Vero cell cultures. In addition, we used anti-LAC virus hyperimmune mouse ascitic fluid and horseradish peroxidase-conjugated anti-mouse IgG as secondary antibody, whereas Jamnback et al. used a more direct alkaline phosphatase-conjugated anti-LAC virus hyperimmune mouse ascitic fluid. Although our technique requires additional washings and incubation and more frequent addition of reagents, we found that the higher the dilutions of reagents that can be used (in these tests secondary antibody was used at a dilution of 1:10,000, and conjugated antibody was used at a dilution of 1:5,000), the lower the background readings will be. In addition, there is an economy of valuable reagents and no loss in sensitivity. We found all of this to be dependent on calibration of the system by using purified virus for preliminary standardizations. The use of enzyme-conjugated monoclonal antibodies of high titer may, in the near future, provide us with a method that is even more rapid and specific.

Because of the rapidity with which this test can be performed, it is possible to detect IgM antibody in individuals in prodromal or premorbid states during epidemics. This could be of particular value when antiviral compounds become available. However, because IgM antibody appears to persist for 2 to 3 months or longer after infection, it cannot be determined with certainty that the presence of IgM antibody to LAC virus in single serum samples from individuals in an endemic area is indicative of recent infection with that virus. Thus, serologic surveys for the determination of antibody prevalence should continue to be done with the neutralization or HI test.

For primary serodiagnosis of LAC virus infections in humans, the MAC ELISA can be used to replace the more conventional HI and neutralization tests because it is rapid, sensitive, and less expensive to perform than the neutralization test. Application of IgG assays to routine serodiagnosis and detection of antibody to LAC virus for other purposes does not appear to have advantages over the HI and neutralization tests. It would be more appropriate for state, local, and other public health agencies to use LAC and JC virus hemagglutinins for HI tests in primary serodiagnosis than to use CF, the least sensitive of the tests compared in this study and previously (3). The CF test should be excluded as a routine serodiagnostic procedure for detecting infections caused by California serogroup viruses.

IgM antibody appears to be complex- but not type-specific in humans infected with alphaviruses (4) and flaviviruses (13). Because LAC and JC viruses belong to separate antigenic complexes within the California serogroup (2), we can only assume that the MAC ELISA is sufficiently specific to determine the infecting serotype, either by type-specific reactivity or by comparison of relative titers; this remains to be proven. Serodiagnostic problems of IgM antibody cross-reactivity can be minimized. First, the MAC ELISA would likely detect IgM antibody to either LAC or JC virus; the exquisite sensitivity of this test is recommendation enough for its use in routine serology. Second, an as yet unpublished serologic study by H. Artsob, P. Grimstad, and C. Calisher, with hemagglutinins for LAC and JC viruses prepared by a modification of the method of Chappell et al. (6), demonstrated the usefulness of these antigens in distinguishing between human infections caused by the two viruses. One solution to the problem of choosing between a less sensitive system (HI), a sensitive and specific but relatively slow system (neutralization), and a highly sensitive system with unknown specificity (MAC ELISA) may be to use the MAC ELISA for primary serodiagnosis, with HI and neutralization reserved for confirmatory tests. The availability of type-specific antigens for use in the MAC ELISA would likely eliminate these complications.

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LITERATURE CITED

1. **Beaty, B. J., T. L. Jamnback, S. W. Hildreth, and K. L. Brown.** 1983. Rapid diagnosis of La Crosse virus infections: evaluation of serologic and antigen detection techniques for the clinically relevant diagnosis of La Crosse encephalitis. Prog. Clin. Biol. Res. **123**:293–302.
2. **Calisher, C. H.** 1983. Taxonomy, classification, and geographic distribution of California serogroup bunyaviruses. Prog. Clin. Biol. Res. **123**:1–16.
3. **Calisher, C. H., and R. E. Bailey.** 1981. Serodiagnosis of La Crosse virus infections in humans. J. Clin. Microbiol. **13**: 344–350.
4. **Calisher, C. H., A. O. El-Kafrawi, M. I. Al-Deen Mahmud, A. P. A. Travassos da Rosa, C. R. Bartz, M. Brummer-Korvenkontio, S. Haksohusodo, and W. Suharyono.** 1986. Complex-specific immunoglobulin M antibody patterns in humans infected with alphaviruses. J. Clin. Microbiol. **23**:155–159.
5. **Calisher, C. H., T. P. Monath, N. Karabatsos, and D. W. Trent.** 1981. Arbovirus subtyping: applications to epidemiologic studies, availability of reagents, and testing services. Am. J. Epidemiol. **114**:112–131.
6. **Chapell, W. A., P. E. Halonen, R. F. Toole, C. H. Calisher, and L. Chester.** 1969. Preparation of La Crosse virus hemagglutinating antigen in BHK-21 suspension cell cultures. Appl. Microbiol. **18**:433–437.
7. **Deering, W. M.** 1983. Neurologic aspects and treatment of La Crosse encephalitis. Prog. Clin. Biol. Res. **123**:187–191.
8. **Deibel, R., S. Srihongse, M. A. Grayson, P. R. Grimstad, M. S. Mahdy, H. Artsob, and C. H. Calisher.** 1983. Jamestown Canyon virus: the etiologic agent of an emerging human disease? Prog. Clin. Biol. Res. **123**:313–325.
9. **Fauvel, M., H. Artsob, C. H. Calisher, L. Davignon, A. Chagnon, R. Skvorc-Ranko, and S. Belloncik.** 1980. California group virus encephalitis in three children from Quebec: clinical and serological findings. Can. Med. Assoc. J. **122**:60–63.
10. **Gundersen, C. B., and K. L. Brown.** 1983. Clinical aspects of La Crosse encephalitis: preliminary report. Prog. Clin. Biol. Res. **123**:169–177.

11. **Jamnback, T. L., B. J. Beaty, S. W. Hildreth, K. L. Brown, and C. B. Gundersen.** 1982. Capture immunoglobulin M system for rapid diagnosis of La Crosse (California encephalitis) virus infections. J. Clin. Microbiol. **16**:577–580.
12. **Monath, T. P.** 1979. Arthropod-borne encephalitides in the Americas. Bull. W.H.O. **57**:513–533.
13. **Monath, T. P., R. R. Nystrom, R. E. Bailey, C. H. Calisher, and D. J. Muth.** 1984. Immunoglobulin M antibody capture enzyme-linked immunosorbent assay for diagnosis of St. Louis encephalitis. J. Clin. Microbiol. **20**:784–790.