Comparison of Three Tests for the Serological Diagnosis of Lymphocytic Choriomeningitis Virus Infection

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Received for publication 28 May 1975

Levels of lymphocytic choriomeningitis virus antibody were assayed in 62 infected persons. The three tests used were indirect fluorescent antibody (IFA), complement fixation, and neutralization in mice. The sera first became positive by the IFA test, and IFA titers rapidly rose to a relatively high level, with the sera remaining positive long after the antibody detectable by complement fixation had disappeared. The IFA test appeared to be specific. The sera became positive last by the mouse neutralization test; with this test, antibody first appeared several weeks after infection. Virus-infected cells were stable when stored at -60°C , allowing diagnostic sera to be tested promptly by the IFA test. The IFA test for lymphocytic choriomeningitis antibody should increase the number of serological diagnoses, since it is not only rapid and specific, but detects cases not diagnosed by the other methods.

Lymphocytic choriomeningitis (LCM) virus infection is generally diagnosed serologically by two types of tests. Complement fixation (CF), the most widely used test, frequently fails to detect antibody after the infection (9), and with the neutralization test, antibody can only be first detected several weeks after infection (4).

These inadequacies are apparently not encountered in the indirect fluorescent antibody (IFA) test. Cohen et al. (4) reported all 12 sera from human LCM cases as IFA positive, one as early as 24 h after the onset of meningeal symptoms. During a recent national epidemic of LCM (P. D. Walter et al., manuscript in preparation), we had the opportunity to compare the IFA, CF, and neutralization tests with a large number of sera. Multiple sera obtained from two laboratory-acquired cases with precisely known dates of onset permitted especially useful comparisons of the three tests.

MATERIALS AND METHODS

Sera. The sera positive for LCM virus antibody came from individuals infected by hamsters from infected colonies (32 cases), from persons exposed to the virus by employment in the infected hamsteries (28 cases), and from two persons who developed laboratory-acquired infection. The 50 negative (control) sera were from 50 individuals with neither clinical nor epidemiological evidence of infection.

IFA test. BHK-21 cells were suspended ($10^6/\text{ml}$) in the medium of MacPherson and Stoker (10), modified by adding $\text{Fe}(\text{NO}_3)_3$ (0.1 mg/ml) and containing 10% fetal bovine serum. The cell suspension was stirred with a magnetic bar overnight at 35°C , after

which the cells were collected by centrifugation, resuspended at their original concentration in the modified medium containing only 2% serum, and inoculated with the WE (12) strain of LCM virus. The inoculum, passed twice in BHK-21 cells, was 10% of the volume of the suspension culture and contained 0.01 infectious particles/cell. A control culture was similarly inoculated with medium containing no virus.

Stirring of the cultures was continued at 35°C for an additional 48 h, by which time approximately 85% of the cells contained fluorescing antigen. The cells were then collected, washed once in 0.01 M phosphate-buffered saline, pH 7.4, and resuspended in phosphate-buffered saline to one-fifth of the original volume of medium. The suspensions (infected and control cells) were dispensed in 0.05-ml drops onto uncoated circular areas (5-mm diameter) arranged four each in two rows on epoxy-coated slides (5); one row of infected cells, and one of companion control cells. After the drops were allowed to air-dry, the cells were fixed in acetone (-20°C) for 20 min and then stored at -60°C . They remained satisfactory for use for at least 10 months, the longest storage period tested.

The cells were stained by the procedure (3) generally used for IFA tests. Fluorescein-conjugated anti-human globulin from several commercial sources was employed; each proved to be satisfactory, although at differing dilutions. Each diagnostic serum dilution tested was added to infected and uninfected cells on the same slide. Known positive and negative sera were included with every test.

CF test. The Laboratory Branch Complement Fixation microtiter method (1) was used for the CF test with reagents from the Biological Products Division, Center for Disease Control, Atlanta.

Neutralization test. The viral inoculum was a 10% mouse brain suspension of a recent virus isolate

containing 100 intracerebral lethal doses 50/0.03 ml. This dilution was mixed with equal volumes of serial twofold dilutions of test serum, the mixtures were incubated in a 37 C water bath for 1 h, and 0.03 ml was then inoculated intracerebrally into each of five mice (ICR strain). The number of mice that died between 3 and 21 days after inoculation was used to calculate the 50% neutralization end point dilution of the sera by the Karber method (8).

RESULTS

The appearance of cells stained positively by the IFA test was so distinctive that little time was required for microscopy examination. The fluorescence of positively stained cells was predominately granular and was restricted to the cytoplasm. Although occasionally uninfected control cells displayed a dull uniform fluorescence after being treated with low dilutions of test sera, the infected cells were unquestionably distinguishable.

Titers of sera collected from 30 cases at known periods after clinical onset are shown in Table 1. All of the seven sera obtained during the first week of illness were IFA positive, with a geometric mean titer of 47. This antibody persisted for some time; geometric mean titers for sera collected 2, 3, 4, and over 4 weeks after onset were 91, 72, 128, and 69, respectively. In contrast, some sera remained negative by the CF test even 2 or 3 weeks after clinical onset. The CF test geometric mean titers at 2, 3, 4, and over 4 weeks were <4, 4, 9, and 9, respectively. The antibody response measured by the neutralization test was even slower; of the sera collected earlier than 11 weeks after onset, only two (4 and 6 week) had a detectable titer. Although all 39 postinfection sera (acute and convalescent) were IFA positive, 7 had no detectable CF antibody, and 31 were negative by the neutralization test.

Although the cases recorded in Table 2 as well as those in Table 1 had experienced symptoms suggesting LCM virus infection, reliable information was lacking for the cases in Table 2 to define precisely the interval between onset of symptoms and collection of sera. All but 6 of the 39 sera listed in Table 2 contained neutralizing antibody; in contrast, only 8 of 41 sera listed in Table 1 had neutralizing antibody. This difference probably occurred because all persons listed in Table 2, except cases 31 and 32, were employed in LCM virus-infected hamsteries (P. D. Walter et al., manuscript in preparation) and most likely had contracted the infection long before their sera were collected; slowly developing neutralizing antibody, therefore, had time to appear. Late collection of specimens could also explain why six of the nine paired

TABLE 1. *LCM virus antibody titers of sera at varying periods after clinical onset*

Case	Serum collection ^a	LCM virus antibody titer ^b		
		IFA	CF	Neutralization
1	<0 ^c	<8	<4	<5
	4	256	4	8
2	<0	<8	<4	<5
	6	32	16	<5
3	<1	32	<4	<5
	1	32	<4	<5
4	3	64	8	<5
	1	256	8	<5
5	4	256	QNS ^d	<5
	1	128		<5
6	4	64	16	<5
	1	64	4	<5
7	4	64	32	<5
	1	16	16	<5
8	4	128	8	<5
	1	16	8	<5
9	8	16	8	<5
	2	512	<4	<5
10	2	256	4	<5
	2	64	8	<5
11	2	64	<4	<5
	2	64	8	<5
12	2	64	<4	<5
	2	16	<4	<5
13	3	128	8	<5
	3	64	<4	<5
14	4	128	8	<5
	3	64	16	<5
15	3	64	<4	<5
	7	128	8	<5
16	3	64	4	<5
	4	128	4	<5
17	6	128	4	<5
	6	64	8	8
18	7	128	4	<5
	8	128	16	<5
19	11	128	16	10
	12	64	16	40
20	13	128	16	32
	14	64	8	25
21	17	32	4	50
	24	16	8	40

^a Week after onset when serum collected.

^b Dilution factor of serum end point dilution.

^c Preinfection serum.

^d Quantity not sufficient to test.

sera showed a decline in IFA titer and four showed a disappearance of CF antibody. The geometric means of the Table 2 sera were 33, <4, and 11 by the IFA, CF, and neutralization tests, respectively. Control sera from 50 individuals with no clinical or epidemiological evidence of infection were negative by all three tests.

To examine the serological response to virus

TABLE 2. *LCM virus antibody titers of sera at unspecified periods after infection*

Case	Serum	LCM virus antibody titer ^a		
		IFA	CF	Neutralization
31	1	32	<4	<5
	2 (3) ^b	128	16	10
32	1	32	<4	<5
	2 (3)	64	8	<5
33	1	256	8	10
	2 (5)	64	<4	10
34	1	128	4	10
	2 (5)	32	<4	13
35	1	128	4	8
	2 (5)	32	<4	16
36	1	128	<4	16
	2 (5)	32	<4	13
37	1	128	4	6
	2 (5)	32	<4	6
38	1	<8	<4	<5
	2 (6)	32	8	<5
39	1	128	4	13
	2 (11)	32	4	20
40	Single	64	16	79
41	Single	64	16	25
42	Single	64	4	20
43	Single	64	4	20
44	Single	32	8	79
45	Single	32	8	20
46	Single	32	<4	19
47	Single	32	<4	<5
48	Single	16	<4	40
49	Single	16	<4	16
50	Single	16	<4	15
51	Single	16	<4	12
52	Single	16	<4	8
53	Single	16	<4	6
54	Single	8	<4	63
55	Single	8	<4	43
56	Single	8	<4	20
57	Single	8	<4	14
58	Single	8	<4	14
59	Single	8	<4	11
60	Single	8	<4	10

^a Dilution factor of serum end point dilution.^b Weeks between collection of sera 1 and 2.

infection more closely, we collected and tested several specimens from two workers infected in the laboratory. Results confirmed the conclusions derived from the data of the tables. By the IFA test, antibody was detected within a few days after clinical onset, rose rapidly to a titer as high as 256, and was still present in the specimen taken almost a year after onset (Fig. 1a and b). The CF antibody appeared after about a month, remained at a low titer, and disappeared within a few months. Neutralizing antibody required 30 to 60 days to develop but

remained detectable in the last specimens collected, 300 days after infection. Interestingly, the person whose infection was clinically most prolonged and severe had the greatest antibody response.

Cohen et al. (4) have reported the slow development and long persistence of neutralizing antibody for LCM virus, but the titers we obtained were unexpectedly low. Hotchin et al. (6) found that sensitivity of the neutralization test was increased by use of bovine serum albumin in water as diluent, incubation of the virus-serum mixture overnight at 4 C, and inoculation into the footpads of mice. Neutralization was shown by inhibiting foot swelling caused by the virus. When we applied these modifications with three sera remaining in sufficient volume for a comparison, titers obtained were indeed fivefold to 20-fold higher than by the intracerebral neutralization test. A disadvantage of the footpad neutralization method as a routine diagnostic laboratory test, however, was the extensive time required daily to accurately measure the degree of swelling.

DISCUSSION

Our results, as well as the experience of Cohen et al. (4), indicate that the IFA test will improve the serological diagnosis of LCM virus infection. Antibody is not detectable by the CF test in sera of many patients after infection with LCM virus (4), and even those sera which become CF positive remain so for a relatively short period. The practical usefulness of the neutralization test is limited because neutralizing antibody does not appear until long after infection. Furthermore, many laboratories lack facilities for the neutralization test or hesitate to introduce the virus into their animal quarters. Antibody was not only detected earlier by immunofluorescence than by the CF or neutralization test, but the IFA titers rapidly increased to a level substantially higher than those determined by CF, then declined slowly over several months. Since the LCM-infected acetone-fixed BHK cells and all other test reagents could be stored for months until needed, testing could be completed within 2 h after specimens arrived.

Several observations indicated that the positive IFA test results were specific. All 62 persons with positive sera had been exposed to infected animals, and 34 of these individuals had a clinical diagnosis of LCM; on the other hand, 50 persons with negative sera had neither known exposure nor suggestive symptoms. Moreover, uninfected control cells in the IFA test never showed positive reactions. Finally,

FIG. 1. Serological responses of two human cases of LCM virus infection. Symbols: ●, IFA titer; ○, CF titer; ▲, neutralizing antibody titer.

staining of infected cells by positive human sera was inhibited by hamster antisera to LCM virus, but not by normal hamster sera.

Sera became IFA positive as early as 3 days after the onset of symptoms (case 3, Table 1). Early detection of antibody increases the likelihood that control measures can be initiated in time to limit the occurrence of additional infections. Maximal IFA titers are attained so rapidly, however, that a fourfold increase is frequently not demonstrable even when the first serum is collected soon after clinical onset.

Natural inhibitors of LCM virus have been reported (9) to be present in most human sera. In our study, however, all 50 control sera from throughout the United States demonstrated no

neutralizing activity. Moreover, all diagnostic sera which were positive by the neutralization test were also IFA positive and were from individuals with known exposure to infection.

The true incidence of human LCM virus infection has probably been underestimated because of inadequate laboratory testing. In the few instances when thorough testing has been done, the number of infections recognized has been impressive. For example, the Walter Reed Army Institute of Research found (11) that the virus caused 9% of 1,568 central nervous system infections of presumed viral etiology; this was 19% of those cases for which the etiology was determined. At present, however, the causative agent remains unidentified in well over 50% of

central nervous system infections of presumed viral etiology annually reported. In 1972, for example, the etiology in 845 of 1,302 reported cases of encephalitis remained unknown (2). The availability of rapid, reliable test methods should greatly extend our knowledge of the incidence and epidemiology of LCM infections. While this manuscript was being prepared, Hotchin and Kinch (7) reported a microplaque reduction test for LCM virus neutralizing antibody which seems especially useful for large surveys. General use of both the IFA test and the microplaque reduction test for detection of antibody to LCM virus should greatly improve the serological diagnosis of central nervous system infections.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent laboratory assistance of Helen M. Engelman, Helen S. Lindsey, and John H. Shaddock.

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