Laboratory Aspects

THELMA D. SULLIVAN, M.S., J. V. IRONS, Sc.D., and M. MICHAEL SIGEL, Ph.D.

SPECIMENS from persons with encephalitis and those taken at autopsy were tested extensively in laboratory studies of the 1954 outbreak in Texas. Pooled mosquitoes from the affected area, the Lower Rio Grande Valley, were also examined for the presence of St. Louis encephalitis virus.

Methods and Materials

White Swiss mice have proved useful (*1*) for the recovery and identification of the encephalitis viruses and for the performance of serum virus neutralization tests (*2, 3*). Mouse brain antigens also have been useful in the performance of the complement fixation tests (*4–6*) as a diagnostic aid.

Ten percent brain suspension from two fatal cases was employed for attempted recovery of an agent by the inoculation of mice, chick embryos, baby chicks, and HeLa cell cultures. An agent also was sought in a few selected stools, throat swabs, and serums and spinal fluids by the inoculation of tissue cultures and mice.

For the determination of antibody content of selected serums, the virus neutralization test was employed. The screening test was based on 10 and 100 LD_{50} doses of virus mixed with equal quantities of uninactivated undiluted serum; subsequent tests made use of larger doses of virus. Three-week-old white Swiss mice were inoculated intracerebrally and were observed for 2 weeks.

The complement fixation test was performed at the Texas State Department of Health laboratory with 0.25 ml. of twofold serum dilutions, 0.25 ml. of antigen at the optimal dilution, 0.5 ml. of complement (2 exact units), and 0.5 ml. of sensitized sheep cells containing amboceptor in optimal dilutions. At the Virus and Rickettsia Laboratory of the Communicable Disease Center in Montgomery, Ala., the corresponding amounts were 0.1 ml. of serum, 0.1 ml. of antigen, and 0.2 ml. of complement (1½ to 2 units). The primary incubation was done overnight in the cold room. A full set of controls and a supplementary amboceptor titration were included. The second incubation was carried out for 1 hour at 37° C. (½ hour at the CDC laboratory).

The degree of hemolysis was recorded after 2 hours' refrigeration. (In the CDC laboratory the test was read directly without additional refrigeration.) Titers were based on the last tube showing a 3+ or 4+ fixation. Lederle antigens were employed, except that in addition to the Lederle St. Louis antigen, a second St. Louis antigen and control were prepared from mouse brains at the Texas laboratory by the method of España and Hammon (*7*), and the influenza and mumps antigens were made at the CDC laboratory in Montgomery. All serum samples from the same patient were tested simultaneously when they were available. Most of the specimens were split for tests in both laboratories.

Paired serum specimens from cases showing poor or no serologic response to St. Louis antigen were also screened with western equine

Miss Sullivan is virologist, and Dr. Irons, director of laboratories, of the Texas State Department of Health. Dr. Sigel, formerly with the Communicable Disease Center of the Public Health Service in Montgomery, Ala., is now associate professor of bacteriology, University of Miami School of Medicine, Coral Gables, Fla. *Technical assistance was provided by Mrs. Minnie Sung, James Grimes, Mrs. Mayme Colvin, Miss Rachael Gorrie, and Mrs. Patricia Parish.*

encephalitis (WEE), eastern equine encephalitis (EEE), and lymphocytic choriomeningitis (LCM) antigens, and some were screened with mumps, influenza, and other antigens.

More than 2,000 adult mosquito specimens were collected by the entomological team and sent to the laboratory for virus isolation studies. The majority were received in a frozen state, but a few were sent alive to the State laboratory.

In the laboratory, the mosquitoes were pooled for animal inoculation, separation being based on species and place of collection. A suspension of the macerated mosquitoes was spun in a refrigerated centrifuge, and antibiotics were added to the supernatant. Infant mice were inoculated with subpool material by the subdural and intraperitoneal routes, and a guinea pig received an overall pool in a like manner. Two further brain passages were made in mice before a test was considered negative. The guinea pig was bled a month later for serologic studies.

Results

Several isolation attempts were made with brain tissues from R.A. and C.H., two patients

Table 1. Complement fixation antibody pattern of 110 serum samples

Baseline serum taken (days)	Results	Followup serum taken (days)						
		0–7	8–14	15–21	22–36	37–56	57–85	Later
0–7	Negative	3	2	4	2	1		
	<4>4			2		2		
	No change		4	1	3	2		
	Rise		4	5	11	3		
	Decline							
	Total	**3**	**10**	**12**	**16**	**8**		
8–14	Negative			1	1	2		
	No change				8	6		
	Rise			1	6	2	2	
	Decline							
	Total			**2**	**15**	**10**	**2**	
15–21	Negative					3		
	No change					2	3	
	Rise						2	
	Decline							
	Total					**5**	**5**	
22–36	Negative					1		
	No change					2	5	2
	Rise					2	1	
	Decline					1		1
	Total					**6**	**6**	**3**
37–56	Negative						1	
	No change						1	
	Rise							
	Decline							3
	Total						**2**	**3**
57–85	Negative							
	No change							
	Rise							
	Decline							2
	Total							**2**

NOTE: No change means antibodies present, but without significant fourfold rise.

Table 2. Range of complement fixation titers for St. Louis encephalitis, by week after onset of illness

Number of weeks after onset of illness	Total number of serums tested	Range of complement fixation titers		
		Lowest	Highest	Mean
1	56	<1:4	1:16	<1:4
2	55	<1:4	1:128	1:8
3	34	<1:4	1:256	1:16
4–5	54	<1:4	1:128	1:16
6–8	35	<1:4	1:128	1:16
9–12	14	<1:4	1:64	1:16
20–22	8	<1:4	1:16	<1:4

who died. An agent was recovered with brain tissues from R.A. by the intracerebral inoculation of 2-week-old and 17-day-old mice. Two-week-old mice showed evidence of infection by the 7th day and on passage by the 6th day. When one 17-day-old mouse was found dead on the 6th day, two mice which were not ill were sacrificed on the 7th day for "blind passage" in young adult mice. These mice showed evidence of infection by the 5th or 6th day. Three-week-old mice were much less satisfactory for primary isolation. The R.A. agent was not lethal for chick embryos and produced no obvious illness in guinea pigs. It was identified as SLE virus by means of a neutralization test.

Stools from 17 cases, 10 early acute phase serum samples, 7 throat swabs, and 6 spinal fluids failed to yield an agent by inoculation and by attempted "blind passage" in infant or young adult mice. Negative results were also obtained from inoculations of baby chicks and strain HeLa tissue cultures.

A total of 29 pools of mosquitoes, comprised of 1,613 specimens representing 11 species, were tested for presence of virus, with the result that two strains of St. Louis virus were isolated from *Culex quinquefasciatus* collected at Edinburg in September 1954.

The serologic results obtained on the divided specimens in the two laboratories were in essential agreement. The results used for the tables, with the exception of the serum-virus neutralization tests, were obtained primarily at the Texas bureau of laboratories.

In complement fixation tests, fourfold or greater titer rises were demonstrated in 38 cases, representing 43.7 percent of the 87 cases tested. Paired serum samples from 17 cases failed to show complement fixation titers, at least at the 1:4 (3+) level, and 32 cases showed no significant change in titer. Tests on serums collected after the 13th week were excluded. The extent of change in titer of serums collected from 87 patients is shown in table 1. Only 1 of 29 paired serum samples collected up to the 9th week showed a decreasing titer, but 6 of 8 pairs of samples taken 20 to 22 weeks after onset of illness showed greatly decreased or totally absent titers. Mean titers by week after onset of illness are shown in table 2. The titers ranged from less than 1:4 (3+) through 1:256 (4+).

Seventy percent of the serums collected from 50 persons, who presumably were exposed but not ill, had titers from 1:4 through 1:128 (table 3). These specimens were collected at the close of the epidemic, mainly from other members of families in which proved cases of SLE had occurred.

Serum-virus neutralization tests were performed on paired serums from 7 cases and single serums from several cases. The relation of the neutralization indexes to the complement fixation titers on paired serums is shown in table 4.

Paired serum samples from 12 clinically diagnosed cases showed no antibodies or failed to

Table 3. Results of complement fixation tests for St. Louis encephalitis on persons not ill during outbreak of encephalitis in Lower Rio Grande Valley

Number of persons tested	Number with complement fixing titer of—							Total number with titer of 1:4 or greater	Percentage of total with titer of 1:4 or greater
	<4	4	8	16	32	64	128		
50	15	3	10	9	7	4	2	35	70.0

Table 4. Comparative data on complement fixation titers and neutralization indexes on cases in outbreak of St. Louis encephalitis in the Lower Rio Grande Valley

Case No.	Date of onset of illness	Date specimen taken	Complement fixation titer				Neutralization index
			8	16	32	64	
8	9–4	9–4					0
		9–20					0
13	8–12	9–17	4	4			21
		10–20	4	3			32
14	8–25	8–27					0
		9–17	4	4	4	4	14
16	8–31	9–13					0
		9–20					0
19	8–19	8–27					0
		9–16	4	4	4		68
27	9–12	9–23					0
		10–23					0
29	9–4	9–4					14
		10–22	2				68
51	9–2	9–14	4	4			140
		10–22	4	4	3		140
67	8–17	9–20	3				68
		10–23	3				140
82	8–30	9–23	4	3			21
		10–23	4	4	4	4	210

show a significant increase in titer in complement fixation tests in which SLE, WEE, EEE, and LCM antigens were utilized. Single serums from several cases also were negative in the WEE test. Paired samples of serum from 7 cases were negative in hemagglutination-inhibition tests for influenza A, A-prime, and B. Four paired serum samples were negative for mumps, typhus, Q fever, and diseases of the lymphogranuloma venereum and psittacosis group. Paired samples from 2 cases and a single serum from each of 6 cases were negative in the complement fixation test with Venezuelan equine antigen supplied by Dr. E. H. Lennette of the California State Health Department. Similarly, 4 serums were negative for Japanese B encephalitis and herpes. Agglutination tests with the "febrile" bacterial antigens were essentially negative.

Discussion

In our experience positive results are a rarity in serums submitted from sporadic cases of encephalitis. This had been the experience of Casals (*8*) also. In fact, the validity of the CF test in SLE has occasionally been questioned. By contrast, the findings from the current study indicate that CF antibodies to the causative agent are readily demonstrable in the great majority of patients in an epidemic. Thus, 70 of 87 patients (80.5 percent) were found to possess antibodies to the SLE virus, and 38 of these 87 (43.7 percent) showed a significant rise in titer.

These results confirm the observation of Howitt (*4*) and supply more evidence for the value of the CF test in the diagnosis of SLE. Demonstrations of a significant rise in titer required proper spacing of specimens. When the first specimen was taken during the first week of illness and the second specimen was collected during the second and third weeks of illness, 9 of 22 individuals showed a rise in titer. The rise was encountered most frequently between 22 and 36 days (11 of 16). The comparison of results obtained when the baseline serum was taken during the second week of illness and the followup serum samples 3, 4, 5, or more weeks after onset revealed a fourfold or greater rise in 11 of 29 patients. Using as baseline the samples of serum taken during the third week, there were only 2 rises among serum samples of 10 patients.

In 10 of 46 patients, antibodies present during the first week exhibited no significant rise during subsequent weeks. This suggests two possibilities: Either the CF antibodies to SLE develop quite early in some patients or the in-

formation about the date of onset was inaccurate. Howitt (*4*) recorded positive reactions in 12 of 26 serums taken prior to the end of the first week.

The CF antibodies to the SLE virus were found to decline fairly rapidly. Thus, 6 of 8 patients showed a fourfold or greater decrease in titer in serums obtained beyond the 85th day after onset. Unchanged titers were still present, however, in two individuals. The relatively quick antibody decline was also noted by Howitt (*4*).

Among the serum samples collected from persons who presumably were exposed but who were not ill, 70 percent contained CF antibodies. This finding provides evidence that the virus may cause many inapparent infections.

The success in obtaining useful serologic information was primarily due to the availability of multiple specimens. This study reemphasizes the need for comparative testing of two (and sometimes more) specimens from a patient. Although in this study the infection with SLE virus was associated with a pattern consisting of antibody rise after the first week of illness (most rises were detected between 22 and 36 days) and antibody decline after 3 months, the variability in time of antibody appearance, in the rate of antibody rise, and in the rate of antibody fall makes it difficult and often impossible to interpret validly the results of a single serum.

Laboratories that may be called upon to perform similar tests in the future are cautioned about the reliance on standardization of antigens prepared outside their laboratories. Failure to assay each antigen for its CF activity using several positive serums and the investigator's technique which will later be used in the test may lead to weak and inconclusive reactions.

The neutralization test also yielded positive results. However, the number of serum samples tested was too small to warrant specific conclusions.

Summary

In studies of human cases, virologic and serologic tests proved that the epidemic of encephalitis in the Lower Rio Grande Valley of Texas in 1954 was caused by the St. Louis type of virus. Isolation of St. Louis encephalitis virus from two pools of *Culex quinquefasciatus* taken in the areas supported the diagnosis.

The complement fixation test was found to be of great value.

Detection of significant rise in titer required proper spacing of specimens.

It was found that inapparent infections had occurred with great frequency.

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