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Preparation of La Crosse Virus Hemagglutinating Antigen in BHK-21 Suspension Cell Cultures

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Hemagglutinating and complement-fixing antigens of La Crosse virus (California arbovirus group) were produced in serum-free suspension cultures of BHK-21/13S cells. The appearance and production of these antigens were correlated with the titer of infectious virus. No significant differences in antigen titers were produced by varying virus dose 10-fold. Hemagglutinin appeared 6 to 8 hr after inoculation and reached peak titer in 14 to 22 hr. Both β-propiolactone and Tween 80-ether treatment inactivated infectious virus in the antigens. Unlyophilized antigen was stable at −60, 5 and 24 C for at least 117 days but not for 1 year. Lyophilized antigen was stable for at least a year, however, at −20 and 5 C. Cell culture-produced antigen was more sensitive than brain-produced antigen in detecting hemagglutination inhibition antibody in human sera.

Human disease caused by the California group of arboviruses was first reported in 1945 by Hammon and Reeves (9). They attributed three cases of encephalitis to a new virus which they called California encephalitis virus. After these cases were described, several diagnostic laboratories used this virus as antigen in serological screening of suspect cases for the diagnosis of arboviral encephalitis. Since additional cases were not confirmed, the laboratories gradually dropped this virus from their battery of tests (7). No other cases were observed until 1963, when Quick et al. reported a single case of clinical encephalitis in Florida caused by a California group virus (12). Then in 1964 Thompson et al. reported the isolation of a new antigenic member of the group, La Crosse virus, from the brain of a child who had died of encephalitis in La Crosse, Wisc., in 1960 (15). Stimulated by these reports, several diagnostic laboratories again incorporated California virus in their battery of tests. The original California encephalitis strain was used in most laboratories because antigens for La Crosse virus were not initially available. Nevertheless, from 1964 to 1967, 219 cases of encephalitis due to a California group virus were reported (3).

Although standard procedures of Clarke and Casals (2) have been used to produce La Crosse complement-fixing (CF) antigen, they have given inconsistent results in producing satisfactory hemagglutinating (HA) antigens.

Since the BHK-21 continuous line of baby hamster kidney cells had been successfully used to produce antigens for other arboviruses (4) and since Murphy et al. had reported growing La Crosse virus in it (11), this cell line was investigated for the production of La Crosse virus diagnostic antigens. The successful production of infectious La Crosse virus and inactivated CF and HA antigens in serum-free suspension cultures of BHK-21 cells is described in the present report, along with data on the serological sensitivity and storage stability of the antigens.

MATERIALS AND METHODS

Virus. The original strain of La Crosse virus was kindly supplied by Wayne Thompson, The University of Wisconsin Medical Center, Madison, Wisc. A seed stock was prepared from virus which has been passed seven times in the brains of suckling mice and once in BHK-21/C-13 cells. Seed virus was stored in 2-ml portions at −65 C until used. The seed virus had a titer of $10^{8.2}$ plaque-forming units (PFU) per ml in BHK-21 cells.

Cell cultures. A culture of BHK-21/13S was maintained by the methods reported previously (5, 6) with minor modifications. Monolayers were grown in 32-oz (947 ml) prescription bottles (Brockway Glass Co., Brockway, Pa.) containing 50 ml of BHK-21 growth medium which contained 10% heat-inactivated, fetal calf serum, and 10% tryptose phosphate broth. Cul-

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tures were trypsinized and divided into six subcultures twice weekly. To produce cells for suspension cultures, eight 32-oz monolayer cultures were grown at 35 C for 3 to 4 days. These monolayers were trypsinized, and the cells (about 8×10^8) were suspended in 800 ml of growth medium. The suspension cultures were grown for 24 hr in sealed, 1-liter Pyrex bottles at 35 C and then inoculated with virus.

Preparation of antigen. To produce antigen, 24-hr suspension cultures were centrifuged at 4 C for 10 min at $200 \times g$. The supernatant medium was discarded, and the packed cells were resuspended to give about 1.2×10^9 cells in 100 ml of bovalbumin (BA) medium. The BA medium was the same as growth medium except that the fetal calf serum was replaced by 0.4% bovine plasma albumin, fraction V (Armour Pharmaceutical Co., Chicago, Ill.). The cells were then inoculated with the desired dose of seed virus and incubated at 35 C for 2 hr, with gentle agitation every 20 min. Enough BA medium was added to bring each culture up to 800 ml, and the *p*H was adjusted to between 7.0 and 7.2 with 5% $NaHCO_3$ solution. The infected suspension cultures were incubated at 35 C on a magnetic stirrer. To maintain an alkaline *p*H, the screw cap was not tightened. In some instances, a medium change was made after about 24 hr of incubation. This was accomplished by centrifugation, as described above, and by resuspension of cells in 800 ml of fresh BA medium.

Inactivation of infectious antigens. Two methods were tested for their ability to inactivate residual infectious virus in the final antigens. In one method, tissue culture derived antigens were extracted with Tween 80 and ether, as described elsewhere (6). In the second method, antigens were treated with the following final concentrations of β propiolactone (BPL): 0.01, 0.025, 0.05, 0.1, and 0.2% (14).

Storage stability. To determine the most suitable conditions for storing Tween 80-ether-extracted antigen, the antigen preparation was divided into two equal volumes and treated as follows: one-half was lyophilized and the other half was dispensed in screw-cap vials in the liquid state. Ampoules of dried antigen were stored at 24, 5, −20, and −60 C. Samples of each were tested for HA activity at 0, 7, 13, 27, 61, 117, and 365 days.

Virus and antigen assays. Viable virus was assayed in BHK-21/C-13 cells by plaquing in 3-oz (89 ml) prescription bottles (Chappell, Coleman, and Elliott, *in preparation*). In this procedure two overlays were used. The first overlay medium, which was applied 1 hr after inoculation, consisted of 1% agarose prepared in Earle's balanced salt solution with a final concentration of 330 μg of yeast extract and 1,650 μg of lactalbumin hydrolysate per ml. The second overlay medium, which was added 2 days after inoculation, was the same as the first overlay except that it contained neutral red and that agarose was replaced by Noble agar.

Hemagglutination and hemagglutination-inhibition tests were carried out in plastic microtiter plates (8) by the method of Clarke and Casals (2). Optimal hemagglutination by La Crosse antigen occurred at *p*H 6.0 at 37 C. Four hemagglutinating units of each antigen were used, and serum-antigen mixtures were held at 5 C overnight before the addition of goose erythrocytes. The immune ascitic fluids used were prepared in mice after vaccinations with La Crosse, Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), and St. Louis encephalitis (SLE), according to the method of Tikasingh et al. (17). All sera were adsorbed with kaolin and goose erythrocytes.

CF tests were performed by the microtiter Laboratory Branch Complement Fixation method by using five 50% units of complement (1). Undiluted cell culture supernatant fluid was used as the CF antigen.

Comparison of mouse brain and cell culture HA antigens. HA antigen derived from infected mouse brains was compared with that prepared in BHK-21 suspension cultures. The brain antigen was prepared by the sucrose-acetone extraction method of Clarke and Casals (2). Human sera for the comparative study were selected on the basis of their neutralization antibody content for La Crosse virus. Undiluted sera that caused at least 90% plaque reduction in BHK-21/C-13 cells were considered positive for neutralizing antibody. All sera were adsorbed with kaolin and goose erythrocytes before use in hemagglutination-inhibition (HI) tests.

RESULTS

Results of initial studies showed that La Crosse HA antigen could be successfully produced in BHK-21/13S suspension cultures. Hemagglutinin was first detected 8 hr after inoculation, and peak titers of 1:64 to 1:128 were reached within 22 to 24 hr. Additional studies were performed to determine (i) the effects of changing the medium and the dose of virus inoculum on HA production, (ii) suitable methods of inactivating the infectious antigen, and (iii) optimal storage conditions for the antigen.

Effect of medium change. Two identical suspension cultures of BHK-21/13S cells were inoculated with La Crosse virus in a ratio of 1 PFU per cell. Both cultures were then treated similarly except that 24 hr after inoculation culture no. 1 was centrifuged, and the medium was replaced with fresh medium. No centrifugation or medium change was made in culture no. 2. Samples were collected from both cultures every 2 hr through 30 hr of incubation and then at 48 and 54 hr. These samples were stored at −60 C until tested for HA and infective virus. The test results (Table 1) show the appearance of hemagglutinin at 8 hr post-inoculation in both cultures, with an increase in titer to 1:128 by the 24th hr. The first sample collected from culture no. 1 after the medium change had a titer of only 1:4, whereas a titer of 1:128 persisted in culture no. 2 until 30 hr after inoculation. A correlating observation concerning the infective virus titer was made. The PFU titer in both cultures increased from about $10^{5.9}$/ml in the first sample to about $10^{7.6}$/ml in the 24-hr sample. After the medium change, the infectivity titer of culture no. 1

decreased 10-fold, but that of no. 2 remained the same until 30 hr after inoculation.

Effect of virus dose on antigen production. Two suspension cultures of BHK-21/13S were prepared with an approximately equal number of cells. Culture no. 1 was inoculated with 0.1 PFU of La Crosse virus per cell, and culture no. 2 with 1 PFU of La Crosse virus per cell. Both cultures were incubated identically and were sampled at the same intervals. Samples were stored at −60 C until they were tested for HA and CF activity and for virus infectivity. The first samples were collected immediately after the 2-hr virus adsorption period, and additional samples were collected at the time intervals shown in Table 2. Table 2 reveals a 10-fold difference in infective virus titers between the two cultures at 2 hr. By the 22nd hr both cultures had about the same titer. At 22 hr the cultures were centrifuged, and fresh BA medium was added. The titer then declined in both cultures; the decline was more rapid, however, in culture no. 2, which had received the higher virus dose.

TABLE 1. *Effect of medium change of La Crosse virus on infective virus and hemagglutinin production in BHK-21/13S cell cultures*

Sample	Hours after inoculation	Culture no. 1		Culture no. 2	
		Reciprocal HA	PFU[a]	Reciprocal HA	PFU[a]
A	2	<2	5.9	<2	5.8
B	4	<2	5.6	<2	5.8
C	6	<2	6.1	<2	6.3
D	8	4	7.3	4	7.4
E	10	32	7.6	16	7.7
F	12	32	7.7	32	7.7
G	14	32	7.8	64	7.8
H	16	64	7.9	64	7.9
I	22	128	7.7	64	7.8
J	24	128[b]	7.6[b]	128	7.6
K	25	4	6.6	128	7.8
L	26	2	6.3	128	7.7
M	28	8	6.7	128	7.8
N	30	4	7.0	128	7.9
O	48	8	6.2	64	7.4
P	54	8	6.0	64	7.3

[a] Expressed as $\log_{10}$ PFU per milliliter.
[b] Culture no. 1 was centrifuged, and fresh medium was added.

In both cultures, HA and CF antigens were present by the 22nd hr. A significant reduction of HA followed the medium change, but the CF titer was unaltered. Within about 24 hr after the medium change, hemagglutinin activity had reappeared, but the titer remained lower than at 22 hr. The titer of CF antigen did not decrease as much as that of the HA with increase in the age of the culture. No significant differences in HA and CF titers were observed between the two cultures.

Inactivation of antigen. Since noninfectious antigens are desirable for serological tests, two methods of antigen inactivation were explored. In one method, five different concentrations of BPL ranging from 0.01 to 0.2% were used. The results (Table 3) indicate that a minimum concentration of 0.025% is required for virus inactivation, and concentrations of BPL as low as 0.01% may lower the hemagglutinin titer.

In the second method, antigens were treated with Tween 80 and ether. In this procedure, all demonstrable infective virus was inactivated, but residual ether interfered with the HA test. Several methods were tested to determine the optimal procedure for removing residual ether. An antigen preparation was extracted and divided into three portions. One portion was treated by bubbling nitrogen through the preparation until all residual ether had been removed, the second was lyophilized to remove ether, and the third was first treated with nitrogen and then lyophi-

TABLE 2. *Effect of dose of La Crosse virus on antigen and infective virus production in BHK-21/13S cell cultures*

Sample	Hours after inoculation	Culture no. 1 (1 PFU/10 cells)[a]			Culture no. 2 (1 PFU/cell)[a]		
		Reciprocal HA	Reciprocal CF	PFU[b]	Reciprocal HA	Reciprocal CF	PFU[b]
A	2	<2	<2	5.9	<2	4	6.9
B[c]	22	64	32	7.9	32	16	7.5
C	23.5	<2	32	6.8	<2	16	6.6
D	47.5	16	16	6.2	8	16	5.4
E	71.5	16	16	5.3	4	16	3.5
F	95.5	8	16	4.0	2	16	<3.0
G	119.5	8	16	<3.0	4	16	<3.0

[a] Virus inoculum.
[b] Expressed as $\log_{10}$ PFU per milliliter.
[c] Fresh medium was added to both cultures after B samples were collected.

TABLE 3. *Inactivation of infectious La Crosse virus in antigens treated with β Propiolactone or ether*

Antigen sample	Treatment	PFU titer[a]	Reciprocal HA titer
A	Untreated	8.3	32
B	0.2% BPL	<1.0	4
C	0.1% BPL	<1.0	16
D	0.05% BPL	<1.0	16
E	0.025% BPL	<1.0	16
F	0.01% BPL	4.2	16
G	Tween 80-ether	<1.0	32

[a] Expressed as $\log_{10}$ PFU per milliliter.

TABLE 4. *Effect of residual Ether on the stability of La Crosse virus hemagglutinin*

Code	Treatment of antigen	HA titer
A	Untreated	1:32
B	Tween-ether extracted	1:64
C	Tween-ether + lyophilization	1:16
D	Tween-ether + nitrogen	1:64
E	Tween-ether + nitrogen + lyophilization	1:32
F	C stored at −20 C for 2 months	1:4
G	E stored at −20 C for 2 months	1:16

lized (Table 4). In this study the Tween 80-ether extraction increased the titer of the untreated antigen from 1:32 to 1:64. Bubbling with nitrogen did not alter the titer, whereas lyophilization decreased the titer from 1:64 to 1:16, and nitrogen treatment plus lyophilization decreased the titer to 1:32. There was a further decrease in titer of both lyophilized preparations after storage for 2 months at −20 C.

Storage stability. To determine the optimal storage conditions for antigen extracted with Tween 80 and ether and then treated with nitrogen, both lyophilized and liquid preparations were stored at various temperatures for 1 year (Table 5). There was comparable stability of unlyophilized antigen at all temperatures tested for at least 27 days; however, by 61 days the titer of antigen stored at −20 C had decreased about fourfold, whereas antigens stored at −60, 5, and 24 C remained unchanged. No HA activity of unlyophilized antigen was obtained at the end of 1 year. Titers of lyophilized antigens were unchanged for at least 117 days at −20, 5, and 24 C and, at the end of 1 year, low titers were still obtained.

Antigen specificity and sensitivity. To determine antigen specificity, HI tests were conducted by using 4 to 8 HA units of the BHK-21/13S cell-derived La Crosse antigen and immune ascitic fluids for La Crosse, EEE, WEE, and SLE viruses. Only the homologous ascitic fluids inhibited hemagglutination.

To determine antigen sensitivity, HI tests were carried out on 25 human sera comparing La Crosse cell-produced antigen with antigen made from brains of suckling mice. Of 19 sera positive

TABLE 5. *Stability of wet and dried La Crosse virus hemagglutinins*

Days of storage	HA titer (reciprocal)						
	Lyophilized antigen			Unlyophilized antigen			
	24[a]	5	−20	24	5	−20	−60
0		64			128		
7	32	32	32	32	64	64	32
13	32	32	32	64	64	32	32
27	32	32	32	64	64	32	64
61	32	32	32	32	64	8	64
117	32	32	32	32	64	8	64
365	4	16	16	<2	ND[b]	<2	<2

[a] Storage temperature (C).
[b] Not determined.

TABLE 6. *Comparison of La Crosse virus mouse brain antigen and BHK-21/13S cell culture antigen by HI tests*

Human serum	Reciprocal HI titer		La Crosse virus neutralization
	BHK-21 antigen	Mouse brain antigen	
1	80	10	+[a]
2	40	10	+
3	40	10	+
4	20	10	+
5	20	10	+
6	40	10	+
7	10	<10	+
8	20	<10	+
9	40	10	+
10	40	10	+
11	40	10	+
12	40	10	+
13	40	10	+
14	40	10	+
15	40	10	+
16	20	10	+
17	80	10	+
18	20	<10	+
19	40	<10	+
20	<10	<10	−
21	<10	<10	−
22	<10	<10	−
23	<10	<10	−
24	<10	<10	−
25	<10	<10	−

[a] Positive, +; negative, −.

by neutralization test, all were HI positive with the cell culture-produced antigen, whereas only 15 were positive with mouse brain-produced antigen (Table 6).

DISCUSSION

One of the important North American arboviruses associated with human disease is La Crosse virus, a member of the California group (15). Serological surveys have been carried out by our laboratories and by others (16) to determine the extent of human involvement with this virus in certain areas. In these surveys, either complement fixation or neutralization tests have been used to detect La Crosse virus antibody; HI tests have not been used extensively in human surveys for La Crosse virus antibody because hemagglutinating antigen was not available. Production of La Crosse virus hemagglutinating antigen from infected mouse brain is difficult, and the production of a good quality product is not reproducible.

Suspension cultures of BHK-21 cells, containing serum-free medium, have been used to produce hamagglutinins of rubella (6), rabies, vesicular stomatitis, Cocal, Kern Canyon, and Flanders viruses (4). We have found that this method for the production of La Crosse virus hemagglutinin yields a better quality and is more reproducible than the procedure in which mouse brain is used as an antigen source. In our laboratories, we have successfully produced 16 consecutive batches of antigen with this procedure. Furthermore, hemagglutinin can be produced within 22 to 24 hr after virus is added to cell cultures. Although the highest HA titer of antigen obtained was 1:128, the volume produced in each lot was 800 ml. By treating this product with Tween 80 and ether, all apparent virus infectivity is destroyed without essentially decreasing HA activity. The product is then safe for use in any laboratory as a CF or HA antigen.

One of the most important properties of a HA antigen is its sensitivity in detecting homologous antibody. In the tests to compare cell culture-produced antigen with brain-derived antigen in detection of HI antibody in human sera, the cell culture antigen was more sensitive. Titers obtained were as much as eightfold greater than those obtained with the brain antigen. Furthermore, with the brain-derived antigen, about 20% fewer sera were positive.

The antigenic patterns among the members of the California group of viruses have been studied by complement fixation, neutralization (13), and immunodiffusion (10); however, complete analysis of the group has not been possible because of lack of hemagglutinins. It is hoped that the method of antigen production reported in this study for La Crosse virus will lead to the production of HA antigens for other members of the California group so that more complete studies of antigenic relationships of these viruses can be conducted.

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