Application of Cell Culture to Production of Japanese Encephalitis Virus Hemagglutinin[1] (35027)

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In a previous communication (1), we reported that cell cultures derived from embryonic and newborn hamster tissues support propagation of Japanese encephalitis virus (JEV) and showed definite cytopathic effects (CPE) after infection. Continuous cell lines were developed in which JEV propagated consistently to high titers, as measured by both infectivity and hemagglutinin (HA), when serum-free medium was used. Since the demonstration of HA is comparatively easy, and HA titer of the infected culture fluid correlated with potency of the formalin-inactivated vaccines, HA titration of the virus harvests has become an indispensable tool for assessment of vaccine preparations (2).

The HA antigen for serological diagnosis of JEV infection has been traditionally prepared from infected suckling mouse brain (3). Although high HA titers can be obtained, the method is cumbersome and hazardous. The cell culture method yields moderately high titers by much simpler methodology.

This paper describes the production, characteristics, and specificity of the HA from JEV infected hamster diploid cell culture.

Materials and Methods. Virus strains. JEO strain (1) in the 3rd to 4th passage in suckling mouse brain was used as the cell culture inoculum. The Nakayama strain (unknown high passages) in mouse brain was used as a source of mouse-brain HA. For assay of infectivity, litters of suckling mice were inoculated intracerebrally, using 0.02 ml of serial tenfold dilutions of virus. The mice were observed for 14 days and the LD_{50} was calculated by the Reed-Muench formula.

Cell culture methods. The cell line used in these studies was HS-5 derived from continuous hamster embryonic skin and muscle cells by methods previously reported (1, 2). HS-5 remained diploid through at least 35 passages, the last one tested. It was grown in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum and maintained in MEM with 10% calf serum at 36°. The monolayer cultures were dispersed with 0.1% trypsin and split 1:2 for passage every 3–4 days.

Production of hemagglutinin. Mouse-brain HA was prepared by the method described by Chanock and Sabin (4). For production of HA in cell culture, monolayer cultures of HS-5 cells in 32-oz bottles after discarding the growth medium were inoculated with approximately $10^{7.0}$ LD_{50} of JEO strain in 2 ml of MEM with 10% calf serum. The virus was allowed to adsorb for 2 hr while the bottles were rocked in an incubator at 36°. Then the monolayers were washed twice in Hanks' balanced salt solution (BSS) at pH 7.3, fed with 100 ml of MEM plus 5% calf serum and incubated at 36°. About 36 to 40 hr after virus inoculation, when early CPE had appeared, the monolayers were washed 3 times with BSS at pH 7.3, and the medium was replaced by 70 ml of MEM containing 0.4% bovine albumin at pH 7.5 and incubation continued at 36°. The pH of the medium was maintained during incubation by adding 5.6% sodium bicarbonate. The culture fluid was harvested about 20 hr later, when more than 50% of the cells showed CPE, and was centrifuged at 7000 rpm for 30 min.

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The supernatant fluid, containing HA in titer of 1:384 or greater per 0.5 ml, was diluted 1:3 with 0.016 *M* borate buffer saline at pH 9.0, distributed into vials, sealed and frozen at —60°. The final pH of the HA was about 8.4.

Titration of HA. The method of Buescher *et al.* (5) was followed with the modifications indicated. The reaction was read 2 hr after addition of red blood cells and recorded as +, ±, and — for complete, partial, and negative agglutination. The highest dilution of antigen causing complete agglutination was taken as the final titer.

Hemagglutination inhibition (HAI) test. The HAI test followed Buescher *et al.* (5), when mouse brain antigen was used, except that goose erythrocytes replaced chick red cells (6). For the HAI test with cell culture antigen, serial 2-fold dilutions of acetone-extracted sera were prepared in 0.01 *M* PBS of pH 7.3. The antigen was diluted in PBS to contain 8 units/0.25 ml. To 0.25 ml of diluted sera, antigen was added in equal volume, and the mixture was incubated at room temperature for 60 min. Then 0.5 ml of 0.25% goose red cells in PBS at pH 4.3 were added, shaken, and left at 4° for 2 hr. The HAI titer was expressed as the reciprocal of the highest serum dilution inhibiting hemagglutination. The lowest serum dilution tested was 1:20. A total of 244 sera obtained from animals were studied. Of these 217 were collected from monkeys (*Macaca cyclopsis*) either before or after vaccination with 3 to 4 doses of inactivated JEV vaccine or experimental infection with live JEV. The remaining 25 sera were collected in Shin-chu, Taiwan, from sows with abortion of undetermined etiology. Sera were frozen at —20° until tested. Before HAI testing they were inactivated at 56° for 30 min, and the nonspecific inhibitors removed by acetone extraction (5).

Results. 1. Production of virus and HA in cell cultures. Hamster embryonic cells (HS-5) monolayers in two 32 oz bottles were infected with JEV. In order to demonstrate HA activity in the early phase of the virus infection, the serum-containing maintenance medium was discarded 16 hr after virus inoculation, the monolayers were washed three times with PBS at pH 7.3 and 70 ml of MEM containing 0.4% bovine albumin was added. Two-ml samples of culture fluid were removed from each bottle every 24 hr after infection until only a few cells remained attached to the bottle. The samples were centrifuged and the supernatant fluids were tested for infectivity and HA titers. Both infectivity and HA titers increased rapidly and reached a peak about 48 hr after infection, when less than half the cells showed definite signs of CPE (Fig. 1). Thereafter, as CPE progressed, infectivity dropped continuously while HA remained at the peak level for another day and declined slightly as the cells detached from the glass.

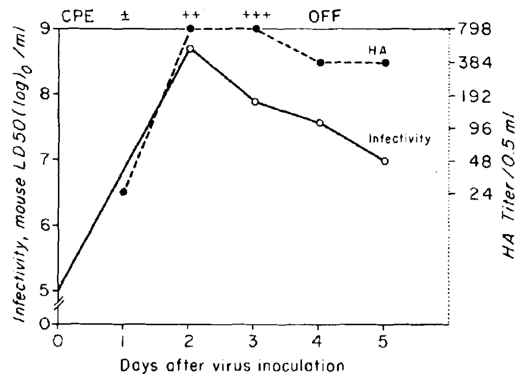

FIG. 1. Growth of JE virus and production of hemagglutinin in HS-5 cell culture.

Various concentrations of calf serum, bovine albumin, or human albumin were incorporated into the maintenance medium and tested for ability to support HA production. HA titers were low with calf serum but high when human or bovine albumin were employed in either of two concentrations (Table I).

2. Demonstration of HA Activity. The influence of pH on HA titer was investigated by reacting HA diluted at 4 different pH values (from 7.1 to 7.7) with goose red cells suspended in diluent of 3 different pH values (4.1 to 4.5). For both red cells and HA the diluent was 0.01 *M* PBS.

Table II shows that the final pH of the HA-red cell mixtures was chiefly influenced

TABLE I. Relation of Protein Content in Medium to the Titer of Hemagglutinin.

Content of protein in the medium	HA titer/0.5 ml of the harvest
Human albumin, 0.2%	768
1.0%	768
Bovine albumin, 0.2%	768
1.0%	768
Fetal calf serum, 2.0%	192
5.0%	97
Calf serum, 3.0%	48

by the pH of the PBS used to dilute the HA. Maximum titers were obtained repeatedly when pH 7.3 PBS was used as the HA diluent regardless of the pH of the red cell diluent within the range tested. The optimal pH of the red cell-HA mixture was about 6.5.

Our experience suggests that HA titers are more reproducible at 4° than at any higher temperatures tested up to 36°, but the differences were small.

HA activity was tested with erythrocytes from newborn chicks (hatched within 24 hr), roosters and geese at pH 6.5 and 4°. The titer of the cell culture HA was highest (1:192) with goose red cells, slightly lower with chick cells (1:96) and lowest with rooster cells (1:6).

3. Stability of the HA. Cell culture HA diluted with borate-buffered saline at pH 9.0 was distributed into screw-capped vials, which were sealed tightly and stored at −60, 4, 25, and 36°. Stored samples were titrated at regular intervals for HA activity.

Cell culture HA was stable at −60° for more than a year. The HA titer was also stable at 4° for at least 3 months, but dropped progressively at higher temperatures (Fig. 2). The half-life of HA activity at pH 8.4 was 5 days at 36° and 15 days at room temperature (about 25°).

4. Specificity of Cell-Culture HA. Parallel tests of 244 animals' sera for HAI antibody were completed using mouse brain and cell culture antigens (Table III). Antibody titers obtained with cell culture antigen corresponded closely with those obtained with mouse brain antigens (correlation coefficient = 0.95). Thirteen, or 5.3%, of the sera showed titer differences greater than 4-fold. Of three specimens positive with mouse brain antigen but negative with cell culture antigen, two were also negative when tested for neutralizing antibody by the cell culture metabolic inhibition test (7), whereas both specimens positive with tissue culture antigen but negative with mouse brain antigen were shown to possess neutralizing antibody.

Discussion. The hemagglutination inhibition test is a simple and useful test in laboratory diagnosis and epidemiological study of arbovirus infections. The one drawback to

TABLE II. Relation of pH to Titer of JEV HA Produced.

pH of diluents			Dilution of cell culture fluids[a]					
HA	RBC	Mixture	1/24	1/48	1/96	1/192	1/384	1/768
7.7	4.5	6.62	+	+	+	±	±	—
	4.3	6.62	+	+	+	±	—	—
	4.1	6.62	+	+	+	±	±	—
	4.5	6.57	+	+	+	±	—	—
7.5	4.3	6.57	+	+	+	±	—	—
	4.1	6.57	+	+	+	±	—	—
	4.5	6.52	+	+	+	+	+	—
7.3	4.3	6.52	+	+	+	+	+	—
	4.1	6.52	+	+	+	+	+	—
	4.5	6.44	+	+	+	+	+	—
	4.3	6.44	+	+	+	±	—	—
	4.1	6.44	+	+	+	±	—	—

[a] +, Complete hemagglutination; ±, incomplete hemagglutination.

TABLE III. Correlation of HAI Antibody Titers with Cell Culture HA and with Mouse Brain HA.

Mouse brain antigen	HAI titer:	Cell culture antigen				
	20	20	40	80	160	320
20	49	2				
20	2	7	4			
40	1	7	16	2	1	
80				12	6	2
160				12	28	7
320				3	18	65

the use of the test has been the complicated and dangerous processing required to prepare antigen from infected suckling mouse brain (3).

Since development of cell culture methods, efforts have been made to utilize cell culture virus as a source of HA. JEV has been shown to multiply in cultures of many cell types, but development of high HA titer usually did not occur, presumably due to limited viral multiplication, thermal instability of the virus, and the presence of nonspecific inhibitors in the culture medium. Cholesterol, phospholipids and free fatty acid are among these nonspecific inhibitors (7) and their elimination by acetone extraction has been described for dengue virus (8). Diercks *et al.* (9) reported that HA could be detected in hamster kidney cell cultures infected with JEV, when the medium contained low concentration of horse sera selected for their minimal content of nonspecific inhibitors. They also reported that, compared with mouse brain HA, hamster kidney cell culture HA was more sensitive to pH but less sensitive to temperature change. Salminen (10) also was able to demonstrate HA activity in cultures of continuous human amnion cells infected with JEV by using serum-free medium containing 0.2% bovine albumin, but the titers were not high enough for practical use. High HA titers have been achieved with a few selected group A and B arboviruses propagated in cell cultures by the time consuming process of concentration by ultrafiltration (11). More recently, high HA titers have been obtained by growth of JEV in cultures of continuous hamster cell lines (1) and in primary hamster kidney cells (13), simply by maintaining the infected cell cultures with serum-free medium containing bovine or human albumin. Darwish and Hammon (12) concluded that use of reduced amounts of serum-free maintenance medium containing 4% human albumin at pH of 8.0 gave optimum HA titers.

The pattern of viral growth in the HS-5 strain of hamster embryonic cells was similar to that in the HL strain described earlier (1). The rise of HA activity was proportional to the titer of infectivity during the first 2 days of infection. However, thereafter the infectivity titer declined whereas the HA activity remained at a high level. This suggests that HA activity is more stable than infectivity and may represent the accumulation of both live and dead viruses as suggested by Salminen (13). Its usefulness as a predictor of the potency of the cell culture vaccine has been reported (2).

The biological properties of JEV HA from mouse brain tissue have been described by Sabin and Buescher (14, 15). This HA agglutinates red cells from 1-day-old chicks

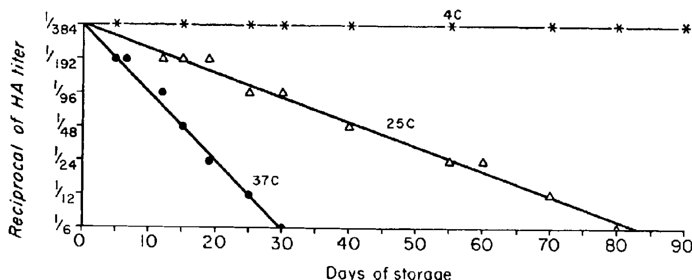

FIG. 2. Stability of hemagglutinin at different temperatures (culture fluid was diluted 1:3 with borate-buffered saline at pH 9.0 before tested).

only in a narrow pH range between 6.5 to 6.8, and is markedly unstable at temperatures above 4–6° at pH 6.5. Casals and Brown (16) indicated that group B arboviruses agglutinate chick red cells more effectively at pH 7.0 than at the pH 6.5 used for group A arboviruses.

In this study, cell culture HA was more influenced by pH but less influenced by temperature (4 to 36°) than mouse brain HA. These results are in accordance with those previously reported for HA from infected hamster kidney cell culture (9, 12).

Cell culture HA offers many advantages over mouse brain antigen. A titer of 1:384 to 1:1536 per 0.5 ml can be attained within 2 days of virus inoculation without laborious or risky manipulations. The cell culture HA is stable at 4° for at least 3 months and at —60° for 1 year or longer, if the pH is adjusted to about 8.4. Since the results obtained with cell culture antigen are very similar to those with mouse brain antigen, we recommend that mouse brain antigen be replaced by cell culture HA in routine HAI testing. The relatively unlimited supply of continuous embryonic cells and the ease of production compensate for the lower HA titers obtained in cell culture.

Summary. Hemagglutinin of titer 1:384 or more could be produced regularly in continuous hamster embryonic cell culture 2 to 4 days after injection with JEV, provided that the culture was maintained with serum-free medium containing bovine or human albumin. The cell culture HA was stable for more than 3 months at 4° and 1 year at —60°.

Titers of cell culture HA were highest when phosphate buffer salines of pH 7.3 and 4.3 were used as diluents for HA and red blood cells, respectively. The optimal pH of the HA-red cell mixture was about 6.5. Goose red cells yielded better results than cells from day-old chicks or roosters.

Two hundred and forty-four sera from monkeys and pigs were tested in parallel with both cell culture and mouse brain HA for HAI antibody. There was a high degree of correlation between the titers obtained (0.95).

Replacement of traditional mouse brain HA by cell culture antigen in the HAI test of JEV infection is recommended.

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