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NOTES

Detection of Antibodies to Alphaviruses by Enzyme-Linked Immunosorbent Assay

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Cell culture-derived antigens detected antibodies to alphaviruses in human sera with the enzyme-linked immunosorbent assay technique. Results correlated with those from hemagglutination inhibition and neutralization tests.

Antibody for alphaviruses has classically been measured by the complement fixation, hemagglutination inhibition (HI), and neutralization tests (3, 4). Each of these tests has drawbacks involving sensitivity, specificity, rapidity, or expense. The enzyme-linked immunosorbent assay (ELISA) has been widely applied to the study of viruses (1, 10) and for two togaviruses, rubella (6) and tick-borne encephalitis (7), has proved highly sensitive, reliable, quick, and inexpensive. The present communication reports the adaptation of ELISA for alphaviruses, its application to the evaluation of serological response to Venezuelan equine encephalitis (VEE) vaccine, and its use without elaborate equipment as a field technique for serological survey and diagnosis.

The coating antigens for both Sindbis and VEE were prepared in the following manner. Virus was grown in CER cells (9) in Eagle minimal essential medium with Hanks balanced salts. The culture medium was harvested at the first evidence of cytopathic effect, centrifuged at 11,000 × *g* for 20 min at 4°C, and filtered through a 0.45-μm filter. The virus was concentrated by precipitation with polyethylene glycol, using 10% (wt/vol) polyethylene glycol 6000 and 2.3% (wt/vol) NaCl. The precipitate was suspended in a STE buffer [NaCl, 5.84 g/liter; ethylenediaminetetraacetic acid, 0.37 g/liter; tris-(hydroxymethyl) aminomethane-hydrochloride, 1.21 g/liter, pH 7.2], and 10 ml was placed on top of a gradient consisting of 3 ml of 60% sucrose, 2 ml of 25% sucrose, and 1 ml of 5% sucrose in STE buffer. After centrifugation for 2 h at 95,400 × *g*, the visible band was harvested, and the material was stored at −70°C.

Antigen was diluted in coating buffer (Na_2CO_3, 1.59 g/liter; $NaHCO_3$, 2.93 g/liter; pH 9.6), and 0.21 ml was added to each well of a micro ELISA plate (Cooke Engineering Co., Alexandria, Va.). The antigen was allowed to absorb overnight at 4°C, and then the plate was washed three times with a solution of phosphate-buffered saline–Tween 20 (PBST) (0.5 ml of Tween 20 per liter, pH 7.4). Material was aspirated from the plates with a plastic pipette tip connected via rubber tubing and a series of traps to the house vacuum. PBST was added manually with a 250-ml plastic squeeze bottle. Human sera which had been stored at −20°C were used without further treatment except where noted. Sera were diluted in PBST, and 0.21 ml was incubated in each well for 2 h at room temperature. The plate was washed with PBST three times before 0.21 ml of the conjugate, alkaline phosphatase-labeled sheep anti-human immunoglobulins (Microbiological Associates, Bethesda, Md.), was added to each well. After a 2-h incubation at room temperature, the plate was washed three times with PBST. The *p*-nitrophenyl phosphate substrate was dissolved (1 mg/ml) in diethanolamine buffer (97 ml/liter, pH 9.8) and was added to the plate. The color was allowed to develop until reference positive and negative sera showed the desired contrast. The reaction was stopped by the addition of 0.05 ml of 3 M NaOH. The intensity of the color was rated on a four-point scale by visual examination. Wells showing a +3 or +4 intensity were considered positive. Repeat titrations with several sera resulted in titers that varied by one twofold dilution or less. Results read by two investigators were within one twofold dilution, with one set being consistently higher for positive sera, and agreement was noted with the negative sera.

The optimal dilution of coating antigen was determined by titration of the antigen with reference negative and positive sera and selection

of the dilution that gave the best resolution between the two sera. This dilution did not change in antigen preparations used repeatedly for a year. Inactivation of infectivity by treatment of the final antigen with 0.3% beta-propiolactone did not detectably alter its properties in ELISA.

The amount of virus that attached to the plates was determined as a function of time and virus concentration, using Sindbis virus labeled with [^{3}H]uridine (gift of A. L. Smith). The results are shown in Fig. 1. The amount of attachment increased appreciably with time in the 1:100 to 1:400 dilution range, which included the optimal dilution of most coating antigens. Plates coated with high concentrations of virus for short periods of time gave a high background with negative sera, and this interfered with the visual evaluation of the test. When intensity of the ELISA reaction was correlated with the number of counts, plaque-forming units, and particles attached for two strains of Sindbis, a direct correlation between the amount of material attached and the intensity of the reaction was found. All positive wells contained more than 2×10^4 plaque-forming units of virus as titrated in CER cells and more than 10^6 viral particles (8).

A Sindbis antigen was used to test 24 human sera collected in 1977 in connection with the

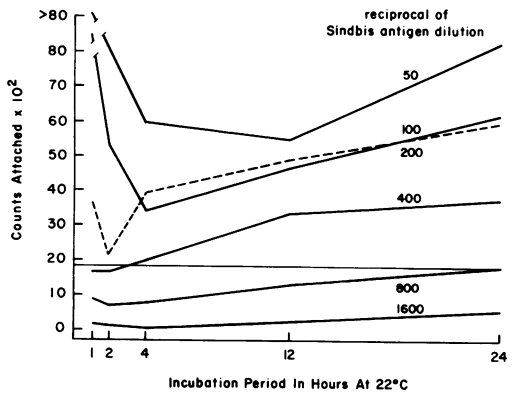

FIG. 1. *Counts attached as a function of time and dilution of Sindbis antigen on ELISA plates. Each point is the average of three determinations. All points above solid line parallel to x-axis gave positive readings.*

TABLE 1. *Comparison of anti-Sindbis ELISA and HI titers in Senegalese human sera*[a]

Serum	Titer	
	ELISA	HI
51	10	10
385	160	20
398	80	40
585	160	40
664	80	40
701	80	20
775	40	40
866	40	40
997	80	>80
385[b]	160	20

[a] Fourteen additional sera were <10 by HI and ELISA.
[b] Internal repeat.

TABLE 2. *Anti-VEE ELISA titers after VEE vaccination*

Vaccinee	Days after vaccination	ELISA	HI	NT[a]
1	0	0[b]	0	–
	+286	>80	640	+
2	−588	0	0	
	+29	160	>80	
3	−3	10	80	
	+36			+
	+192	20	20	
4	−193	0	0	
	+31	40	10	+
5	−1,120	0	0	–
	+261	0	0	–
6	0	0	0	–
	+286	40	40	
	+741	20	40	
7	0	0	0	–
	+38	>320	>80	+
8	−1,233	0	0	
	+132	20	10	
9	−344	10[c]	10	
	+28		0	–
	+342	10	0	
10	−2,049	80[d]	10	
	+28	40	40	–
	+1,913	80	20	
	+2,778[e]	80	10	
11	Normal[f]	0	0	
12	Normal	0	0	
13	+1,360	80	20	
	+2,192	80	20	

[a] NT, Neutralization test.
[b] Reciprocal of serum titer; 0 = <10.
[c] Received Eastern equine encephalitis virus vaccine 8 years before.
[d] Infected with Mayaro virus 3 years before.
[e] Revaccinated day +2,763.
[f] Unvaccinated control.

Senegal River Pilot Health Research Program supported by the United States Agency for International Development. The data shown in Table 1 correlated completely with results of the HI test (5) as far as the presence or absence of antibody was concerned, but ELISA was generally more sensitive. A VEE antigen was reacted with pre- and postvaccination sera (Table 2). The results were compared with HI and neutralization test (2) data and vaccination status; ELISA, HI, and neutralization test results corresponded, and ELISA detected all vaccination conversions.

Nonspecific reactions occurred when the test was performed with several sucrose–acetone-extracted baby mouse brain antigens prepared by the method of Clarke and Casals (5). Experiments with normal mouse brain antigens showed that the sera were reacting with the mouse brain. Extraction of the sera with either acetone or acetone-ether yielded no detectable decrease in non-specificity, but when sera were absorbed with normal mouse brain, a slight improvement was noted. Postcoating of the plates with bovine albumin, fetal calf serum, and rabbit serum resulted in no appreciable reduction in the nonspecific background.

The ELISA technique requires little complicated equipment, utilizes small amounts of serum and antigen, and uses reagents that are readily obtained and have a long shelf life. The key to the successful use of ELISA with alphaviruses is the preparation of a pure coating antigen. Results with cell culture-grown, gradient-purified antigens have established that ELISA detects antibody to alphaviruses in human sera. The antigen can be inactivated for safe use in the field. A high correlation with results of HI and neutralization tests was found.

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