NOTES

Hemagglutination and Hemagglutination Inhibition Tests with Enterovirus Type 70

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Human type "O," guinea pig, and chicken erythrocytes were agglutinated by enterovirus type 70 at 4°C or room temperature. A hemagglutination inhibition test, using human "O" erythrocytes, is described for the serological diagnosis of acute hemorrhagic conjunctivitis caused by enterovirus type 70.

Hemagglutination (HA) by enterovirus type 70 (EV70) was examined during our initial studies on the general characteristics of the virus (4), but our early attempts to demonstrate HA by EV70 proved unsuccessful. Esposito et al. (1) were also unable to demonstrate HA in similar studies.

However, we have now been able to demonstrate HA activity with EV70, and a HA inhibition (HI) test has been developed as a simple means for diagnosing acute hemorrhagic conjunctivitis (AHC).

The prototype strain of EV70, J 670/71, was used as the HA antigen in all experiments. To prepare the HA antigen, the stock virus, which had been passaged three times in human embryonic kidney cell cultures, was subjected to three further passages in LLCMK2 cells (3) at 33°C (6). The last passage was made in cells grown in roller bottles. The growth medium was Eagle minimal essential medium containing 5% calf serum, and the maintenance medium was the same without serum. Infected culture fluids were harvested 18 h after inoculation, frozen and thawed three times, and then centrifuged at $15,000 \times g$ for 30 min. The supernatant fluid was stirred with 8% polyethylene glycol 6000 at 4°C overnight and centrifuged at $15,000 \times g$ for 30 min. The resulting pellet was resuspended in 1/50 the original volume of phosphate-buffered saline (pH 7.6) (137.9 mM NaCl-2.7 mM KCl-8 mM Na_2HPO_4 -1.5 mM KH_2PO_4), and coarse debris was removed by low-speed centrifugation. CsCl was added at 0.5 g/ml of supernatant fluid, and the solution was centrifuged in a Beckman SW 50.1 rotor at 40,000 rpm for 42 h at 4°C. Fractions of 0.3 ml were collected by piercing the bottom of the tube. Three of the fractions

with buoyant densities close to 1.34 g/ml were pooled and used as the source of HA antigen; the infectivity titer of the pooled fractions was $10^{9.5}$ 50% tissue culture infective doses per ml.

Hyperimmune sera against the prototype J 670/71 strain were prepared in monkeys and rabbits. The immune monkey sera were prepared as reported previously (5), and the immune rabbit serum was prepared by five intravenous injections of 5 ml of purified EV70 at intervals of 3 days. A booster injection was given 36 days following the primary course of immunizations, and the serum was collected 10 days later. Eight different 0.4% suspensions of erythrocytes (human "O," guinea pig, chicken, swine, cattle, horse, sheep, and rabbit) were prepared according to the method of Hierholzer and Suggs (2).

In the first experiments, twofold serial dilutions of HA antigen were distributed in 0.2-ml amounts to small test tubes, to which the same volume of 0.4% erythrocyte suspension was added, using phosphate-buffered saline (pH 7.6) as the diluent. The mixtures were incubated at 4°C for 18 h, at room temperature (20 to 25°C) for 2 h, or at 37°C for 1 h. HA titers were determined from the sedimentation pattern of the erythrocytes.

As shown in Table 1, the purified preparation of J 670/71 virus agglutinated human "O," guinea pig, and chicken erythrocytes, but did not agglutinate those of the other five species. HA of human and guinea pig cells was positive at 4°C and room temperature, but negative at 37°C. On the other hand, chicken erythrocytes were agglutinated at all three temperatures, although HA at 37°C was only seen at a dilution of 1:10. HA titers were highest with human "O"

TABLE 1. HA titers of EV70 (J 670/71 strain) obtained with erythrocytes from various animals at three different incubation temperatures

Erythrocytes ^a	HA titer ^b at temp:		
	4°C	Room temp ^c	37°C
Human type "O"	3,200	1,600	<10
Guinea pig	1,600	800	<10
Chicken	400	100	10
Swine	<10	<10	<10
Cattle	<10	<10	<10
Horse	<10	<10	<10
Sheep	<10	<10	<10
Rabbit	<10	<10	<10

^a Samples were 0.4% suspensions in phosphate-buffered saline (pH 7.6).

^b Reciprocal of highest antigen dilution showing HA. Since a 100-fold dilution was HA negative, 10-fold dilutions were retested, but remained HA negative.

^c 20 to 25°C.

cells, followed by guinea pig cells, and lowest with chicken erythrocytes at 4°C or room temperature.

After determining the optimal conditions for HA by EV70 in tubes, tests were then done by the microtiter method. Serial twofold dilutions of HA antigen were made in 0.05-ml volumes in wells of disposable U-plates (Cooke) to which an equal volume of 0.4% human "O" erythrocyte suspension was added. The mixture was mixed thoroughly and incubated at room temperature for 2 h or 4°C for 18 h. HA titers were expressed as the reciprocal of the highest antigen dilution showing complete HA. The diluent used for the microtiter method was phosphate-buffered saline (pH 7.6) supplemented with 0.0005% gelatin and 0.005% bovine serum albumin fraction V. HA titers obtained by the microtiter method were identical to those obtained with the tube method. Appropriate controls were run in both the tube and microtiter tests; the diluents used in each test and the supernatant fluid from uninfected cell cultures had no HA activity.

Figure 1 is a scanning electron micrograph showing the adsorption of EV70 onto the surface of human "O" erythrocytes. Washed erythrocytes (0.4% suspension) were mixed with an equal volume of purified EV70 (128 U of HA) and allowed to adsorb the virus for 10 min at room temperature, before fixation with 2% glutaraldehyde in 0.2 M cacodylate buffer, followed by dehydration with varying concentrations of ethanol. Subsequently, the preparations were taken through isoamyl acetate before critical-point drying, using liquid CO₂. They were coated with a gold-palladium alloy while the stage was rotated, and samples were observed with a JEM-

100 CX/ASID electron microscope.

For HI, each serum was pretreated by the Rivanol (2-ethoxy-6,9-diamino-acridine lactate) method of Styk et al. (8) to remove nonspecific inhibitors. The animal sera were adsorbed with the various erythrocyte suspensions to remove natural agglutinins. Serial twofold dilutions of serum were then made in 0.025-ml volumes and mixed with 4 U of HA antigen in 0.025 ml. The mixtures were incubated at 4°C for 18 h, and 0.05 ml of 0.4% human "O" erythrocyte suspension was added to each well. The immune and nonimmune animal sera served as positive and negative controls in each HI test after the specificity of HI had been established. If the control immune serum showed the expected HI titer, the HI titers of test sera were taken as the reciprocal of the highest dilution showing complete HI. The other controls for HI were as follows: a back titration of hemagglutinin known to contain 4 U in the test; and a mixture of erythrocyte suspension and diluent as a control on the sedimentation pattern of the cells.

HI antibody titers of normal and hyperimmune sera from monkeys and rabbits are shown in Table 2. Preimmune animal sera had no inhibitory activity, but two monkey and one rabbit hyperimmune sera showed antibody titers up to 1:512 to 1:4,096, which were comparable to those seen in neutralization tests described previously (5).

With guinea pig or chicken erythrocytes, HA was also inhibited by immune sera against EV70 (data not shown). However, we employed only human "O" cells in the standard HI procedure, since the lower HA titers obtained with guinea pig and chicken cells were considered disadvantageous.

HI tests were performed on 10 pairs of sera of patients in acute and convalescent phase of AHC (Table 3). A significant increase of HI antibody titer against EV70 was shown with all serum pairs, and the results were in complete accordance with those of neutralization tests.

Since HA by EV70 was found to be highly sensitive to nonspecific inhibitors in serum, we used Rivanol to remove them; this technique seemed to be satisfactory for serological diagnosis of AHC as reported here. Kaolin and sodium periodate treatments were found to be unsatisfactory. However, Rivanol treatment destroyed immunoglobulin M antibody and often gave lower HI antibody titers than were seen by neutralization tests (unpublished data). Therefore, this should be considered when sera supposed to contain immunoglobulin M are examined.

It is likely that our initial failure to detect HA was due to a low concentration of the virions or

FIG. 1. Scanning electron micrographs of EV70 adsorbed onto the surface of human "O" erythrocytes and control preparations. (a) Agglutinated erythrocytes at low magnification (×5,000). (b) Surface of a cell in (a) at a higher magnification (×50,000). Numerous particles of 30- to 35-nm diameter are seen on the surface. The particles are considered to be EV70 virions [see micrograph of virions in (e)]. (c) Untreated erythrocytes at low magnification (×5,000). (d) Surface of a cell in (c) but at a higher magnification (×50,000). (e) Purified EV70 virions in a higher magnification (×50,000), prepared by the freeze-drying method (7).

TABLE 2. Results from HI and neutralization tests on normal and hyperimmune sera from monkeys and rabbits against EV70

Serum	Antibody titer ^a	
	Neutralization	HI
Normal monkey	<4	<8
Immune monkey A	4,000	512
Immune monkey B	3,200	1,600
Normal rabbit	<4	<8
Immune rabbit	5,120	4,096

^a Reciprocal of the highest serum dilution giving a positive response.

TABLE 3. Results of HI and neutralization tests of paired sera from patients with AHC

Pair no.	Phase ^a	Titer ^b	
		Neutralization	HI
1	A	4	<4
	C	32	16
2	A	<4	<4
	C	256	32
3	A	<4	<4
	C	128	64
4	A	<4	<4
	C	16	32
5	A	<4	<4
	C	256	128
6	A	<4	<4
	C	16	16
7	A	64	16
	C	256	128
8	A	<4	<4
	C	64	64
9	A	<4	<4
	C	128	128
10	A	<4	<4
	C	32	64

^a A, Acute, phase; C, convalescent phase.

^b Reciprocal of serum dilution.

to the presence of inhibitors in cellular or residual serum components of the crude extract used

as HA antigen. With crude extracts of infected-cell cultures, HA was usually negative in lower dilutions but became positive in higher dilutions. No such phenomenon was encountered with purified HA antigen. Cell substrates used for virus cultivation would likely not be the cause of negative HA results, because HA activity has been demonstrated in extracts of HeLa, as well as LLCMK2, cell cultures infected with EV70.

In summary, we conclude that HA is specific to EV70 and that HI is valuable in studies on the viral etiology of AHC.

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