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Encephalomyocarditis Virus Hemagglutination-Inhibition Test Using Antigens Prepared in HeLa Cell Cultures. (27080)

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Hemagglutinins can be demonstrated in suspensions prepared from the brain tissue of mice infected with the encephalomyocarditis (EMC) virus(1). Although cell cultures of many types support the growth of the virus (2), they have been utilized to only a limited extent for production of hemagglutinating (HA) antigen(3). During our investigations on occurrence of EMC hemagglutination-inhibition (HI) antibody in human sera, a need arose for large quantities of antigen. When reproducible results were not obtained with mouse brain suspensions, cell cultures were tried as a source of hemagglutinin. This report describes a method for preparation of EMC HA antigen in HeLa cells. Conditions necessary for optimal hemagglutination have been investigated and the findings applied to development of an HI test.

Materials and Methods. Virus. The EMC strain was recovered in this laboratory from the lung tissue of a naturally infected pig(4). It was isolated in HeLa cells and identified by neutralization tests employing a hyperimmune antiserum prepared against the American Type Culture prototype strain of EMC virus.‡ At the time of these studies the virus had received 5 consecutive HeLa passages.

HeLa Cells§ were grown as monolayers in 36 oz bottles in Eagle's medium containing 10% horse and 10% calf serum. Before virus inoculation cell sheets were washed twice with Hanks' solution; Eagle's medium containing 4% chicken serum was then added. Heated serum (56°C for 30 minutes) and antibiotics were routinely used in all media.

Diluents. The following solutions were prepared in distilled demineralized water: 0.15 M NaCl, 0.15 M KCl, 0.05 M H_3BO_3 in 0.12 M NaCl, 0.2 M Na_2HPO_4 − 0.2 M NaH_2PO_4 in 0.15 M NaCl (PO_4 buffer − NaCl) or 0.15 M KCl (PO_4 buffer − KCl) over the range pH 7.0 to 8.0, and 0.05 M H_3BO_3 in 0.12 M NaCl (BO_3 buffer − NaCl) or 0.12 M KCl (BO_3 buffer − KCl), adjusted with 1.0 M NaOH to pH 8.0 and 8.5. Occasionally, these solutions contained non-specific hemagglutinins which were readily removed by adsorption with small amounts of kaolin.

Red Blood Cells. (RBC) were collected from sheep, guinea pigs and humans in an acid-citrate-dextrose solution(5), and washed 3 times with isotonic saline. They were stored as a pack at 4°C for not longer than one week and resuspended in diluent as needed.

HA Tests. Four-tenths ml of antigen was serially diluted in 2-fold steps in 12 × 100 mm tubes. Four-tenths ml of a 0.4% suspen-

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sion of washed RBC was then added and the mixture thoroughly agitated. Several different diluents were tried (Table I). Sedimentation was allowed to occur at 4°C or at 25°C and titers were read after 2 hours.

HI Tests. Serum was adsorbed with a 25% suspension of kaolin or extracted twice with acetone(5). To remove non-specific agglutinins serum was also adsorbed with packed RBC from the same source as those used in the test. Two-tenths ml of serum was diluted by 2-fold steps and 4-8 units of antigen in 0.2 ml was added.

After incubation of the antigen-serum mixture at room temperature for 1 hour 0.4 ml of a 0.4% suspension of RBC was introduced and the tubes thoroughly agitated. The antigen and RBC diluent are discussed below. Tests were read by the pattern technique following sedimentation at room temperature for about 90 minutes. Serum controls were included in all tests.

Virus Titrations. Serial ten-fold dilutions of virus were inoculated in 0.1 ml amounts into each of 2 HeLa tube cultures and endpoints were read after 5 days by cytopathic effect (CPE). Titers (TCD_{50}) were calculated by the method of Reed and Muench.

Results. Hemagglutinin Preparation. In attempts to demonstrate hemagglutinin in EMC-infected HeLa cell cultures, the medium overlying the cell sheets was HA tested at various times after the appearance of CPE. Hemagglutinin was usually not present in cultures exhibiting CPE, even though the fluid often contained moderate amounts of infectious virus ($10^{5.5}$ — $10^{6.5}$ TCD_{50}/ml). However, if the cultures were first subjected to alternate cycles of freezing and thawing, hemagglutinin at titers of 1:64 to 1:128 was readily demonstrated in the culture fluids. Because this suggested that physical destruction of infected cells was associated with the release of the hemagglutinating antigen, the following procedure was developed for routine preparation of antigen pools.

Monolayer bottle cultures were inoculated with 10^3 to 10^4 TCD_{50} of virus, a dosage known to initiate CPE in about 48 hours. When CPE was apparent cell sheets were washed with Hanks' solution and 50 to 60 ml of Eagle's medium without serum were added. After incubation for 6 hours at 36-37°C the cells and overlying medium were subjected to 2 quick cycles of freezing and thawing. This was accomplished by alternately immersing the bottle in baths of acetone-dry ice and 37°C water. The cell remnants and culture fluid were homogenized in a TenBroeck grinder and the preparation clarified by centrifugation. Supernatant fluids were stored at −20°C.

Diluents. Different solutions were tried with the purpose of determining whether the chemical composition or pH of the diluent influenced the hemagglutination reaction. Hemagglutination occurred at antigen dilutions greater than 1:4 only when diluents containing potassium (K) or boron (B) ions were used (Table I). Optimal results were obtained in the presence of both ions. The reaction appeared not to be pH dependent as it occurred in K or B solutions over a wide pH range. Interestingly, hemagglutination was not demonstrated in phosphate buffered solutions even when K or B ions were added. As a result of these findings, the BO_3 buffer-KCl solution adjusted to pH 8.0 was used routinely as the diluent in subsequent studies.

TABLE I. Effect of Chemical Composition of Antigen and RBC Diluent on Hemagglutination by EMC HeLa Antigen.

Antigen Diluent*	RBC Diluent*	Final pH	HA Titer‡
NaCl	NaCl	ND†	4
idem	PO_4 buffer—NaCl	7.0	<4
"	idem	7.5	<4
"	"	8.0	<4
"	PO_4 buffer—KCl	7.0	<4
"	idem	7.5	<4
"	"	8.0	<4
"	BO_3 buffer—NaCl	8.0	4
"	idem	8.5	<4
H_3BO_3—NaCl	NaCl	ND	8
BO_3 buffer—NaCl	PO_4 buffer—KCl	8.0	<4
idem	NaCl	8.0	8
"	BO_3 buffer—NaCl	8.0	16
"	KCl	8.0	64
KCl	idem	ND	16
idem	BO_3 buffer—NaCl	8.0	64

* Diluent composition and concentration defined in text.
† Not done.
‡ Expressed as reciprocal of antigen dilution.

RBC. It has been reported(1,6) that optimal results with EMC mouse brain antigens were obtained by using sheep RBC at low concentrations (0.25%) and overnight sedi-

mentation of cells at 4°C. Since in our locality sheep RBC were not readily available, guinea pig and human Type "O" erythrocytes were tried. Titers with these cells did not substantially differ from those obtained by us with sheep RBC. HA tests employing guinea pig or human RBC gave similar results at sedimentation temperatures of 4° and 25°C. While guinea pig RBC were used in most experiments, human "O" cells were found to be useful in HI tests of human serum because adsorption for nonspecific agglutinins was not necessary. The HI testing of large numbers of sera was facilitated by using RBC concentration of 0.4% and carrying out tests at room temperature, since it was possible to read endpoints after cell sedimentation for 90 to 120 minutes.

Hemagglutinin Stability. The antigen was stable at pH 8.0 in the BO_3 buffer-KCl diluent: no significant change in titer was noted after several months' storage at −20°C, up to 8 weeks in the refrigerator at 4°C and after standing overnight at room temperature.

Serum Inhibitors. In HI tests with human and swine sera known to be free of EMC-neutralizing antibodies, naturally occurring inhibitors were frequently found. Removal of these inhibitors was readily accomplished by treatment with kaolin but not by acetone extraction (Table II). HI titers were easily read after kaolin treatment of serum since hemagglutination patterns were smooth and slippage of cells rarely occurred.

TABLE II. Removal of Non-Specific EMC Hemagglutination Inhibitors by Kaolin but not Acetone.

Serum	Treatment		
	None	Kaolin	Acetone
Human	16*	<8	32
	64	<8	16
	8	<8	S1†
Swine	32	<8	16
	8	<8	8
	16	<8	16
	16	<8	16

* Reciprocal of serum dilution completely inhibiting hemagglutination by 4 units of antigen.
† Slippage.

HI Tests. We have employed the methods described to survey human sera for EMC antibody and to follow the serological response of swine experimentally infected with the virus. Of 33 human sera shown to possess EMC neutralizing antibody, 19 were found to have HI titers of 1:4 or greater. Titers of 1:64 or higher were demonstrated in several of these sera. Only one of 35 sera negative by neutralization test had HI antibody. HI antibody was found in the serum of swine as early as 8 days following the oral administration of EMC virus. In these animals an increase in HI titer was associated with the appearance of serum neutralizing antibody.

Discussion. Jungeblut and Kodza(7) were unable to obtain HA titers greater than 1:4 with the Col SK strain of EMC virus adapted to HeLa cells. These workers suggested that the relatively low concentration of infectious virus in the medium overlying the cell sheet ($10^{5.5}$ in tissue culture fluids as compared to 10^8-10^9 in the mouse brain) accounted for the low HA titer. Our prelimintary tests using culture fluids as antigens gave similar results. Antigen pools with high HA titers were regularly obtained only when the cells exhibiting CPE were alternately frozen and thawed.

Suspensions of EMC infected mouse brain have been used to determine some of the physical and chemical conditions necessary for hemagglutination(1). This reaction was found to occur optimally in the presence of K but not Na or the divalent cations, Mg and Ca. Using tissue culture antigen, we confirmed the K ion requirement. It is of interest that hemagglutination was also demonstrated in presence of the trivalent B ion. The diluent adopted for routine use in the HI test gave consistently satisfactory results and made possible standardization of pH without phosphate buffer. Gard(6) has reported that PO_4, as well as several other polyvalent anions, inhibits EMC hemagglutination. Our tissue culture antigen did not hemagglutinate in phosphate buffered solutions even when K or B were added.

The EMC HI test described here offers certain definite advantages over previously reported methods(1,6). Large amounts of a relatively "clean" antigen can be prepared in tissue cultures. The use of higher concentrations of RBC and room temperature for sedimentation makes the test quicker to perform and less cumbersome. Kaolin adsorption of serum adds to the clarity of results and effectively removes non-specific inhibitors. In our

hands, the test has been no more time consuming or difficult than procedures now employed to detect myxovirus HI antibodies(8). Preliminary results suggest that the HI test can be utilized for purposes of diagnosis and population survey.

Summary. 1. A stable EMC hemagglutinating antigen was prepared by alternate freezing and thawing of virus-infected HeLa cell cultures. 2. Optimal hemagglutination titers were obtained at 4° and 25° C with human and guinea pig erythrocytes when diluents containing potassium and boron ions were used. 3. A hemagglutination-inhibition test employing kaolin treated serum and tissue culture antigen is described and its practical usefulness in detecting antibody in human and swine serum is demonstrated.

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Quantitative Aspects of Friend Leukemia Virus in Various Murine Hosts.*† (27081)

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The leukemia virus described by Friend (FV) is considered to have a narrow and specific adult murine host range(1,2,3), severely limiting the type of experiments which may be carried out. This virus has a relatively short latent period characterized by splenomegaly about one week after inoculation, with blood changes occurring later in the disease. Because of the obvious advantages of utilizing a virus producing early onset of overt disease, it seemed desirable to reinvestigate the host range with particular attention to the reliability of splenomegaly as an early indicator of infection and susceptibility. It was thought possible that inapparent infection might occur in some strains of mice without the usual early pathognomonic signs.

Materials and Methods. C57BL/Crgl, C3H/Crgl, C3H/Crgl/2, DBA/2NCrgl, A/Crgl mice were obtained from Cancer Research Genetics Laboratory, Univ. of California, Berkeley. BALB/c mice were either from our own colony or Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and are so indicated. The random bred Swiss mice were from the colony of the Hooper Foundation. All animals were 6-8 weeks old at time of inoculation.

The Friend virus used in these studies was received from Dr. C. F. T. Mattern of the Virus Laboratory, Univ. of California, Berkeley. He had received it from Dr. Charlotte Friend after an unstated number of passes in Swiss mice. The stock virus used was from a large pool of spleens prepared from the 11th pass in Swiss mice in this laboratory. Spleens were homogenized in a sucrose-phosphate buffer solution as a 20% suspension. This was centrifuged at 2,000 rpm for 10 minutes and the supernate distributed in sealed ampules

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