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Demonstration of Encephalomyocarditis Virus Antibody in Human Serums From Panama. (28714)

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The importance of the encephalomyocarditis (EMC) virus as a cause of disease in man is not known. Apparently unrecognized human infections occur inasmuch as antibody has been found in serums collected in many parts of the world. In 1958 EMC virus was recovered for the first time in Panama from swine dying with myocarditis(1). As a result of the isolation of this virus from a common domestic animal, we undertook an investigation to determine whether EMC serum antibody was present in the human population of the Isthmus. We report here results of studies on 180 sera and describe our experience with the hemagglutination inhibition (HI) test and neutralization (NT) tests employing HeLa cell cultures and mice.

Materials and methods. Serum. Blood specimens were obtained from inhabitants of 4 widely separated, geographically distinct areas of the Republic of Panama. Blood was aspirated with B-D vacutainer tubes or by syringe and was stored at environmental temperatures for 1 to 3 days. After transportation to the laboratory, serum was separated and stored for varying periods at –20°C.

Virus. The EMC virus was isolated in both mice and HeLa cells from the lung tissue of a naturally infected pig(1). It was identified in NT tests employing antiserum prepared against the American Type Culture strain of EMC virus.

Tissue culture (TC) NT tests. Virus which had been passaged 5 and, in some instances, 6 times in HeLa cell cultures was used in all tests. The medium overlying the cell monolayer was harvested after the appearance of cytopathic effect (CPE) and served as the virus pool. Tube cultures of HeLa cells which had been grown in Eagle's medium, containing 10% horse and 10% calf serum, were washed 2 times with Hanks' solution and maintained in Eagle's medium with 5% chicken serum. Antibiotics and heat-inactivated (56°C for 30 minutes) sera were used in all media. Cultures were incubated in stationary racks at 36-37°C.

Two types of NT tests were carried out. In the population survey, equal quantities of virus and inactivated whole serum were incubated together at 37°C for 30 minutes and 0.1 ml aliquot of the mixtures were introduced into each of 2 tube cultures of HeLa cells. Four virus dilutions were routinely used and CPE titers were determined on the 4th day after inoculation. The culture medium was not changed during the test. Several human sera lacking neutralizing activity were included in each test run and the titer(2) obtained with the majority of these controls was used to calculate the neutralization index (NI). In some instances sera were screened against 100 tissue culture infectious doses (TCD_{50}) of virus and those exhibiting neutralizing activity were retested against 4 virus dilutions.

In the second type of NT test, sera were diluted in 2-fold steps and tested against 100 TCD_{50} of virus. Serum-virus mixtures were

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handled in the manner described above and the serum antibody endpoint was calculated on the 4th day.

Mouse NT tests. The virus had received 6 intracerebral passages in suckling mice and was prepared as a 20% brain suspension. Equal quantities of inactivated whole serum and decimal dilutions of virus were incubated together at 30°C for 30 minutes. A 0.03 ml aliquot of the mixture was then inoculated intracerebrally into each of 5 or 6 recently weaned mice. Individual serums were tested against 4 or more virus dilutions. In this test virus titers(2) were determined on the 6th and 12th days. Two antibody-negative human serums were included in each test and these controls were used for calculation of the NI.

Hemagglutination inhibition tests. The method for preparation of antigen in HeLa cell cultures and the details of the testing procedure have been reported(3). In brief, 0.2 ml of unheated serum was adsorbed with a suspension of 10% kaolin and diluted in 2-fold steps. Four units of hemagglutinin in 0.2 ml were added to 0.2 ml of the treated serum and the mixture incubated for 60 minutes at room temperature. Four-tenths ml of a 0.4% suspension of washed, type O human erythrocytes was then added and sedimentation allowed to occur. A diluent containing 0.05 M H_3BO_3 and 0.12 M KCl adjusted to pH 8 was used routinely. The last serum dilution completely inhibiting hemagglutination was considered the antibody endpoint.

Results. One hundred and eighty human serums were tested for EMC neutralizing antibody in HeLa cell cultures using decimal dilutions of virus and heat-inactivated, whole serum. Thirty-six (20%) of the specimens had NI of 100 or more, with several exceeding NI 1000; although a few sera neutralized less than 100 TCD_{50}, the majority exhibited no activity. On the basis of the distinct differences in neutralization by individual sera and the results of the HI test described below, NI of 100 or more was considered evidence of specific antibody.

Additional studies were carried out to evaluate the usefulness of other serologic

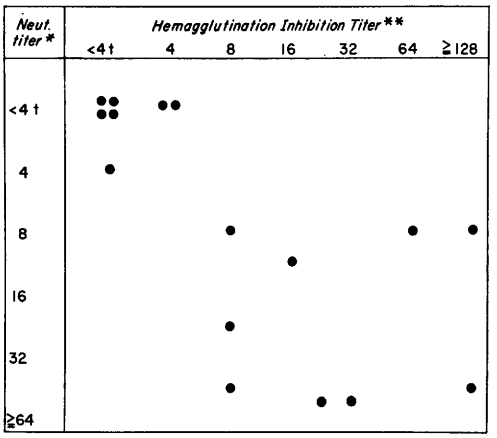

FIG. 1. Comparison of EMC neutralization and hemagglutination inhibition titers of human serums. * Neutralization titer: serial, 2-fold dilution of inactivated serum against 100 TCD_{50} of virus in HeLA cell cultures. ** Serial, 2-fold dilution of serum against 4 hemagglutinating units of antigen. † Reciprocal of serum dilution.

tests. These studies were limited by the quantity of individual sera available. Sixteen of the 36 sera positive in the NT test were diluted in 2-fold steps and tested in HeLa cell cultures against 100 TCD_{50} of virus. Ten of the 16 neutralized the virus at a dilution of 1:4 or higher, and 2 were positive at 1:64, the maximal dilution tested (Fig. 1).

Twenty-two serum specimens which had been tested previously in TC were evaluated in mouse neutralization tests. Ten of the 17 TC positive sera neutralized 100 lethal doses (LD_{50}) or more of virus by the 6th day of the mouse test, whereas on the 12th day only 4 specimens inactivated this amount of virus. Thus, with many antibody-containing sera there appeared to be a "break-through" of virus between the 7th and 10th day of the test. All 5 specimens which lacked activity in the TC test neutralized less than 100 LD_{50} of virus in mice.

HI tests were carried out on 33 of the 36 sera positive in the TC NT test and 35 other sera which lacked evidence of neutralizing antibody. HI titers of 1:4 or higher were found in 19 (58%) of the 33, whereas only 1 of the 35 controls (3%) was positive (Table I). The rough correlation between titers obtained by the HI and NT tests with the 16 sera tested after serial dilution can be

TABLE I. Comparison of Neutralization Indices and Hemagglutination Inhibition Titers of Human Serums.

Neutralization index*	No. tested	Hemagglutination inhibition titer							
		<4†	4	8	16	32	64	128	≧256
≧100	33	14	6	5	2	2	2	1	1
<100	35	34		1					

* Undiluted, inactivated serum tested in HeLa cell cultures.

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

seen in Fig. 1. Specimens with high HI titers were consistently positive in the NT test. Neutralizing activity after dilution was not demonstrable in 4 of the 5 serums lacking HI antibody.

As noted under *Materials and methods,* 180 blood specimens were collected from inhabitants of 4 typical, but topographically different, rural areas of Panama in an attempt to obtain a representative sample of the population. The study groups from 2 of the 4 areas (Chiriqui and Panama Provinces) were made up of residents of the savannah which borders the Pacific coast of the Isthmus. The third group (Darien Province) comprised persons from isolated communities in a heavily forested region of the Isthmus and the fourth (San Blas Province) consisted of inhabitants of coastal and island villages on the Caribbean. No attempt was made to sample major urban centers. Although persons of all ages were bled, the very young and old were usually excluded.

Table II summarizes results of NT tests on these sera. A substantial proportion of each of the 4 population groups was found to have neutralizing antibody. Although the incidence in individual groups varied from 15 to 33%, the differences are of doubtful significance because of the size of the samples.

Antibody was found more often in the serum of children than adults. Eighteen of the 74 (24%) persons under the age of 20 possessed antibody whereas only 10 of 84 (12%) over 20 years were positive. Moreover, the incidence in children under 10 years was more than 4 times that in adults of 30 and over. Irrespective of age, the incidence in males was similar to that in females. Although the age distribution of HI antibody was similar to that of neutralizing antibody, the HI titers appeared to bear no relationship to age or sex of the donors.

Discussion. We considered a serum to contain antibody if it neutralized 100 TCD_{50} of EMC virus. The observation that individual sera, in most instances, either neutralized this amount of virus or exhibited little or no activity, suggests that the test was specific. The results of the HI test support this conclusion. Fifty-eight per cent of the sera neutralizing 2 or more logs of virus were positive by HI. Although there can be little doubt that specific antibody was present in sera reactive in both tests, the significance of neutralization by an HI negative serum is unknown. Evidence accumulated in this laboratory indicates that HI titers in experimentally infected animals fall with time(4) and it appears likely that the absence of HI antibody in sera with neutralizing activity may only reflect the interval from time of exposure.

In our hands, the mouse NT test was an

TABLE II. Neutralization Test* Results on Serums from Republic of Panama by Geographic Location and Age.

Location (province)	Age (yr)						Total	%
	<10	10–19	20–29	30–49	>49	Unknown		
Chiriqui	2/5 †	1/8	2/12	1/5	0/2	—	6/32	19
Panama	4/18	3/19	3/15	0/7	0/9	—	10/68	15
Darien	2/7	6/17	2/14	1/17	1/3	1/1	13/59	22
San Blas	—	—	—	—	—	7/21	7/21	33
Total	8/30 (27%)	10/44 (23%)	7/41 (17%)	2/29 (7%)	1/14 (7%)	8/22 (36%)	36/180	20

* Undiluted, inactivated serum tested in HeLa cell cultures.

† No. of serums with NI ≧100/No. tested.

unsatisfactory method for detecting antibody. Often the NI on the 12th day of the test was substantially lower than on the 6th day, presumably due to "break-through" of virus. Our tests were carried out routinely using the IC route of inoculation and incubation of the virus-serum mixtures for only 30 minutes. Other workers(5) have successfully employed the intraperitoneal route of inoculation and longer periods of incubation, and it is possible that our procedure was relatively insensitive. The work reported here does not exclude the possibility that the mouse test would detect antibody in sera which were negative in TC.

The NT test in HeLa cell cultures using whole serum appears to be a highly sensitive means for detecting EMC antibody in human serum. Certainly it is preferable to similar tests carried out in mice. Although the HI test is less sensitive, the results show that it could be effectively applied in serological survey work.

EMC virus has been isolated in both North and South America(6,7,8,9,10). Antibody has been found in human and animal sera collected in the United States(11,12), Mexico(13), Surinam(14), Peru(12) and Brazil(10). In addition, we have demonstrated antibodies in human sera from Guatemala, Costa Rica and British Guiana. Our findings document further the widespread occurrence of EMC virus in the western hemisphere.

Summary. Encephalomyocarditis (EMC) virus neutralizing antibody was found in 36 (20%) of 180 human sera collected in 4 geographically separated, rural areas of the Republic of Panama. Hemagglutination inhibition antibody was present in over half (58%) of the sera positive in the neutralization test. Sera from more than 15% of the population sampled in each of the 4 areas contained neutralizing antibody. Children had a significantly higher incidence than adults, a difference which is unexplained.

The sera from San Blas Province were kindly supplied by Dr. E. A. Cleve.

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