An Evaluation of Various Tissues in Culture for Isolation of Eastern Equine Encephalitis Virus.* (23673)

DONALD N. MEDEARIS, JR.† AND SIDNEY KIBRICK (Introduced by J. F. Enders)
Research Division of Infectious Diseases, The Children's Medical Center, Boston

In 1936, Cox found that suspended fragment cultures of embryonic chick tissue could be employed to detect eastern equine encephalomyelitis virus in dilutions which were inactive on intracerebral inoculation of mice and guinea pigs(1). The presence of virus in such cultures was demonstrable by intracerebral inoculation of the supernatant fluid into mice. It was subsequently noted that this agent would multiply in cultures of other tissues as well, and that such multiplication was associated with a cytopathogenic effect on the cells(2,3,4,5,6). Despite these observations, however, tissue cultures have not been commonly employed for diagnostic or epidemiologic studies of this disease. Instead, 3-week-old mice, suckling mice or 9-12-day-old embryonated hens' eggs are usually recommended for such purposes(7). The propagation of poliovirus in cultures of human tissue by Enders, Weller and Robbins(8) served to reemphasize the value of tissue culture for the study of viruses, and this method has subsequently been extensively developed and widely applied. Accordingly, when epizootics and epidemics of EEE occurred in Massachusetts during 1955 and 1956, it was decided to evaluate the efficacy of such cultures for the isolation of these agents by comparing them with embryonated hens' eggs and suckling mice. The results of this study are herein presented.

Materials. Virus. Specimens of human, horse, pheasant, pigeon and sparrow cerebral tissue, naturally infected with EEE virus, were supplied by the Department of Pathology, Children's Medical Center, Boston, or through the courtesy of Mrs. Joan Daniels, Virus Section, Division of Laboratories, Massachusetts Department of Public Health. Four specimens of mouse brain artificially infected with this agent were also employed. *Aedes aegypti* mosquitoes infected with EEE strain AR-167 were kindly provided by Dr. Roy W. Chamberlain, Communicable Disease Center, Public Health Service, Montgomery, Ala. This virus, originally isolated from specimens of *Culiseta melanura* was inoculated as second mouse-brain and first chick-embryo-passage materials into half day-old chicks. The latter, during the stage of viremia, served as the source of infection for the Aedes mosquitoes. The identity of these agents in the different materials was confirmed by neutralization tests using specific antisera and cultures of chick embryonic tissue. The mosquitoes were preserved in the CO_2 icebox. The remaining specimens were either stored in the form of tissue fragments at −15°C or as supernatant fluids of 10 or 20% suspensions of the tissues in the CO_2 chest. Such fluids will hereafter be referred to as "viral suspensions" or simply "suspensions." *Tissue Cultures.* The following types of cultures were utilized during this study: stationary tube cultures of trypsinized monkey renal, human amniotic, human renal, HeLa, Detroit 6, embryonic chick, embryonic mouse, and newborn mouse brain cells, and roller tube fragment cultures of human embryonic skin and muscle and embryonic chick intestine. For HeLa and Detroit 6 cell cultures, Eagle's medium with 5% or 10% inactivated horse serum was used(9). The nutrient for newborn mouse brain tissue consisted of bovine amniotic fluid 45%, Hanks' balanced salt solution 40%, inactivated horse serum 10% and beef embryo extract 5%. This medium was adjusted to pH 7.2 with CO_2 immediately prior to use. For cultures of the remaining tissues, the bovine amniotic fluid medium was also employed, but the concentration of Hanks' balanced salt solution was increased to 45% at the expense of the horse serum constituent. In addition 0.1 ml of a 1% solution of soybean inhibitor

* Aided by a grant from The National Foundation for Infantile Paralysis.

† Holder of a Fellowship in Tuberculosis administered for The National Tuberculosis Association by The Nat. Acad. Sci., Nat. Research Council.

per 20 ml of media was added to the cultures of human embryonic skin and muscle. All media contained 100 units of penicillin and mycostatin and 100 micrograms of streptomycin per ml. The media were usually changed before inoculation of the virus and whenever the pH fell below 7.0. When cellular outgrowth was abundant, the inoculum was added and the cultures incubated at 35°-37°C in a rotating drum. The cultures were then examined at intervals for cytopathogenic changes during a minimum period of 7 days. Simplified methods for preparation of embryonic chick and embryonic mouse tissue cultures are presented in detail below. A method for growing newborn mouse brain cells developed by Dr. Simon Thiery in this laboratory, is also given. Cultures of the remaining tissues were prepared by generally accepted procedures(10).

i. *Embryonic chick tissue cultures.* 7- to 9-day-old chick embryos were washed with isotonic phosphate buffer solution and incubated with agitation (magnetic stirrer) for 1 hour at room temperature in 0.25% trypsin (Difco) solution adjusted to pH 7.8. The released cells were separated by centrifugation at 1000 rpm for 10 minutes, washed once and then resuspended in nutrient medium. In one experiment approximately 30 ml of a suspension containing 8 million cells per ml was obtained from 7 embryos which had been treated with 60 ml of trypsin. Cultures initiated with 600,000 cells suspended in 1 ml of medium were usually ready for use after 24 hours. When the media was changed at appropriate intervals the cultures remained suitable for inoculation during at least one month. Suspensions of chick embryo cells have yielded satisfactory cultures after storage at 4°C for periods up to 1 week, although formation of the cell sheet was somewhat delayed. ii. *Embryonic mouse tissue cultures.* 6 to 8 embryos obtained from a 12- to 15-day gravid Webster strain Swiss mouse were usually employed as the source of tissue. The cell suspension was prepared in a manner similar to that for the embryonic chick cultures. However, trypsinization was allowed to proceed at 37°C instead of at room temperature. Tubes prepared with a 1 ml inoculum consisting of 600,000 to 800,000 cells were suitable for use after 5 to 7 days. iii. *Newborn mouse brain tissue cultures.* Ten ml of 0.25% trypsin solution adjusted to pH 7.5 were added to a brain mince prepared from 4 to 8 ether-anaesthetized newborn mice. After 1 to 2 minutes incubation at room temperature the trypsin was discarded, a fresh aliquot added and trypsinization allowed to proceed for 5 to 10 minutes with occasional shaking. The cells were removed by centrifugation in the cold at 300 rpm for 30 minutes, washed once and resuspended in nutrient medium at a concentration of 1 million cells per ml. Cultures prepared with 1 ml of this suspension per tube were ready for use in 4-5 days.

Procedures. The evaluation of the various procedures for the isolation of the virus was carried out in 3 ways. First, cultures of various tissues were tested for susceptibility to virus strains as indicated by the appearance of cytopathic changes. Secondly, the infectivity titers of suspensions of tissues infected with a human, equine, avian and arthropod strain were determined in suckling mice, embryonated hens' eggs, and in cultures of certain tissues. Thirdly, aliquots of the same suspensions were titrated in cultures of embryonic chick and embryonic mouse tissue, and in the case of the arthropod material in cultures of newborn mouse cerebral tissue. Experiments with a given suspension of virus were carried out simultaneously in each of the systems to be compared. Dilutions of the suspension were prepared immediately prior to use and held in the cold until added to the cultures.

Comparative susceptibility of tissue cultures to EEE virus strains. The inocula were prepared by grinding specimens of infected cerebral tissue with sufficient isotonic phosphate buffer solution or bovine amniotic fluid medium to give a 10% suspension. Penicillin and streptomycin in concentrations of 100 μ and 100 μg per ml respectively were included. Each specimen was centrifuged at 2000 rpm for 10 minutes at room temperature. Cultures of various tissues, usually in duplicate, were

TABLE I. Susceptibility of Various Tissues in Culture to EEE Virus from Human, Equine, Avian and Murine Sources.*

	Embryonic chick	Monkey kidney	Human amnion	Human kidney	Human embryonic skin & muscle	HeLa
No. specimens tested	19	19	18	18	12	15
No. positive	19	15	12	0	10	0
% positive	100	79	67	0	83	0
No. cultures employed	36	36	35	35	23	25
No. positive	35	28	23	0	17	0
% positive	97	78	66	0	74	0

* Two or more strains of virus from each source were tested in each tissue.

inoculated with 0.1 ml amounts of the supernatant fluid and incubated in the roller drum.

Titration of representative strains in the suckling mouse, embryonated hens' egg and in tissue culture. Isotonic phosphate buffer solution with 2% inactivated horse serum and antibiotics was employed as the virus diluent. Ten percent suspensions were prepared as described above from specimens of infected human, equine and avian cerebral tissue. Five infected mosquitoes were ground in 5 ml of diluent to provide a virus suspension from an arthropod source. Each suspension was centrifuged in the cold at 5000 rpm for 1 hour and 10-fold serial dilutions were prepared from each supernatant fluid. Six to 16 Webster strain Swiss mice, 1 to 3 days of age, were inoculated intracerebrally with 0.02 ml and intraperitoneally with 0.03 ml of each dilution tested. Concurrently, the same quantity of virus (0.05 ml) diluted to 0.1 ml was inoculated into the yolk sac of each of five 9- to 12-day eggs and into each of 4 or 5 cultures of the tissues selected for testing. The mice were observed during a period of 2 weeks for failure to thrive, inactivity, vomiting, convulsions or death; the eggs were examined at intervals during 7 to 10 days for decreased activity, hemorrhage or death of the embryos. Tissue cultures were observed for a minimum of 7 days. Dead mice and embryos were stored intact at −15°C, and appropriate specimens were subsequently examined to confirm the presence of virus. This was done by inoculating cultures of embryonic chick tissue with 0.1 ml of the supernatant fluid from a 10% suspension of the heads or brains and noting any cytopathic effect.

Titration of the 4 representative strains in cultures of embryonic chick, embryonic mouse, and newborn mouse brain tissue. The inocula wer prepared as indicated in the above paragraph and consisted of 10-fold dilutions of the viral suspensions. Each of 3 to 5 tissue cultures was inoculated with 0.1 ml of each dilution.

Results. In the first part of this evaluation cultures of 6 different kinds of cells were examined for cytopathic changes following the addition of 10% suspensions of 19 specimens representing 15 strains of virus. These suspensions were prepared from infected cerebral tissue of 4 human beings, (7 specimens), 3 horses, 5 birds (2 pheasants, 2 pigeons, 1 sparrow) and 4 mice. Of the murine specimens, 3 represented 1st mouse brain passage of the virus from 2 human cases and the 4th consisted of the 141st mouse brain passage of a stock laboratory strain. The results are presented in Table I and indicate that of the tissues tested cultures of embryonic chick cells were the most sensitive for detection of this agent, regardless of the source of the inoculum. In these cells, moreover, the cellular destruction was marked and usually progressed rapidly to completion, although areas of epithelial growth, when present, remained unaffected by the virus. Cultures of monkey renal and human amnion cells were not only less sensitive but in these tissues, the cytopathic effect was frequently limited to specific areas at the periphery of the cell sheet and progressed slowly or not at all.

In certain instances cultures of these tissues appeared to develop a resistance to the agent as manifested by rapid regrowth of the cell sheet after the appearance of cytopathic changes. This phenomenon was also noted to

TABLE II. Infectivity Titers of Representative EEE Virus Strains in the Suckling Mouse, Embryonated Hens' Egg and Tissue Cultures.

Test system	Virus source					
	Human brain	Horse brain	Pheasant brain*		Mosquito pool*	
Tissue cultures						
Embryonic chick	7.2†	3.5	7.5	6.8	3.7	3.7
Monkey kidney	6.8	2.5	6.6		3.6	2.8
Human amnion	4.4	<2.0‡	6.6		3.8	3.8
Embryonated hens' eggs	5.3	3.4		7.2	5.3	4.5
Suckling mice	7.8	3.6		7.7	5.9	5.9

* Titrations of 2 samples prepared from the same specimen and tested at different times.
† Negative log ID_{50}/0.1 ml; endpoints calculated by the method of Reed and Muench.
‡ Lowest dilution tested.

a lesser extent in cultures of human embryonic skin and muscle. The nutrient media in several of these cultures were replaced often enough to preclude persistence of the virus originally introduced. Nevertheless, subculture and titration of the fluid from these cultures provided evidence that multiplication of the virus was continuing in the absence of a discernible cytopathic effect even after 3 months. Similar observations have been reported with this agent in cultures of rat normal and tumorous cells and in HeLa cells by Gey and his associates(3,5) and by Chambers with western equine encephalomyelitis virus (11).

Human renal cell cultures were found to be unsatisfactory for isolation of the virus, since no grossly detectable cytopathic effect was observed. When HeLa or Detroit 6 cells were employed a slight increase in granularity and occasional areas of cellular degeneration were noted with certain specimens of virus. These changes, however, were not unlike those occasionally found, though in lesser degree, in uninoculated control cultures and were therefore considered as probably non-specific. When aliquots of the fluid phase from 9 of the HeLa cell cultures were tested for the presence of virus by subinoculation into cultures of embryonic chick tissue, 5 proved to be positive. The fluids from 9 human renal cell cultures were similarly tested and virus was found in 3 of them. Thus, in certain of the cultures where no clear cytopathic changes were observed, proliferation of the agent probably occurred.

The occasional presence of uninjured epithelial cells in infected cultures of embryonic chick tissue suggested that these cells were resistant to the destructive effect of the virus. This observation was confirmed by adding large doses of the virus to cultures of embryonic chick intestine which yields an outgrowth that is primarily epithelial. The few non-epithelial elements present were quickly destroyed, but the epithelial cells remained apparently unaffected.

In the second part of the evaluation the relative susceptibilities of the suckling mouse, embryonated hens' egg and cultures of various tissues to 4 specimens consisting of infected human, equine, avian and arthropod tissue were defined by determining infectivity endpoints in each of these systems. For these experiments cultures of embryonic chick, human amnion and monkey renal cells were selected since in them the cytopathic effect is marked and they are readily available in many laboratories. The results are presented in Table II and indicate that the suckling mouse, following combined intracerebral and intraperitoneal inoculation, was the most sensitive in revealing the presence of the virus. This finding was constant with each of the 4 strains tested. Results with the remaining test systems were less consistent and varied with the virus strain. With 3 of the specimens, values obtained in cultures of embryonic chick tissue closely approximated those found in the mice. With the arthropod strain of virus, however, the cultures proved less sensitive than either mice or eggs, an observation which was confirmed in 2 separate tests.

Sharp titration endpoints were usually evident within 2-3 days in cultures of embryonic chick tissue, and in fertile eggs. The endpoints in mice were also apparent in most instances at this time, but occasionally animals, in which the cause of death was later confirmed by subculture, did not succumb until 4 to 12 days after inoculation.

Cultures of monkey renal and human amnion cells were less sensitive than those of embryonic chick tissue. It was also noted that in these cells, addition of large viral inocula frequently was followed by less widespread cellular degeneration. A similar inverse relationship between the size of the inoculum and the extent of cellular degeneration was recently described by Chambers for WEE virus in cultures of strain L cells(11).

From the results of the titrations given in Table II it can be calculated that the volume of viral suspension required to produce infection in the suckling mouse in the case of 3 of the specimens was less by 0.1 to 0.9 log than that necessary to induce cytopathic changes in cultures of chick cells. From the practical standpoint, however, it seemed that this disadvantage of the tissue culture could be overcome by using in this system a larger volume of inoculum. Accordingly, the following experiment was performed:

To each of 20 cultures of chick cells was added in a volume of 0.1 ml 3 TCD_{50} of virus as calculated from the data obtained in a previous titration of the infected horse brain suspension. The same amount of virus in a volume of 1.0 ml was added to each of 20 similar cultures. In the preparation of the inocula a mixture consisting of 2% inactivated normal horse serum in isotonic buffer solution was employed.

To determine whether the amount of tissue extractions present in a 10% suspension of nervous tissue (as usually employed in isolation attempts) might prove toxic for the cultures or impair their susceptibility to small quantities of virus, a comparable experiment was simultaneously performed in which the virus was diluted in the supernatant fluid from a suspension of normal horse brain prepared in horse serum-isotonic phosphate buffer solution. At the same time the infectivity endpoint of the virus was also confirmed in 2 separate titrations in which as diluent normal horse brain suspension and horse serum-isotonic phosphate buffer were respectively employed.

TABLE III. Failure of Variation in Volume of Inoculum to Influence Sensitivity of Chick Cell Cultures to Eastern Equine Encephalomyelitis Virus.

Vol inoculum containing 3 TCD_{50} virus (ml)	Chick cell cultures showing cytopathic changes	
	Virus suspended in horse serum-buffer	Virus suspended in normal horse brain extract
.1	17/20*	13/20
1.0	19/20	8/20

* $\frac{\text{Cultures with cytopathic changes}}{\text{Cultures inoculated}}$.

The results summarized in the second column of Table III indicate that a 10-fold increase in volume of the inoculum does not diminish the number of cultures that become infected by small doses of virus. A large quantity of brain extractives accompanying the virus may slightly reduce the chances of infection as suggested by the somewhat lower proportion of cultures showing pathogenic changes among those inoculated with virus suspended in normal horse brain extract. It is clear, nevertheless, from these data taken as a whole that a significant increase in the recovery of these agents from specimens of low infectivity may be expected if the volume of the inoculum is increased.

In view of the marked sensitivity of the suckling mouse to strains of this virus, the susceptibilities of cultures prepared from embryonic mouse tissue and newborn mouse brain were investigated to determine whether they might be more effective than those of embryonic chick cells. In Table IV are presented the infectivity endpoints in cultures of murine tissues and in chick cells of human, equine, avian and arthropod specimens. In all cultures a marked and rapidly progressive degenerative effect was observed. The sensitivity of the cultures of embryonic mouse tissues to the virus present in these materials was in general equal to or greater than that of the chick cells.

TABLE IV. Infectivity Titers of Representative EEE Virus Strains in Cultures of Mouse and Chick Cells.

Cells	Virus source					
	Human brain	Horse brain	Pheasant brain*		Mosquito pool*	
Embryonic chick	6.8†	4.5	8.3	7.5	4.5	5.3
Embryonic mouse	7.0	5.3	7.3	7.5	5.4	5.8
Mouse brain			N.D.			5.3

* Titrations of 2 samples prepared from the same specimen and tested at different times.
† Negative log ID_{50}/0.1 ml; endpoints calculated by method of Reed and Muench.

Discussion. Despite the recent adoption of tissue culture technics as routine in the primary isolation of many viruses, they have been little used in attempts to recover the virus of eastern equine encephalomyelitis from infected materials of mammalian, avian, or insect origin. Instead, mice and embryonated eggs have been usually employed. In the present investigation, therefore, experiments have been carried out to determine more precisely the value for this purpose of the tissue culture method. The capacity of certain isolated cell systems to detect small amounts of virus in a variety of infected materials was compared with that of embryonated hens' eggs and suckling mice. Although the latter were found to be most efficient in this respect, cultures of trypsinized chick cells and embryonic mouse cells proved nearly as effective.

In practice the size of the inoculum that can be added to the tissue culture may be varied within wide limits. *A priori,* therefore, it would seem possible to render it as effective as the suckling mouse simply by increasing the volume of the inoculum. Indeed by adding in succession several portions of the material under examination one might increase the actual efficiency of the culture far beyond that of the suckling mouse which, of course, can tolerate injection of only small quantities of fluid. If it be accepted that the culture of chick cells is equivalent to the suckling mouse in its capacity to reveal the presence of minimal amounts of virus in specimens of the kind we have studied, then it offers certain advantages over all other systems now available. Thus cultures of chick cells may be prepared quickly and easily and in any number desired from inexpensive and readily available materials. The virus grows rapidly in these cells where it produces cytopathic changes that are sufficiently characteristic to permit a tentative identification within 24-72 hours. The identity of the agent can then be readily confirmed by subsequent neutralization tests with specific immune serum done in cultures of chick cells. This system can also be conveniently applied to the demonstration of specific antibody formation during convalescence from illness suspected to be caused by the EEE virus as we have found in the study of several patients(12).

The ease with which these cultures may be transported, maintained and inoculated makes them eminently suitable for use in the field for such purposes as epidemiological surveys and studies of host reservoirs, as Sanders has pointed out(13). Finally, their use would eliminate for the investigator the risk of infection inherent in the use of hypodermic needles required in the inoculation of mice.

Summary and conclusions. An evaluation of embryonated hens' eggs, suckling mice and cultures of various tissues for the primary isolation of EEE virus from human, equine, avian and arthropod specimens indicated that suckling mice were the most effective. The evaluation was based on a comparison of the ID_{50}s obtained by concurrently inoculating suckling mice intracerebrally and intraperitoneally, 9-12-day-old embryonated hens' eggs into the yolk sac, and cultures of various tissues with equal quantities of virus. Evidence is presented that cultures of embryonic chick tissue can be made as efficacious as suckling mice for the primary isolation of this virus by increasing the volume of the inoculum. On the basis of these findings and in view of the practical advantages of the tissue culture method it is concluded that cultures of embryonic chick cells should provide a satisfactory technic for use as routine in the etio-

logic diagnosis and in epidemiologic studies of eastern equine encephalomyelitis.

1. Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1936, v33, 607.

2. Bang, F. B., Gey, G. O., *ibid.*, 1949, v71, 78.

3. ———, *Bull. Johns Hopkins Hosp.*, 1952, v91, 427.

4. Scherer, W. F., Syverton, J. T., *Am. J. Pathol.*, 1954, v30, 1075.

5. Gey, G. O., Bang, F. B., Gey, M. K., *Ann. N. Y. Acad. Sci.*, 1954, v58, 976.

6. Eiring, A. G., Scherer, W. F., unpublished observations with L strain of mouse cells (Earle), quoted in *Ann. N. Y. Acad. Sci.*, 1955, v61, 806.

7. Hammon, W. McD., in *Diagnostic Procedures for Virus and Rickettsial Diseases*, 2nd Edit., APHA, N. Y. City, 1956, 169-206.

8. Enders, J. F., Weller, T. H., Robbins, F. C., *Science*, 1949, v109, 85.

9. Eagle, H., *J. Exp. Med.*, 1955, v102, 37.

10. Melnick, J. L., in *Diagnostic Procedures for Virus and Rickettsial Diseases*, 2nd Edit., APHA, N. Y. City, 1956, pp97-152.

11. Chambers, Velma C., *Virology*, 1957, v3, 62.

12. Unpublished data.

13. Sanders, M., Iris Kiem, M. S., Lagunoff, D., *AMA Arch. Path.*, 1953, v56, 148.

Received October 25, 1957. P.S.E.B.M., 1958, v97.

Effect of Chlorophyll Derivative upon Experimentally Produced Lymphedema in Rats. (23674)

J. Q. GRIFFITH, JR. AND HENRY T. STANTON, JR.

J. Q. Griffith, Jr., Fn. for Medical Research, Phila., Pa., and Research Department, Rystan Co., Mount Vernon, N. Y.

It has previously been shown(1) that edema of the foot and lower leg in the rat can be produced by injecting a suspension of colloidal kaolin about the ankle. If, at the height of the edema, thorotrast be injected subcutaneously into the dorsum of the foot, dilated lymphatic channels can be demonstrated by roentgenogram. These channels extend upward toward the regional lymph nodes, and it is believed, therefore, that the edema is due to lymphatic block near the lymph nodes. This edema will subside 14-17 days after the initial injection and the foot will appear grossly normal, unless infection involves it, in which case the edema may continue indefinitely, and ulceration may result.

Beef hydrolysates might be expected to exert an effect upon the lymphatic channels comparable with the products of infection. In preliminary studies it was found that no sustained edema appeared following injection of beef hydrolysates into the foot of the rat. However, if the foot had been previously conditioned by an injection of colloidal kaolin, an injection of beef hydrolysate either caused the appearance of edema (if none was originally present) or augmented an already present edema. Edema due to beef hydrolysate also tended to disappear after 14-17 days, but if repeated injections were given, some chronic swelling might be grossly apparent, or, if not grossly apparent, might be shown by surface area measurements.

Materials and methods. Colloidal kaolin was suspended in 3 times its volume of distilled water. At the beginning of the experiment, 1 cc was injected in the form of a ring completely encircling each ankle. At no time was chlorophyll given along with the kaolin. Beef hydrolysate was prepared from Bacto Beef Powder (Difco). The powder was hydrolyzed in aqueous solution for 3 hours at 37°C, using papain as the digestant. A portion of this was mixed with a derivative of chlorophyll (potassium sodium copper chlorophyllin) so that the resultant solution contained 0.2% chlorophyll by weight. Injections were given into the dorsum of the foot, always paired, so that one foot received the beef hydrolysate without chlorophyll, and the other beef hydrolysate with chlorophyll. When repeated injections were given, the same order was maintained in the feet. The volume of each injection was 0.2 cc.