Preparation of ECHO Complement-Fixing Antigens in Monkey Kidney Tissue Culture and Their Purification by Fluorocarbon. (23796)

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Increasing number of newly recognized ECHO viruses made it desirable to find a simple technic, such as the complement fixation (CF) test, for serological studies of these viruses, for determining antigenic relationships between types, and for typing newly isolated strains. In initial attempts to use CF antigens prepared in monkey kidney tissue culture in tests with ECHO antisera prepared in guinea pigs, difficulties were encountered in obtaining antigens with suitable titers which reacted specifically.

The present report describes the effect of fluorocarbon purification on ECHO complement-fixing antigens prepared in monkey kidney culture, the effect of maintenance media on the ECHO complement-fixing antigen yield, and the results obtained in CF tests using purified antigens from ECHO prototype strains and ECHO antisera prepared in guinea pigs and in monkeys.

Materials and methods. Viruses. The prototype strains of ECHO viruses passed in monkey kidney culture were furnished by Drs. J. L. Melnick, William McD. Hammon, and A. B. Sabin. *Tissue cultures.* Monkey kidney cultures were obtained from Microbiological Associates, Bethesda, Md. The cultures were grown for 6-8 days in a medium containing 2% calf serum, 0.5% lactalbumin hydrolysate, and 97.5% Hanks' solution. At the time of inoculation, the cultures in 32-oz. prescription bottles were washed twice with 40 ml of Hanks' solution. The maintenance media which were used during the study in bottle cultures appear in Table III. After comparison of CF antigen yield in various media, a medium containing 1% calf serum, 0.5% lactalbumin hydrolysate, and 98.5% Earle's solution was selected for routine use. The maintenance medium in tube cultures was Mixture 199(1). *Antisera. Guinea pig sera.* Guinea pigs were immunized with ECHO viruses grown in monkey kidney culture in Mixture 199. In some cases, crude tissue culture fluid, in others concentrated tissue culture material after treatment by sonic vibration, were used as immunization antigens. For anti-monkey kidney serum preparation, normal monkey kidney tissue culture cells in Mixture 199 were used. Detailed descriptions of these procedures and the immunization schedules will be published elsewhere. For CF tests, guinea pig sera were inactivated at 56°C for 30 minutes. *Monkey sera.* The lyophilized sera were obtained from the National Foundation for Infantile Paralysis.† The procedures used for preparation of these sera have been published by Archetti, Weston, and Wenner(2). Sera were reconstituted with distilled water and diluted 1:16 with buffered saline. The diluted sera were stored at −20°C. For CF tests, monkey sera were inactivated at 60°C for 20 minutes. Since the reconstituted monkey sera were anticomplementary, the lowest monkey serum dilution used was 1:32. *CF antigens.* Monkey kidney bottle cultures containing 40 ml of maintenance medium were inoculated with 0.3 ml of each of the prototype strains of ECHO types 1 to 14(3). The bottles were incubated at 37°C. After complete degeneration of tissue the bottles were stored at −20°C for 1-7 days. The majority of antigens were prepared as follows: 1) One part of fluorocarbon‡ was added into 2 parts of frozen and thawed tissue culture material. 2) The chilled mixture was homogenized in a VirTis Homogenizer at 20,000 rpm for 5 minutes. 3) The homogenate was centrifuged at 2,000 rpm for 10 minutes. 4) The aqueous phase contain-

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‡ Genetron 113, obtained from Allied Chemical & Dye Corp., New York City.

TABLE I. Effect of Fluorocarbon Treatment on Concentrated ECHO Type 12 CF Antigen. Homologous and heterologous CF reactions of ECHO guinea pig sera with untreated and fluorocarbon-treated E-12 antigens.

ECHO guinea pig antiserum	E-12 antigen, no treatment + (1:2) Serum dilutions 1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	E-12 antigen, treated by fluorocarbon (1:2) Serum dilutions 1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
E – 4	4	4	4	4	4	4			0	0	0	0	0	0		
5	4	4	4	4	3	0			0	0	0	0	0	0		
6	4	4	4	3	0	0			0	0	0	0	0	0		
12	4	4	4	4	4	4	2	0	4	4	4	4	4	4	2	1
14	4	4	4	4	4	4			0	0	0	0	0	0		

4 = no hemolysis; 3, 2, 1 = different degrees of hemolysis; 0 = complete hemolysis.

ing the purified antigen was collected and stored at –20°C in screw-cap vials. In the early experiments when the maintenance medium in bottles was Mixture 199 without serum, and the virus yield was relatively low, concentration of antigens was necessary. The thawed tissue culture material was centrifuged in a Spinco ultracentrifuge at 10,000 rpm for 30 minutes, in Rotor #30. One part of fluorocarbon was added to 9 parts of supernatant, and the mixture was homogenized as described above. The aqueous phase was collected, 0.6% gelatin was added, and the material was concentrated by centrifugation in the Spinco at 30,000 rpm for 3 hours. The gelatin was added in order to keep the pellet compact, which made it possible to remove the supernatant without losing the pellet.§ The pellet was resuspended to 10X concentration in Mixture 199. All CF antigens were used unheated. *CF test.* The Bengtson complement fixation technic(4) with some modifications was used. Each new lot of commercial glycerinated hemolysin was titrated against 1:40 dilution of complement. Commercial lyophilized guinea pig complement was restored each Monday and titrated with a new lot of sheep cells. The same lot of restored complement and the same lot of cells were used in the tests of 5 following days. The restored complement was stored at 4°C. For the test 0.2 ml each of serum dilution, antigen, and complement (2 units) were mixed and incubated overnight at 4°C. Sensitized cells (0.4 ml) were added to give a final volume of 1.0 ml. The hemolysin and 2% sheep cells were mixed 10 minutes previously, during which time the test was removed from the refrigerator and held at room temperature. The tubes were incubated at 37°C for 1 hour, then held overnight at 4°C. The test was read the following morning. The titer was the highest dilution of serum or antigen giving 3 or 4+ fixation. A control titration of complement was included in each test, as well as the cell control and the anticomplementary controls of the lowest dilutions of antigens and sera.

Results. Effect of fluorocarbon treatment on ECHO antigens. Initially, fluorocarbon purifications were made with concentrated an-

§ This technic was suggested to us by Dr. S. Baron.

tigens prepared in Mixture 199 without serum, using 1 part of fluorocarbon in 9 parts of concentrated tissue culture material. This treatment removed sufficient non-specific reacting antigens that the purified antigens gave only specific fixation in CF tests when titrated against several ECHO guinea pig sera (Table I).

A more detailed study of the effect of fluorocarbon was made with unconcentrated ECHO antigens. It was found that 1 part of fluorocarbon in 2 parts of tissue culture material, homogenized at 20,000 rpm for 5 minutes, usually removed the reaction with host antigens (Table II). The effect, however, depended on the maintenance medium in the antigen. If maintenance medium was Mixture 199 without serum, one fluorocarbon treatment completely removed the host antigen reaction, whereas, if the maintenance medium was Mixture 199 + calf serum, many lots of antigens retained some host antigen reaction after one fluorocarbon treatment. In these cases a second fluorocarbon treatment was sufficient to abolish host antigen reaction. The purification was slightly improved if the cells were centrifuged down from antigens before fluorocarbon treatment, but in order to keep the purification procedure as simple as possible, and because even in the presence of cells, one or 2 fluorocarbon treatments removed the host antigen reaction completely, the precentrifugation of tissue culture materials was not adopted as standard procedure.

The effect of fluorocarbon on reducing the specific reaction of ECHO CF antigens seemed also to depend on the maintenance medium. Table II shows the effect of one and 2 treatments with fluorocarbon on ECHO type 4 antigens prepared in Mixture 199 and in Mixture 199 + 2% calf serum. One treatment did not decrease the antigen titer significantly in either antigen, but after the second treatment the antigen in Mixture 199 without serum did not have any specific reaction; in contrast to the antigen in Mixture 199 + 2% calf serum, which had practically the same antigen titer as after the first treatment. In similar experiments with other ECHO strains in Mixture 199 without serum, the decrease in the specific antigen titer after one fluorocarbon treatment was 2-fold or more. In the antigens containing serum, the decrease in the antigen titer after one or even 2 fluorocarbon treatments was less than 2-

TABLE II. Effect of Fluorocarbon Treatment on ECHO Type 4 CF Antigen in Mixture 199 and in Mixture 199 + 2% Calf Serum. Antigens titrated in CF test against type 4 monkey serum and anti-monkey kidney serum prepared in guinea pigs.

Antigen	Treatment of antigen	E-4 monkey serum dil. 1:128 — Antigen dilutions 1:1	1:2	1:4	1:8	Anti-monkey kidney guinea pig serum dil. 1:16 — Antigen dilutions 1:1	1:2	1:4	AC*	Infectivity titer (TCD_{50}/ml)
E-4 in Mixture 199, unconc.	F & T†	4	1	1	0	4	4	4	0	5.0
	F & T; homogenized	3	2	0	0	4	4	3	0	5.5
	F & T; 33% fluorocarbon ×1	3	2	0	0	0	0	0	0	5.0
	F & T; 33% fluorocarbon ×2	0	0	0	0	0	0	0	0	2.5
E-4 in Mixture 199 + 2% calf serum, unconc.	F & T	4	4	3	0	4	4	3	0	5.0
	F & T; homogenized	4	4	3	0	4	4	4	0	5.0
	F & T; 33% fluorocarbon ×1	4	4	2	0	0	0	0	0	4.5
	F & T; 33% fluorocarbon ×2	4	4	1	0	0	0	0	0	4.5

4 = no hemolysis; 3, 2, 1 = different degrees of hemolysis; 0 = complete hemolysis.

* AC = anticomplementary reactions of antigens. † F & T = frozen and thawed.

TABLE III. Homologous and Heterologous CF Titers of ECHO Type 4, 5, 6, 11, 12, 13, and 14 Guinea Pig Sera.

Fluorocarbon treated ECHO CF antigens	ECHO guinea pig sera						
	E–4	E–5	E–6	E–11	E–12	E–13	E–14
E – 4	1024	0	0	0	0	0	0
5	0	4096	0	0	0	0	0
6	0	0	4096	0	0	0	0
11	0	0	0	2048	0	0	0
12	0	0	0	0	128	0	0
13	0	0	0	0	0*	1024	0
14	0	0	0	0	0	0	512
Homologous neutralization titer against 100 TCD_{50}	512	8000	64,000	8000	2048	512	2048

* $<1:2$ serum dilution. In all other instances, $0 = <1:8$ serum dilution.

fold.

Effect of maintenance medium on ECHO CF antigen titers. ECHO Type 4 antigen prepared in Mixture 199 had a titer of 1:1 before fluorocarbon treatment, whereas antigen prepared in a replicate bottle with Mixture 199 plus 2% calf serum had a titer of 1:4 (Table II). Similarly, ECHO Type 6 antigens prepared in replicate bottles with different media indicated that small amounts of calf serum increased both infectivity titers and antigen yields. Lactalbumin hydrolysate (0.5% to 1.0%) also increased antigen yields; however, the addition of cysteine (0.01%) and glucose (0.25%) to lactalbumin hydrolysate and calf serum had no apparent additional effect on antigen titers.

Medium containing 98.5% Earle's solution, 0.5% lactalbumin hydrolysate, and 1% calf serum was finally selected as the most desirable medium, since the use of Earle's solution eliminated the necessity (often encountered with Mixture 199 and Hanks' solution) for adjusting pH levels during the growth period. However, Earle's solution appeared to offer no other obvious advantages.

CF titers of ECHO antisera prepared in guinea pigs and monkeys. The homologous titers of guinea pig sera ranged between 1:128 and 1:4096 (Table III). The heterologous titers were all less than 1:8. No cross reaction (less than 1:2) could be demonstrated in repeated tests between type 13 antigen and type 12 guinea pig serum. The fluorocarbon purified antigens were also used in CF tests with monkey sera. However, the untreated antigens gave nonspecific reactions only with low dilutions (1:32 or less) of some sera.

The homologous and heterologous titers of monkey sera are shown in Table IV. The

TABLE IV. Homologous and Heterologous CF Titers of ECHO Monkey Sera.

ECHO CF antigen	ECHO monkey sera													
	E–1	E–2	E–3	E–4	E–5	E–6	E–7	E–8	E–9	E–10	E–11	E–12	E–13	E–14
E – 1	4096	0	0	0	0	0	0	32	0	0	32	32	512	0
2	0	1024	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	1024	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	1024	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	4096	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	4096	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	2048	0	0	0	0	0	0	0
8	512	0	0	0	0	0	0	8192	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	1024	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	2048	0	0	0	0
11	32	0	0	0	0	0	0	0	0	0	4096	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	512	0	0
13	8192	0	0	0	0	0	0	512	0	0	0	512	8192	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	2048

$0 = <1:32$ serum dilution.

homologous titers ranged between 1:512 and 1:8192. Two-way cross reactions occurred mainly between types 1, 8, and 13. Type 13 antigen had high titer (1:512) one-way cross reaction with type 12 antiserum.

Discussion. Human and monkey tissue cultures are at present the only source of certain viruses for experimental studies; hence, tissue culture grown virus preparations must be used for CF antigens as well as for antiserum production in laboratory animals. Since animals homologous with the type of tissue culture used in the propagation of viruses (*e.g.* monkeys) are not always available, and since epithelial human and monkey tissue cultures apparently have common antigenic properties(5), difficulties will be encountered in CF tests in obtaining only specific fixation between virus antigen and antibody without fixation between tissue culture host antigens and antibodies. The purification of virus preparations with fluorocarbon reported by Gessler *et al.*(6) and application of the technic to tissue culture materials reported by Manson *et al.*(7) and by Hummeler and Hamparian(8), may solve these tissue culture host antigen problems in some cases. In the present study, fluorocarbon treatment proved a useful method for removing host antigen reaction in CF tests from ECHO antigens prepared in monkey kidney cultures. The effect of fluorocarbon on host antigens and also on specific ECHO virus antigens seems to depend largely on the amount of serum in tissue culture medium. In the present study, 2 fluorocarbon treatments (1 part of fluorocarbon to 2 parts of tissue culture material) decreased by 2.5 logs the infectivity titer of ECHO type 4 antigen in Mixture 199 without serum. Manson *et al.*(7) using 10% serum in medium, were able to treat HeLa grown poliovirus 3 times with fluorocarbon (9 parts of fluorocarbon in 1 part of tissue culture materials) before they could demonstrate decrease in infectivity titer. A preliminary study varying the amount of fluorocarbon and the number of treatments, therefore, may be necessary when a new type of medium and presumably a new type of virus will be exposed to fluorocarbon purification.

With the objective of typing newly isolated ECHO strains by the CF test, the purification of every isolation with fluorocarbon, although a simple procedure, might not be convenient. A more practical use of fluorocarbon purification may be the purification of immunization antigens. Whether or not fluorocarbon-purified antigens, which do not give host antigen fixation in CF tests, would produce any host antibodies in repeated immunization of guinea pigs, and whether these antigens would still be sufficiently active to produce specific antibodies, is unknown.

It is interesting to note a lack of correlation between infectivity titers and CF antigen titers of different types of ECHO viruses. For instance, type 5 antigen had 4.5 logs higher infectivity titer than type 4 antigen, but type 5 had 4 times lower CF antigen titer. A similar lack of correlation between neutralizing and CF antibody titers in ECHO antisera prepared in guinea pigs was observed (Table III).

The homologous and heterologous CF titers of ECHO monkey sera are similar to the results reported by Archetti, Weston, and Wenner(2), using ECHO antigens prepared in HeLa cells with the same sera. Minor differences are mainly in some cross reactions with lower titers. As with Archetti, Weston, and Wenner, the main two-way cross reactions were encountered between types 1, 8, and 13. The highest one-way cross reaction in both studies was obtained with type 13 antigen and type 12 antiserum. Since this cross reaction could not be obtained with type 12 guinea pig serum, the significance of this cross reaction in type 12 monkey serum may be questionable.

Summary. The fluorocarbon purification technic was applied to purification of complement-fixing antigens prepared in monkey kidney cultures from ECHO prototype strains. The effect of serum in tissue culture medium on the effect of fluorocarbon was studied. Adding serum to synthetic tissue culture medium improved the titers of ECHO CF antigens. Lactalbumin hydrolysate media containing serum represented the best media of several tried for the preparation of ECHO CF antigens in monkey kidney culture. The untreated antigens, when tested with guinea

pig sera prepared by immunizing animals with monkey kidney grown virus, gave strong monkey kidney tissue reactions. However, fluorocarbon treatment removed the nonspecific tissue reaction and "purified" antigens gave specific reactions in CF tests with ECHO antisera prepared in guinea pigs and in monkeys.

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1. Morgan J. F., Morton, H. J., Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

2. Archetti, J., Weston, J., Wenner, H. A., *ibid.*, 1957, v95, 265.

3. Com. on ECHO Viruses of the Nat. Fn. for Infantile Paralysis, *Science,* 1955, v122, 1187.

4. Bengtson, I. A., *Pub. Health Rep.,* 1944, v59, 402.

5. Habel, K., Hornibrook, J. W., Gregg, N. C., Silverberg, R. J., Takemoto, K. K., *Virology,*

6. Gessler, A. E., Bender, C. E., Parkinson, M. C., *N. Y. Acad. Sci. II,* 1956, v18, 701.

7. Manson, L. A., Rothstein, E. L., Rake, G. W., *Science,* 1957, v125, 546.

8. Hummeler, K., Hamparian, V., *ibid.,* 1957, v125, 547.

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Functional Adrenocortical Homotransplants in the Golden Hamster.* (23797)

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No previous reports of transplantation of adrenal cortex in the hamster have been found in the literature. In this laboratory we have had no success in autologous transplantation of the adrenal cortex in adult hamsters, although such procedures are routine in the rat(1). However, we have found that fetal hamster adrenal cortex transplanted homologously to the cheek pouch of the adult hamster will grow, differentiate and function. This paper describes the technic and presents evidence for the first known cases of successful adrenocortical transplantation in the hamster.

Materials and methods. Two female golden hamsters (Mesocricetus auratus) of 14 days pregnancy were obtained from the Boston University Mammalian Genetics and Breeding Laboratory, following their observed matings, one with a sire of albino phenotype, the other with a sire of golden phenotype. The golden hamsters used as hosts were procured from a local independent hamstery and were about 3 months old, weighing from 60 to 80 g. The pregnant female was anesthetized with Nembutal, and the gravid uterus removed *in toto* to Ringer's solution. One by one, each fetus was excised from uterus and membranes, and the adrenal glands (where recoverable) were dissected out and placed on sterile gauze moistened with Ringer's solution. While the dissections were being performed by one person, the other operator prepared the cheek pouches of the anesthetized hosts and did the transplantations according to the technic for tumors described by Lutz *et al.*(2). No more than 5 minutes elapsed between excision of a fetal adrenal gland and its transplantation into a cheek pouch. Instruments were sterilized by boiling. Host cheek pouches were swabbed with a dilute concentration of Zephiran chloride (Winthrop) in Ringer's solution prior to transplanting the fetal organs. Eight fetal adrenal glands were transplanted homologously to cheek pouches of 6 intact host hamsters (Table I). On the 22nd day after transplantation the hosts were bilaterally adrenalectomized by way of 2 small dorsolateral incisions, in a single operation under light Nembutal anesthesia. Over a period of 15 to 28 days (Table I) after adrenalectomy these animals received 6 subcutaneous doses of cortisone (Cortone acetate, Sharp &

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