BIKEN'S JOURNAL 2, 295-303 (1959)

Cultivation of Coxsackie Virus (Group A, Type 2) in Mouse Tissue Grafted to Chorioallantois

TOSHIKAZU FUJITA, TOSHIJI SUGAI AND YOSHIOMI OKUNO

Department of Virology, The Research Institute for Microbial Diseases Osaka University, Osaka

(Received for publication, December 15, 1959.)

SUMMARY

1. Coxsackie virus, Group A Type 2 an be serially cultivated over long periods by inoculating adult mouse muscle tissue grafted to chick embryo chorioallantoic membrane. An egg adapted strain of the virus was obtained using this method.

2. Cultivation for a longer period was more possible with the EK system (using adult mouse kidney tissue grafted in egg) than in the control using egg only, but an egg adapted strain could not be obtained.

3. The egg adapted strain was proved by neutralization tests using immune serum to be a Group A, Type 2 virus.

4. Interference tests showed that a positive result was obtained when the titer of the interfering virus (Coxsackie virus) was greater than $10^{5.0}$ MLD_{50} at the time of inoculation irrespective of the culture system.

5. Infectivity tests in the adult mouse failed to show any significant difference between the original and the adapted strain, but in suckling mice, the adapted strain had a longer incubation period.

6. No cytopathogenic effect was detected on L-cells but a trend to propagation of the adapted strain in L-cell cultures was observed.

INTRODUCTION

Various investigators in the past 10 years have attempted to adapt Coxsackie virus to the chick embryo but all have been unsuccessful and cultivation has succeeded for only 5-8 generations at the most. Dalldorf and Sickles (1949) have reported that cultivation of Group A, Type 1 in eggs was impossible, while Godenne and Curnen (1952) stated that Group A, Type 8 (Easton-10) was cultivated for 5 generations by inoclation on chorioallantoic membrane of the developing chick embryo (CAM). Shaw (1952) reported that Group A, Type 2 was successfully cultivated for 8 generations in the yolk sac.

Huebner and Ranson (1950) have cultivated Group A, Type 2 for 10 generations in the yolk sac and recently Sugai (1958), one of the present authors, has cultivated Group A. Type 2 (Shida strain) for 12 generations under certain conditions.

Group A, Type 4 (Minnesota-1) Coxsackie virus has been successfully cultivated in tissue cultures of mouse embryo tissue by Slater and Syverton (1950), and in flask culture (No. 50376 strain) of chick embryo tissue by Shaw (1952) while Crowell

and Syverton (1955) and Sickles et al. (1955) have investigated the cytopathogenic effect of Group A and B virus on HeLa cells and monkey kidney and trypsinized monkey testes cells respectively.

The technique for cultivation of tissues on the CAM have been reported by Cameron and Oakley (1938) who successfully transplanted chick embryo liver tissue to the CAM. Goodpasture (1938) has reported that human skin tissue could grow on the CAM. Blank and Coriell (1948) have used this technique and serially cultivated herpes simplex and vaccinia viruses and Li, Schaeffer and Nelson (1954) have cultivated poliovirus for long periods in monkey kidney and monkey skin tissue grafted to CAM.

There are no reports however, on the adaptation of virus to eggs following such cultivation methods as above

It was considered that if the tissue culture method and the cultivation of CAN were combined and the tissues susceptible to virus were utilized as an intermediate step in the cultivation, egg adapted Coxsackie virus (C-virus) could be obtained. Studies were therefore conducted in an attempt to verify this consideration. The results presented here show that the chick embryo adapted C-virus could be obtained and serially cultivated for a long period without reduction in titer.

MATERIALS AND METHODS

1. *Virus*

Group A, Type 2, Shida strain isolated by Sugai (1958) from the stool of a suspected poliomyelitis patient.

2. *Suckling mice*

NA2 strain was used within 3–4 days after birth.

3. *Immune serum*

Group A, Type 2 (FL 49190) supplied by the National Institute of Health of Japan.

4. *Tissue culture material grafted to CAM*

a) *Tissue:* Thigh muscle and kidney taken from 4–5 week old mice (ddo strain), minced to 2–3 mm^2

b) *Culture media*

Exp. 1	Hanks' solution	60%
	Bovine serum	30%
	Chick embryo extract	10%

Containing 100 μg/ml streptomycin and 100 units/ml of penicillin

Exp. 2	YLH solution	60%
	Bovine serum	40%

YLH Hanks' solution containing 1 per cent yeast extract and 0.5 per cent lactalbumine

5. *L-cell*

Maintained in our laboratory by stationary culture in 5 percent bovine serum-YLH solution.

6. *Virus passage in developing chick embryos grafted with mouse tissue*

The kidney and thigh muscles of adult mice were removed aseptically, cut up with a scissor so that the pieces were ca 2 mm^3 in size and washed several times with Hanks'

solution. They were then suspended in culture media and inoculated onto the CAM of 7 day eggs, about 10 pieces of them being placed together with culture media in each egg through an artificial window (ca. 8–10 mm in diameter). The window was closed aseptically with cellophane and sealed with paraffin and the eggs were incubated for 24 hours at 37°C. The window was reopened, and 0.2 ml of C-virus material was dropped onto the tissue on the CAM and the window resealed. After incubation for 6 days at 37°C, the CAM was excised and emulsified with an equal volume of Hanks' solution. After centrifugation at 3000 rpm for 20 minutes, 0.2 ml of the supernatant was used as an inoculum for serial passage.

Another aliquot of the same material was diluted with physiological saline and back-inoculated into suckling mice. The MLD_{50} was determined by the method of Reed and Muench.

The MLD_{50} of the control material (C-virus was inoculated directly to CAM without tissue graft) was also measured.

7. *Neutralization tests and interference tests*

To ascertain whether the derived virus was Group A, Type 2, C-virus, the neutralization tests were performed on experimental material Exp. 2, E'll. The material was serially diluted, and an equal volume of immune serum was added. After 2 hours incubation at 37°C. 0.04 ml aliquots of the serum-virus mixture were inoculated intrapeitoneally into 3 day old suckling mice. Normal mouse serum was used in the control experiment and the neutralizing antibody titer was determined.

As an index for the evaluation of C-virus growth in the chick embryo, interference tests with mumps virus (Enders strain) (Sugai, 1958) were conducted simultaneously.

To investigate any changes occurring with adaptation in the developing eggs, the pathogenicity against adult and suckling mice and the effect on L-cells of the adapted and original strains of C-virus were compared.

A 10 per cent emulsion of the thigh muscle of infected mice in physiological saline was used for serial passage of C-virus throughout the experiments. For inoculation of suckling mice, both in serial passage and back-inoculation, a 0.03 ml inoculum was injected intraperitoneally.

8. *Experiments with adult mice*

The pathogenicity of C-virus in 5 week old mice was examined by inoculating five mice from each group intracerebrally (0.03 ml) and intraperitoneally (0.2 ml) with a 10 diluted inoculum of mouse passaged $10^{6.0}$ MLD_{50} virus or $10^{6.0}$ E' 8th virus of Exp. 2 and the animals were observed for 3 weeks.

9. *Experiment with L-cell*

L-cells were distributed in short tubes and after 3 days inoculated with virus. One L-cell sheet was inoculated with a 10^{-2} diluted inoculum of $10^{7.0}$ MLD_{50} of thigh muscle emulsion infected with mouse passaged virus $10^{6.5}$ MLD_{50} of CAM emulsion infected with egg passaged virus (Exp. 2, E'17). The culture media was removed every 24 hours and back-inoculated in suckling mice and the growth curve was determined. The cytopathogenic effect of the C-virus was checked for 14 days.

To ascertain the reproducibility of the results, the experiment was divided into 2 parts. In Part 1, 60 per cent Hanks' solution, 30 per cent calf serum and 10 per cent chick serum was used as the culture medium up to the 10th generation, while from the eleventh generation of Part 1 and in Part 2, 60 per cent YLH and 40 per cent calf serum was used.

RESULTS

1. *Developing chick embryo cultivation*

The following abbreviations are used throughout.

E=Egg culture
EK=Adult mouse kidney tissue culture on CAM
EM=Adult mouse muscle tissue culture on CAM
E′=Egg culture after 11 of 12 EM culture generations (without tissue graft)

Table 1. Experiment 1

Mice→Mice→Mice 10% Thigh Suspension (MLD_{50}–7.24)

Host Range	Egg Culture	Adult Mice Kidney Tissue Culture on Chorioallantoic Membrane	Adult Mice Muscle Tissue Culture on Chorioallantoic Membrane		
Generation					
1	5.75 (a)	5.75 (a)	6.50 (a)		
2	3.25	3.50	4.24		
3	2.75	4.25	4.00		
4	2.00	4.00	4.00		
5	2.50	3.74	4.50		
6	2.50	3.74	4.24		
7	1.74	3.24	4.50		
8	0.74	3.50	4.50		
9	0.74	3.50	5.00		
10	0	2.75	5.24		
11		1.50	6.00		
12		0.74	5.50	→ Egg Culture	Generation
13		1.00	4.75	4.50 (a)	1
14		1.25	4.59	4.24	2
15		0.50	4.75	4.75	3
16		0	5.50	4.75	4
17			5.00	4.50	5
18			5.24	5.24	6
19			5.00	5.00	7
20			6.00	5.50	8
21			6.24	6.00	9
22			6.24	6.50	10
				6.50	11
				6.24	12
				⋮	⋮
				6.75	15

7 day old chick embryo used throughout
(a) : 50% lethal dose in suckling mice (back inoculation)

Table 2. Experiment 2

Mice→Mice→Mice 10% Thigh Suspension (MLD_{50}–7.24)

Host Range	Egg Culture	Adult Mice Kidney Tissue Culture on Chorioallantoic Membrane	Adult Mice Muscle Tissue Culture on Chorioallantoic Membrane
Generation			
1	5.50 (a)	6.00 (a)	6.00 (a)
2	3.74	4.24	4.50
3	3.50	4.00	4.50

4	2.59	4.00	4.24		
5	2.50	3.75	4.00		
6	2.00	3.50	4.24		
7	1.50	3.50	5.24		
8	1.24	2.50	5.24		
9	1.00	3.00	5.00		
10	0.74	2.74	5.24		
11	0.50	1.50	5.50 ⟶	Egg Culture	Generation
12	0	1.50	5.00	4.50 (a)	1
13		1.24	4.24	3.59	2
14		1.00	5.00	4.50	3
15		0.50	4.75	4.75	4
16		0.75	5.50	5.24	5
17		0	6.00	5.75	6
18			6.24	5.74	7
19			6.50	6.00	8
20			7.00	6.24	9
21			6.50	6.50	10
				7.24	11*
				6.50	12
				6.75	13
				⋮	⋮
				6.50	17
				⋮	⋮
				6.74	20

7 day old chick embryos used throughout

(a) : 50% lethal dose in suckling mice (back inoculation)

* : neutralized by Type 2 Antiserum (Table 3)

a) A comparison of the E, EK and EM systems in Exp. 1 and 2 show that the EM could be serially passed for the longest time. In the E and EK systems, the MLD_{50} shows a gradual decrease with each successive passage. In Exp. 1, the MLD_{50} of the E and EK systems dropped from $10^{5.75}$ and $10^{6.0}$ respectively to zero. Comparison of the E and EK systems in Exp. 1 and 2 revealed that the EK system could be passaged longer. That is, in Exp. 1, the MLD_{50} of E becomes zero at the tenth generation whereas that of EK is $10^{2.75}$, and in Exp. 2, the MLD_{50} of E is $10^{0.75}$ at the tenth generation compared to $10^{2.74}$ for the EK.

In Exp. 1, the MLD_{50} of the EK system dropped to zero at the sixteenth generation and in Exp. 2, the MLD_{50} was zero at the seventeenth generation. It was thus observed that the virus titer of the E and EK systems gradually decreased, finally becoming zero, and prolonged serial passage was impossible.

b) The MLD_{50} of the EM system dropped from $10^{5.6}$ to $10^{4.0}$ in Exp. 1 and from $10^{6.0}$ to $10^{4.0}$ in Exp. 1 with serial passages in the chick embryo, but from the 6-8th generation, the titer again rose slowly and a fairly constant level was maintained there after (MLD_{50} $10^{5.5}$ -$10^{6.5}$).

c) The twelfth generation of the EM system in Exp.1 and the eleventh generation in Exp. 2 were inoculated directly (without graft) to the CAM of a developing chick embryo. The results show that when the system changes (EM→ E'), the MLD_{50} first dropped from $10^{5.5}$ to $10^{4.24}$ in Exp. 1 and from $10^{5.5}$ to $10^{3.59}$ in Exp. 2, but then gradually rose again from the 5th-6th generation and

become $10^{6.5}$ at the 10th generation in both Exp. 1 and 2, and no drop was noted thereafter.

The results suggest that the C-virus was adapted to the devloping chick embryo.

Prior to this experiment, material of the EM system showing a drop in the MLD_{50} and then a trend to a rise (Exp. 1, 5th generation, $10^{4.5}$ MLD_{50}, Exp. 2, seventh generation $10^{5.24}$ MLD^{50}) was inoculated in to a chick embryo but the MLD_{50} dropped suddenly showing that no adaptation occurred.

d) Neutralization tests

The results of neutralization tests are given in Table 3. As can be seen, the neutralization antibody titer was 3.5 showing clearly that the virus of the eleventh generation of E′ in Exp. 2, is Group A, Type 2, Coxsackie virus.

Table 3. Neutralization Test of Coxsackie Virus (see Table 2-*)

Serum (mouse)	Dilution of Virus	Mortality Rate of Suckling Mice	MLD_{50}
normal	10^{-5}	3/3	6.74
	10^{-6}	3/3	
	10^{-7}	1/3	
immune	10^{-3}	2/3	3.24
	10^{-4}	0/3	
	10^{-5}	0/3	

Serum virus mixture incubated at 37°C for 2 hours; 0.04 ml. of the mixture inoculated intraperitoneally into suckling mice

Table 4. Interference Test of Coxackie Virus and Mumps Virus

Experiment	H. A. Titer	Result	Original Titer of Coxsackie Virus (-log)
Exp. A	2^2	positive	5.50
Control	2^7		
Exp. B	2^0	positive	5.74
Control	2^8		
Exp. C	2^6	negative	0.74
Control	2^7		
Exp. D	2^7	negative	0
Control	2^7		

Material of Exp. A: 10% chorioallantoic membrane (CAM) suspension of 12 generation of adult mouse muscle tissue culture on CAM (Exp. 1)
Material of Exp. B: 10% CAM suspension of 7 generation of egg culture after 11 EM generation (Exp. 2)
Material of Exp. C: 10% CAM suspension of 9 generation of egg culture (Exp. 1)
Material of Exp. D: 10% CAM suspension of 12 generation of egg culture (Exp. 2)
Interval of Ist (Coxsackie virus) and 2nd (Mumps virus) virus: 96 hours
site of interference test: allantoic cavity

e) Interference test

Table 4 shows the results of the interference tests. The 12th generation EM in Exp. 1 and the 7th generation E′ showed positive results while the 9th generation E in Exp. 2 showed negative results. The MLD_{50} of the inoculum was $10^{5.5}$ for 12th generation EM in Exp 1, $10^{5.74}$ for the 7th generation E′ in Exp. 2, $10^{0.74}$ for the 9th generation E′ in Exp. 1 and zero for the 12th generation E in Exp. 2. In other words, a positive result was obtained when the MLD_{50} of the virus was greater than $10^{5.0}$. This suggests that the interference pheomenon is affected by the MLD_{50} level and not by the cultivation system. The results coincide with those of Sugai (1958).

f) The overall mortality rate of the developing chick embryos was 15-20 per cent and contamination by Fungus reached as high as 30 per cent on the 6th day. Fungus-contaminated material was not used in the study, regardless of the embryo.

2. *Experiments in the adult and suckling mice*

The original and egg-adapted strains were inoculated intracerebrally or intraperitoneally in 5 adult mice, and no appreaciable change was observed during 3 weeks.

Intraperitoneal inoculation in suckling mice however, resulted in infection after an average of 48 hours incubation in the case of the original strain and 84 hours with the egg-adapted strain.

It can be seen that the incubation time is prolonged in the egg adapted strain.

3. *Experiments in L-cells cultures*

L-cells were inoculated with the egg adapted and the original strains, and culture fluid was removed every 24 hours, and back-inoculated into mice and the growth curve was determined. The curve was compared to that of control (cell-free medium inoculated with the original strain).

As can be seen in Table 5, the MLD_{50} becomes zero 144 hours after inoculation of the virus in the control, whereas the MLD_{50} are $10^{4.0}$ and $10^{3.0}$ respectively 168 hours after inoculation of L-cells with the egg adapted and original strains. The MLD_{50} 72 hours after inoculation of virus, are $10^{4.59}$ and $10^{4.5}$, both showing

Table 5. Growth Curve of Coxsackie Virus (C Virus) in L Cell Culture

Material of Inoculation in L Cell Culture / Time (Hour)	Mouse Passage C Virus	Egg Passage C Virus*	Control (contained no Cell
24	4.24	4.50	4.00
48	4.00	4.50	
72	4.50	4.59	2.24
120	3.74	4.00	
144	3.59	3.74	0
168	3.00	4.00	
Original Titer	7.00	6.50	7.00
	MLD_{50}(−log)		

* Material of 17 Generation in Exp. 2 (Egg Culture after 11 EM Culture Generation)

Table 6. Subculture Titer (MLD_{50}) of Coxsackie Virus in L Cell Culture

Generation	Mouse Passage C Virus	Egg Passage C Virus	Comparable Dilution Rate to Original Material
1	4.50	4.59	10^{-2}
2	3.00	4.24	10^{-3}
3	2.74	3.74	10^{-4}
4	2.50	4.00	10^{-5}
Original Titer	7.00	6.50	

a high titer. The optimum time for the L-cells was therefore 72 hours and after 4 successive passages, the viruses were back-inoculated in suckling mice and the MLD_{50} was measured. As shown in Table 6, the MLD_{50} of the 4th generation of the two strains were $10^{4.0}$ and $10^{2.5}$ respectively. The results show that there is a trend for Coxsackie virus to grow in L-cell, especially the egg adapted strain. No cytopathogenic effect on the L-cell was noted during a period of 14 days with either strain.

DISCUSSION

With improvement in the tissue culture technique, it has become possible to cultivate some strains of Coxsackie virus in tissue cultures of HeLa cells, human embryo and monkey kidney cells but there has been little progress in their cultivation in embryonated eggs. We have been able to obtain an adapted strain which can be maintained over a prolonged period without loss in titer by combining the tissue culture method and the egg culture method. That is, Coxsackie virus (Group A, Type 2) sensitive mouse muscle tissue was utilized as an intermediate host in adapting the strain to the egg.

The major part of the mouse tissue, grafted to the chorioallantoic membrane of the chick embryo, is engulfed and absorbed by the chorioallantoic membrane with time, but a part remains affording a medium for the virus to start growing, and the virus then in supposed to spread out to the adjoining chorioallantoic tissue. By repeating these processes, it may be that the virus is adapted to the egg. In other words, it is possible to adapt a virus to a non-susceptible host by gradually altering the environmental conditions. There is a close relationship between the virus and host (host range) and if the host is altered, it may be possible to change the affinity of the virus to the tissue. It is believed that this may provide a suggestion on the relationship between host and virus affinity. The egg adapted strain may be used for the preparation of a live avirulent Coxsackie virus vaccine similar to the egg adapted vruses of measles, poliomyelitis and influenza which are now being studied in our laboratory.

REFERENCES

Blank., Coriell, L. L. and Mc. Nair Scott, T. F. (1948). Human skin grafted upon the chorioallantois of the chick embryo for virus cultivation. *Proc. Soc. Exptl. Biol. Med.* **69**, 341-345.

Crowell, R. L. and Syverton, J. T. (1955). The viral range in vitro of a malignant human epithlial cell (Shtrain HeLa, Gey) IV. The cytopathogenicity of C viruses *J. Immunol.* **74,** 169–177.

Curnen, E. C. and Godenne, M. O. (1952). Propagation of Coxsackie virus on the chorioallantoic membrane of embryonated eggs. *Proc. Soc. Exptl. Biol. Med.* **85,** 81–85.

Dalldorf, G. and Sickles, G. M. (1948). An unidentifed, filtrable agent isolated from the faces of children with paralysis. *Science* **108,** 61–62.

Dalldorf, F. and Sickles, G. M. (1949). A virus recovered from the faces of "Poliomyelitis" patients pathogenic for suckling mice. *J. Exptl. Med.* **89,** 567–582.

Goodpasture, E. W. Douglas, B. and Anderson, K. (1938). Study of human skin grafted upon the chorioallantois of chick embryo. *J. Exp. Med.* **68,** 891–904.

Huebner, R. J. Ranson, S. E. and Beeman, E. A. (1950). Studies on Coxsackie Adaptation of a strain to chick embryos. *Public. Health Rep.* **65,** 803–807.

Li, C. P. Schaeffer, M. and Nelson, D. B. (1954). Multiplication of poliomyelitis virus in monkey tissues grafted onto chorioallantois of chick embryos. *Proc. Soc. Exptl. Biol. Med.* **87,** 153–157.

Oakley, C. L. (1938). Chorioallantoic grafts of liver. *J. Pathol. Bacteriol.* **46,** 109–120.

Shaw, M. (1952). Cultivation of Coxsackie virus in embryonated eggs and in chick tissue cultures. *Proc. Soc. Exptl. Med.* **79.** 718–720.

Sickles, G. M. Mutterer, M. Feorino, P. and Plager, H. (1955). Recently classified types of Coxsackie virus, group A. Behavior in tissue culture. *Proc. Soc. Exptl. Biol Med.* **90,** 529–531.

Slater, E. A. and Syverton, J. T. (1950). The cultivation of Coxsackie virus. *Proc. Soc. Exptl. Biol. Med.* **74,** 509–510.

Sugai, T. (1958). Studies on the propagation and interference phenomena of Group A Type 2 Coxsackie virus in developing chick embryos. *Med. J. Osaka Univ.* **8,** 685–694.