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Cultivation of Egg-Adapted Theiler's Mouse Encephalomyelitis (TO) Virus in Chick Tissue Culture. (20175)

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The present report describes the successful propagation of an egg-adapted strain of Theiler's mouse encephalomyelitis virus (TO) (1) in chick embryo tissue culture. The work was undertaken as part of an investigation of the problems involved in the cultivation of neurotropic viruses of low mouse infectivity. Poliomyelitis virus may be cultivated in human(2) and monkey(3,4) tissue. Maintenance in culture on tissues of other species has, however, presented difficulties. Similarly the TO type of Theiler's virus has not lent itself to cultivation, though the highly mouse-infective GD VII type may be readily grown(5).

Recent successful use of chick tissue for strains of Coxsackie virus which failed to grow on mouse tissue(6) prompted the investigation of the same medium for TO mouse encephalomyelitis virus.

Methods and materials. Viruses. The mouse encephalomyelitis (TO) strains were isolated in this laboratory from the intestines of albino mice(7). Stock strains have been maintained as infected mouse brain in 50% glycerol. The 40th mouse brain passage of strain No. 4727 was used to inoculate embryonated eggs. A suspension of infected embryo legs and wings from the 11th egg passage initiated the tissue

culture series. *Adaptation to eggs.* Fertile hens' eggs, incubated for 6 days at 37°C, were inoculated by the yolk sac route and reincubated for 10 days at approximately 35°C. The inoculation, harvesting of tissues, and preparation of suspensions were essentially as described for the Coxsackie viruses(6). Brains, legs and wings, and the abdominal and thoracic viscera were usually harvested. *Tissue cultures.* Suspended cell tissue medium in Erlenmeyer flasks, as used for the Coxsackie viruses, was used for the TO strains. Chick embryos, 6 to 8 days of age, furnished the tissue. Older embryos were less satisfactory. Usually the whole embryo, minus the eyes, was used; in certain experiments minced tissue from heads and legs was tested separately for its suitability as a substrate for viral growth. The first 14 passages were incubated for 10 days, subsequent passages for 7 days at 34°C. *Infectivity titers.* Mouse infectivity was demonstrated by inoculating 10- to 12-gram mice intracerebrally with 0.03 ml of the test material. To determine egg-infective titers, serial dilutions were inoculated into the yolk sac of 6-day embryonated eggs using as inoculum 0.1 ml of the chick embryo suspensions or 0.5 ml of tissue culture fluids. *Identity of strain.* The identity of the strain after egg or tissue cultivation was established by injection of the virus into 10- to 12-gram and infant mice, by histopathologic examination of the tissues of infected animals, and by challenge with the mouse passage strain No. 4727, with a representative strain of Theiler's virus (TO) obtained from the American Type Culture Collection and with the Lansing strain of poliomyelitis virus. For comparison the A.T.C.C. strain of Theiler's virus was also subjected to the above procedures for adaptation to eggs and tissue culture.

Results. Adaptation of virus to embryonated eggs. Strain No. 4727 adapted readily to growth in eggs. Virus was present throughout the embryo. A similar distribution of virus in the embryo has been reported by Riordan and Sá-Fleitas who carried a TO strain through 4 egg passages by inoculation on the chorioallantoic membrane(8). A suspension of the viscera of embryos of the second egg passage induced paralysis in 6 of 10 mice injected intracerebrally with 0.03 ml of a 10^{-4} dilution and in 1 of 10 injected with the 10^{-5} dilution. Subsequent passages were of similar titer, the brains containing less virus than the limbs and the viscera. Infective titers for eggs were approximately one log higher than for mice, possibly in part because of the larger inoculum used for eggs. The strain was transferred through 14 successive egg passages. In two passages some embryos were harvested 6 days, others 10 days, after inoculation; the longer incubation period gave a better yield of virus.

Infected embryos appeared to be stunted in comparison with uninoculated embryos of the same age but otherwise showed no signs of infection. Histopathologic examination revealed damage to the striated muscles but no lesions of the central nervous system. Mice inoculated with egg passage virus showed the typical signs of disease and lesions characteristic of the TO type of Theiler's virus(1). After 12 egg passages, the strain underwent a sharp drop in mouse virulence, which was not reversed by subsequent mouse brain passage. Two other strains were introduced into embryonated eggs. Strain No. 4771 grew readily, No. 47218 failed to adapt though repeated attempts were made. Both have approximately the same mouse infective titer. The A.T.C.C. strain of Theiler's virus also adapted readily to eggs.

Tissue cultures. Strain No. 4727 has been carried through 42 passages, the first 14 transferred at intervals of 10 days, subsequent passages after 7-day incubation periods. The infectivity titer of the 10th passage in tissue culture was determined both in mice and in eggs. Seven of 9 mice became paralyzed after intracerebral injection with 0.03 ml of a 10^{-1} dilution of culture fluid, 2 of 9 when a 10^{-2} dilution was used. Virus was present in quantity in chick embryos harvested from eggs inoculated with 0.5 ml of a 10^{-3} dilution, indicating the usefulness of the embryonated egg for detection of virus present only in minimal quantity. With *subsequent passages* increase in mouse-infective activity was suggested by the fact that the first paralyzed mice were occasionally noted 7 to 8 instead of 10 to 12 days after inoculation. The 31st passage was

TABLE I. Challenge of Mice Previously Injected with Egg-Adapted Theiler's Virus No. 4727.

Preliminary inj.	Challenge virus*: Theiler's virus No. 52102† 10^{-2}	Theiler's virus No. 52102† 10^{-4}	Lansing poliomyelitis virus No. 4701 10^{-1}	Lansing poliomyelitis virus No. 4701 10^{-1} + anti-Lansing mouse serum
Controls				
None	8/8	5/8	7/8	0/7
Test mice				
No. 4727, gen. E-13				
2×10^{-1}	0/4		4/4	
10^{-1}	0/4		4/4	
10^{-3}	1/3		2/4	
10^{-5}	3/4		4/4	

* Challenge viruses were suspensions of infected mouse brains and cords. An interval of 5 wk elapsed between preliminary and challenge doses.

† American Type Culture Collection strain TO virus.

Denominator = No. of mice injected. Numerator = No. of mice paralyzed.

The previously injected mice weighed 25-28 g at time of challenges; mice of similar wt were used as controls. Mice were not paralyzed prior to inj. with challenge viruses.

TABLE II.

Challenge of Mice Previously Injected with Theiler's Virus No. 4727, Tissue Culture Strain.

Preliminary inj.	Survivors of 1st inj. P†	Survivors of 1st inj. NP‡	Challenge virus*: Theiler's virus, No. 52102 10^{-1}	10^{-2}	10^{-3}	10^{-4}
Controls						
Tissue culture medium		18		5/6	6/6	3/6
10% normal mouse brain		18		5/6	4/6	2/6
Test mice						
Culture fluid No. 4727,		12	3/6	3/6		
40th tissue culture passage	11		0/4	0/7		

* The challenge virus was a suspension of infected mouse brains and cords. Interval of 5 wk elapsed between preliminary and challenge doses.

† Paralyzed in one or more legs. Suitable markings were used to identify paralyzed leg before injecting with challenge virus.

‡ Not paralyzed.

titrated both for mouse and egg infectivity. The highest dilution infective for mice was found to be 10^{-2} as before. On the other hand, virus was present in quantity in chick embryos inoculated with a 10^{-4} dilution of the tissue culture. Higher dilutions were not tested.

Minced tissue from legs of 8-day chick embryos, from heads or from the whole embryo served equally well as substrate for the propagation of the virus, differing from the findings of Pearson for the GD VII type which failed to grow in chick embryo body tissues other than brain and spinal cord(9). The *egg passage virus* was again adapted to tissue culture in a second series of transfers. On the other hand, success was never attained with mouse-brain virus similarly transferred to chick tissue culture. The A.T.C.C. strain of Theiler's virus also adapted to cultivation *in vitro*, using embryo tissue of the 1st egg passage as inoculum.

Identity of strain. The egg passage virus and the strain grown *in vitro* maintained the characteristics of the TO type of Theiler's encephalomyelitis virus. Infected mice developed flaccid paralysis after a relatively long incubation period (6 days or more) and otherwise showed little evidence of infection. The initial paralysis noted was usually in the hind legs. This localization of paralysis has characterized strain No. 4727 since its isolation, in contrast to other strains previously described(7). Infant mice showed no paralysis until 18 or more days following intracerebral

inoculation. Neither infant nor 10- to 12-g mice could be infected by the intraperitoneal route. *Histopathologic* examination of mice paralyzed after infection with tissue culture material revealed lesions similar to those induced by mouse passage virus.

Mice injected with egg or tissue culture virus were challenged 5 weeks later with Theiler's virus strains No. 4727, No. 52102 (A.T.C.C. strain), or the Lansing strain of poliomyelitis virus.

Discussion. With other viruses that we have investigated (MM, GD VII, Coxsackie) the titer of tissue cultures, even when well established, has been at least 2 logs lower than the mouse passage or egg passage virus used to initiate the series. In the case of strains of low titer such as Lansing poliomyelitis virus or the TO strains of mouse encephalomyelitis such a decrease may bring the viral content near or below the minimal infective level for mice. With strains which may be egg adapted this difficulty may be partially obviated by the inoculation of relatively large amounts of the cultures into embryonated eggs and subsequent testing in mice for the presence of virus.

The failure of one of 3 strains of similar mouse-infective titer to adapt either to egg or tissue culture suggests that strain differences exist within this group of viruses as well as among the Coxsackie viruses(6).

Strain No. 4727, *in vitro* or in the developing chick embryo, had no particular predilection for nervous tissue. In tissue cultures it grew on leg and skeletal tissue; in chick embryos infected via the yolk sac, lesions were found in the muscles and not in the central nervous system. This suggests that non-nervous tissue may offer a better source of essential metabolites for the multiplication of the virus than does brain tissue.

Summary. 1. An egg-adapted strain of Theiler's mouse encephalomyelitis virus(TO) has been carried through 42 passages *in vitro* on embryonic chick tissue. Attempts to adapt the mouse passage virus directly to chick tissue culture were unsuccessful. Preliminary growth in embryonated eggs, followed by tests in mice of 20% embryo suspensions, facilitated the titration of tissue culture fluids. 2. One of 3 strains of approximately the same mouse-infective titer failed to adapt either to egg or tissue culture. 3. A representative strain of Theiler's mouse encephalomyelitis (TO) virus obtained from the American Type Culture Collection also adapted to egg and tissue cultivation.

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