specific fluorescence in tissues and tissue cultures. The possibility of a nonspecific protein-protein reaction is discussed. This reaction apparently plays no part in the serological system to which it has been added.

1. Chadwick, C. S., McEntegart, M. G., Nairn, R. C., *Lancet*, 1958, v1, 412.
2. Chadwick, C. S., McEntegart, M. G., Nairn, R. C., *Immunol.*, 1958, v1, 315.
3. Marshall, J. D., Eveland, W. C., Smith, C. W., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 893.
4. Hughes, P. E., *Cancer Res.*, 1958, v18, 426.
5. King, E. S. J., Hughes, P. E., Louis, C. S., *Brit. J. Cancer*, 1958, v12, 5.
6. Louis, C. J., *idem.*, 1958, v12, 537.

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Characteristics of Growth of Encephalomyocarditis Virus in Cultures of Mouse Muscle.* (25183)

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Encephalomyocarditis virus (EMC) has been cultivated in embryonic mouse tissues by several investigators. Sanders and Jungeblut (1) cultured the Columbia SK strain through 200 consecutive passages in suspended embryonic mouse brain. Both the Columbia SK and MM agents have been propagated in suspensions of embryonic mouse brain, lung, small intestine, cardiac muscle, and tail and in minced chicken embryo by Shean and Schultz(2); no evidence of multiplication was detected when tongue, liver, kidney, spleen or skeletal muscle of embryos were used. Similar observations have been reported by Chambers, Smith, and Evans in tissue cultures of mouse embryo, embryonic intestine, and urinary bladder of hamsters(3). Growth did not occur in mouse intestine or in hamster subcutaneous tissue and skeletal muscle in plasma clot preparations. The encephalomyocarditis virus has also been grown in non-embryonic tissues. Chambers, Smith and Evans(4) recorded its cultivation in suspended human or mouse testicle. Smith and Evans(5) noted that it grew rapidly and produced incomplete degeneration of roller-tube cultures of monkey testicular tissue. Similar findings were reported by Fabiyi(6) using mouse testes as source of cells. Although Mengovirus multiplies in and disrupts Ehrlich ascites tumor cells *in vivo*(7), no evidence of a cytopathogenic effect could be demonstrated *in vitro* by Flanagan and Colter(8). On the other hand, frequent oncolysis of these tumor cells by the encephalomyocarditis virus was observed by Levy and Snellbacker(9). Multiplication of this virus with development of a cytopathic effect in cultures of KB (human epidermoid carcinoma), L, and HeLa cells has been described by Eagle, *et al.*(10), and Jungeblut and Kodza(11): the latter were unable, however, to cultivate the Columbia SK and MM strains on KB cells and the F virus on any but L cells. Our purpose is to report the successful propagation of encephalomyocarditis virus in cultures of abdominal wall muscle of mice and to describe the characteristics of regularly observed cytopathic changes.

Methods. The strain of encephalomyocarditis virus used, was obtained from Dr. Monroe Eaton, Harvard Medical School. It was passed by repeated intraperitoneal injection into mice and a pool of infected brain prepared and stored at –20°C. The muscle for making tissue cultures was obtained from *adult* mice killed by ether. Immediately after death, the skin was reflected and the entire mass of anterior abdominal musculature removed aseptically. After washing twice in Hanks' solution containing 100 units of penicillin and 50 μg of streptomycin, the tissue was minced with sharp scissor until all frag-

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ments were 1 mm or less in diameter, then washed 3 times in Hanks' solution and suspended in a small quantity of this fluid. One drop of this suspension containing 10 to 20 tissue fragments was embedded in 0.1 ml of fresh heparinized chicken plasma on the wall of Pyrex tubes (15 x 150 mm) and a drop of chicken embryo extract added to clot the plasma. Two ml of medium (10 ml of inactivated horse serum, 10 ml of beef embryo extract, 40 ml of calf amniotic fluid, 40 ml of Hanks' solution, 50 μg of streptomycin and 100 units of penicillin/ml, and 0.5 ml of 1% soybean antitrypsin) were added and the cultures incubated at 37°C in stationary horizontal position for 6 to 7 days. Most tissue fragments showed considerable outgrowth at this time; the medium was changed and 0.2 ml of 10^{-7} dilution of encephalomyocarditis virus-infected mouse brain pool ($LD_{50} = 10^{-8.3}$) inoculated into each tube. The cultures were then incubated in horizontal position at 37°C and examined daily for cellular changes. To determine the curve of viral growth, aliquots of fluid were removed from cultures at 10 minutes, 24, 48, 72, 96, and 120 hours; the material from several tubes was pooled and titrated for virus content by intraperitoneal injection in mice. To establish the fact that the observed cytopathic effect was due to encephalomyocarditis virus, studies using specific immune serum prepared in rabbits by intraperitoneal injection of mouse brain pool containing the virus were carried out. The serum was diluted 4-fold serially and an equal volume of fluid containing 200 CP_{50} of 6th passage virus added to each serum dilution. After mixing and incubating at 37°C for 2 hours, the serum-virus mixtures were inoculated intraperitoneally into mice and into tissue cultures of mouse muscle. Controls consisted of virus alone, normal rabbit serum mixed with virus, and rabbit immune serum alone. Cultures were observed for development of cytopathic changes and the mice for paralysis or death, for 2 weeks.

Results. Cultures of mouse muscle inoculated with encephalomyocarditis virus infected mouse brain pool revealed a clear-cut cytopathic effect after 48 hours incubation at 37°C. These changes were characterized by extensive destruction of the cellular outgrowth from tissue fragments. Normal cells were replaced by rounded ones which exhibited cytoplasmic granulations and nuclear condensation. The cultures were completely destroyed in about 72 hours. Figs. 1 (prior to inoculation of virus) and 2 (48 hours after infection)

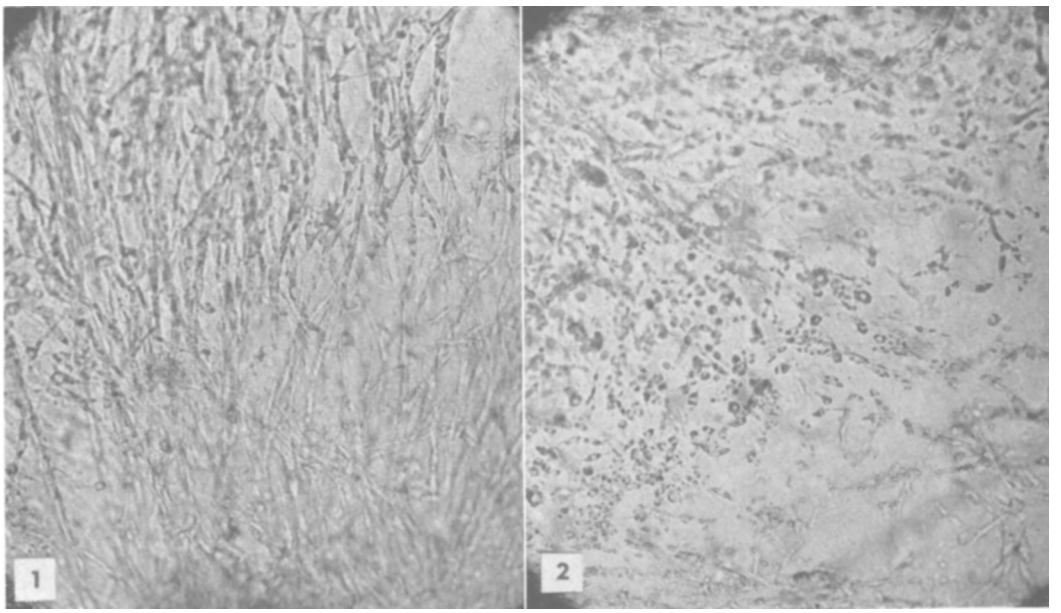

FIG. 1. Appearance of uninoculated mouse muscle tissue culture.
FIG. 2. Appearance of tissue culture 48 hr after infection with EMC virus.

TABLE I. Neutralization of Encephalomyocarditis Virus in Tissue Culture by Specific Immune Serum.

Serum dilution	Positive cytopathic effect*	Death in mice
1:4	0/4	0/6
1:16	"	"
1:64	1/4	1/6
1:256	4/4	4/6
Normal serum	"	6/6
Virus alone	"	"

* Numerator = No. of tubes showing cytopathic effect or No. of deaths in mice. Denominator = Total No. of tissue cultures or mice.

illustrate type and intensity of cellular changes.

Repeated passage of virus in mouse muscle tissue cultures using a 100-fold dilution of the fluid from preceding culture resulted in reproduction of the cytopathic effect. Increase in rapidity of cellular destruction was noted, complete degeneration occurring in about 48 hours.

Mixing of virus and rabbit immune serum resulted in complete elimination of the cytopathic effect and in failure of agent to kill mice following intraperitoneal inoculation. The results are presented in Table I.

Fig. 3 illustrates growth curve of virus in muscle tissue cultures. After an initial drop in titer, the quantity of virus reached a maximum at 48 hours. Control tubes containing virus plus medium but no cells were free of detectable infectivity for mice after 48 hours

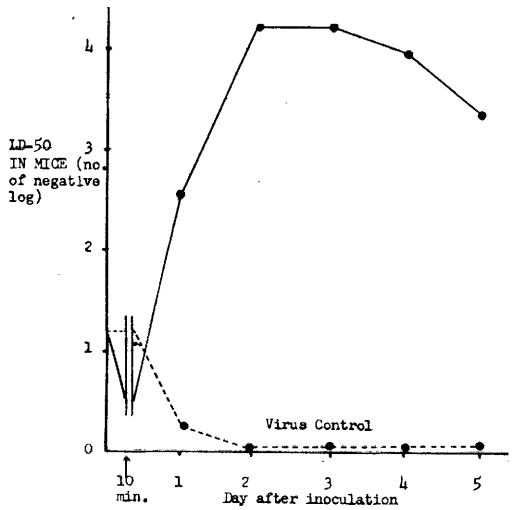

FIG. 3. Growth curve of EMC virus in mouse muscular tissue.

incubation at 37°C.

Discussion. Our observations indicate that encephalomyocarditis virus grows well in cultures of abdominal muscle of mice. Although this agent has been propagated in other types of cells, the method described here has the advantage of employing tissues of an animal, almost always available in microbiological laboratories and does not require constant propagation of a cell line such as with HeLa cultures, or use of material obtained from humans or monkeys.

The question may be raised whether the cytopathogenic effect observed, was due directly to encephalomyocarditis virus or to activation of a latent agent present in mouse muscle and carried along in serial cultures. Complete inhibition of cell destruction by addition of specific antiserum suggests strongly that the tissue changes were due to invasion by the virus being studied and not by some unknown organism.

Conclusions. 1. Encephalomyocarditis virus grows readily in cultures of abdominal muscle of mice. 2. A characteristic cytopathic effect is produced; complete destruction of cultures occurs in about 72 hours. 3. The cellular changes are not due to activation of a virus latent in mouse muscle but to the agent of encephalomyocarditis.

1. Sanders, M., Jungeblut, C. W., *J. Exp. Med.*, 1942, v75, 631.

2. Shean, D. B., Schultz, E. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 622.

3. Chambers, V. C., Smith, W. M., Evans, C. A, *J. Immunol.*, 1950, v65, 605.

4. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 76.

5. Smith, W. M., Evans, C. A., *J. Immunol.*, 1954, v72, 353.

6. Fabiyi, A., *Experienta,* 1955, v11, 156.

7. Koprowski, I. Koprowski, H., *J. Nat. Cancer Inst.*, 1953, v14, 627.

8. Flanagan, A. D., Colter, J., *Cancer Res.*, 1955, v15, 657.

9. Levy, H. B., Snellbacker, *J. Infect. Dis.*, 1956, v98, 270.

10. Eagle, H., Habel, K., Rowe, W. P., Huebner, R. J., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 361.

11. Jungeblut, C. W., Kodza, H., *ibid.*, 1957, v96, 133.

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