OBSERVATIONS CONCERNING THE PERSISTENCE OF LIVING CELLS IN MAITLAND'S MEDIUM FOR THE CULTIVATION OF VACCINE VIRUS

By T. M. RIVERS, M.D., E. HAAGEN, M.D., and R. S. MUCKENFUSS, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

PLATE 7

(Received for publication, June 7, 1929)

The fact that vaccine virus is capable of multiplying in cultures of susceptible tissues is well established. Some investigators (1), however, have claimed that it is possible to obtain an increase of this infectious agent in the absence of living host cells. These claims have not been substantiated. Indeed, Harde (Steinhardt) (2, 3) and Nye and Parker (4) have reported that tissues killed by freezing and thawing and by hypotonic salt solutions did not support the survival or multiplication of vaccine virus.

Recently Maitland and Maitland (5) recorded observations concerning "cultivation of vaccinia virus without tissue culture." Their medium consisted of minced chicken kidney suspended in a mixture of chicken serum (1 part) and Tyrode's solution (2 parts). These workers found that the virus increased in the absence of any detectable growth of cells in the cultures. In fact, they state that "after 24 hours the small pieces of kidney had begun to disintegrate, and by the third day autolysis of the tissue was extensive." There is no reason to doubt that vaccine virus multiplied in Maitland's medium. Moreover, Eagles and McClean (6) and Andrewes (7) have shown that certain viruses are capable of increasing under such conditions. Inasmuch as most workers have been unable to cultivate vaccine virus in the absence of living cells, and since it is known that certain cells remain viable under many conditions (8, 9), there are reasons for ascertaining whether living cells can persist or whether growth of cells can occur in Maitland's medium. It is with this problem that the present communication is chiefly concerned.

EXPERIMENTAL

Methods and Materials

Vaccine Virus.—Levaditi's neurovaccine was injected in the testicles of a rabbit. 4 days later the testicles were removed[1] and ground in a mortar with sand and M/50 phosphate solution, pH 7.6. The emulsion was thoroughly centrifuged and the supernatant fluid was used as an inoculum. The titer of the virus was determined in rabbits by means of intradermal inoculations of 0.2 cc. of serial dilutions of virus emulsions.

Culture Medium.—Throughout this work Maitland's technique of preparing and testing cultures was followed.

> "Blood was withdrawn from a hen and after it had clotted the serum was collected The hen was killed with chloroform and ether, and the kidneys, which were removed aseptically, were minced finely with scissors. Into a flask was put 0.66 c. cm. (approximately) of minced kidney and 1.33 c. cm. of inoculum diluted 1 in 6.6 with Tyrode's solution. The mixture was allowed to stand in the cold room for four hours. Then were added 12 c. cm. of Tyrode's solution and 6 c. cm. of hen's serum. The final dilution of the inoculum was thus 1 in 100. The mixture was distributed in 2 c. cm. into Carrel's tissue culture flasks, type D, which were incubated aerobically at 37°C., without caps. The cultures were tested after various periods of incubation by grinding the whole of the contents of each flask with sand and centrifugalising."

Controls.—In addition to the normal kidney tissue, controls with minced kidney tissue that had been frozen (CO_2 snow) and thawed 10 times were employed. To determine whether the tissues were living or dead at the beginning of each experiment, just prior to the distribution of the cultures in flasks, bits of the normal and of the frozen kidney tissue were placed in hen plasma and embryo extract on mica coverslips which were inverted and sealed over hollow ground slides. These preparations were then incubated at 37°C. After 5 or 6 days they were examined for evidences of cell growth.

To ascertain whether living cells persisted in the cultures prepared and handled according to Maitland's method, bits of tissue were removed from the Carrel flasks at 3, 4, and 5-day intervals, washed in Ringer's solution, and planted in plasma and embryo extract on mica coverslips These preparations were examined frequently for evidences of cell growth.

Inasmuch as it seemed possible that certain cells might at times multiply in a mixture of serum and Tyrode's solution, bits of normal kidney tissue were placed in such a mixture on mica coverslips over which hollow ground slides were placed and sealed. The slides were not inverted, since it is well known that in a liquid medium cells usually require a surface along which to grow. After incubation at 37°C., these preparations were examined for evidences of cell migration or cell growth.

---

[1] All operations were performed under ether anesthesia.

To test the viability of bacteria one usually resorts to subcultures rather than to examinations of fresh and stained specimens. Consequently, in determining whether cells are able to remain alive in the medium employed by Maitland for the cultivation of vaccine virus, subcultures, as described above, of bits of tissue in a favorable medium were made. Under these conditions, it is appreciated that positive results are more significant than negative ones. In this work 4 experiments were performed, 3 of which will be described in detail. The fourth will be omitted, since the results were similar to those of the others.

Experiment I

Jan. 15, 1929.—Fresh hen kidney was minced. One portion was frozen (CO_2 snow) and thawed 10 times. Then both portions were placed in contact with vaccine virus in the ice box for 4 hours. After the exposure to virus, 10 pieces of unfrozen and frozen tissue respectively were cultured as controls in plasma and embryo extract on mica coverslips. The inoculated frozen and unfrozen tissues were then added to a mixture of serum and Tyrode's solution and distributed in Carrel flasks (2 cc. each)—3 with unfrozen and frozen tissue respectively. Titer of vaccine virus in the cultures at this time: frozen = 1:100; normal or unfrozen = 1:100.

Jan. 17.—Each of the 10 control cultures made on coverslips from the unfrozen bits of tissue showed growth of cells, while in those made from frozen tissue no evidence of cell growth was observed at this time or upon subsequent examinations.

Jan. 18.—The cultures were removed from the Carrel flasks. Bits of tissue from 2 of the flasks with unfrozen kidney and from 2 of the containers with frozen kidney were washed in Ringer's solution and subcultured in hen plasma and embryo extract. None of the subcultures of frozen tissue showed growth in 6 days (Fig. 2), while in all of those made from unfrozen tissue growth of cells (Fig.1) was evident. The predominant cells were fibroblasts, yet cells of the macrophage type evidencing phagocytosis were observed.

The titer of vaccine virus in the flasks with frozen (F) and unfrozen (N) tissues was as follows: N1 = 1:500, N2 = 0, N3 = 1:5,000; F1 = 0, F2 = 0, F3 = 1:50.

The results of the above experiment indicate that vaccine virus did not multiply in the presence of kidney tissue killed by freezing and thawing, while it did persist or increase in amount in cultures set up with normal kidney tissue in a mixture of serum and Tyrode's solution. Furthermore, it is quite obvious that living cells persisted for at least 3 days in Maitland's medium.

Experiment II

Feb. 13, 1929.—Cultures were prepared with frozen and unfrozen tissue as described in the previous experiment. From the respective mixtures 2 cc. were placed in each of 4 Carrel flasks. The titer of virus at this time: frozen = 1:1,000; normal or unfrozen = 1:1,000.

Feb. 16.—10 bits of tissue were removed respectively from each of 2 Carrel flasks containing unfrozen (N) tissue and from each of 2 flasks with frozen (F) kidney, washed in Ringer's solution, and subcultured in plasma and embryo extract. The results of the subcultures were as follows:

N1: 8 showed growth of cells, while 2 did not.
N2: 5 " " " " " 5 " "
F2: 10 " no growth of cells.
F3: 10 " " " " "

Feb. 18.—To test again the viability of cells, 20 subcultures were made from N3 and N4 respectively and 10 from F1 and F2 respectively. The results of the tests were as follows:

N3: 14 showed growth of cells, while 6 did not.
N4: 15 " " " " " 5 " "
F1: 10 " no growth of cells.
F2: 10 " " " " "

Titration of virus from 2 Carrel flasks containing unfrozen (N) tissue and from 2 containers with frozen (F) kidney resulted as follows: N1 = 1:1,000, N3 = 1:100,000, F3 = 0, F4 = 0.

From the results of Experiment II it is evident that certain cells are able to survive for at least 5 days in a mixture of serum and Tyrode's solution. It also appears that vaccine virus survived or multiplied in the presence of living cells while it ceased to be active in the cultures prepared with tissues killed by freezing and thawing.

Experiment III

Feb. 25, 1929.—The vaccine virus used in this experiment was obtained from N3 of Experiment II. It had been diluted ten times and stored on ice for 7 days. As previously described, cultures were prepared with frozen and unfrozen minced kidney tissue. Prior to distributing the cultures in flasks, the following controls were set up on mica coverslips:

10 cultures of frozen tissue in plasma and embryo extract.
10 " " " " " serum and Tyrode's solution.
10 " " unfrozen tissue in plasma and embryo extract.
10 " " " " " serum and Tyrode's solution.

Subsequent examinations of the above controls revealed the following facts: None of the frozen tissues showed growth; 9 of the unfrozen tissues in plasma and embryo extract presented signs of growth; 5 of the unfrozen tissues in serum and Tyrode's solution showed evidences either of definite cell growth (Fig. 3), of wandering out of surviving round cells, or of beginning giant cell formation (Fig. 4) through cell apposition.

March 2.—30 pieces of unfrozen tissue were taken from each of 2 Carrel flasks (N1 and N3) and 10 of frozen were removed from each of 2 containers (F1 and F2). To test the viability of cells, these bits of tissue were washed in Ringer's solutions and subcultured in plasma and embryo extract. The results of the tests were as follows:

N1: 10 cultures showed growth, while 20 did not.
N3: 29 " " " " 1 " "
F1: 10 " " no growth.
F2: 10 " " " "

At the beginning of the experiment, titration of the virus resulted in no vaccinal reactions in the rabbit. After incubation at 37°C. for 5 days the contents of the flasks were examined for the presence of vaccine virus. None was found.

The virus used in this experiment was culture virus from Experiment II. It had been diluted and stored on ice for 7 days. During that time it had ceased to be active. This experience coincides with the findings of Eagles and McClean (6) who have had difficulty in preserving culture virus. The results of Experiment III indicate that cells are not only able to survive but may at times multiply in a mixture of serum and Tyrode's solution.

DISCUSSION

The results of the experiments described above are in agreement with those obtained by other workers who found that tissues killed by freezing and thawing failed to support *in vitro* the multiplication of vaccine virus. No evidence was secured to cast doubt upon Maitland's observations concerning the increase of vaccine virus in a medium consisting of minced fresh normal kidney tissue suspended in a mixture of serum (1 part) and Tyrode's solution (2 parts). In this medium, however, which Maitland considered not to be a tissue culture and in which he thought autolysis of the cells to be extensive within 3 days, it was possible to show that many cells remain viable for at least 5 days. This was accomplished by subculturing bits of the

tissue in a favorable medium of plasma and embryo extract. Furthermore, it was found that a medium of serum and Tyrode's solution is capable at times of supporting multiplication of certain cells (Fig. 3).

From the work here presented it appears that the increase of vaccine virus obtained by Maitland did not occur in the absence of living cells. Nevertheless, Maitland has made a definite contribution to the study of viruses in that he has found a medium for the easy cultivation *in vitro* of vaccine virus and other infectious agents of a similar nature (7).

SUMMARY

Cells survive for at least 5 days and at times are capable of multiplying in a mixture of serum and Tyrode's solution used by Maitland for the cultivation *in vitro* of vaccine virus.

BIBLIOGRAPHY

1. Fornet, W., Ueber die Reinkultur des Pockenerregers. VI. Mitteilung, *Cent. Bakt., Abt. 1, Orig.*, 1921–22, **87**, 36.
2. Steinhardt, E., and Lambert, R. A., Studies on the cultivation of the virus of vaccinia. II, *Jour. Infect. Dis.*, 1914, **14**, 87.
3. Harde, E. S., Some observations on the virus of vaccinia, *Anns. Inst. Past.*, 1916, **30**, 299.
4. Nye, R. N., and Parker, F., Jr., Studies on filterable viruses. III. Further observations on vaccine virus, *Am. Jour. Path.*, 1929, **5**, 147.
5. Maitland, H. B., and Maitland, M. C., Cultivation of vaccinia virus without tissue culture, *Lancet*, 1928, **2**, 596.
6. Eagles, G. H., and McClean, D., Cultivation of vaccinia virus, *Brit. Jour. Exp. Path.*, 1929, **10**, 35.
7. Andrewes, C. H., Virus III in tissue cultures. I. The appearance of intranuclear inclusions *in vitro*, *Brit. Jour. Exp. Path.*, 1929, **10**, 188.
8. Carrel, A., Some conditions of the reproduction *in vitro* of the Rous virus, *Jour. Exp. Med.*, 1926, **43**, 647.
9. Strangeways, T. S. P., The technique of tissue culture "in vitro," W. Heffer and Sons, Cambridge, 1924.

EXPLANATION OF PLATE 7

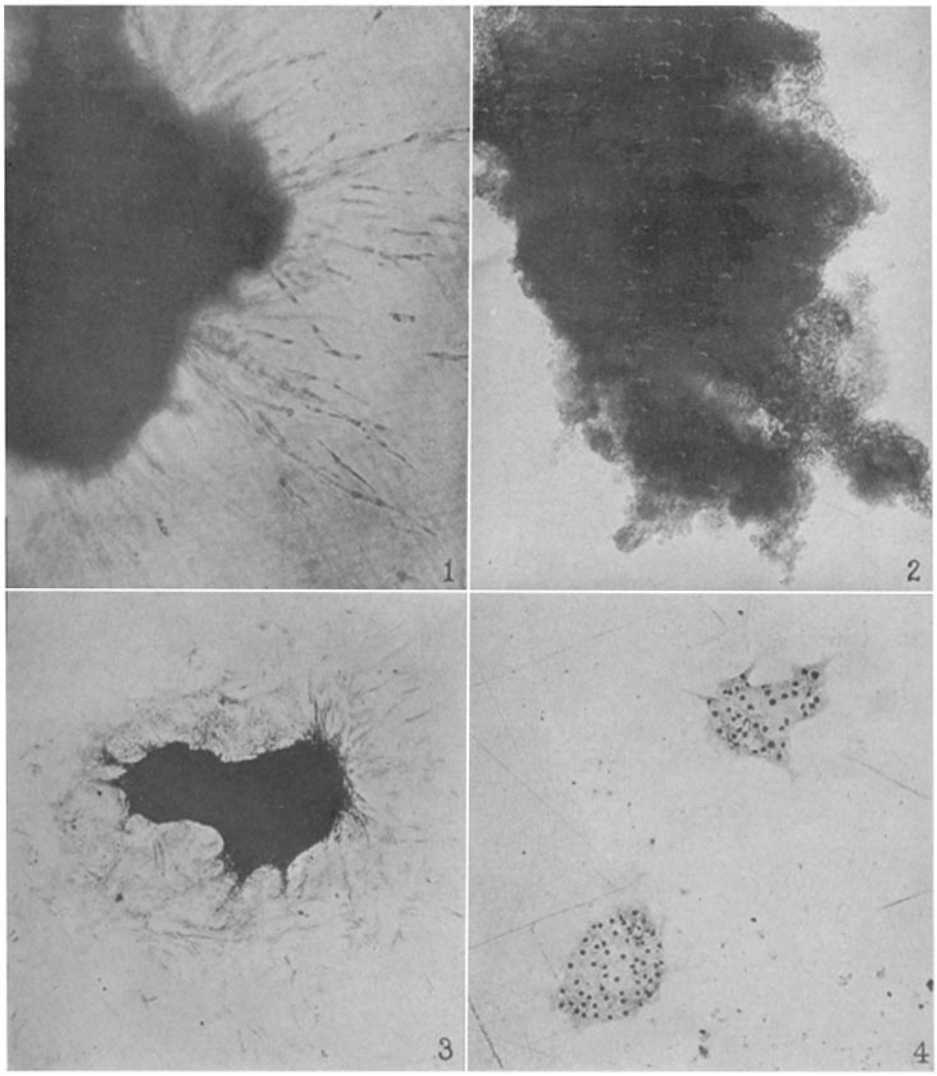
FIG. 1. A small piece of kidney tissue removed from a 3-day Maitland culture and subcultured in plasma and embryo extract. Note growth of cells. × about 95.

FIG. 2. Tissue treated similarly to that in Fig. 1 with the exception that it was

frozen and thawed before being used in a Maitland culture. Note absence of cell growth. × about 95.

FIG. 3. Fresh kidney tissue cultured 4 days in a mixture of serum and Tyrode's solution on a mica coverslip. Note growth of cells. Fixed preparation stained with Delafield's hematoxylin. × about 85.

FIG. 4. Giant cells formed by cell apposition in a medium of serum and Tyrode's solution. Fixed preparation stained with Delafield's hematoxylin. × about 85.



(Rivers *et al.*: Persistence of living cells in Maitland's medium)