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Age of Tissue in Agar-Slant Cultures and Multiplication of Virus of Herpes Simplex.

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The rate of multiplication and decline of a number of virus agents in association with living cells under various conditions of tissue culture has been studied by numerous investigators. The literature has been reviewed by Feller and his associates[1] who found that the quantity of vaccinia virus remained at or near its maximum in roller tube tissue cultures for at least 9 weeks, during which time the cells continued to live and to proliferate. Subsequently Enders and Florman[2] have shown that in suspended cell cultures of the Maitland-Rivers type this virus persists for about 4 weeks in association with viable cells. These observations are in contrast to those of Zinsser and Schoenbach[3] on the virus of Western equine encephalitis. In suspended cell cultures the titre of this agent reached its maximum within 3 days and then declined rapidly, more or less coincidentally with the decline in tissue metabolism. Pang and Zia[4] found that the maximal activity of the virus of St. Louis encephalitis on Zinsser's agar tissue medium was reached about the 6th day. In one case virus was detected as long as 36 days following inoculation although the titre was low from the 10th day on.

The experiments described in this paper were undertaken with the purpose of determining more precisely the relationship between the age of the tissue components in the agar-tissue medium and viral activity.

Materials and methods. The technique of cultivating the virus of herpes febrilis on Zinsser's agar slant tissue cultures has been previously described.[5] The same strain of virus was used in the present experiments. The brain of a moribund mouse was removed aseptically and ground in sand with the addition of 2 cc of broth. The resulting mixture was spun at 2800 rpm for 10 min and the clear supernatant fluid pipetted off. The intracerebral inoculation of an 0.04 cc amount into 24-day-old white mice resulted in death with typical signs within 36-72 hr. Inoculation of a series of agar slant tissue cultures was effected by permitting 0.2 cc of the supernatant fluid to flow over the tissue fragments spread on the surface of the agar slants. To transfer the virus to a fresh culture, 0.5 cc of Simm's solution[6] were added to an old slant and the tube gently rocked on its long axis for several minutes. During this process numerous small tissue fragments were washed down to the bottom of the tube. About 0.2 cc of this tissue fragment-containing fluid were then removed with a Pasteur pipette and added to an uninoculated tube. Titration of the viral activity of any given culture was carried out by scraping off the tissue fragments, placing them in a sterile mortar and then rinsing the tube with 2 cc of Simms' solution which were added to the contents of the mortar which likewise contained a small amount of sterile sand. After thorough grinding, followed by centrifugation at 2800 rpm for 10 min, the clear supernatant was pipetted off and tenfold dilutions made in infusion broth. Two mice were inoculated with each dilution. The highest dilution causing the death of both mice was taken as the end point.

1 Feller, A. E., Enders, J. F., and Weller, T. H., *J. Exp. Med.*, 1940, **72**, 367.

2 Enders, J. F., and Florman, A. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 153.

3 Zinsser, H., and Schoenbach, E., *J. Exp. Med.*, 1937, **66**, 207.

4 Pang, K. H., and Zia, S. H., *Chinese Med. J. Suppl.*, 1937, **3**, 446.

5 Cheever, F. S., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 113.

6 Sanders, M., *Arch. Path.*, 1939, **28**, 541.

TABLE I.

Age of culture in days at time of inoculation of virus	Additional days of incubation after inoculation	Age of culture in days at time of testing for viral activity	Ratio No. of mice dying to No. of mice inoculated
0	4	4	8/8
1	4	5	6/6
2	4	6	6/6
3	4	7	10/10
4	4	8	6/6
5	4	9	6/8
6	4	10	6/9
7	4	11	6/11
8	4	12	9/9
9	4	13	3/7
10	4	14	6/8
11	4	15	3/5
12	4	16	7/8
13	4	17	3/3
14	4	18	7/7
15	4	19	3/4
16	4	20	5/5
17	4	21	5/6
18	4	22	0/3
19	4	23	3/5
20	4	24	0/4
21	4	25	0/3

Autopsies were performed on mice dying with atypical signs, and cultures made of heart's blood and of emulsified brain. When possible, the latter material was inoculated into fresh mice. Deaths not obviously due to the herpes virus were classified as "no virus demonstrable."

I. *Survival of the virus on tissue cultures of different ages.* Agar slant tissue cultures were set up daily and incubated at 37°. After the first 24 hr the reaction in the tube became acid to phenol red due to the products of tissue metabolism. These tissue cultures of varying ages were then inoculated simultaneously with the same batch of virus, the tubes incubated for an additional 96 hr and then tested for the presence of the virus.

The results of several series of experiments are summarized in Table I.

Thus the virus survived for at least 4 days with a fair degree of regularity on tissue cultures as old as 17 days at the time the virus was inoculated.

Subsequently the activity of the virus on tissue cultures of varying ages was titrated. The results, summarized in Table II, indicated a fall from a titre of $10^{-3.5}$ in the case of virus inoculated into fresh tissue to 0 in that of virus inoculated into cultures of 18 days or older. The curve of viral activity, while showing a general downward course, failed to approximate a smooth line.

II. *Attempts to propagate the virus on tissue cultures of varying ages.* Since the virus could survive for 4 days at least on tissue as old as 17 days, we proceeded to determine whether cultures composed of aging cells would support the multiplication of the virus in serial transfer. Cultures were prepared at intervals of 4 days and stored at 37°. At the end of 12 days, fresh, 4-day-old, 5-day-old and 12-day-old cultures respectively were inoculated with the same batch of virus. The tubes were replaced in the incubator during an additional period of 96 hr and then removed. The material from each tube was transferred to a tissue culture 4 days younger, *i.e.*, the original fresh tissue culture now 4 days old was transferred to a fresh tissue culture; the original 4-day-old culture—now 8 days old—transferred to a 4-day-old tissue culture—etc. The viral activity of each tube was titrated at intervals of 8 days.

From the results graphically presented in Fig. 1, it is apparent that the virus can be propagated indefinitely on fresh tissue without significant loss of activity. In cultures

TABLE II.

Age of culture in days at time of inoculation of virus	Additional days of incubation after inoculation	Age of culture in days at time of testing for viral activity	Titre of virus
0	4	4	$10^{-3.5}$
1	4	5	10^{-3}
3	4	7	10^{-3}
7	4	11	10^{-2}
8	4	12	10^{-1}
10	4	14	$10^{-.5}$
12	4	16	10^{-2}
14	4	18	10^{-1}
15	4	19	10^{-1}
16	4	20	10^{-0}
17	4	21	10^{-1}
18	4	22	0
19	4	23	0
20	4	24	0
21	4	25	0

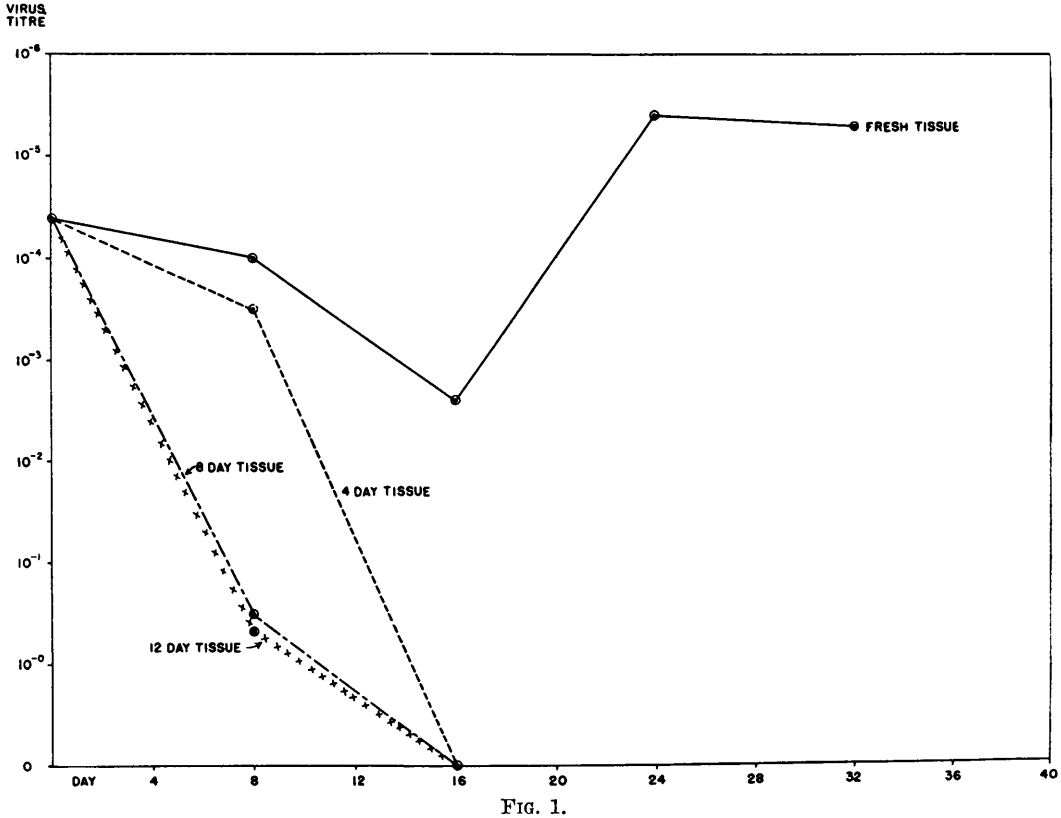

FIG. 1.
Behavior of virus of herpes simplex in serial passage on cultures composed of tissues of different age.

composed of older tissue, the titre of the virus declined in rough proportion to the relative age of the tissue culture. In no instance could the virus be demonstrated after the second passage, *i.e.*, when tested at 16 days.

Discussion. Since it has been shown[5] that the virus of herpes simplex does not survive for 96 hr at 37° if the tissue cells on the agar slants have been previously killed, it follows that some cells remained viable for as long as 17 days. In support of this conclusion, cultivation of fragments removed

from uninoculated agar cultures varying in age from 1 to 16 days have been found to result in the outgrowth of fibroblasts. A few observations on younger cultures inoculated 4 days previously with herpes virus also revealed fibroblastic proliferation when transferred to the plasma drop.

Accordingly we may be assured that the presence of living cells in agar slant tissue cultures as old as 17 days permits the survival of a portion of the virus for at least an additional 96 hr following inoculation. The titre, however, of viral activity of a culture after 4 days appears to vary approximately with its age at the time of inoculation. In this correlation between age of culture and titre of virus is to be found, we believe, the explanation for the fact that in freshly prepared cultures, when transferred at intervals of 4 days the virus may be propagated indefinitely without significant loss in titre, whereas the activity diminishes rapidly and soon disappears if cultures of 4 days or older are employed as the media for transfer.

These observations, then, represent further evidence in support of the intimate interdependence between the activity of the virus and the state of the tissue cell. In addition they suggest that multiplication of virus and survival may depend upon different cellular factors, since the former occurs only in the presence of cells freshly removed from the living embryo, but survival to a greater or less degree is afforded by cells whose vital functions have presumably been impaired in some manner. This conception has been stated by Plotz[7] and has received additional support in the experiments of Feller and his coworkers,[1] but the evidence presented here lends it greatly increased probability.

What the nature of such factors might be awaits further analysis. Multiplication of virus may depend upon the sum total of the normal metabolic processes of the cell as suggested by the experiments of Zinsser and Schoenbach.[3] Survival or preservation, on the other hand, may be correlated with the continued functioning of certain cellular enzymatic systems during the temporary suppression of others.

It is possible that the technique described of maintaining virus without multiplication through the use of pre-incubated tissue cultures may offer a means of investigating this problem, since it should permit observations of the effect on the virus and cells of the addition of various growth-promoting factors to the system.

Summary. 1. Agar slant tissue cultures as old as 17 days will permit the survival of the inoculated herpes simplex virus for at least 96 hr. 2. There appears to be a rough inverse relation between the age of the inoculated tissue culture and its virus content 96 hr later. 3. The virus may be propagated indefinitely on fresh agar slant tissue cultures; such is not the case if cultures 4 days or older are employed as the media for transfer.

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7 Plotz, H., *Compt. rend. Soc. Biol.*, 1937, **125**, 603, 719.