STUDIES ON FILTERABLE VIRUSES *

I. CULTIVATION OF VACCINE VIRUS

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One of us [1] in a preliminary note has reported the successful cultivation of vaccine virus in vitro. The medium employed was a tissue culture of rabbit testis. The virus was successfully carried through a maximum of nine transfers, representing a total time of fifty-four days in the incubator. The virus showed a definite increase on comparing the first and third generations of two of the cultures. We wish to report below further work along the same general lines.

Growth of Virus in Normal Tissue

In the work referred to above each culture was started with pieces of fresh, vaccine-infected testis. In view of Wolbach's [2] experience with the cultivation of Rocky Mountain spotted-fever virus, in which he gained the impression that only cells originally infected in the animal, or descendants of these cells, contained the virus in cultures, we thought it of interest to attempt to discover if the same held true of vaccine virus.

For this purpose pieces of normal testis were soaked for five minutes in a suspension of glycerinated virus-infected testis, that had been kept in the ice box for seventy-one days, after which time of course no cells could be expected to survive. These pieces of testis were then covered with drops of normal rabbit plasma, diluted with two parts of Ringer's solution, incubated for five days, transferred, reincubated, etc., as described in the preliminary note. The fourth generation of this culture was tested on a rabbit's cornea and found markedly positive macro- and microscopically. This virus, VC 6 A, was carried through 36 transfers (Table I). The experiment would appear to show that the growth of this virus, unlike that of spotted fever, does not take place only in the originally infected cells, but

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occurs either in or near normal tissue cells. Other experiments carried out in a somewhat different way also confirm this conclusion. In these experiments pieces of normal testis were covered with drops of normal plasma to which had been added small amounts of a heavy undiluted suspension of glycerinated, vaccine-infected testis. Here again, after several transfers, virus could be demonstrated and shown to be growing in all of the cultures (VC 10 — Table II — represents one of these cultures).

Numerous investigators in studying vaccinia in animals and man have attempted to demonstrate the virus in the blood of the infected animal, but always without success until quite recently. Ohtawara,[3] by using intratesticular inoculations with comparatively large amounts of blood, has shown conclusively that the virus does occur in the blood stream and remains there several days following inoculation on the skin with vaccine virus. This was true not only of rabbits, but in one instance of a human. This being true, the virus in the blood must occur in very small numbers, for ordinary methods, such as corneal and skin inoculations with blood, are always negative. In view of these facts we felt that it would be of interest to use as an inoculating material the plasma of an infected rabbit, for should our cultures prove positive on corneal or skin inoculation with this plasma as the sole source of the virus, it would show that definite growth of the virus had taken place presumably in the absence of primarily infected cells. Therefore, cultures were set up using the plasma of a rabbit inoculated (cornea, skin and testis) with vaccine virus five days previously, and pieces of normal rabbit testis. The cultures were incubated and transferred in the usual way. The third generation, inoculated on the cornea of a rabbit, was strongly positive macroscopically and showed many typical Guarnieri bodies microscopically.

Duration of Virus Growth in Artificial Medium

In the former piece of work, vaccine virus was successfully cultivated for a total period of fifty-four days, at which time the work was discontinued. We decided to attempt to discover how long we could cultivate the virus. A culture, VC 6 A, was started June 25, 1924, using normal plasma and pieces of normal rabbit testis that had been soaked for five minutes in a heavy emulsion in Ringer's solution of a seventy-one-day-old, glycerinated, virus-infected rabbit

testis. This culture was incubated, transferred, and tested in the usual way (Table I).

TABLE I

Growth Record of Vaccine Virus Culture, VC 6 A

Date		Generation	Result of testing
6/24/24	Started	I	
7/1	Transfer	II	
7/7	"	III	
7/11	"	IV	Cornea +
7/15	"	V	
7/21	"	VI	
7/25	"	VII	
7/30	"	VIII	
8/5	"	IX	Cornea +; Skin +
8/11	"	X	
8/15	"	XI	
8/20	"	XII	
8/27	"	XIII	
9/3	"	XIV	
9/10	"	XV	
9/15	"	XVI	
9/19	"	XVII	
9/24	"	XVIII	Cornea +; Skin +
9/29	"	XIX	
10/3	"	XX	
10/9	"	XXI	
10/15	"	XXII	
10/22	"	XXIII	
10/28	"	XXIV	Cornea o; Skin o; Testis + Emulsion of this rabbit's testis inoculated on another rabbit's cornea, skin and testis; all were +.
11/3	"	XXV	
11/7	"	XXVI	
11/13	"	XXVII	
11/19	"	XXVIII	
11/25	"	XIX	
12/1	"	XXX	
12/5	"	XXXI	Cornea o; Skin o
12/11	"	XXXII	Cornea o; Skin o; Testis o Emulsion of this rabbit's testis inoculated on another rabbit's cornea, skin and testis; all were o.
12/16	"	XXXIII	
12/22	"	XXXIV	
12/27	"	XXXV	
1/2/25	"	XXXVI	Cornea o; Skin o; Testis o Tested 3/6/25 for immunity as described below.

As will be seen from the above table, the fourth generation, inoculated on a rabbit's cornea, was positive macro- and microscopically; the ninth generation tested on the cornea was positive and a dilution equivalent to $\frac{1}{4600}$ of the culture injected intradermally was positive. The twenty-fourth generation was negative on the cornea and skin, but positive macroscopically in the testis. This testis was removed, an emulsion made and inoculated on another rabbit's cornea, skin and testis. All of the latter were positive macroscopically and the cornea and skin were positive microscopically. This result with the twenty-fourth generation may have been due to decreasing virulence of the virus, or to a diminished number of organisms, for the amount inoculated into the testis was several times that inoculated on the cornea and skin; however, the possibility of the development of a tissue affinity on the part of the virus due to continued cultivation in testicular tissue should be taken into consideration. The thirty-first generation, inoculated on the cornea and skin, was negative macroscopically. The thirty-second generation, inoculated on the cornea, skin and testis, was negative macroscopically in all; however, the testis was emulsified and inoculated on another rabbit's cornea, skin and testis. All these were negative macro- and microscopically. The thirty-sixth generation was negative on the cornea, skin and testis; this rabbit was tested for immunity on March 6, fifty-seven days after inoculation. This was done by inoculating on both corneas, skin and testis with an emulsion of glycerinated, vaccine-infected testis, and also on the skin with commercial vaccine virus. Both corneas and both testes were positive macro- and microscopically; the skin both with the commercial virus and the testis emulsion showed what was similar to an accelerated take in the human; that is, a marked take at the end of twenty-four hours, which was fading at seventy-two hours. Another rabbit, previously inoculated with positive results, and a normal rabbit were inoculated at the same time with both viruses; the revaccinated animal also showed an accelerated take while the reaction in the normal did not reach its height until the fourth day. Therefore it would appear that the rabbit inoculated with the thirty-sixth generation showed evidence of immunity as far as the skin was concerned. We carried out this experiment in this way as we felt that there was a possibility either that the virus might be present in too small amounts to give a positive take but in sufficient amounts to confer immunity, or that it

had lost its infecting power but had preserved its immunizing power. To sum up this cultivation experiment, the virus was demonstrated to be present in the fourth, ninth, eighteenth and twenty-fourth generations, covering a period of one hundred and thirty-two days of incubation; it did not give a positive take with the thirty-first, thirty-second and thirty-sixth generations; but the last apparently brought about some skin immunity on inoculation into a rabbit. The thirty-sixth generation represents a period of one hundred and ninety-eight days' incubation.

One experiment was undertaken to determine the amount of virus present in the ninth generation. The tissue of one of the cultures was weighed (5.9 mg.), ground with sand and 1.0 c.c. of Ringer's solution and centrifuged. Various dilutions of the supernatant fluid were made. One-tenth c.c. of each of these dilutions were injected intradermally into a rabbit. A dilution representing $\frac{1}{4600}$ of the culture (0.0013 mg. of culture) gave a positive skin test. Unfortunately this represented the maximum dilution and so the exact limit of positivity was undetermined.

Proof of Multiplication of Virus

We have felt all along that, while experiments, such as the one just mentioned and the one where we started with infected plasma, proved that the virus actually multiplied in our cultures, we should endeavor to devise some method whereby the amount of virus present in any particular culture could be determined. Obviously corneal and skin inoculations with scarification are unsatisfactory, as the amount of inoculated material that actually comes in contact or remains in contact with the tissues long enough to infect, must vary tremendously; furthermore the depth and number of scarifications are other variable factors. Intradermal injection seemed to offer a method wherein the amount injected and site of injection could be most accurately controlled, and this method has proved most satisfactory.

The technique of titration is as follows: the whole culture (tissue and plasma) is ground up with 1.0 c.c. Ringer's solution in a sterile test-tube using a sterile glass rod and sterile sand; it is then centrifuged at moderate speed for five minutes, the clear or slightly opalescent supernatant fluid pipetted off and the desired dilutions of the latter made; these dilutions are injected intradermally in 0.1 c.c.

(rarely 0.2 c.c.) amounts using a tuberculin syringe and a fine (No. 26 gauge) needle.

This method of titration was carried out with culture VC 10 and the results are given in Table II. The original culture was started with pieces of normal rabbit testis and normal plasma to which had

TABLE II

Growth and Titration of Vaccine Virus Culture, VC 10

Generation	Date	Split	Titration			Taking Skin Doses Contained per Culture	Virus Content Relative to Original Inoculum
			Date	Amt. Used	Result		
				1	0.2 c.c undil. +	$2\frac{1}{2}$*	1
I	11–25–24		11–25–24	culture*	0.1 c.c. undil. −		
				1			
II	12– 1–24		12– 5–24	culture*	0.2 c.c. undil. −	$<2\frac{1}{2}$*	<1
III	12– 5–24						
				2	0.1 c.c. 1–50 +	250	10
IV	12–11–24		12–16–24	cultures	0.1 c.c. 1–100 not done		
V	12–16–24	Yes					
				2	0.1 c.c. 1–200 +		
VI	12–22–24		12–27–24	cultures	0.1 c.c. 1–400 −	1,000	800
VII	12–27–24						
				$4 \times \frac{1}{2}$	0.1 c.c. 1–400 +		
VIII	1– 2–25	Yes	1– 8–25	culture	0.1 c.c. 1–800 −	2,000	1,600
IX	1– 8–25						
X	1–14–25	Yes					
				2	0.1 c.c. 1–3200 +		
XI	1–20–25		1–26–25	cultures	0.1 c.c. 1–6400 not done	16,000	51,000

* These two cultures contained originally 2 drops of virus plasma mixture instead of 1 drop. In each instance the "taking skin dose" is calculated on the assumption that only 1 drop was used.

been added such an amount of glycerinated vaccine-infected testis that the plasma virus mixture represented a dilution of about 1: 10 of the undiluted testicular suspension. Each piece of testis was covered with one drop of the plasma virus mixture, except for two pieces which received two drops apiece. One of the latter cultures, as soon as clotting had taken place, was removed, ground in 1.0 c.c. of Ringer's solution and titrated on a rabbit; 0.2 c.c. of the undiluted suspension gave rise to one vesicle. The other inoculations were all negative. Hence, this control culture contained a sufficient amount of virus to cause five positive intradermal reactions and all of the

other cultures, with the exception of the second one, which received 2 drops of plasma virus mixture, contained theoretically on the basis of this titration sufficient virus to cause two and a half positive intradermal reactions.

The cultures were then incubated and transferred as shown in Table II. When transferring, the tissue was freed as much as possible from its surrounding plasma. Obviously this method resulted in the loss at each transfer of a certain amount of virus; this loss has not been taken into account when figuring the relative strength of the original culture and the subsequent generations. If the whole culture could have been transferred each time, instead of only the tissue, the virus content of each generation would have been greater than that found. By the term "split" is meant that, as the amount of tissue in a culture became too large for satisfactory growth, this tissue was divided in half as evenly as possible and each half carried on as an individual culture, with the result that each culture of the subsequent generation contained only one half as much virus as before the splitting occurred.

Inspection of the column in Table II labeled "Taking Skin Doses per Culture" reveals the fact that a continuous increase in the virus content per culture took place from the third generation on. The reduction in virus content in the second generation was probably due to the fact that a considerable part of the virus had died, for it is known that incubation of the virus in plasma diminishes its strength rather rapidly. Between the second and fourth generations the virus evidently began growing well, for the fourth generation contained ten times as much virus as the original. From this generation onward multiplication occurred until in the eleventh generation the virus content per culture was 51,000 times that of the original. This figure was calculated as follows:

$$\frac{16{,}000\left\{\begin{array}{l}\text{number of skin taking}\\ \text{doses in 11th generation}\end{array}\right\}}{2.5\left\{\begin{array}{l}\text{number of skin taking}\\ \text{doses in first culture}\end{array}\right\}} \times 8\left\{\begin{array}{l}\frac{1}{8}=\text{portion of first}\\ \text{culture in 11th gen-}\\ \text{eration due to}\\ \text{splitting}\end{array}\right\} = 51{,}000$$

The average weight of our cultures was 10 mgm. Therefore, in the eleventh generation, $\frac{1}{1600}$ mgm. of the culture contained one taking skin dose, or one gram of this culture would yield 1,600,000 taking skin doses, whereas one gram of the original contained only 250 such doses.

This culture after the eleventh generation was unfortunately contaminated with moulds and had to be discarded.

Location of Virus Growth

There has been throughout the work a question in our minds as to whether the virus grows within the cells, near the cells or at a distance from the cells in the plasma clot with which the tissue is covered. In an experiment reported in the preliminary note, it was shown that a normal piece of testis was infected by being placed in the same clot as an infected piece of testis although a space of 2 or 3 mm. of plasma intervened. In this experiment infection of the piece of normal testis might have taken place through (1) growth of the virus through the plasma, (2) migration of the virus through the plasma, (3) migration of infected cells through the plasma, or (4) transfer of the virus in the serum squeezed out of the clot. In order to throw light on the region of growth, a piece of virus tissue culture was placed in a plate, and covered with plasma, a projection of plasma about 1.0 cm. long being made at one side. After seven days' incubation the tissue and the plasma prolongation were removed separately and each was weighed, emulsified in Ringer's solution and similar dilutions made with regard to the weight of each piece. These various dilutions were then tested on a rabbit. The testis emulsion was positive with a dilution representing $\frac{1}{4600}$ of the whole, whereas the plasma emulsion was negative throughout. This experiment suggests that the growth of the virus takes place in intimate connection with the tissue. Histological investigations of our cultures have failed to throw any light on location of the virus or its morphology, so at present we cannot say whether growth is intra- or extracellular.

Unsuccessful Attempts to Cultivate Virus

It seems worth while to record some of our unsuccessful attempts to cultivate vaccine virus in media other than the one used in the above experiments.

1. Pieces of culture testis from VC 6 A, sixth generation, were cultivated in serum agar. These were transferred every sixth day to fresh medium of the same composition. The sixth transfer was tested on a rabbit's cornea and proved negative macro- and microscopically.

2. A piece of culture testis from VC 6 A, sixth generation, was placed on the side of a 100 × 31 mm. test tube, about 0.5 cm. from the bottom. To this was added normal diluted rabbit plasma, the tube was slanted and the plasma allowed to clot. The tube was then placed upright and 2.0 c.c. of Ringer's solution added. After six days' incubation two drops of the Ringer's solution were transferred to another tube of the same composition, except that normal rabbit testis was used instead of culture testis. Two drops were also added as controls to a tube of Ringer's solution and plasma, and to another of Ringer's solution and normal testis. After five days' incubation the Ringer's solution in the original tubes and the Ringer's solution in the various tubes of the last transfer were tested on rabbits' corneas and all found negative macro- and microscopically.

This experiment was repeated incubating the tubes anaerobically, as well as aerobically, using as virus VC 6 A, ninth generation. After three transfers the fluids in both the anaerobic and aerobic cultures were tested intradermally in a rabbit and were negative.

3. As Carrel and other investigators have shown that chick embryonic tissue can be cultivated in artificial media for long periods of time, we thought this tissue might prove useful for our purposes. In a Carrel[4] "D" plate were placed several pieces of a fourteen day old chick embryo; these were covered with a mixture composed of 1.0 c.c. normal rabbit plasma, 0.15 c.c. chick embryonic juice, 0.15 c.c. emulsion of glycerinated vaccine-infected testis and 0.5 c.c. Ringer's solution. After clotting, 1.0 c.c of a Ringer's solution dilution (1:15) of chick embryonic juice was added. A second plate similar to the first except that no virus was added was set up as a control on tissue growth. The supernatant fluid was removed every three days, the cultures washed with Ringer's solution as recommended by Carrel and fresh chick embryonic juice diluted with Ringer's solution added. Tissue growth in both plates was good. At the end of 19 days' incubation (6 washings) the supernatant fluid, plasma and tissue of the virus culture were each tested separately by intradermal injections on a rabbit and all were found negative.

4. As embryonic juice is known to be a growth stimulant, it seemed advisable to try its effect on the virus using normal rabbit tissue rather than embryonic chick tissue. The cultures were set up in 100 × 13 mm. test-tubes, a piece of normal rabbit testis being

placed in the bottom. To each tube was added a mixture containing 0.25 c.c. 1: 5 Ringer's solution dilution of virus culture VC 10, tenth generation, ground up in 1.0 c.c. Ringer's solution, 0.25 c.c. chick embryonic juice and 2.5 c.c. Ringer's solution. One tube was incubated aerobically, the other anaerobically. At the end of six days 0.25 c.c. of the fluid from the bottom of each tube was transferred to a tube containing similar amounts of normal testis, chick embryonic juice and Ringer's solution, and the tubes were incubated aerobically and anaerobically as before. At the same time some of the fluid from each of the original tubes was tested intradermally on a rabbit and found to be negative. The cultures were transferred after three days and again at the end of six days; at this time the fluid in each tube was again tested intradermally on a rabbit with negative results. After two more days' incubation, the piece of testis in each tube was emulsified in the fluid part of the culture and these emulsions were injected intradermally into a rabbit, and found to be negative.

Summary

1. Vaccine virus can be cultivated in tissue cultures composed of normal tissue; the presence of previously infected, living cells is not necessary.

2. Vaccine virus was successfully cultivated in an artificial medium for one hundred thirty-two days. After one hundred ninety-eight days, the virus was not demonstrable by the usual methods, but apparently gave rise to a certain degree of skin immunity as tested by revaccination.

3. A method has been devised by means of which the virus content of any culture can be determined with a fair degree of accuracy. Using this method, the eleventh generation of one culture was shown to contain 51,000 times as much virus as the material from which it was started.

4. Our results suggest that the virus grows only in close proximity to the cells. Whether this growth takes place intra- or extracellularly cannot be stated at this time.

5. Various other methods for the cultivation of vaccine virus were tried without success.

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