THE ADAPTATION OF UNMODIFIED STRAINS OF YELLOW FEVER VIRUS TO CULTIVATION IN VITRO

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(Received for publication, March 18, 1937)

Using a technique similar to that devised by Rivers (1) for the cultivation of vaccinia virus, Haagen and Theiler (2) reported successful cultivation of a strain of yellow fever virus which had been greatly modified through many mouse passages by intracerebral inoculation. The medium consisted of chick embryo tissue and Tyrode solution containing 10 per cent of normal monkey serum. However, efforts to cultivate unmodified strains of yellow fever virus failed (3). After many attempts, Lloyd, Theiler, and Ricci (4) finally succeeded in establishing the unmodified Asibi strain in tissue cultures in which mouse embryo was used as the tissue component of the medium in place of chick embryo tissues. This strain of virus has now been under continuous cultivation in this laboratory for over 3 years.

The Asibi strain of yellow fever virus is the most virulent both in viscerotropic and in neurotropic properties so far isolated. Through continued cultivation *in vitro*, it has lost much of its pathogenicity (4, 5) and is used at present for human vaccination against yellow fever (6). However, as practically nothing is known regarding the permanency of the changes that take place in the virulence of the virus during prolonged *in vitro* cultivation, it seemed desirable to adapt strains of less initial pathogenicity to cultivation in tissue cultures. Many of the strains of virus recently isolated from human cases of yellow fever are of relatively low virulence and only moderately pathogenic for *rhesus* monkeys. It was considered probable that these strains would lose their virulence in tissue cultures within a shorter time and with less danger of reversibility than the highly virulent Asibi strain.

Method

The technique, in general, was briefly as follows:

All cultures were made in 50 cc. Erlenmeyer flasks closed with cork stoppers wrapped with lead foil. The fluid component of the tissue culture medium consisted of 10 per cent normal monkey serum in Tyrode solution. 4 cc. of this mixture were used as routine in each flask. To this were added 2 large drops of tissues, which had been minced with a tissue crusher or chopped finely with scissors. In a few experiments the plasma clot technique was tried in place of liquid medium. The source of virus used to inoculate the cultures consisted of either serum from an infected monkey or a Seitz filtrate of a 10 per cent infected mouse-brain suspension. With a few exceptions 1.0 cc. of the original virus-containing material was generally used to inoculate each flask and a similar amount of an old culture was added to fresh medium when subsequent transfers were made. Subcultures were usually made at intervals of 3 to 7 days. The cultures were incubated at 37°C.

EXPERIMENTAL

This technique which was so successfully applied to the cultivation of the Asibi strain was repeatedly tried with four additional unmodified strains of virus. Twenty-nine unsuccessful attempts to adapt the F. W. strain were made. In only nine experiments could virus be demonstrated in the primary culture after 3 to 7 days incubation. In one instance virus was present in the second subculture. At least fourteen similar attempts were made with three other strains of virus. None of the experiments gave promise of success.

As all these efforts to cultivate new strains of virus had failed, it was decided to approach the problem from a different angle. One possibility that suggested itself was the use of tissues of a susceptible animal such as the *rhesus* monkey. In order to determine to which particular tissue of the monkey the virus had greatest predilection, the following experiments were carried out.

Experiment 1.—A monkey was given intraperitoneally 3 cc. of serum from another monkey infected with the F. W. strain of yellow fever virus. On the 3rd day after inoculation the monkey had fever. On the 4th day it was exsanguinated under anesthesia. The blood was allowed to clot and the serum removed. On intracerebral test in mice the serum was found to have a moderate amount of virus, and proved infective for mice when inoculated intracerebrally with a dilution of 1 in 1,000. The spleen, ovaries, a piece of the liver, and both femurs were removed

aseptically from the monkey. The bone marrow was then extracted from the femurs under sterile precautions. A series of cultures was then made using the infected monkey tissues as the source of virus, as well as the tissue component in the serum-Tyrode medium. All the cultures were incubated at 37°C. for 7 days, after which their infectivity was tested in mice by intracerebral inoculation. The results showed that among the cultures with various tissues those which contained bone marrow seemed most favorable for virus preservation. This experiment was repeated several times, using F. W. as well as the French strain of virus, and similar results were obtained. Although the virus seemed to have a greater predilection for the bone marrow, when attempts were made to maintain it in a medium containing bone marrow tissues, it was invariably lost after the second or third subculture.

Experiment 2.—It occurred to us that monkey tissues taken shortly after the animal had been infected with virus might give a more favorable cellular medium for virus multiplication. Thus the use of cells rendered necrotic by the infection and the possible presence of antibodies in the animal tissues on the 3rd or 4th day of infection might be avoided. Accordingly, three monkeys were each given a large dose of F. W. strain of virus intravenously, and were killed by exsanguination under anesthesia at 5, 24, and 48 hours, respectively, after inoculation. The bone marrow, spleen, testicle, and an adrenal were removed from each monkey and culture made in exactly the same manner as in the preceding experiment.

The virus content of all the tissues used for culture purposes was determined by the intracerebral inoculation of mice. With the exception of the blood, which showed only a trace, no virus was demonstrated in any of the organs of the monkey killed 5 hours after inoculation. The monkey killed 24 hours after infection had demonstrable virus in the blood and liver but in none of the organs used for culture purposes. The third animal of the series killed 48 hours after infection with the F. W. virus showed considerable amounts of virus in the bone marrow and only small quantities in the spleen and testicle.

After 4 to 7 days' incubation, subcultures were made by using as an inoculum a suspension of the ground up tissue fragments of the original culture. The tissue used for preparing the subcultures was obtained from a normal monkey. At the time when subcultures were made, mice were inoculated intracerebrally with the material used for making transfers. The results of these inoculations showed virus to be present in only three of the cultures. None of the cultures prepared from the tissues of the monkey killed 5 hours after infection showed virus. In the cultures made from the 24 hour monkey, only those having adrenal gland showed virus. Cultures prepared from spleen and bone marrow of the 48 hour monkey showed a small amount of virus. Infectivity tests with material from the first subcultures

demonstrated virus to be present only in the cultures containing adrenal gland tissue, in the series initiated from the monkey killed 24 hours after infection.

Infection of Mouse Embryos in Utero with Yellow Fever Virus.—The successful cultivation by Lloyd, Theiler, and Ricci (4) of the unmodified Asibi virus in a medium containing mouse embryo tissues, suggested that in the mouse embryo there must be some groups of cells which serve as a favorable medium for the propagation of the virus. On the other hand, the fact that the concentration of the virus in mouse embryo tissue culture was never very high seemed to indicate that the proportion of such cells was relatively small in the whole embryo. With a view to determining to which particular organ the virus has the greatest predilection, embryos were infected *in utero*, and later the relative virus content of the various organs determined as follows:

The technique used for inoculation of the mouse embryos *in utero* was similar to that described by Woolpert (7) in his study of bacterial infections in mammalian embryos. The Swiss mice about the 13th day of pregnancy were used. The mouse was anesthetized with ether and tied on a board so as to expose the abdomen. An incision was made in the skin over one side of the abdomen and by careful dissection an area of the peritoneum about 1 square cm. was exposed. The embryos could be seen through the peritoneum and by some manipulation all the embryos in one uterine horn could be inoculated. Using a tuberculin syringe and a 26 gauge needle, each embryo was inoculated with 0.02 cc. of the virus-containing material.

These experiments were carried out with three different strains of the virus. At the end of 4 days, virus could be recovered from the inoculated embryos in considerable amounts as demonstrated by the intracerebral mouse test of a suspension of embryo tissue. After inoculation, however, the pregnant mice aborted so often that the continuous passage of virus from embryo to embryo was found to be very difficult. This study was consequently limited to the determination of the distribution of virus in the infected embryos, in order to learn which tissue was the most suitable for virus multiplication.

Experiment 1.—The embryos contained in the right uterine horn of three Swiss mice were inoculated on Feb. 20, 1936, with the serum of a monkey infected with the Asibi strain of virus. 4 days later one mouse aborted. From the other

two mice a total of seven embryos were removed aseptically. They were carefully dissected and various organs were removed separately, which included the brain, liver, kidney, a large proportion of the skin, the abdominal and thoracic viscera, and legs and tail. The homologous organs of the seven embryos were pooled and weighed. They were then ground finely in a sterile mortar and suspended in a diluent consisting of 10 per cent normal monkey serum in saline. The infectivity of each organ pool was tested for virus by intracerebral injection of 1:10 and 1:100 dilutions into mice.

The results indicated that with one exception all suspensions were infective in a dilution of 1 in 100, the exception being the suspension made of the abdominal and thoracic viscera of the seven embryos, which proved to be non-infective.

As no satisfactory conclusion could be drawn from this experiment with regard to the relative concentration of virus in the different mouse embryo tissues, the same procedure was repeated, using higher dilutions of the infected tissues.

Experiment 2.—The experiment just described was repeated with another group of embryos infected *in utero*. The Asibi strain of yellow fever virus was again used and the embryos were removed for titration at the end of a 4 day period after inoculation. In this experiment the decimal serial dilutions of the pooled organ suspensions were tested for presence of virus by intracerebral inoculation in mice up to 1 in 100,000.

The results are shown in Table I. It will be seen from the results that while the titration of tissues showed a wide variation in the distribution of the virus throughout the body, the mouse embryo brain contained a higher concentration than any other organ, as no end-point had been reached in a dilution of 10^{-5}. The thoracic and abdominal viscera which gave entirely negative results in the preceding experiment contained relatively small amounts of virus in this test.

The Cultivation of Unmodified Strains of Virus in Vitro in the Presence of Mouse Embryo Brain Tissues.—In accordance with the information obtained from the above experiment, attempts were made to cultivate unmodified strains of virus in a medium in which the tissue component consisted of minced mouse embryo brains. The brains were removed from 2 weeks old embryos under strict sterile precautions, and cultures prepared in the usual manner. Attempts to cultivate unmodified strains of virus in this medium proved highly successful.

Seven different strains of yellow fever virus were tried. They ranged in pathogenicity from the highly virulent French strain to newly isolated viruses from human cases of the so called jungle yellow

TABLE I

Titration of the Virus Content of Various Organs of Mouse Embryos Infected in Utero

Mouse embryo tissue tested	Dilutions of tissue tested	Result of intracerebral test for virus
Brain	10^{-1}	5/6*
	10^{-3}	6/6
	10^{-4}	6/6
	10^{-5}	6/6
Liver	10^{-1}	7/7
	10^{-3}	3/6
	10^{-4}	0/7
	10^{-5}	0/6
Skin	10^{-1}	5/5
	10^{-3}	5/6
	10^{-4}	4/6
	10^{-5}	2/6
Placentae	10^{-1}	6/6
	10^{-3}	6/6
	10^{-4}	0/5
	10^{-5}	0/6
Legs and tails	10^{-1}	6/6
	10^{-3}	6/6
	10^{-4}	5/6
	10^{-5}	2/6
Other viscera	10^{-1}	5/5
	10^{-3}	3/6
	10^{-4}	0/6
	10^{-5}	0/6

* The numerator represents the number of mice that succumbed to infection; the denominator, the number of mice used in the test.

fever which are low in neurotropic and viscerotropic properties. Of these, five were successfully adapted to tissue culture on the first attempt. Suspensions of infected mouse brains were used as a source

of virus to initiate the strains in the mouse embryo brain tissue cultures, and subcultures were made at 3 to 5 day intervals. In comparison with our former experience this was a striking success.

In our tissue culture work with yellow fever virus, the supernatant fluid of the centrifuged culture is tested as a routine procedure in mice for presence of virus by intracerebral inoculation each time a subculture is made. It was soon noted that the time required for encephalitis to develop in the mice used for these tests became shorter and shorter as the number of subcultures progressed, indicating an increase of neurotropism due to multiplication in the embryo brain tissue. As we wished to avoid an increase in neurotropism of the newly adapted strains by cultivating them too long in a medium rich in nerve tissue, an effort was made to replace the brain tissue with that of whole mouse embryo as early as possible during the *in vitro* cultivation. This substitution was unsuccessful in four different strains of virus when attempts were made at the fifth and sixth subcultures. When the virus strains had grown from twenty to twenty-five subcultures in mouse embryo brain, however, they were adapted to the whole mouse embryo with greater facility.

As the greatest attenuation of the Asibi virus had occurred in a medium, the tissue component of which was minced chick embryo tissue containing minimal amounts of nervous tissue (5), attempts were made to cultivate these new strains in similar media. The first attempts were unsuccessful. After longer periods of cultivation, however, it was found possible to maintain the virus in media, the tissue components of which were prepared from chick embryos from which the brain and spinal cord had been cut away before mincing. In a similar manner successful cultivation was obtained in mouse embryo tissue containing minimal amounts of nervous tissue.

SUMMARY

1. In a search for suitable tissues for the cultivation of yellow fever virus *in vitro*, mouse embryos were inoculated with this virus *in utero*. A titration for virus content of the various organs of the embryos indicated that the virus was present in the brain in greatest concentration.

2. Unmodified strains of yellow fever virus were readily adapted to

cultivation *in vitro* in a medium consisting of minced mouse embryo brain tissue and Tyrode solution containing 10 per cent normal monkey serum.

3. After a continued cultivation in mouse embryo brain tissue cultures for twenty to twenty-five subcultures, these strains were readily adapted to cultivation in whole mouse embryo tissue medium.

4. There is evidence to indicate that a prolonged cultivation of the virus in mouse embryo brain medium increases its neurotropic properties.

5. Attempts to employ monkey tissues for *in vitro* cultivation of yellow fever virus gave entirely negative results.

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