THE EFFECT OF PROLONGED CULTIVATION IN VITRO UPON THE PATHOGENICITY OF YELLOW FEVER VIRUS

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All strains of yellow fever virus so far studied (about thirty in number) exhibit two major tissue affinities, namely, viscerotropic and neurotropic. By viscerotropic affinity is meant that affinity which is responsible for the classical lesions of yellow fever. The organ chiefly attacked is the liver. The experimental animal generally used to demonstrate this affinity is the *rhesus* monkey (1). The final outcome of an infection in the monkey depends upon the virulence of the virus. Highly virulent viscerotropic strains produce death in most of the animals. The majority of strains of yellow fever virus isolated from human cases exhibit a mild degree of virulence for the *rhesus* monkey and seldom produce death. That such monkeys undergo an infection can readily be shown by the demonstration of virus in the blood.

To demonstrate this, advantage is taken of the neurotropic affinity of the virus (2). Mice are inoculated intracerebrally with serum obtained from the infected monkeys at regular intervals after inoculation (3). Presence of virus is shown by the development of a fatal encephalitis in the inoculated mice. This method is sufficiently accurate and sensitive to allow a quantitative estimation of the virus in the circulating blood. Numerous studies along these lines have clearly shown that the quantitative study of virus in the circulation gives a reasonably accurate index of the viscerotropic affinity of the virus. The more virulent the virus the higher its concentration in the circulating blood during infection. By appropriate methods the viscerotropic affinity of the virus can be diminished. This can be most readily achieved by serial propagation of the virus in mouse brains (2, 4). This procedure, however, not only reduces the viscerotropic properties but also enhances the neurotropic affinities (5, 6).

Through serial passages by direct intrahepatic inoculation of *rhesus* monkeys, Findlay and Clarke (7) state that they have restored the viscerotropic affinity which had been lost through prolonged mouse brain passages. It would appear, therefore, that the inherent tissue affinities of yellow fever virus can be increased or decreased by appropriate procedures. This was clearly demonstrated by Lloyd, Theiler, and Ricci (8) when they employed tissue culture methods for determining the effect of prolonged cultivation in various types of tissues upon the yellow fever virus. The tissues chiefly used in their studies were minced chick

and mouse embryos, and minced testicles of guinea pigs and mice. Their results indicated that a prolonged cultivation *in vitro* of a highly virulent strain of the virus had resulted in a marked loss of the power to produce a fatal infection with typical visceral lesions in monkeys. On the other hand, there was no evidence to indicate that the degree of neurotropism of the virus had increased during cultivation in the various tissue media. Most of the experiments reported by these authors were carried out with virus grown in mouse embryo tissues. Although there was no clear evidence to indicate that the virus grown in one type of tissues had undergone much greater change than in another, certain suggestive differences were noticeable.

The purpose of the present paper is to report the changes that have taken place in yellow fever virus in the course of continued cultivation *in vitro* for a period of over 3 years without intervening passage through a living host. These observations were made upon the changes induced in the virus cultivated in media containing three different types of tissues. As the three different series of cultures were all derived from the same original virus, it would seem reasonable to conclude that the changes in the pathogenicity and the tissue affinity that occurred during the prolonged cultivation are in all probability attributable to the different cellular components of the tissue culture media. This work is essentially the continuation of that reported by Lloyd, Theiler, and Ricci (8), and the present authors owe a deep sense of gratitude to the late Dr. Wray Lloyd, through whose untiring efforts the success of initiating these experiments is largely due, and regret that by his untimely death he was unable to see the fruits of his labors.

Methods and Materials

Strains of Virus.—The strain of virus used to initiate these series of tissue culture experiments was the so called Asibi strain (1). The history of this strain from the time of its isolation from man to adaptation and growth in tissue culture has already been reported in full (8). It was first successfully established in tissue culture containing mouse embryonic tissue and 10 per cent normal monkey serum in Tyrode solution. Up to the time of writing it has been maintained without interruption in this medium for more than 240 subcultures during a period of over 3 years. For the purpose of reference, this strain is designated as 17 E.

After cultivation through eighteen subcultures in this medium, a separate branch was initiated from this virus in a medium containing minced whole chick embryo. After further cultivation through 58 subcultures in the latter medium, the tissue component was modified to the extent that instead of using the whole

minced chick embryo, the brain and spinal cord were removed from the embryo before mincing. The virus has been maintained continuously in this medium containing minimal amounts of nervous tissue for over 160 subcultures. This strain is designated as 17 D. The third strain of virus included in this study and referred to as 17 AT was obtained from 17 E after 27 subcultures. The tissue component of the medium used for the cultivation of this series was minced adult mouse testicle. After 70 subcultures in this medium, adult guinea pig testicle was substituted for the mouse testicular tissue. The virus was grown for another 90 subcultures in this medium, after which the experiment was discontinued.

Methods of Cultivation.—The methods of virus cultivation used have been fully described previously and are essentially similar to the technique used by Rivers (9) and Rivers and Ward (10) for the propagation of vaccinia virus *in vitro*. The culture medium generally consisted of minced tissues and Tyrode solution containing 10 per cent of normal monkey or human serum. The cultures were incubated at 37°C. and subcultures were made every 3 or 4 days. At every passage the infectivity of the supernatant fluid of the centrifuged tissue culture was tested by the intracerebral injection of mice. At regular intervals portions of the culture virus were desiccated in vacuum in the frozen state for the purpose of preservation (11).

Titration of Virus and Antibody.—Mice of susceptible strains were used for titrating virus preparations. Groups of at least six mice were inoculated intracerebrally with 0.03 cc. of decimal dilutions. From the resulting mortality the theoretical dilution which would produce a 50 per cent mortality was determined statistically by Muench's method (12). In titrating the antibody content, the intracerebral protection test was used (13). This method consists essentially of mixing serial dilutions of the serum with a standard amount of virus, incubating the mixture for 1 hour at 37°C., and inoculating groups of mice with each mixture. From the resulting mortality in mice, the theoretical dilution of serum which would protect one-half of the mice is determined. Each protection test is controlled by the titration of a known immune serum. The titer of a serum is expressed as that theoretical dilution of the serum which, under the conditions of the test, protects one-half of the mice inoculated.

Tests for Immunity.—The immunity of all monkeys which survived inoculation with tissue culture was tested by two procedures. In almost all animals the antibody titer of the serum 1 month after inoculation was determined by means of the intracerebral protection test in mice. In addition, the actual immunity of the monkeys was tested by the injection of a highly virulent virus. In the great majority of instances the virus used for this purpose was virulent Asibi strain, which was injected intraperitoneally. This virus produces a mortality from visceral lesions in about 95 per cent of normal monkeys. The immunity of some monkeys was tested by the intracerebral inoculation of the French neurotropic virus. This virus variant was produced by the serial passage of the so called French strain (2) through more than 200 mice by direct brain to brain transmission. When inoculated intracerebrally, this virus invariably produces death from encephalitis in normal monkeys (6).

Tests for Circulating Virus.—There is a distinct correlation between the amount of circulating virus in the blood of a monkey and the virulence of the virus. The titer of the virus in the circulating blood is highest in those animals which die of viscerotropic lesions (3). Consequently, the duration and the relative concentration of the virus in the peripheral blood of monkeys during infection serve as an index of the viscerotropic affinity of the virus. The presence of virus in the blood of monkeys is determined by intracerebral inoculation of mice with the serum of monkeys obtained at regular intervals after infection. In the studies reported here, no effort was made to determine the titer of the virus in the peripheral circulation, and only its presence or absence was tested.

Pathogenicity of the Cultivated Strains of Yellow Fever Virus for Mice

As a rule at each subculture mice were inoculated intracerebrally with the supernatant fluid used for making a transfer to fresh medium and thus the presence of virus in the cultures was verified. The impression was obtained from the incubation period and time of death of the mice that there was a marked difference in the same strain of yellow fever virus grown for prolonged periods of time in various media. In general, mice inoculated with virus grown in chick embryo tissue exhibited a longer incubation period and a longer period of sickness than mice inoculated with virus grown in mouse embryonic tissue. As the incubation period in mice inoculated intracerebrally depends not only on the strain of virus used but also on its concentration, the results of numerous titrations were analyzed. These showed quite clearly that the virus grown in chick embryo tissue was less neurotropic for mice than that cultivated in mouse embryonic tissue.

Another index of neurotropism available is the rate at which a virus becomes fixed when passed serially through a number of mice by intracerebral inoculation. Such serial passages were initiated with the virus grown in three different types of tissue culture media as well as with the unmodified Asibi strain which, it will be recalled, is the parent strain from which the tissue culture series were branched off. The subcultures used to initiate the serial passages in mice were the 176th subculture of the virus 17 D grown in chick embryo, the 157th subculture of the virus 17 E grown in mouse embryo, and the 160th subculture of the virus 17 AT grown in testicular tissue.

Six to eight mice were used for each passage and the time of death was noted. The average time of death in the mice inoculated at each subsequent passage with the different viruses was calculated, and a

statistical analysis of the figures was made.[1] All four series of mouse passages showed a progressive shortening of the time from inoculation to death. The rate of fixation varied considerably, however. It was greatest with the parent Asibi strain and lowest with virus 17 D grown in chick embryo tissue.

Several points of interest were brought out in this analysis. The first was that there was no marked difference between the parent strain and the strain grown in mouse embryo tissue for 157 subcultures. The second was that the strain of virus grown for 160 subcultures in testicular tissues and the strain maintained for 176 subcultures in chick embryo tissue were significantly less neurotropic than the parent Asibi strain. At the time that this serial passage in mice was commenced, the virus grown in chick embryo (17 D) had been cultivated through 176 subcultures in a medium containing chick embryo tissue. During the last 100 of these subcultures only minimal amounts of nervous tissue were incorporated in the medium. It seems hardly a coincidence that the two least neurotropic viruses were obtained in media containing also the least amounts of nervous tissue.

In order to obtain information as to the time when the loss of neurotropism for mice appeared in the virus 17 D, serial passages in mice were begun with its 114th subculture. At this time the virus had been maintained for 96 subcultures in the chick tissue and during the last 38 subcultures only minimal amounts of nervous tissue were present in the medium. Although at the time of writing only 15 serial passages have been made with this virus, the results nevertheless suggest that the virus at this stage of cultivation is far more neurotropic than it was in the 176th subculture. The average time of death in the first 15 passages in mice with this virus in the 114th and 176th subcultures is shown in Table I. The average time of death of mice inoculated in series with virus from the 176th subculture is significantly later than that of those inoculated in series commencing with the 114th subculture. This loss of neurotropism for mice is evidently progressive in a medium containing chick embryonic tissue, and as will be shown below, the virus grown in this medium loses its power to produce fatal encephalitis in monkeys between the 89th and 114th subcultures.

[1] This analysis was kindly made for us by Dr. H. Muench.

Pathogenicity of Cultivated Strains of Virus for Rhesus Monkeys

Virus 17 E Grown in a Medium Containing Mouse Embryo Tissue.—In the previous communication by Lloyd, Theiler, and Ricci (8), the results of inoculating *rhesus* monkeys intraspinally and intracerebrally with virus grown in the presence of mouse embryo tissue have been presented. The supernatant fluid from the 35th to the 82nd subculture

TABLE I

Average time of death in mice infected with virus 17 D grown in chick embryo tissues; mice inoculated with 114th and 176th subcultures, and subsequent passages made in two parallel series by means of intracerebral inoculation of infected brain suspension.

No. of serial passage in mice	Average time interval between inoculation and death			
	Series initiated from 114th subculture		Series initiated from 176th subculture	
	Average for each passage	Average for 5 passages	Average for each passage	Average for 5 passages
	days	*days*	*days*	*days*
2	7.5		9.1	
3	6.8		10.1	
4	6.7		10.0	
5	7.7	7.1	9.7	9.8
6	8.5		9.7	
7	7.0		10.4	
8	7.9		9.3	
9	7.4		9.0	
10	7.5	7.0	10.4	9.6
11	6.3		10.8	
12	6.8		9.1	
13	7.4		8.2	
14	7.4		8.2	
15	7.8	7.2	8.0	9.1

was used. Three monkeys inoculated intracerebrally died presenting the histopathological lesions of encephalitis. Two of them also showed hepatic necrosis typical of visceral yellow fever. One of the latter had been inoculated with virus from the 82nd subculture. Ten monkeys were inoculated intraspinally with material from the 41st to the 76th subculture. All ten animals showed a febrile reaction. Three monkeys died, two presenting lesions of visceral yellow fever

and one of encephalitis. In six monkeys the circulating blood was examined for virus, which was shown to be present in all for a period of from 3 to 5 days. The significant features of these animal experiments were the production of visceral lesions of yellow fever even after 82 subcultures, the presence of circulating virus in the blood of all animals examined, and the relatively few animals which died of encephalitis following intraspinal injection of virus.

TABLE II

Results of Intracerebral Inoculation into Rhesus Monkeys of Virus 17 E Grown in Mouse Embryo Tissue Cultures

Monkey No.	Inoculum		Test for virus in circulating blood: mortality ratio in mice inoculated with serum*							Fever on days after inoculation	Time to death after inoculation	Mortality ratio in mice inoculated with brain suspension*	Histopathology of brain
	Subculture	Amount	Days after inoculation										
			1	2	3	4	5	6	7				
		cc.									*days*		
1	201	1.0		6/6		4/7		0/4		3, 4, 5, 7	10	5/6	Encephalitis
2	"	"		4/5		7/7		0/7		4, 5, 6, 7	9	7/7	"
3	203	"		7/7	7/7		3/6		0/6	6, 7, 8	12	Not tested	"
4	"	"		3/6	7/7		4/7		0/6	3, 4, 7	9	4/6	Not examined
5	"	"		4/7	5/6		3/5		1/6	5, 6, 7, 8	11	4/6	Encephalitis
6	"	"		5/7	7/7		4/6		0/6	3, 4, 5, 6, 7	10	5/5	Appears normal
7	"	"		3/6	6/7		1/6		0/5	6, 7, 8	10	5/6	Encephalitis

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

In the present study the neurotropism of the cultivated strain of virus for *rhesus* monkeys was tested by the far more severe test of intracerebral inoculation. In all, seven monkeys were inoculated intracerebrally, two with the supernatant fluid from the 201st subculture, and five with the supernatant fluid from the 203rd subculture. The results are shown in Table II. All the monkeys responded with a febrile reaction commencing on the 3rd to the 6th days after inoculation and lasting for 3 to 5 days. In their blood circulating virus was shown to be present for 3 to 4 days, and all the animals died in from

9 to 12 days after inoculation. The diagnosis of yellow fever virus encephalitis was confirmed in all the animals by histopathological study or by isolation of the virus from their brains after death, or by both methods. In all the animals the virus had disappeared from the blood at the time of death.

These studies give no indication of an apparent loss of neurotropism for monkeys of the virus cultivated in mouse embryo tissue. It must be emphasized that the strain of yellow fever virus used to initiate the culture is not only highly viscerotropic but also neurotropic to a high degree. This latter quality in the unmodified virus can be readily demonstrated in the *rhesus* monkey by intracerebral inoculation of the virus if the animal is given simultaneously an injection of immune serum intraperitoneally. Under these conditions the monkey develops a fatal encephalitis.

Virus 17 AT Grown in Testicular Tissue.—The amount of experimental evidence available concerning the affinity of the virus grown in testicular tissue for the central nervous system of monkeys is meagre. Lloyd, Theiler, and Ricci (8) reported the results obtained on inoculating four monkeys intraspinally with virus from the 30th and 35th subculture in testicular tissue. This virus had previously been maintained for 27 subcultures in mouse embryo tissue. All four monkeys died of yellow fever virus encephalitis in from 10 to 13 days. Noteworthy is the fact that in three of these monkeys, which were studied for the presence of virus in the blood, only minimal amounts could be demonstrated.

In the present study the pathogenicity for *rhesus* monkeys on intracerebral inoculation of the virus grown in testicular tissue was tested with the 90th subculture in guinea pig testicular tissue. This virus had been in tissue cultures for a total of 187 passages, during the last 160 of which testicular tissue was used. Two monkeys were inoculated intracerebrally with 1.0 cc. amounts of supernatant fluid. The results are shown in Table III. Both monkeys died of encephalitis, one on the 9th and the other on the 12th day following inoculation. In the blood of both only small amounts of circulating virus were demonstrated.

Virus 17 D Grown in Chick Embryo Tissue.—Three sets of observations are available concerning the pathogenicity of the virus grown in chick embryo tissue. The first experiment was performed with the 89th subculture, and has been previously presented (8). This experiment is included here for the sake of completeness. In this test six monkeys were inoculated intraspinally and three of them, Nos. 13, 14, and 15, received in addition 2.0 cc. of the culture intraperitoneally (see Table IV). All six animals died of encephalitis in 10 to 13 days after inoculation. Virus was shown to be present in the blood of all during a period of from 3 to 6 days.

The second experiment deals with four *rhesus* monkeys inoculated intracerebrally with virus from the 114th subculture. The concentration of virus in tissue culture is not very great. In order, therefore, to use a greater amount of virus for the inoculation of monkeys, several mice were inoculated intracerebrally with rehydrated material representing the 114th subculture. When these mice became sick as a result of yellow fever virus encephalitis, they were killed and from their brains a 10 per cent suspension was made. Four monkeys were inoculated intracerebrally with 1.0 cc. of the suspension. Titration of the suspension in mice showed that each monkey received approximately two million average lethal doses for mice. The results are shown in Table IV. All four monkeys lived, and the blood of two was followed for the presence of circulating virus. In the blood of one animal very small amounts were demonstrated on the 2nd and 3rd days; whereas in the blood of the other sufficient virus was present on the 2nd day fol-

TABLE III

Results of Intracerebral Inoculation into Rhesus Monkeys of Virus 17 AT Cultivated in Testicular Tissue Medium

Monkey No.	Inoculum		Test for virus in circulating blood: mortality ratio in mice inoculated with serum				Fever on days after inoculation	Time to death after inoculation	Mortality ratio in mice inoculated with brain suspension	Histopathology of brain
	Subculture	Amount	Days after inoculation							
			3	5	7	9				
		cc.						*days*		
8	160	1.0	0/6	3/6	1/7	0/5	4, 5, 6, 7	9	5/6	Encephalitis
9	160	1.0	0/5	2/6	0/6	0/5	Continuous 3rd to 10th	12	6/7	"

lowing inoculation to kill all of the five mice. Neutralizing antibodies were demonstrated in the serum of the four monkeys 1 month after inoculation. Their immunity was tested by an intracerebral inoculation of the most highly neurotropic yellow fever virus. All four monkeys lived, though one responded with a fever. It may be noted that this animal had the lowest antibody titer of the four monkeys in its serum at the time of the immunity test.

The third series of monkeys consisted of seven inoculated with material representing the 186th to the 216th subcultures in a medium containing chick embryo tissue. All responded with febrile reaction. Two of the monkeys died of intercurrent disease, No. 21 from peritonitis on the 29th day and No. 25 from tuberculosis on the 17th day. The brain of the latter, though free of virus as tested by intracerebral inoculation of mice, nevertheless showed histopathological signs of encephalitis in the form of a very mild perivascular infiltration. This histopathological evidence, as well as the febrile reaction in all the monkeys following

TABLE IV

Results of Inoculation of Virus 17 D Grown in Chick Embryo Tissues into Rhesus Monkeys by Neural Route

Monkey No.	Sub-culture used for inocu-lation	Route	Test for virus in circulating blood: mortality ratio in mice inoculated with serum									Fever on days after inoculation	Time to death after inoculation	Antibody titer 4 wks. after inoculation	Result of immunity test	
			Days after inoculation												Asibi virus subcu-taneously	French neurotropic virus intra-cerebrally
			1	2	3	4	5	6	7	8	9					
													days			
10	89th	i.s.		3/6		5/5		2/6				7, 8	10			
11	"	"		4/6	4/5		4/4		5/6			4, 5, 6, 7, 8	13			
12	"	"		5/6		5/6		5/6				6, 7, 8	11			
13	"	i.s. and i.p.		5/6	6/6		3/3		4/6			Continuous 4th to 10th	13			
14	"	"		6/6		5/5		3/6				1, 3, 5, 6, 7	12			
15	"	"		5/6	5/5		5/5		5/5			—	10			
16	114th	i.c.										2, 3, 4, 6, 7, 9, 10	Lived	1:2		Lived
17	"	"										8	"	1:3		"
18	"	"	0/6	2/6	2/6	0/2	0/3	0/6	0/4	0/6	0/3	Continuous 5th to 10th	"	1:4		"
19	"	"	0/6	5/5	3/6	0/4	0/6	0/5	0/6	0/6	0/6	7, 8, 9	"	1:2		"
20	186th	"										1, 2	"	1:28	Lived	
21	"	"			0/5		1/6		2/5		0/5	Continuous 3rd to 10th	" *			
22	"	"			0/5		0/6		2/7		0/6	8, 9, 10	"	1:25	Lived	
23	216th	"										6, 7, 8, 9	"	Positive not titrated		
24	"	"										Continuous 4th to 10th	"	"		
25	"	"										6, 7	†			
26	"	"										6, 7, 8, 9, 10	Lived	"		

i.s., intraspinally. i.p., intraperitoneally. i.c., intracerebrally. s.c., subcutaneously.

* Died of peritonitis 29 days after inoculation.

† Died of tuberculosis 17 days after inoculation.

the intracerebral inoculation of the virus grown in chick embryo tissue, on the one hand, and the absence of febrile reaction when the same virus was injected subcutaneously, on the other hand, clearly indicate that the virus is capable of producing a mild non-fatal encephalitis.

The remaining five animals showed protective antibodies in their serum obtained 4 weeks after inoculation. Two of the monkeys, Nos. 20 and 22, were given a test dose of highly virulent viscerotropic Asibi virus subcutaneously and showed no reaction.

From these three experiments it is evident that a marked change was induced in the virus grown in chick embryo tissue. This change had a comparatively sudden onset. The virus from the 89th subculture was highly neurotropic for monkeys and still had the ability to produce a visceral infection characterized by an abundance of virus in the circulating blood. The virus in the 114th subculture had lost both of these qualities.

Extraneural Inoculation of Rhesus Monkeys with Cultivated Strains of Virus

Virus 17 E Cultivated in Mouse Embryo Tissue.—The progressive loss of the power of the pantropic yellow fever virus grown in mouse embryo tissue to produce death in monkeys has been reported in full previously (8). Of seventeen monkeys inoculated intraperitoneally or subcutaneously with supernatant fluid from the 49th to the 109th subculture, fourteen responded with a febrile reaction of 1 to 5 days' duration. All survived. Virus was consistently demonstrated in the blood of all the animals on 2 or more days during the first week following inoculation.

In the present study the results of inoculating ten monkeys with material from the 179th and 206th subcultures are shown in Table V. In this experiment emphasis was placed on the presence of circulating virus, as it affords a far more delicate index of the viscerotropic affinity of a virus. Examination of the table shows that virus was present for 3 to 7 days in each animal. Of the ten monkeys, only No. 30 had a febrile reaction which could be attributed to the inoculated virus. Three monkeys, Nos. 27, 28, and 35, had irregular fever; in Nos. 27 and 28, it was attributed to tuberculosis, as both showed extensive tuberculous lesions when submitted to autopsy at the termination of the experiment. Monkey No. 33 died of intercurrent disease, and the remainder lived and were shown to be immune when tested by inoculation of virulent yellow fever virus 34 to 42 days after the injection of the tissue culture virus.

Virus 17 AT Cultivated in Testicular Tissue.—No specific experiments were undertaken to determine the pathogenicity of this strain of virus for monkeys by extraneural inoculation. From previous work and also the study of circulating virus in monkeys inoculated intracerebrally, the conclusion seems justified that

TABLE V

Results of Inoculation of Virus 17 E Grown in Mouse Embryo Tissue Cultures into Rhesus Monkeys Subcutaneously

Monkey No.	Inoculum		Test for virus in circulating blood; mortality ratio in mice inoculated with monkey serum										Fever on days after inoculation	Result	Antibody titer 30 days after inoculation	Test for immunity with Asibi virus
	Sub-culture	Amount	Days after inoculation													
			1	2	3	4	5	6	7	8	9	10				
		cc.														
27	179th	0.1	1/6	4/4	10/11	10/12	9/9	8/12	4/11	0/12		0/12	Irregular	Lived	Not tested	Lived
28	"	"	0/4	1/4	9/11	10/11	6/8	12/12	2/12	1/11		0/8	"	"	"	"
29	"	"	0/4	1/4	10/11	11/12	11/12	6/12	4/12	2/12		0/12	—	"	"	"
30	"	"	0/6	4/4	7/9	9/12	7/10	7/10	2/12	0/12		0/12	4, 5, 7	"	"	"
31	"	"	0/6	1/3	9/11	10/10	7/9	7/11	2/12	0/13		0/12	2	"	"	"
32	"	"	0/4	1/4	7/8	10/11	10/12	8/12	2/12	0/12		0/11	—	"	"	"
33	206th	1.0	0/5	1/5	6/7	6/6	6/6	3/3	3/7				—	*		
34	"	"	2/5	3/4	5/6	2/5	2/6	0/3	0/6				—	Lived	1:110	"
35	"	"	0/5	0/4	4/5	6/6	6/7	3/4	0/6				Irregular	"	1:38	"
36	"	"	0/5	0/5	5/6	5/5	4/6	0/4	0/7				—	"	1:45	"

* Died of colitis 14 days after inoculation.

this virus has a tendency to produce mild systemic infections with only minimal amounts of virus in the circulating blood.

Virus 17 D Cultivated in Chick Embryo Tissue.—Data on three groups of monkeys inoculated extraneurally with virus cultivated in chick embryo tissue are presented. The first group consists of three monkeys inoculated intraperitoneally with 2.0 cc. of supernatant fluid from the 89th subculture. Virus was shown to be present in the circulating blood of two of these animals for a duration of 5 and 6 days. The third animal was not studied from this point of view, though on one occasion when mice were inoculated intracerebrally with its serum, virus was shown to be present. Monkey 38 responded with a febrile reaction of 5 days' duration beginning the 3rd day after inoculation. This animal died on the 16th day and at autopsy showed lesions of tuberculosis. Histopathological studies, however, revealed encephalitis also. The other two animals remained well and resisted a test dose of virulent Asibi virus.

The second group of animals consists of four monkeys inoculated subcutaneously with material representing the 114th subculture of the virus in chick embryo tissue. The inoculum consisted of 1 in 20 suspension of infective mouse brains prepared from the brains of mice sick after an intracerebral inoculation of the 114th subculture. The same material was used to inoculate four monkeys intracerebrally, the results of which have been previously mentioned (monkeys 16 to 19, Table IV). Titration in mice by intracerebral inoculation of decimal dilutions showed that each monkey received approximately one million average lethal doses of virus for mice. All four monkeys were bled daily for the first 10 days from the vein, and the serum tested in mice for presence of virus. It will be seen from a study of Table VI that virus was shown to be present in the blood of three animals, though only in minimal amounts, the serum in no instance having sufficient concentration of virus to kill more than one mouse of the group inoculated. Protection tests with the sera taken 7 and 14 days after inoculation show that at 7 days antibodies were beginning to be present and were well developed by 14 days. All four animals survived. Their immunity was tested 41 days after the original inoculation by intracerebral injection of the French neurotropic virus. The four monkeys responded to this test inoculation with a febrile reaction commencing on the 4th and 6th days and lasting from 3 to 5 days. All lived, though No. 43 showed signs of encephalitis on the 8th day following the test inoculation.

The third set of observations concerns nine monkeys, of which four were inoculated with 0.5 cc. and five with 1.0 cc. of the supernatant fluid from the 186th, 195th, and 197th subcultures. Monkey 48 responded with a febrile reaction of 1 day's duration on the 5th day. Virus was proved to be present in the blood of five of these animals, although in two (Nos. 44 and 49) in very small amounts as only one mouse in a group became ill. In one animal, No. 50, virus was shown to be present for 5 days commencing on the 5th day after inoculation. Another monkey, No. 47, showed presence of virus on the 6th and 7th days in fair amounts, the last 2 days on which this animal's blood was tested. Monkeys 47 and 50 died

TABLE VI

Results of Inoculation of Virus 17 D Grown in Chick Embryo Tissue Culture into Rhesus Monkeys by Extraneural Routes

Monkey No.	Inoculum			Test for virus in circulating blood: mortality ratio in mice inoculated with serum										Fever on days after inoculation	Result	Antibody titer 4 wks. after inoculation	Result of immunity test	
	Sub-culture	Amount	Route	Days after inoculation													Asibi virus subcutaneously	French neurotropic virus intracerebrally
				1	2	3	4	5	6	7	8	9	10					
		cc.																
37	89th	2.0	i.p.		4/6		6/6		2/6					—	Lived		Lived	
38	"	"	"		6/6									Continuous 3rd to 7th	*			
39	"	"	"		5/5	4/4		4/4		2/5				—	Lived		Lived	
40	114th	Each received	s.c.	0/4	1/6	1/6	0/5	0/4	0/6	0/6	0/5	0/5	0/6	2	"	1:10		Lived
41	"	about 1,000,000	"	0/6	0/5	0/6	0/5	0/4	0/5	0/6	0/6	0/7	0/5	2	"	1:45		"
42	"	mouse M.L.D.	"	0/5	0/5	0/5	1/4	0/5	0/6	0/6	0/3	0/5	0/6	2	"	1:28		"
43	"		"	0/6	0/6	1/6	1/4	0/4	0/4	0/5	0/3	0/8	0/6	2	"	1:23		"
44	186th	1.0	"		1/6	0/6	0/6							—	"	1:40	Lived	
45	195th	"	"	0/5	0/5	0/5	1/7	1/6	0/6	0/5				—	"	1:8	"	
46	"	"	"	0/4	0/6	0/4	1/7	0/6	1/6	2/4				—	"	1:54	"	
47	"	"	"	0/4	0/5	0/5	0/5	0/5	4/6	4/5				—	†			
48	"	"	"	0/6	0/6	0/4	1/6	0/6	0/6	0/2				5	Lived	1:16	"	
49	197th	0.5	"	0/6	1/6	0/7	0/7	0/6	0/8	0/7	0/6	0/8	0/8	—	"	Positive, not titrated	"	
50	"	"	"	0/6	0/8	0/8	0/8	5/7	5/6	6/8	4/7	2/8	0/8	—	‡			
51	"	"	"	0/7	0/7	0/8	0/8	0/7	0/6	0/8	0/7	0/8	0/8	—	Lived	" "	"	
52	"	"	"	0/7	0/7	0/7	0/8	0/7	0/7	1/8	5/6	2/8	0/8	—	"	" "	"	

Underlined mouse groups show instances where death from yellow fever was verified.

* Monkey 38 died 16 days after inoculation showing tuberculosis as well as lesions of yellow fever.

† Monkey 47 died of colitis 10 days after inoculation and showed no evidence of yellow fever.

‡ Monkey 50 died of colitis 26 days after inoculation and showed no evidence of yellow fever.

of intercurrent diseases. The remainder lived, and all were shown to have developed neutralizing antibodies and to be immune to a test dose of virulent virus 36 to 37 days after the original inoculation.

Pathogenicity of the Cultivated Strains of Virus for Hedgehogs

Findlay and Clarke (14, 15) made the observation that the common European hedgehog is susceptible to the virus of yellow fever. Not only do these animals die after an inoculation with virulent viscerotropic strains of yellow fever virus but they also succumb to the infection after subcutaneous inoculation of modified neurotropic French strain. This latter strain, the character of which will be discussed in another paper (16), has been serially passed through a large number of mice by intracerebral inoculation and has lost its original viscerotropic affinity for monkeys to the extent that it rarely produces death with visceral lesions in them. Findlay showed, however, that in the hedgehog this virus still has the capacity to produce hepatic necrosis. It seems that the hedgehog therefore offers a more delicate means of determining the index of the viscerotropic potentialities of the virus than the *rhesus* monkey. It was decided to test the pathogenicity of the tissue culture virus in this species of animal. As the hedgehog has to be imported from Europe, the number of observations are limited.

Only two of the cultivated strains were tested in hedgehogs. They all were inoculated subcutaneously and the presence of virus in the liver and brain of those which died was tested by intracerebral inoculation of the organ emulsion in mice. The results are shown in Table VII. Of seven animals inoculated with virus 17 E grown in mouse embryonic tissue, No. 1 which had received virus from the 84th subculture died of yellow fever, whereas the six inoculated with the supernatant fluid from the 212th subculture all lived. The blood of these six hedgehogs was tested for the presence of neutralizing properties before inoculation and 25 days after they had received the injection of virus. The sera taken before the injection of virus were shown to be free of antibodies, whereas those obtained 25 days after inoculation showed the presence of antibodies.

The results in another series of six hedgehogs which were inoculated with the 198th subculture of virus 17 D grown in chick embryonic tissue were marred by the fact that five of the six animals died of intercurrent infection 12 to 14 days after inoculation. Intracerebral inoculation of mice with suspensions of liver and brain from these hedgehogs failed to reveal the presence of virus. An epidemic disease spread through all our hedgehogs at this time, and the mortality

among those under experiment was not higher than among those which were known to be immune to yellow fever.

In Table VII are also presented the results on eight normal hedgehogs inoculated subcutaneously with unmodified Asibi strain, the parent virus from which the tissue culture strains are derived. All eight animals died in 3 to 7 days. In the liver or brain, or both, of all the animals, virus was found to be present at death.

TABLE VII

Pathogenicity of Tissue Culture Strains of Yellow Fever Virus for Hedgehogs

Hedgehog No.	Inoculum		Result	Test for presence of virus at death	
	Virus	Subculture		Brain	Liver
1	17 E	84	Died of yellow fever	Positive	Positive
2	"	212	Lived		
3	"	"	"		
4	"	"	"		
5	"	"	"		
6	"	"	"		
7	"	"	"		
8	17 D	198	Died of intercurrent infection	Negative	Negative
9	"	"	Lived		
10	"	"	Died of intercurrent infection	Negative	Negative
11	"	"	" " " "	"	"
12	"	"	" " " "	"	"
13	"	"	" " " "	"	"
14	Asibi, unmodified		Died on 7th day, yellow fever	"	Positive
15	"	"	" " 7th " " "	Positive	"
16	"	"	" " 3rd " " "	"	"
17	"	"	" " 7th " " "	"	"
18	"	"	" " 7th " " "	"	"
19	"	"	" " 5th " " "	"	"
20	"	"	" " 5th " " "	"	"
21	"	"	" " 7th " " "	"	Negative

Though marred by the accidental deaths of five hedgehogs, the results give some definite information. It is apparent that after cultivation for 212 subcultures in mouse embryonic tissue the virus has lost its power to produce a fatal infection in hedgehogs. The results obtained with the virus 17 D grown in chick embryonic tissue can only be interpreted as indicating that this virus is avirulent for hedgehogs also. Although nearly all the animals had died of inter-

current disease, no virus could be demonstrated in the liver and brain at death and the conclusion seems warranted that no fatal infection followed inoculation. This conclusion is all the more probable when it is considered that in *rhesus* monkeys this virus has been shown to have less viscerotropic affinity than the variant grown in mouse embryonic tissue (17 E).

DISCUSSION

The results of these experiments show clearly that the modification induced in cultivated strains of yellow fever virus is determined by the nature of the tissue used in the medium. By prolonged cultivation in minced whole mouse embryonic tissue there is a progressive loss of the viscerotropic affinity of the virus but no obvious change in the neurotropic. Cultivation in testicular tissue induced a marked loss of viscerotropic affinity. Loss of neurotropic virulence was also induced but not to a marked extent. The loss of neurotropic affinity could be shown in mice but not in monkeys. Cultivation in a medium containing chick embryonic tissue induced a marked loss of both the viscerotropic and neurotropic affinities.

For a correct understanding of the changes induced, a clear knowledge of the pathogenicity of the virus before it was propagated in tissue culture is essential. The Asibi virus, the parent strain, is the most virulent yellow fever virus so far studied. When inoculated subcutaneously into monkeys, it produces death in 4 to 7 days in 95 per cent of these animals. Maximal amounts of virus are present in the blood. Not only does this virus exhibit a high viscerotropic affinity, but its neurotropic affinity is well marked, not only for mice but also for monkeys. Prolonged cultivation of the Asibi virus in a medium containing chick embryonic tissue leads to a marked loss of both of these two tissue affinities. Subcutaneous inoculation rarely produces febrile reaction and the quantity of virus demonstrable in the blood of infected monkeys is usually minimal. On intracerebral inoculation into monkeys, a non-fatal encephalitis is induced.

This marked loss of neurotropic affinity in the virus grown in minced chick embryonic tissue is in all probability due to the use of minimal amounts of nervous tissue in the medium. This was achieved by cutting away the brain and spinal cord of the chick embryos before

TABLE VIII

A Comparison of the Pathogenicity of Cultivated Yellow Fever for Rhesus Monkeys and Hedgehogs

Tissues used in culture medium	Results in monkeys		Results in hedgehogs by subcutaneous inoculation
	By extraneural inoculation	By intracerebral inoculation	
Whole mouse embryo—virus 17 E	Monkeys survive, but show a considerable amount of virus in the circulating blood.	Death from encephalitis	Survive
Mouse and guinea pig testicular tissue—virus 17 AT	Monkeys survive and show only traces of virus in the circulating blood	" " "	Not tested
Chick embryo tissue with head and spinal cord removed—virus 17 D	Monkeys survive and show only traces of virus in the circulating blood	Non-fatal encephalitis	Survive
Unmodified Asibi virus	About 95 per cent of monkeys die of yellow fever showing typical visceral lesions; virus present in the circulating blood in high concentration	Death usually from generalized infection with typical visceral lesions. Death from encephalitis only when immune serum is given intraperitoneally at the time of intracerebral injection of virus	Death from yellow fever in 3 to 7 days with typical visceral lesions

mincing. It must be realized, however, that the experiments do not exclude the possibility that these marked changes were induced by the chick tissue itself and not by the relative absence of the nervous tissue. The pathogenicity of the three cultivated strains for monkeys and hedgehogs is summarized in Table VIII. Included in this table are the pathogenic characteristics of the Asibi strain of virus, the parent strain from which the three cultivated variants are derived.

SUMMARY

1. Experimental evidence is presented to show that prolonged cultivation of yellow fever virus *in vitro* results in a change in its pathogenicity, and that this change varies with the type of tissues used for the cultivation.

2. In the tissue cultures used for the propagation of the virus, three different types of tissues were used. They included whole mouse embryo, chick embryo from which the head and spinal cord had been removed, and testicular tissues of mice and guinea pigs.

3. The changes in the pathogenicity of the virus cultivated for a period of over 3 years in a medium containing the tissues of whole mouse embryo were not striking. The viscerotropic virulence of the virus appeared somewhat diminished, in that when injected subcutaneously into *rhesus* monkeys or hedgehogs it failed to produce a fatal infection, although there is evidence to indicate that a generalized infection takes place as demonstrated by the appearance of virus in the circulating blood in relatively high concentration during infection. The neurotropic virulence of the virus remained unaltered during the cultivation in this medium.

4. The changes in the pathogenicity of the virus cultivated in medium containing tissues of chick embryo from which the head and spinal cord had been removed were very pronounced. The viscerotropic virulence of the virus was lost to a large extent. When injected subcutaneously into monkeys there was as a rule a very mild generalized infection, as demonstrated by the minimal quantities of virus found in the circulating blood. Its neurotropism was also much diminished. When injected into monkeys intracerebrally, it no longer produced a fatal encephalitis but only a moderate febrile reaction, followed by recovery and solid immunity to reinoculation with a highly

virulent strain of virus. When injected intracerebrally into mice, the mortality ratio was not diminished but the incubation period was markedly prolonged.

5. The changes in the pathogenicity of the virus cultivated in medium containing testicular tissues were somewhat similar to those observed after cultivation in chick embryo medium which contained only a minimal amount of nervous tissue. Its viscerotropic affinity had been largely lost and only very small amounts of virus were found in the circulating blood of monkeys inoculated subcutaneously. Given intracerebrally, it produced death from encephalitis in monkeys. The incubation period in mice inoculated intracerebrally with this virus was also prolonged but somewhat less so than with the virus grown in chick embryo tissues without the central nervous system.

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