THE SURVIVAL OF YELLOW FEVER VIRUS IN CULTURES

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During the period from January to June, 1929, blood and tissues from many *rhesus* monkeys, experimentally infected with yellow fever, were planted in a variety of artificial media. Cultures were made, also, from several samples of human blood from patients with yellow fever; but these were prepared under less favorable circumstances than the cultures of monkey material, since there was usually a lapse of several days between the withdrawal of the sample and its arrival at the laboratory. The great majority of the cultures remained bacteriologically sterile, as determined by the absence of visible growth and our failure to find organisms by dark field examination and in stained smears.

Injection of Cultures into rhesus Monkeys

Cultures were injected into *Macacus rhesus*, and some of the inoculated animal subsequently had febrile temperatures; in a few instances it was possible to prove that yellow fever had really been contracted. In Table I a brief summary is given of nine experiments in which the proof was complete.

The longest period intervening between inoculation of culture medium and injection therewith of a test monkey, with resulting yellow fever, was 12 days. In this instance, McNeal-Marchoux medium had been employed, but the most consistently satisfactory results were obtained with a special egg medium. This was a modification of McCoy's egg-yolk medium (1) for the cultivation of *B. tularense*. Its composition and mode of preparation were as follows:

Egg yolk	110 gm. (approximately)
Rabbit blood (defibrinated)	40 cc.
Distilled water	50 cc.

The ingredients were thoroughly mixed, coagulated in a slanted position in the Arnold sterilizer, and sterilized fractionally at a temperature of 75°C. In later

* This paper was prepared by Dr. N. C. Davis from the laboratory notes of Dr. Paul A. Lewis, who died of yellow fever in Bahia, Brazil, June 30, 1929.

TABLE I

Yellow Fever Infections in Monkeys Following Injection of Cultures Inoculated with Virus

Material cultured	Source of inoculum	Medium	Length of time in culture (days)	Quantity infectious blood injected (cc.)	Monkey inoculated	Strain of virus	Outcome	Criteria of infection
Liver	*Rhesus* H	Egg-Serum	2		*Rhesus* L	Asibi	Killed when moribund	Typical lesions in liver
Blood	*Rhesus* A	Egg-Serum	4	0.25	*Rhesus* B	Asibi	Killed on 2nd day of fever	Typical lesions in liver
Blood	*Rhesus* A	Egg-Serum	4 + 4*	?	*Rhesus* C	Asibi	Killed on 4th day of fever	Typical lesions in liver
Blood	*Rhesus* A	Egg-Serum	8	0.5±	*Rhesus* D	Asibi	Killed when moribund	Typical lesions in liver
Blood	*Rhesus* A	Marchoux	12	0.5±	*Rhesus* E	Asibi	Killed on 2nd day of fever	Positive transfers of blood. (Liver lesions not definite)
Blood	*Rhesus* B	Egg-Serum	9	0.2	*Rhesus* J	Asibi	Killed when moribund	Typical lesions in liver
Blood	*Rhesus* E	Egg-Serum	4	0.000033‡	*Rhesus* K	Asibi	Killed when moribund	Typical lesions in liver
Blood	*Rhesus* U	Noguchi† (semi-solid)	7	0.1	*Rhesus* Z	S.R.	Fever. Recovered	Serum protected against virus
Blood	*Rhesus* U	Noguchi (semi-solid)	7	0.1	*Rhesus* AA	S.R.	Fever. Recovered	Positive transfer of blood. Animal afterward immune

* First condensation water from culture tubes injected into *Rhesus* B. Fluid replaced and injected into *Rhesus* C at end of second 4-day period.

† Culture also contained *Leptospirae*.

‡ Only condensation water and washings were injected; clot of original blood (diluted with normal blood) was not used.

experiments part of the egg yolk in this mixture was replaced by monkey liver and the medium was heated to 100°C. However, in the experiments in which the latter mixture was used, the results were negative.

A number of trials were made with the "MON" medium recommended by Kuczynski (2). It was impossible, however, to obtain the "normosal" called for in this formula, so a 1:5 dilution of sea-water was used instead. It is believed that none of the animals which were injected with cultures made on this medium contracted yellow fever. In one series the original quantity of blood was small (0.0001 cc.), but if the medium had been as favorable to the multiplication of the causative organism as its originator claimed, there should have been no trouble in securing an infection at the end of 4 days. In another series, the quantity of infectious blood in each tube was about 0.1 cc., but the incubation of the cultures lasted 7 days.

One series of experiments was made with Noguchi's semisolid *Leptospira* medium. Some of the inoculated tubes were left unsealed; the others were sealed with vaseline (partial anaerobiosis). None of the monkeys injected with these cultures developed yellow fever. In a second series, half of the tubes were inoculated with *Leptospira icteroides* (strain Palmeiras V) in addition to the infectious blood, since it was thought that the virus might maintain itself better in symbiosis with *Leptospirae*. At the end of 1 week mild infections were obtained by the injection of media both with and without *Leptospirae*; injections at the end of 2 weeks did not produce infection.

In a series of fifteen tubes of coagulated egg medium, the condensation fluid was supplemented by an egg-liver-brain extract with a trace of some substance for enrichment, either dextrose, lactose, maltose, sucrose, mannitol, glycerol, glycogen, peptone, or tryptic digest; and to this was added 0.001 cc. of infectious blood diluted to 0.05 cc. with normal monkey blood. No infections resulted from injections of this material at the end of 1 week.

The virus used in cultures represented three strains (Asibi, B.B., and S.R.) in routine use at the laboratory in Bahia (3). Except for the two cultures in semisolid medium with S.R. strain virus, all successful inoculations were with the Asibi strain. Cultures in Noguchi's medium were maintained at room temperature (22 to 25°C.); the other cultures were incubated at 35°C.

In addition to the experiments in which yellow fever was produced (see Table I), there were a number of others in which, without definite proof of infection, suggestive evidence was obtained.

The most interesting of these concerned *Rhesus* Q, which was injected with material from a culture on egg medium 18 days old. The animal had fever on the second and third days following injection; the maximum temperature was 105.2°F. 16 days after the first injection he was given an immunity test with fresh infectious blood and again had fever, this time for 6 days, with a maximum temperature of 104.6°F. Recovery followed.

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Injections of Dilutio

Quantity of blood (cc.)	Experiment I. May 28, 1929			Experiment II. June 7, 1929		
	Strain of virus	Animal inoculated	Outcome	Strain of virus	Animal inoculated	Outcome
3.0						
1.0	Asibi	*Rhesus* F	Killed on 2nd day of fever. Lesions indefinite			
0.1	Asibi	*Rhesus* G	Fever. Dead on 4th day. Typical yellow fever			
0.01	Asibi	*Rhesus* H	Fever. Killed when moribund on 4th day. Typical yellow fever			
0.001	Asibi	*Rhesus* I	Fever. Killed when moribund on 5th day. Typical yellow fever	Asibi	*Rhesus* P	Fever for 5 days. covered
0.0001				Asibi	*Rhesus* O	Fever. Died on 5t Typical yellow f
0.00001				Asibi	*Rhesus* N	Fever. Killed moribund on 4t Typical yellow fe
0.000001				Asibi	*Rhesus* M	No fever. Died c day. Probable fever

II

of Infectious Blood

	Experiment III. June 11–14, 1929			Experiment IV. June 20–24, 1929		
	Strain of virus	Animal inoculated	Outcome	Strain of virus	Animal inoculated	Outcome
	S.R.	*Rhesus* S	Fever. Killed when moribund on 4th day. Typical yellow fever			
	S.R.	*Rhesus* U	Fever. Killed when moribund on 6th day. Typical yellow fever	S.R.	*Rhesus* BB	No fever. No reaction to immunity test
	S.R.	*Rhesus* R	Fever. Killed when moribund on 5th day. Typical yellow fever	S.R.	*Rhesus* Y	Killed on 2nd day of fever. Typical yellow fever
e-	S.R.	*Rhesus* T	Fever. Killed when moribund on 5th day. Typical yellow fever	S.R.	*Rhesus* X	Fever. Killed when moribund on 6th day of fever. Typical yellow fever
w				S.R.	*Rhesus* W	No fever. No reaction to immunity test
en y. l ow				S.R.	*Rhesus* V	Fever. Killed when moribund on 7th day. Typical yellow fever

Many animals that showed no temperature reaction to the original injection later proved partly or wholly refractory to test doses of virus; the assumption is that some immunity was called forth by the dead or attenuated virus present in the cultures.

Rhesus B was inoculated with the condensation water from two culture tubes at the end of 4 days' incubation, containing in all about 0.25 cc. of infectious blood. The fluid from these tubes was replaced with that from uninoculated medium, and incubation was continued for 4 days more. *M. rhesus* C was inoculated with the fluid from these same tubes at the end of the second 4-day period. Although *Rhesus* B and *Rhesus* C were sacrificed while still in the febrile stage, both had liver lesions typical of yellow fever. It was thought at first that there might have been reproduction of the virus in the culture medium, but the results of dilution experiments suggested that probably there was merely a survival from the original inoculum.

Injections of Infectious Blood Highly Diluted

A summary of the dilution experiments is given in Table II. It will be noted that while Experiment III and Experiment IV each represents a single series of dilutions, in neither case were all injections made on the same day or with blood from the same monkey. Each of these experiments might be subdivided, making six in all, except that additional space would be needed for recording them and the results are sufficiently clear in the present scheme. Dilutions of infectious blood were made in every case with either citrated or defibrinated blood from normal monkeys. Injections were made intraperitoneally.

It is evident that both the Asibi and S.R. strains of virus were fatal in quantities of 0.00001 cc. of infectious blood. There is no reasonable doubt that the monkey which received 0.000001 cc. of blood containing Asibi strain virus also succumbed to yellow fever. The liver section showed a considerable number of postmortem changes, but there was undoubtedly a severe injury preceding death. Aragão (4) records positive results from the injection of a millionth part of an infected mosquito.

DISCUSSION

It has been shown by Sawyer, Lloyd, and Kitchen (5) that citrated or clotted blood from animals infected with yellow fever retains

a certain amount of virulence after storage for at least 35 days and that glycerinated blood is capable of infecting animals after preservation for 60 days. Their specimens were kept at refrigerator temperature. The dosage which produced yellow fever was 1 cc.

From the results of the dilution experiments recorded above, it would seem certain that the infections obtained from the injection of cultures were due to the survival of virus from the original inoculum. The only surprising point is that virus survived in infective quantities for at least 12 days at a temperature of 35°C., when usually it dies out quite rapidly in citrated blood at room temperature.

It is believed that such of the infections reported by Kuczynski (2) as were actually yellow fever, were caused by virus which survived in the medium and not by the visible organisms under cultivation. Since extremely minute quantities of virus will produce infection, it is easy to account for the virulence of subcultures. This explanation does not preclude the possibility that the bacteria of Kuczynski, although probably not the cause of yellow fever, may have had some pathogenic properties of their own.

SUMMARY AND CONCLUSIONS

1. The virus of yellow fever has been found to survive in artificial culture media for at least 12 days at a temperature of 35°C. No visible growth has been present and no reproduction of the virus has been demonstrated.

2. Infections have been obtained in *rhesus* monkeys with two strains of virus in quantities as small as 0.00001 cc. of infectious blood, and with one strain in an amount probably as minute as 0.000001 cc.

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