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Virulence and Pathogenesis of Yellow Fever Virus Serially Passaged in Cell Culture

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Viscerotropic virulence of the Asibi strain of yellow fever virus (YFV) for monkeys has been known to be lost after serial passage in HeLa cell monolayers. This phenomenon was investigated in several other mammalian and insect tissue cell lines. Assay in monkeys of original seed virus and of virus after 7 and 11 passages in a porcine kidney cell line (PK) indicated essentially equal infectivity and mortality. Moreover, monkeys receiving the passaged virus exhibited more rapid onset of disease and death than animals infected with original seed virus. Histological changes in animals inoculated with passaged virus were identical to those in animals receiving the seed virus. Virus from later passages in PK cells was also lethal for approximately 50% of the monkeys; however, evidence for progressive attenuation was seen in these preparations. Similar results were obtained with a mosquito (*Aedes aegypti*) cell line. In contrast to results obtained in PK and mosquito cells, YFV became essentially avirulent (nonlethal and less infective) for monkeys after only seven passages in HeLa cell cultures.

After Stokes discovered in 1928 (12) that the rhesus monkey was susceptible to yellow fever virus (YFV), Bearcroft (1) and others reported on the pathology of the virus in various organs of this experimental animal (*Macaca mulatta*). Theiler (15) showed that the pathology of yellow fever in the monkey is similar to that in man. Tigertt et al. in 1960 (16) presented a detailed and comprehensive study of the pathology of yellow fever in the liver of *M. mulatta*.

The Asibi strain of YFV has long been known to lose its virulence upon continued passage in cell culture, as indicated by Lloyd et al. in 1936 (7). Theiler corroborated this virulence change in experimentally infected monkeys in 1937 (13), as did Schindler and Hallauer in 1963 (11).

In more definitive investigations, Hardy in 1963 (4) and Hearn et al. in 1966 (5) reported that, although intracerebral virulence in mice remains at a high level, intraperitoneal virulence for the monkey is drastically reduced during serial passage of YFV in HeLa cell monolayer cultures. The reduction in virulence was noted as early as the third passage and progressed to the point at which monkeys were able to resist inoculation of doses as high as 700,000 mouse intracerebral median lethal doses ($MICLD_{50}$) after only seven passages.

It is of great interest to understand the mechanisms involved in the rapid loss of viral virulence during serial passage in animal cell cultures. It was believed that differences in rates of virus attenuation during passage in various cell lines might be related to some characteristics of these cells, such as their nutritional or metabolic differences. This report deals with the search for a cell line with markedly different ability to cause attenuation of YFV from that found with the HeLa cell line. The screening of several mammalian and insect cell lines for resulting virulence of YFV during serial passage, a detailed study of changes in the virulence pattern of YFV in one of the more promising cell lines, and comparative pathology of passaged and unpassaged virus are described.

MATERIALS AND METHODS

Agent. The Asibi strain of YFV from blood serum of moribund infected monkeys (*M. mulatta*), stored at −60 C, was used for studies on pathogenesis and as inoculum for cell cultures. The titer of this monkey serum was 10^8 to 10^9 $MICLD_{50}$/ml.

Cell cultures. Cell lines reported on in this study were: a mouse embryo intestinal line (MEI) developed in this laboratory; a clonal line of porcine kidney stable cells, PK (PS-15), initiated by Inoue in Japan

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(6) from the PK2A cell line; and a mosquito cell line, initiated by Grace (3) in Australia from *Aedes aegypti*, a known vector of yellow fever. HeLa cell monolayers were included as controls.

Growth and maintenance of cell cultures. Mammalian cells were grown and maintained as monolayer cultures throughout the experiment in the defined medium of Nagle (9) without insulin but with 10% fetal bovine serum added. *A. aegypti* cultures were adapted to growth in suspension in Nagle's hemolymph-free insect cell medium (8). This medium also contained fetal bovine serum.

Inoculation and adsorption. Cell cultures were inoculated with approximately 10^7 $MICLD_{50}$ of virus (virus-cell multiplicity of infection of 10 per 1) and incubated for 1 hr at 35 C, the inoculum was removed, the cell cultures were washed with balanced salts solution (BSS), and the medium was replaced. Incubation temperatures for the experiments were 35 C for mammalian cells and 25 C for insect cells (2). Approximately neutral *p*H was maintained in the mammalian cultures throughout incubation by addition of bicarbonate when necessary. Insect cell cultures maintained a constant *p*H of approximately 6.6 without adjustment.

Assay of virus. Twelve- to 14-g Swiss Webster, Detrick strain mice were inoculated intracerebrally (ic) with 0.03 ml or intraperitoneally (ip) with 0.25 ml of appropriate dilutions of virus in beef heart infusion broth. Animals were observed for 21 days, and the LD_{50} titers were calculated by the method of Reed and Muench (11). Ten mice were used for each dilution.

In preliminary studies to determine virulence in monkeys, 0.5 ml of the seed virus or virus suspensions from various passages was inoculated ip and lethal effects were noted. Surviving animals were rechallenged (ip) at 30 days postinoculation with 5×10^5 $MICLD_{50}$ of the seed virus. All animals surviving the challenge dose were considered to have been infected and immunized by the original inoculation; those not surviving the challenge with seed virus were not considered as infected by the primary injection. For final evaluation in monkeys, 0.5 ml of appropriate dilutions of passaged virus from various passage numbers was inoculated ip, and the animals were observed several times daily for onset of disease and day of death. Survivors were tested for subclinical infection by challenge as described above. This method of determining infection (by rechallenge) could safely be used since, originating in India, it is highly unlikely that the monkeys could ever have been exposed to yellow fever before this test. Complete necropsies were performed on all monkeys at death or upon completion of the experiments, gross pathology was noted, and histological sections were prepared by the usual methods for final diagnosis.

Studies on pathogenesis. Mice and monkeys were inoculated with YFV ip, ic, or intravenously (iv), both before and after six serial passages in HeLa or MEI monolayer cultures. Monkeys received 0.5 ml of virus by all three routes; mice received 0.03 or 0.25 ml ic or ip, respectively. In addition, monkeys were inoculated with appropriate dilutions of various passages in PK monolayers, up to and including the 20th passage, for comparative histopathological changes. All animals were subjected to complete necropsies to examine for gross and histopathological changes and to determine the effects of route of inoculation on the disease.

RESULTS

Preliminary screening of cell lines. Data in Table 1 indicate the possible superiority of the mammalian cell lines PK and MEI and of the mosquito cell line over the HeLa cell controls relative to stability of virulence for monkeys during serial passage of the virus. Moreover, inoculation of several dilutions of the virus from the seventh passage in PK cells indicated lethality with <16 $MICLD_{50}$. Because of fast growth, ease of handling, and consistently higher titers, the PK line was chosen for additional study.

Studies with the PK cell line. Figure 1 shows representative curves of YFV replication during passage in HeLa and PK monolayer cultures. In general, virus yields measured by ic inoculation in mice showed increases through the seventh passage in PK cells. On the other hand, the titer in HeLa cells dropped to a low point at the third or fourth passage and then steadily increased.

In the first trial, the seed virus and virus from the seventh passage in PK monolayers were titrated in monkeys. As shown in Table 2, no apparent loss of virulence for the monkey occurred at the seventh passage in PK cells. The pattern of infection was identical for the two preparations, and lethality was essentially equal; approximately 6 $MICLD_{50}$ of virus resulted in 100% mortality in each preparation. The lower mortality in the two higher doses of virus (Tables 2 and 4) can be explained by the observations of

TABLE 1. *Preliminary assay (ip) of passaged yellow fever virus in monkeys*

Cell line	Passage no.			
	Sixth		Seventh	
	Dose ($MICLD_{50}$)	Mortality[a]	Dose ($MICLD_{50}$)	Mortality[a]
HeLa	200,000	1/2		
Mouse embryo intestine	16,000	1/2		
Aedes aegypti			4,000	2/2
			400	2/2
Porcine kidney			4,000	2/2
			<16	1/2
HeLa[b]			700,000	0/2

[a] Dead total.

[b] Hearn et al. (5).

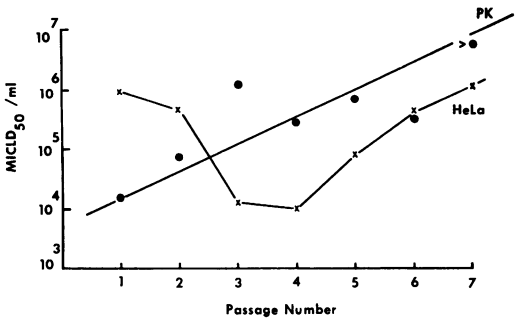

FIG. 1. *Virus titers of yellow fever virus during serial passage in pig kidney (PK) and HeLa cell monolayers.*

TABLE 2. *Assay of yellow fever virulence (ip) in monkeys*

Inoculation dose ($MICLD_{50}$)	No. infected (infected/total)		No. dead (dead/total)[a]	
	Seed virus	7th Passage virus[a]	Seed virus	7th Passage virus[a]
600	5/5	5/5	4/5	4/5
60	5/5	5/5	5/5	4/5
6	5/5	5/5	5/5	5/5
0.6	2/5	2/5	1/5	0/5
0.06	1/5	1/5	0/5	0/5

[a] Virus passaged in porcine kidney monolayers.

Theiler (14), who indicated the importance of using several dilutions of suspected material to exclude the phenomenon of lower mortality in lower dilutions of YFV.

Both infection and death of monkeys were inconsistent below one $MICLD_{50}$; therefore, a dividing line was established between the 0.6 $MICLD_{50}$ dose and the 6 $MICLD_{50}$ dose. Only data obtained in monkeys in the range between 6 and 60,000 $MICLD_{50}$ were used to compare virulence or attenuation of various passages. As shown in Table 3, onset of disease occurred 2 to 3 days earlier and death occurred 1 to 2 days sooner in animals receiving the passaged virus than in animals inoculated with the seed virus.

A repetition of the above passage of YFV in PK cells, extended to include 15 passages, confirmed the first results and established that virulence for the monkey had not changed at the 11th passage but indicated some degree of virus attenuation at the 15th passage. This was evidenced by decrease of both infectivity of the virus and resulting mortality. Further studies, confirming this attenuation, were carried to the 20th passage.

In all four of these studies, agreement among experiments for the 7th, 11th, and 15th passages was very close for infectivity, mortality, onset of disease, and time of death postchallenge. Composite data are shown in Table 4. Since the number of monkeys per point ranged from 4 to 14 animals, values are expressed as per cent for clarity.

As shown in Table 4, YFV produced during 11 serial passages in PK cell cultures caused practically 100% infection and more than 90% mortality in monkeys when doses in the range of 6 to 60,000 $MICLD_{50}$ were inoculated. In contrast, at the 15th passage, the infectivity of the virus had decreased to 90%, and only approximately 50% of the infected animals died. The virulence pattern for the 20th passage was similar to that of the 15th, as indicated by lack of correlation of dose with mortality, decrease of infectivity to 80%, and a mortality value only one-half the corresponding morbidity value. A subsequent test of 13th passage virus indicated that attenuation had already begun at this time, as shown by very slight decrease in the infectivity of the virus (94%) and a decrease in mortality to approximately 60%.

Examination of virus from the HeLa cell control cultures indicated complete loss of lethality for monkeys and 30% loss of infectivity of the virus as early as the 7th passage, in contrast to no loss of virulence in the PK cultures through 11 passages.

Studies on pathogenesis. The disease in monkeys was characterized by clinical illness occurring usually on the 3rd or 4th day but sometimes as late as the 7th day postinoculation. Inappetence, extreme debilitation, and ruffled fur were the

TABLE 3. *Pathogenesis of yellow fever virus in monkeys*

Inoculation dose ($MICLD_{50}$)	Mean incubation period (days postinoculation)[a]		Mean day of death (postinoculation)	
	Seed virus	7th Passage[b]	Seed virus	7th Passage[b]
600	5 (4–6)	3.2 (3–4)	5.75 (5–6)	4.75 (4–5)
60	5 (4–6)	3.25 (3–4)	6.2 (5–7)	5 (4–7)
6	5.8 (5–7)	4.2 (3–5)	6.8 (6–8)	5.2 (5–6)
0.6	6[c]	3[c]	7[c]	
0.06	7[c]			

[a] Day of obvious clinical illness. Figures in parentheses indicate span.

[b] Virus passaged in porcine kidney monolayers.

[c] One animal each.

TABLE 4. *Composite data of intraperitoneal virulence of yellow fever virus in monkeys*[a]

Inoculation dose ($MICLD_{50}$)	Seed		PK-7[b]		PK-11		PK-13		PK-15		PK-20		HeLa-7	
60,000									6/9	67	1/4	25	0/4	0
6,000	2/2	100	10/11	91			1/4	25	4/9	44	1/4	25	0/4	0
600	6/7	86	12/14	86	9/9	100	4/4	100	7/9	78	3/4	75	0/4	0
60	7/7	100	13/14	93	9/9	100	4/4	100	3/9	33	2/4	50	0/4	0
6	7/7	100	12/14	86	7/9	78	0/4	0	1/4	25	1/4	25	0/4	0
0.6	1/7	14	5/14	36	3/9	33	0/4	0						
0.06	0/7	0	0/10	0	0/5	0								
Per cent infection[c]	23/23	100	52/53	98	27/27	100	15/16	94	36/40	90	16/20	80	14/20	70
Per cent mortality[c]	22/23	96	47/53	89	25/27	93	9/16	57	21/40	52	8/20	40	0/20	0

[a] Four experiments: total of 254 monkeys (4 to 14 monkeys per value). Values in top section of table: per cent mortality. Fractions indicate dead/total; whole numbers indicate per cent.
[b] Virus passaged in pig kidney cells; numbers indicate passage number.
[c] Values in bottom section of table include animals receiving doses of 6 to 60,000 $MICLD_{50}$ of virus.

usual clinical signs, although death sometimes occurred without illness being detected. Death generally occurred on the 4th or 5th day but was delayed as long as 8 days in rare instances.

In the liver, histopathological changes included individualization of hepatocytes, diffuse hepatocellular degeneration, granular and dense eosinophilic intracytoplasmic bodies (Councilman bodies) in hepatocytes and Kupffer cells, and an occasional irregular small eosinophilic inclusion body in hepatocyte nuclei. Necrosis of germinal centers and perifollicular nuclear fragmentation were encountered commonly in the spleen, lymph nodes, and other lymphoid tissue accumulations. Nephrosis characterized by degeneration of the epithelium of proximal and distal tubules and proteinaceous cast formation in collecting tubules was observed in approximately 50% of the kidneys examined. In animals that died, route of infection did not alter severity of the lesions, nor were differences noted in animals infected with YFV seed or the various tissue culture passage inocula.

Histological changes were limited to the central nervous system of mice. Lesions included perivascular cuffing with mononuclear cells, focal and diffuse gliosis, satellitosis, and spongy degeneration. Route of inoculation or tissue culture passage inoculum utilized did not alter the lesion distribution.

An interesting observation was noted in a passage experiment in MEI and HeLa cell cultures. Normally the ic titer of a given YFV suspension is much higher in mice than the corresponding mouse ip titer ($10^{6.0}$ $MICLD_{50}$/ml compared with $10^{1.6}$ $MIPLD_{50}$/ml in a sample of a dilution of the monkey serum virus seed). After six serial passages of the seed virus in MEI cells, the ip titer in mice had increased from $10^{1.6}$ to $10^{3.6}$ $MIPLD_{50}$/ml. On the other hand, the $MIPLD_{50}$ titer of YFV passages in HeLa cells became undetectable during a comparable period. The $MICLD_{50}$ titers of virus passages in both cell lines were in the usually expected range of $10^{6.0}$.

DISCUSSION

In regard to the method of determining infection in monkeys surviving inoculation with YFV (by rechallenge), we must admit the remote possibility of slight protection against yellow fever from preexposure to other group B arboviruses. However, a significant level of protection from such exposure is highly unlikely considering the high challenge dose (10^5 $MICLD_{50}$) of yellow fever. In addition, of the three likely group B viruses occurring in India, Japanese B encephalitis virus was ruled out by serological tests in these monkeys, Kyasnur Forest disease virus occurs in a very limited area, and, to our knowledge, no protection against yellow fever by preexposure to dengue virus has been reported. It might also be stated that the patterns of infection and mortality in these monkeys were very similar in the three repetitive experiments.

The above data have substantiated the findings of Hearn et al. (5) that drastic reduction in virulence of YFV for monkeys follows serial passage in the HeLa cell line and demonstrates a striking difference between the HeLa and PK cell lines in their ability to maintain this virulence. Not only does virulence (both lethality and infectivity for monkeys) remain stable for at least 11 transfers in PK cells, but also substantial

lethality is evident even after 20 passages. Additional evidence to indicate stability of virulence during passage in PK cells was the similarity and equal severity of histopathological lesions in monkeys inoculated with the seed virus and with the passaged virus.

Moreover, there is some evidence that, at least through 11 passages in the PK cell line, the virus may be increasing in virulence. First, let us consider the progressive rise in mouse titer in successive passages (Fig. 1). Since mouse inoculation is the practical method of assay of YFV (a successful plaque assay for unattenuated YFV has not been developed), the possibility exists that the rise in titer may reflect an increase in virulence for mice rather than an increase in the amount of virus. Next, the onset of disease in monkeys and the time to death were shortened after inoculation of a given $MICLD_{50}$ dose of passaged virus.

A possible explanation of the drastic loss of virulence for monkeys during passage in the HeLa cell line is suggested by the data in Fig. 1. Hearn and his co-workers (5) first noted a loss at the third passage, which progressed to essentially an absence of virulence by the 7th passage. This would indicate a mixed population of virulent and avirulent virus in which the virulent portion died out by the third passage, followed by an overgrowth of the avirulent population, and could explain not only the decrease and subsequent increase in titer of the curve in Fig. 1 but also the avirulence for monkeys of seventh-passage material.

At least two separate factors, independent of each other, are involved in loss of virulence for monkeys of YFV (Table 4): (i) a decrease in infectivity of the virus and (ii) a decrease in lethality in the infected animals. Each of these factors progressively decreased at an independent rate.

It is obvious from the data presented above that cell lines vary in their ability to support unattenuated YFV during passage and in their tendency to favor the overgrowth of an avirulent form of the virus. The differences between the PK cell line and the HeLa cell line, in this respect, are very striking.

Although the PK cell line is very hardy and fast-growing, it differs from many other cell lines in a number of ways, i.e., its dependence on serum for growth and the difficulty of getting it to grow in suspension. It also appears to have some unique nutritional requirements. In a large number of cell lines tested, of both human and other mammalian origin, the PK was the only one that required substances such as cholesterol, hematin, and lecithin for optimal growth in a chemically defined medium (K. Higuchi, Abstr. Annu. Meeting, Tissue Culture Ass., Washington, D.C.).

A closer comparative examination of the nutrition and metabolism of the PK and HeLa cell lines may throw some light on the causes of in vitro virulence changes. The nutritional and metabolic differences of these two cell lines will be reported at a future date. Further knowledge of the mechanisms of virulence change in cell culture may have valuable application in improvement of vaccines.

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