Loss in Virulence of Yellow Fever Virus Serially Passed in HeLa Cells. (30167)

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A recent report(1) has indicated that HeLa cell cultures supported the growth of yellow fever virus (Asibi strain) and that the virus grown in such a system was capable of infecting rhesus monkeys at low doses by the respiratory route. Hardy(2) showed that 6 serial passages of the Asibi strain of virus in HeLa cells resulted in a virus population that was not lethal for monkeys but lyzed the cells in culture. This was in contrast to the parent mouse brain preparation which was lethal for monkeys but did not lyze the cells in the culture. Our investigation of these phenomena revealed that serial passage of this virus in HeLa cells was attended by alterations of several more viral properties and that some of these changes took place at earlier passage intervals than had been previously reported. Moreover, some of the genetic markers of the viral populations that were encountered appeared to be closely associated with one another.

Materials and methods. Harvest of virus from HeLa cell cultures. The Asibi strain of yellow fever virus was inoculated into a stock strain of HeLa cells maintained at the U. S. Army Biological Laboratories. Viral inoculum consisted of a suckling mouse brain seed preparation. In one instance a monkey plasma seed preparation was employed. Prior to inoculation of the cell cultures, the monolayers, grown in T-60 flasks, were washed once with Hanks' balanced salt solution (BSS). One ml of inoculum, diluted 1:5, was placed over the cells and allowed to adsorb for 1 hour at 37°C. The cells were washed twice with BSS and overlaid with 12 ml of growth medium consisting of yeast extract and proteose peptone 3 supplemented with 20% horse serum(3). The medium was replaced every day and samples of the supernatant fluid were harvested and frozen at −60°C with egg yolk from 6-day-old embryonated eggs (20% v/v) until they were titrated intracerebrally in 10- to 14-g mice. Samples that were harvested for use as aerosols consisted of the infected supernatant medium and the cells that were scraped off the glass surface mixed with 20% egg yolk.

Aerosol methods. The procedures employed to produce aerosols of the viral preparations into an atmosphere with a relative humidity (RH) between 40% and 80%, the exposure of monkeys to these preparations, the assay procedures, the method by which the initial per cent recovery of virus was determined including replication and analysis have been described(1). For each assessment of infectivity, 9 rhesus monkeys were divided into 3 groups of 3 each. Two additional monkeys were held as controls. Each group received different inhaled doses achieved by aging the virus aerosol. In the cases indicated below, monkeys that survived virus infections were challenged by the intraperitoneal (i.p.) route with a lethal dose consisting of 1000 mouse intracerebral (MIC) LD_{50} of the mouse brain seed.

Results. Eleven experiments were carried out in a similar manner in order to characterize the properties of virus that had been passed once in HeLa cells. Mean values of the data are presented in Table I as a "typical" virus population (Ty-1). Maximal virus titers ranged from $10^{6.9}$ to $10^{7.4}$ $MICLD_{50}$/ml of virus and were obtained in 5 days; no evidence of lysis (cpe) of the HeLa cells was observed. The initial virus recoveries from aerosols of these preparations were not affected by exposure to different relative humidities, as revealed by a statistical analysis at the 5% level of significance. At 40% to 50% RH, recovery values ranged from 24.6% to 64.4%; those at 70% to 80% RH were 28.8% to 69.9%. Ten $MICLD_{50}$ of these virus preparations administered to rhesus monkeys by exposure to aerosols during 3 representative experiments produced lethal infections. Pathologic evidence of liver involvement of infected monkeys was found to

TABLE I. Properties of "Typical" and "Atypical" Yellow Fever Virus Populations Obtained After One Passage in HeLa Cell Monolayers.

Properties	Populations (Ty-1)*	(ATy-1)†
Maximal $MICLD_{50}$/ml (log_{10}) titer in culture	7.1	8.1
Time of maximal titer postinoculation	day 5	day 5
Cytopathic effect in culture	neg	pos
% Initial recovery in aerosol		
40-50% relative humidity	43.2	9.7
70-80% relative humidity	46.7	28.2
Monkey lethality, 40-50% relative humidity	lethal at 10 $MICLD_{50}$	lethal at 10 $MICLD_{50}$

* "Typical" viral population representative of harvests obtained in 11 of 12 experiments.
† "Atypical" viral population representative of a harvest obtained in one of 12 experiments.

be similar to that described for yellow fever (4).

In contrast to the viral populations observed during the 11 tests mentioned above, one additional population was encountered that was atypical (ATy-1). The activity of this population was significant because of a cpe which was found after only one passage in HeLa cells; in the data that are to be discussed later, it will be seen that this type of response (*i.e.*, cpe) was observed usually after 3 serial passages. Because of the rarity of this event, this virus population was studied in the same detail as the "typical" one-passage population (Ty-1) mentioned above. Properties of both Ty-1 and ATy-1 after one passage in HeLa cells are presented in Table I.

Differences shown by ATy-1 include a 10-fold increase in viral yields, cell lysis in culture, and an adverse effect of the lower relative humidity. The recovery of 9.7% that was obtained at 50% RH was significantly lower ($P<5\%$) than 28.2% that was obtained at 80% RH. Similarities between Ty-1 and ATy-1 include the 5-day postinoculation interval of maximal titer and lethality for monkeys at doses of 10 $MICLD_{50}$.

A series of 6 experiments was carried out in which the yellow fever virus was serially passed 3 times in HeLa cells. Populations obtained in 2 experiments were uniformly different from those obtained during the other 4. This suggested that a greater incidence of variation was found among populations obtained after 3 serial passages than after one. The results of the majority (4) of experiments were tentatively considered as "typical" while those of the minority (2) were considered as "atypical." A comparison of the properties representative of the 2 types of third-passage populations is shown in Table II.

The "typical" population (Ty-3) differed from the "atypical" population (ATy-3) by showing higher titers, cell lysis, and an adverse effect of 50% RH. The 2 populations were similar in demonstrating maximal yields at day 3 and by not being lethal for monkeys.

Not included in Table III are data suggesting that infection occurred in the absence of lethality. Monkeys that had survived the exposure to aerosols of virus resisted a challenge with a lethal dose of virus administered by the i.p. route, thereby indicating that the primary respiratory infection was immunogenic.

Discussion. Conversion of the "typical" first-passage population, Ty-1, into the "typical" third-passage population, Ty-3, in these experiments was undoubtedly similar to the phenomenon that Hardy reported after 6 passages in HeLa cells(2). The appearance of the "atypical" first-passage and the

TABLE II. Properties of "Typical" and "Atypical" Yellow Fever Virus Population Obtained After 3 Serial Passages in HeLa Cell Monolayers.

Properties	Populations (Ty-3)*	(ATy-3)†
Maximal $MICLD_{50}$/ml (log_{10}) titer in culture	8.8	6.9
Time of maximal titer postinoculation	day 3	day 3
Cytopathic effect in culture	pos	neg
% Initial recovery in aerosol		
50% relative humidity	9.8	19.7
80% relative humidity	31.5	12.3
Monkey lethality	non-lethal at >52.5 $MICLD_{50}$	non-lethal at 800 $MICLD_{50}$

* "Typical" viral population representative of harvests obtained in 4 of 6 experiments.
† "Atypical" viral population representative of harvests obtained in 2 of 6 experiments.

TABLE III. A Summary of Changes in Properties of Yellow Fever Virus (Asibi Strain) in HeLa Cell Cultures.

HeLa cell preparation	Maximal $MICLD_{50}$ /ml (log_{10}) titer in HeLa cells	Day of maximal titer	Cytopathic effect*	Effect at 50% RH†	Attenuation of virus for monkeys‡
One passage	6.9–7.4	5	negative	resistant	negative
	8 or >	5	positive	sensitive	negative
Three passages	6.8–7.0	3	negative	resistant	positive
	8 or >	3	positive	sensitive	positive

* Cell rounding, increase in density and detachment from glass.
† Effect of aerosolization on virus at 50% RH.
‡ By exposure to infected aerosols.

"atypical" third-passage populations, ATy-1 and ATy-3, respectively, suggest that intermediate populations can occur during that conversion. Such populations might account for the unexplained difficulty that Hardy experienced in stabilizing the virus until 6 passages had been performed(2). Factors responsible for the selection of ATy-1 or ATy-3 rather than Ty-1 or Ty-3 in culture remain undefined but unpublished data suggest that the conversion of Ty-1 to Ty-3 is more rapidly facilitated by using high multiplicities of infection during serial passages of the virus. The intermediate populations appeared unstable in HeLa cells but displayed some order, however, in their appearance. ATy-1, apparently the rarest of all the populations, shared certain of its properties with Ty-1 and Ty-3. ATy-3 which was encountered on 2 out of 6 occasions shared certain of its properties with Ty-1 and Ty-3. Surprisingly, perhaps, ATy-1 and ATy-3 shared no properties with each other.

Of considerable interest is the fact that many of the properties that were established for the various viral harvests appear to be very closely related with one another. An attempt to represent this is shown in Table III. As examples, (a) virus grown after one passage in HeLa cells (approximately 10^7 $MICLD_{50}$ in 5 days) was virulent for monkeys. Conversely, virus grown in HeLa cells after 3 serial passages (10^8 or greater $MICLD_{50}$ in three days) was attenuated for monkeys. (b) Virus that had shown a cpe was adversely affected by aerosolization at 50% RH, but virus that did not induce a cpe was unaffected. (c) Viral harvests that failed to show maximal titers in excess of 10^7 $MICLD_{50}$, despite some degree of increased adaptation in HeLa cells (maximal titer at day 3), did not induce a cpe; harvests that contained titers of 10^8 $MICLD_{50}$ or greater induced a cpe in culture.

One aspect of safety in the laboratory might be mentioned as a result of these studies. As indicated by studies on infections contracted in the laboratory(1,5-7), the inhalation of infectious virus, unaltered by laboratory passage, may constitute a danger to laboratory workers. Our studies with yellow fever virus suggest that after serial passages *in vitro,* the danger of air-borne infections may be markedly reduced. This could be attributed not only to the selection *in vitro* of particles possessing a lowered virulence, but also to the eventual selection of mutants that are unstable when air-borne unless they are in an environment possessing a very high relative humidity. Whether this information can apply to other arboviruses must await the results of further tests. Evidence for a general decline in virulence *in vivo* of at least one other arbovirus by the respiratory as well as other routes as a result of laboratory passage *in vitro,* however, has been reported(8,9).

Summary. Yellow fever virus that was passed one and three times in HeLa cells was characterized with respect to the following: (a) adaptation to growth in cell culture as indicated by the time required to obtain maximal titer, (b) level of titer, (c) production of a cytopathic effect (cpe), (d) stability of virus when aerosolized at either 50% or 80% relative humidity (RH), and (e) the loss of virulence of viral preparations for rhesus monkeys infected by the respiratory

route. Four viral populations which possessed different combinations of these properties were obtained. In 11 of 12 experiments, one passage in cell culture yielded a virus population (Ty-1) that titered to 10^7 $MICLD_{50}$/ml on day 5, that failed to cause cell lysis (cpe), that was equally stable at 50% and 80% RH, and that was lethal for monkeys. In contrast, in 4 of 6 experiments, 3 passages in cell culture yielded a virus population (Ty-3) that titered 10^8 $MICLD_{50}$ or greater on day 3, that induced a cpe, that had a greater sensitivity to 50% than to 80% RH, and that was non-lethal for monkeys. In the remaining experiments, one "atypical" viral population (ATy-1) was obtained from culture after one passage. In 2 other experiments, "atypical" viral populations (ATy-3) that were similar to each other but different from ATy-1 were obtained after 3 passages. The characteristics demonstrated by ATy-1 and ATy-3 suggested that these were intermediate to Ty-1 and Ty-3. The manner in which population characteristics became altered during the conversion of Ty-1 to Ty-3 *in vitro* suggested that some of the characteristics were closely associated with one another.

1. Miller, W. S., Demchak, P., Rosenberger, C. R., Dominik, J. W., Bradshaw, J. L., Am. J. Hyg., 1963, v77, 114.

2. Hardy, F. M., J. Inf. Dis., 1963, v113, 9.

3. Mayyasi, S. A., Schuurmans, D. M., Proc. Soc. Exp. Biol. and Med., 1956, v93, 207.

4. Tigertt, W. D., Berge, T. O., Gochenour, W. S., Gleiser, C. A., Eveland, W. C., Vorder Bruegge, C., Smetana, H. F., Trans. N. Y. Acad. Sci., 1960, v22, 323.

5. Sulkin, S. E., Bact. Rev., 1961, v25, 203.

6. Slepushkin, A. N., Vopr. Virusol., 1959, v4, 54.

7. Taylor, R. M., in Yellow Fever, ed., Strode, G. K., McGraw-Hill Book Co., Inc., 1st ed., 1951, 431.

8. Keuhne, R. W., Sawyer, W. D., Gochenour, W. S., Am. J. Hyg., 1962, v75, 347.

9. Hearn, H. J., Proc. Soc., Exp. Biol. and Med., 1961, v107, 607.

Received February 10, 1965. P.S.E.B.M., 1965, v119.

Mucopolysaccharide Defect in Experimental Lathyrism.* (30168)

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The most striking biochemical alteration in the connective tissue of lathyritic animals has been found by several authors to be a change in the solubility of collagen(1,2,3). Not quite the same agreement exists regarding a change in the connective tissue mucopolysaccharides (MPS). While some authors found a decrease of MPS in epiphyseal plates and fracture callus of aminoacetonitrile (AAN)-treated animals(4,5,6), no change whatsoever was found in skin(7). The MPS composition of skin is known to differ considerably(8,9) from that of epiphyseal plate (10) and bone callus(11,12). A possible explanation of the different results obtained in these tissues could be that only certain types of MPS (for ex. Chondroitin-4-SO_4 and Chondroitin-6-SO_4) are affected by the lathyrogenic agents. In those tissues where these particular MPS are not present or are present only in very small amount, lathyrism would not produce any change in the MPS content. Fracture bone callus has been chosen as a suitable tissue on which to test this hypothesis. Supporting evidence is reported here.

Experimental. In 2 experiments performed under the same conditions, the middle third of both tibiae of 40 Sprague-Dawley male rats weighing approximately 160 g were fractured under ether anesthesia. The fractures were not immobilized. The animals were divided in 2 groups one of which was kept on a Purina chow diet and served as control, while the other group received Purina chow containing 0.075% AAN sulfate. Both groups received daily the same amount of

* Supported by NIH grant 149.