BACTERIOLOGICAL REVIEWS, Sept., 1966
Vol. 30, No. 3

Printed in U.S.A.

Attenuation of Aerosolized Yellow Fever Virus After Passage in Cell Culture

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INTRODUCTION

Reports in the literature, recently reviewed by Mussgay (8), contain numerous examples of alterations in the properties of arboviruses as a result of their passage in various host systems. Countless other observations of this type undoubtedly have been made but not reported. Early accounts of alterations in virulence of yellow fever virus (YFV) as a result of propagation in vitro have been described by Lloyd, Theiler, and Ricci (6) and by Theiler and Smith (11). Studies by Theiler and his associates led to the isolation and eventual use of the well-known 17-D strain for human vaccination. A more detailed description of the development of various attenuated strains of YFV may be found in a review by Theiler (10). More recently, Hallauer (1) reported losses in virulence of the 17-D strain for mice and of the Asibi strain for monkeys after passage in KB cell cultures. Following this, Schindler and Hallauer (9) described additional losses in the viscerotropism of Asibi strain variants obtained after prolonged passage in KB cells. These authors also reported the isolation from human cell lines of a 17-D viral substrain whose virulence for monkeys was reduced to a degree that has apparently not been found in any other 17-D strains. Hardy (2) demonstrated attenuation of the Asibi strain for monkeys with viral isolates obtained after serial passage in HeLa cells. In keeping with the general subject of this symposium, we should like to present some observations on changes in the properties of yellow fever virus that were discernible upon aerosolization of preparations after growth in HeLa cells.

A major consideration in determining our experimental approach was the prevailing lack of information on the behavior of airborne virus that previously had undergone routine passages in cell culture in the laboratory. Information of this kind has broad applications not only for the viral geneticist but for those who are engaged in the problems of laboratory safety. To carry out a meaningful study, it was recognized that a comparison of properties of virus when in suspension with those of virus that was aerosolized was needed. The results will show that the dominant characteristic of virus that was serially passed in cell culture was its decline in virulence. Other properties that were also found to change appeared to vary in parallel with the loss in virulence. It seems probable that some of these alterations may have resulted in, or at least contributed to, the decline in capability of the virus to induce lethal infections.

EXPERIMENTAL APPROACH

Two years ago at Fort Detrick, Hardy (2) demonstrated that his strain of yellow fever virus, which was ordinarily noncytopathic in cell culture and lethal for monkeys, induced cell lysis and became attenuated for these animals after six serial passages in HeLa cells. Subsequently, results reported by Miller et al. (7) indicated the feasibility of performing studies on aerosolized yellow fever virus after it had proliferated in HeLa

TABLE 1. *Properties of typical and atypical yellow fever virus populations obtained after one and three serial passages in HeLa cells*

Population	Titer in culture, $MICLD_{50}$ (log_{10})	Day of maximal titer	Cell lysis in culture	Per cent recovery in aerosol		Monkey lethality	
				40–50% RH[a]	70–80% RH	40–50% RH	70–80% RH
Ty-1[b]	7.1	5	Negative	43.2	46.7	Lethal at 10 $MICLD_{50}$	
ATy-1[c]	8.1	5	Positive	9.7	28.2	Lethal at 10 $MICLD_{50}$	
Ty-3[d]	8.8	3	Positive	9.8	31.5		Nonlethal at >52.5 $MICLD_{50}$
Aty-3[e]	6.9	3	Negative	19.7	12.3		Nonlethal at 800 $MICLD_{50}$

[a] Relative humidity.
[b] "Typical" viral population, representative of harvests obtained in 11 of 12 experiments.
[c] "Atypical" viral population, representative of a harvest obtained in 1 of 12 experiments.
[d] "Typical" viral population, representative of harvests obtained in four of six experiments.
[e] "Atypical" viral population, representative of harvests obtained in two of six experiments.

cells. Encouraged by the results of these efforts, we designed experiments to characterize properties of yellow fever virus in greater detail during serial passage in cell culture. An effort was made during this work to study properties of virus in both the pre- and postaerosolized state and to determine whether any correlation existed among the properties that were altered as a result of passage in vitro. It was found that the viral changes that were reported by Hardy, as well as additional previously undescribed changes, actually began to occur as early as the third serial passage in HeLa cells. We observed alterations in the ability of the virus to grow and induce cell lysis in HeLa cell monolayers, confirming Hardy's published work. We also found changes in the stability of infected cell culture preparations upon aerosolization, and in the level of virulence of such products for rhesus monkeys.

ALTERATIONS IN VIRAL PROPERTIES AFTER CULTIVATION IN HELA CELLS

Recovery of "Typical" and "Atypical" First- and Third-Passage Viral Populations

During the course of our first series of studies, viral products that were prepared in the same or similar manner during either one or three passages did not repeatedly demonstrate the same properties. In other words, although the majority of viral populations behaved in a typical fashion, infrequent atypical populations could be recovered. These proved to be fortuitous events, because this not only allowed for an assessment of the differences between typical one- and three-passage harvests, but, by studying the incidence of variation among properties of typical and atypical viral populations prepared at the same passage level, we were able to gain some insight into possible relationships that existed among individual genetic markers. Of considerable interest was the indication that certain of the properties that were encountered in vitro could be correlated with properties displayed by the virus in aerosols. Examples are shown in Table 1.

It can be seen that typical first-passage preparations possessed titers of approximately 10^7 $MICLD_{50}$ that were obtained on day 5 in the absence of cell lysis. This preparation showed maximal stability during aerosolization at 50% relative humidity (RH) and was lethal for monkeys by the respiratory route. In contrast, typical third-passage preparations, represented in the third column, possessed titers of 10^7 $MICLD_{50}$ (or greater) that were obtained on day 3 attended by cell lysis. The viral recoveries obtained at 50% RH were significantly lower ($P < 5\%$) than those obtained at 80% RH. These preparations were not lethal for monkeys by the respiratory route. In column two, properties of an atypical first-passage preparation are shown. A 1-log increase in titer to 10^8 $MICLD_{50}$ was obtained at day 5, accompanied by cell lysis. This preparation was significantly less stable ($P < 5\%$) at 50% RH than at 80% RH, but it was lethal for monkeys. Atypical third-passage preparations showed maximal titers of about 10^7 $MICLD_{50}$ at day 3 and did not induce cell lysis. This virus was not appreciably affected by changes in RH, although the overall viral recoveries appeared slightly decreased, and it was not lethal for monkeys.

TABLE 2. *Summary of changes in properties of yellow fever virus (Asibi strain) in HeLa cell cultures*

HeLa cell prepn	Maximal $MICLD_{50}$/ml (log_{10}) titer in HeLa cells	Day of maximal titer	Cytopathic effect[a]	Effect at 50% RH[b]	Attenuation of virus for monkeys[c]
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

[a] Cell rounding, increase in density, and detachment from glass.
[b] Effect of aerosolization on virus at 50% relative humidity.
[c] By exposure to infected aerosols.

Comparison of Viral Properties After First and Third Passage

Some of the properties that were established for the various viral harvests appeared to be closely related to one another. An attempt to represent this is shown in Table 2 and by the following examples. (i) 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 three serial passages (10^8 or greater $MICLD_{50}$ in 3 days) was attenuated for monkeys. (ii) Virus that had shown a cytopathic effect was adversely affected by aerosolization at 50% RH, but virus that did not induce a CPE was unaffected. (iii) 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 cytopathic effect; harvests that contained titers of 10^8 $MICLD_{50}$ or greater induced a cytopathic effect in culture.

The results of these experiments also suggested that first-passage viral preparations were stabilized more easily than the third-passage preparations; 1 of 12 of the former populations behaved atypically, whereas 2 of 6 of the latter proved to be atypical. The atypical viral harvests shared properties with both the typical first- and third-passage preparations, and appeared, therefore, to represent intermediate viral populations. Additional work toward the further elucidation of such populations was indicated. Since multiple-passage preparations gave the greatest indication of genetic instability, we concentrated our experimental effort chiefly on characterizing the properties of viral harvests of this type.

FURTHER STUDIES ON VIRAL POPULATIONS AFTER ONE PASSAGE IN HELA CELLS

Studies on virulence were expanded to include a comparison between rates of infectivity after administration of virus by the intraperitoneal (ip) as well as the respiratory route. To accomplish this, graded doses of the same viral preparation isolated from HeLa cells were administered to monkeys by either route; survivors were challenged ip 21 days later with a multiple lethal dose of a mouse brain virus seed to determine whether the original administration of virus had subclinically infected the animals. The first series of tests with this experimental protocol was carried out with first-passage material to obtain base-line information with which to compare the third-passage preparations to be reported later.

TABLE 3. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after one passage in HeLa cells*

Dose ($MICLD_{50}$)	Lethality		Infectivity[c]	
	Resp[a]	ip[b]	Resp	ip
50	6/6[d]		—	
40		6/6		—
5.0	3/6		0/3[d]	
4.0		3/4		1/1
0.5	0/3		0/3	
0.4		3/6		2/3
0.05	NT[e]		—	
0.04		1/3		1/2

[a] Virus administered by respiratory route.
[b] Virus administered by intraperitoneal route.
[c] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[d] Number of monkeys affected per number treated with virus.
[e] Not tested.

Properties of Pre- and Postaerosolized Populations After One Passage

The results of these tests are represented in Table 3. Doses given by the respiratory route represent average values from several experiments. They show that doses of approximately 50 $MICLD_{50}$ given by either route were lethal for the

test monkeys. Doses of approximately 5 $MICLD_{50}$ of aerosolized virus were lethal for one-half of the exposed monkeys. A dose of 4 $MICLD_{50}$, however, was lethal for three of four monkeys injected by the ip route. Three of six monkeys succumbed when given a dose of 0.4 and one of three monkeys succumbed with a dose of 0.04 $MICLD_{50}$.

Animals that survived viral aerosols showed no evidence of having been infected. This was shown by the fact that three monkeys that failed to succumb to respiratory doses ranging from 1 to 10 $MICLD_{50}$ also failed to resist the ip challenge with a multiple lethal dose of suckling mouse brain virus. Thus, the dose that was necessary to infect appeared to be very close if not identical to the dose necessary to cause a lethal illness.

Two of three monkeys resisted a lethal challenge of virus after they survived a dose of 0.4 $MICLD_{50}$ given by the ip route; one of two monkeys that survived the ip dose of 0.04 $MICLD_{50}$ was not resistant. Thus, although there was some evidence that infection by the ip route was not invariably fatal with very low doses, it appears that the median lethal dose and the median infectious dose values obtained by the ip route were much closer to one another with first-passage virus than those obtained with multiple-passaged virus.

FURTHER STUDIES ON VIRAL POPULATIONS AFTER MULTIPLE PASSAGE IN HELA CELLS

Scheme of Tests

The next series of experiments was directed toward elucidating the characteristics of viral populations that arose after multiple serial passages in HeLa cells. Results of tests on two preparations obtained after three serial passages in HeLa cells and one other after seven passages will be presented. The following test scheme was devised for each viral harvest. After inoculation of the virus in culture, that is to say, during preparation of the third- or seventh-passage harvests, samples were obtained from the culture and titrated daily for 6 days postinoculation to establish the maximal titer and the time postinoculation of its occurrence. During that time, the cell sheet was examined microscopically for evidence of cell lysis. Both supernatant fluid and the cells were harvested at or near the time of maximal viral yields, and the material was frozen in glass ampoules. A few days later, a sample of this viral material was titrated intracerebrally in mice and by the ip route in monkeys. Then the preparation was aerosolized at 50 and 80% RH. The amount of virus that could be recovered in the aerosol and the extent to which the property of lethality for monkeys was decreased after the virus was airborne were determined. Values for the former were obtained by plotting recovery values at intervals during the 60-min period following aerosolization.

Virulence for monkeys was ascertained by exposing these animals at various intervals after rendering the agent airborne. Differences in dosage were obtained by exposing the monkeys to clouds of various ages, the older the cloud the smaller the dose. Impinger fluids were collected at intervals corresponding to those during which the monkeys were exposed, and these samples were injected ip into monkeys in such a manner that the same theoretical viral dose was administered to duplicate monkeys by either the respiratory or ip route. As in previous experiments, any monkey that survived the administration of the viral preparations by either route was challenged 21 days later with a multiple lethal dose of a mouse brain virus seed. Two uninfected control monkeys also were challenged in the same manner. The data presented in Table 4 show the results obtained with one third-passage preparation.

Properties of Pre- and Postaerosolized Third-Passage Viral Populations

In Table 4, the data are divided into two main sections. On the left are the results of injecting monkeys intraperitoneally with a third-passage HeLa cell preparation prior to its aerosolization. The most striking feature of these data is the lack of a clear-cut end point in the lethality pattern. Doses ranging from 3 to 3,000 $MICLD_{50}$ were lethal for one-half of the monkeys tested. All survivors were resistant to a multiple lethal dose of virus given 21 days later as an ip challenge. Two other monkeys that were originally injected with 0.3 $MICLD_{50}$ evidently were infected subclinically, since they also resisted the lethal challenge. The dose of 0.03 $MICLD_{50}$ apparently failed to infect the monkeys.

On the right side of Table 4 are the results of administering aerosolized virus to monkeys. Shown are the doses expressed as the number of $MICLD_{50}$, the RH employed during aerosolization of the virus, the lethality and infectivity resulting from exposing the monkeys to aerosolized virus and the lethality and infectivity of the impinger fluid into which the various viral doses were collected postaerosolization.

These data show once again that a clear-cut end point in the lethality pattern did not occur with either route; this was especially evident in monkeys exposed by the respiratory route. Moreover, there was no consistent difference in the incidence of lethality at 50 and 80% RH. This corresponds to the lack of any difference

TABLE 4. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after three serial passages in HeLa cells*

Preaerosolization			Postaerosolization					
Dose (MICLD50)	Intraperitoneal		Dose (MICLD50)	RH (%)	Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]			Leth	Inf	Leth	Inf
3,000	1/2[c]	1/1	2,304–915	50	1/2	1/1	0/2	2/2
300	1/2	1/1		80	1/2	1/1	1/2	1/1
30	1/2	1/1	210–54	50	1/2	1/1	2/2	—
3	1/2	1/1		80	2/2	—	1/2	1/1
0.3	0/2	2/2	12–3	50	2/2	—	1/2	1/1
0.03	0/1	0/1		80	1/2	1/1	0/2	2/2
			1–<1	50	0/2	0/2	0/2	1/2
				80	1/2	1/1	0/2	2/2

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.

TABLE 5. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after three serial passages in HeLa cells*

Preaerosolization			Postaerosolization					
Dose (MICLD50)	Intraperitoneal		Dose (MICLD50)	RH (%)	Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]			Leth	Inf	Leth	Inf
50,000	2/2[c]	—	2,531–1,239	50	0/2	2/2	2/2	—
5,000	1/2	1/1		80	0/2	2/2	2/2	—
500	1/2	1/1	111–54	50	0/2	2/2	0/2	2/2
50	1/2	1/1		80	1/2	1/1	2/2	—
5	0/2	2/2	13–6	50	0/2	2/2	0/2	2/2
0.5	0/2	2/2		80	0/2	2/2	1/2	1/1
0.05	0/2	0/2	2–<1	50	0/2	0/2	0/2	0/2
				80	0/2	2/2	0/2	2/2

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.

between the per cent viral recoveries that were obtained at either humidity. Despite this, however, the RH effect might have been an influencing factor in the incidence of nonlethal infections in monkeys. For example, in the lowest dose range, no animals became infected when exposed to aerosols at 50% RH. At 80% RH, however, one of two monkeys succumbed, and the survivor was resistant to the lethal challenge. After injecting impinger fluids by the ip route, a slight increase may have occurred in the incidence of infectivity at 80% RH. Additional evidence of this is shown in data presented in Table 5.

In Table 5, the results of tests with a second third-passage preparation are shown. As with the previous findings, data on the left side of the table obtained with preaerosolized virus show once again the lack of a clear-cut end point in the lethality pattern. On the right side of the table, it can be seen, in contrast to the previous third-passage preparation, that no appreciable lethality was obtained by the respiratory route. Some lethality was found, however, after injecting the impinger fluids by the ip route; the lethality that occurred with the ip route appeared to be more pronounced at the 80% RH. Similarly, the incidence of infectivity was greater with very low doses of this viral preparation after it was aerosolized at 80% RH and administered by either route than when it was aerosolized at 50% RH.

TABLE 6. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after seven serial passages in HeLa cells*

Preaerosolization			Postaerosolization					
Dose ($MICLD_{50}$)	Intraperitoneal		Dose ($MICLD_{50}$)	RH (%)	Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]			Leth	Inf	Leth	Inf
700,000	0/2[c]	2/2	2,108–1,198	50	0/2	2/2	0/2	2/2
70,000	NT[d]	NT		80	0/2	2/2	0/2	2/2
7,000	0/2	2/2	277–93	50	0/2	2/2	0/2	2/2
700	NT	NT		80	0/2	2/2	0/1	1/1
70	0/2	2/2	12–4	50	0/2	2/2	0/2	2/2
7	NT	NT		80	0/2	2/2	0/2	2/2
0.7	0/2	2/2	1–<1	50	0/2	0/2	0/1	1/1
0.07	NT	NT		80	0/2	2/2	0/2	2/2
0.007	0/2	0/2						

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.
[d] Not tested.

TABLE 7. *Number of $MICLD_{50}$ necessary to produce infectivity with or without lethality after passage in HeLa cells*

Passage no.	Preaerosolization		RH (%)	Postaerosolization			
	Intraperitoneal			Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]		Leth	Inf	Leth	Inf
1st	0.04	0.04	50	5	5	NT	NT
			80	5	5	NT	NT
3rd (I)	3	0.3	50	3	3	3	<1
			80	<1	<1	210	<1
3rd (II)	50	0.5	50	>1,239	6	>1,239	<1
			80	210	<1	12	<1
7th	>700,000	0.7	50	>2,108	12	>2,108	1
			80	>1,098	<1	>1,098	<1

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.

Properties of Pre- and Postaerosolized Seventh-Passage Viral Population

The final test was carried out with a seventh-passage preparation, which resulted from a continuation of serial passages from the first of the third-passage harvests. Results of this test shown in Table 6 revealed that an increase in the attenuation of the virus had occurred; no lethality was encountered with either the pre- or post-aerosolized viral preparations. The incidence of infectivity, as shown by immunity to the lethal challenge, however, did not appear to have declined.

Comparison of Properties of Viral Populations After Multiple Passage in HeLa Cells

In Table 7, we have summarily compared the main points of interest of the three viral preparations. All of the values are expressed as the minimal number of $MICLD_{50}$ that were shown to have induced either a lethal or nonlethal infection in monkeys. The data show that the pre- and post-aerosolized first-passage HeLa cell preparation proved to be a comparatively efficient inducer of lethality, although somewhat more virus appeared to be necessary to infect monkeys by the respiratory route than by the ip route. There did

not appear to be a significant difference between the viral response to either RH.

The first of the third-passage HeLa cell harvests showed a decreased level of lethality for monkeys when administered by either route. As the data in Table 4 had indicated previously, the assessment of the virulence of this preparation was greatly influenced by an apparent interference phenomenon that was expressed as a partial inability of this virus to cause lethality over a wide range of doses. Since the first-passage preparation did not display this phenomenon during its lethal effect in monkeys, it is tentatively concluded that the behavior of the third-passage harvest in this case indicates a weakening of the virulence character as a result of serial passage in HeLa cells. Information derived from other multiple-passage preparations clearly supports this view. The second of the third-passage preparations contained virus possessing a level of virulence that had declined to the extent that it failed to produce lethality by the respiratory route; moreover, it had markedly declined in its efficiency in producing lethal illness by the ip route. After seven passages, all traces of lethality for monkeys had vanished from the viral harvest. In all of the viral preparations, the loss in lethality was not accompanied by a loss in the capability of the virus to immunize the animals.

DISCUSSION AND SUMMARY

Manifestation of the viral "RH" marker was more subtle than expected in the two third-passage and seventh-passage preparations. In prior tests with this virus in our laboratory (4), the effect of RH was readily evident in experimental data showing that statistically significant decreases in viral recoveries were encountered upon aerosolization at 50% RH. This was similarly found to be the case with recovery values for airborne Colorado tick fever, vesicular stomatitis, neurovaccinia, and encephalomyocarditis viruses as reported by Watkins et al. (12). In the present results, the initial recovery values were not as high as those previously encountered in our laboratory, and it became necessary to use other criteria to demonstrate any difference that may have occurred as a result of aerosolization at 50 and 80% RH. In cases in which conditions were such that differences could be demonstrated, serially passed virus aerosolized at 50% RH was the least active.

The first of the third-passage preparations provided, at first glance, what might appear to be an exception to this. The dose of 210 $MICLD_{50}$ of the impinger fluid at 80% RH shown in Table 7 as that necessary to produce lethal illness in monkeys by the ip route is higher than that necessary to produce lethal illness at 50% RH. Two factors appear to have been responsible for this. The first is that the dose of 210 $MICLD_{50}$ was inadvertently higher than that planned. On the basis of other data obtained under similar circumstances, the same clinical response would be expected to have been achieved with a much lower dose. Events such as these illustrate, perhaps, an important disadvantage in using the small number of animals that is usually necessary when monkeys must be employed.

In summary, the data presented in this paper clearly indicate that a pronounced loss of virulence rapidly occurred when yellow fever virus was serially passed in HeLa cells. By as early as the third passage, viral populations become demonstrably weakened in their ability to induce lethal illness in rhesus monkeys by either the ip or respiratory route. At this passage level, very high humidities were necessary to sustain even some semblance of lethality. Passage of the virus in cell culture, however, did not appreciably reduce the ability of the virus to induce an immunity in these animals.

From a genetic viewpoint, it is of considerable significance that the third-passage preparations were highly unstable. It is not surprising, therefore, that many of these viral populations possessed properties that varied to some extent from each other, not only in their degree of attenuation, but also in their response to 50% RH, and in their ability to cause a cytopathic effect in cell culture. In a previous publication (4), we noted that atypical first-passage and atypical third-passage viral populations that had been studied up to that time shared no properties with each other. This was unexpected, since both of these presumably represented viral forms that were intermediate to the virulent, aerosol-stable form and the attenuated aerosol-unstable form. Studies on third-passage populations that were recovered and studied since then have supported the view that these viral populations do represent truly intermediate forms. Furthermore, the data indicate that when a sufficient number of these unstable viral populations were examined, atypical intermediate forms that shared some of their properties with each other could be revealed. The genetic determinants for the viral properties that we have examined, therefore, are probably in close relationship to one another but obviously not linked.

The results of these studies raise the question of whether viral isolates of reduced virulence may be commonly acquired after passage in cell cultures. Viral mutants with either lowered

virulence or decreased stability, or both, might be easily selected in an in vitro system. The use of such mutant populations could reduce the danger of airborne contamination of laboratory workers, experimental animals, and other viral or cell culture materials. Venezuelan equine encephalomyelitis virus, another arbovirus, has been shown to lose its virulence in vivo as a result of its serial passage in vitro (3, 5). The question of whether this applies to other arboviruses can be determined only after an adequate number of suitable tests have been performed.

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Discussion

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Your supposition that a Dutch bacteriologist would have more experience of yellow fields of tulips than of yellow fever is absolutely correct. It is thus with great respect that I have read the careful and laborious experiments of Dr. Hearn on which he certainly is to be complimented.

Dr. Hearn described variations in yellow fever virus after passage in HeLa cells: (i) variation in growth rate, growth capacity, and the appearance of cytopathic effects; (ii) attenuation of virulence for monkeys; and (iii) variation in aerosol stability. These correlations are obvious and important. The HeLa cultures were passed at high multiplicity so that mixed populations were studied. Consequently, the bulk of the particles in the atypical first passage (aTy 1) might well have lost the lethality for monkeys, but the population might still contain a few per cent of virulent particles. Again, the difference between the Ty 3 and aTy 3 might be due to interference or a von Magnus phenomenon. Thus, though the populations seem unstable, the variants might be quite stable genetically. Admittedly, yellow fever virology is very difficult, but, unless these variants are isolated from single plaques or passed at limiting dilutions, it is difficult to discuss these variations in terms of genetic markers. I sincerely hope that Dr. Hearn will find opportunity in the future to work in this direction.

The aerosol work is again of the highest level.