A COMPARISON OF THE GROWTH CURVES OF ADAPTED AND UNADAPTED LINES OF INFLUENZA VIRUS*

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It has recently been reported (1) that the rate of multiplication in the mouse lung of an adapted line of influenza virus is greater than that of an unadapted line of the same strain. In addition it was shown that the adapted line of virus reached a higher infectious titer in mice than the unadapted line. With the exception of the capacity to produce lesions in the lungs after serial passage, no other properties have been described which distinguish an adapted from an unadapted line of virus. Therefore, it seemed important to investigate these phenomena more fully. In this investigation, the rates of multiplication of adapted and unadapted lines of influenza virus, Types A and B, have been contrasted by comparing the growth curves obtained when mice were inoculated with approximately equal concentrations of each line of virus. It will be shown that the shape and slope of the growth curve are determined by the line, strain, and concentration of virus used for inoculation. The lag phase of growth characteristic of the unadapted line of virus and the suppression of multiplication following large doses of unadapted virus will be described.

Materials and Methods

Virus.—Influenza virus, Type A. (Rhodes strain.) This A-prime strain was isolated in this laboratory in 1947. A mouse-adapted line (Rhodes F4M22) passaged 4 times in ferrets and 22 times in mice, and an unadapted line (Rhodes F3E6) passaged 3 times in ferrets and 6 times in eggs were used.

Influenza virus, Type B. (Warner strain.[1]) An adapted line (Warner E7M10) passaged 7 times in eggs and 10 times in mice, and an unadapted line (Warner E13) passaged 13 times in eggs, were employed.

Virus Suspensions.—Infected mouse lungs were ground in a mortar with sterile alundum. A volume of 2 ml. of diluent for each mouse lung was added to yield a 10 per cent suspension. The diluent employed was 10 per cent normal horse serum–saline containing 600 units of penicillin and 1.25 mg. of streptomycin per ml. The suspensions were centrifuged at 2000

R.P.M. for 5 minutes; the supernates were removed, sealed in glass, frozen in an alcohol-solid CO_2 bath, and stored in a solid CO_2 refrigerated cabinet. Infected allantoic fluid was harvested, diluted 10-fold with normal horse serum, sealed in glass, frozen, and stored as above. As needed, ampoules of aliquots of these virus suspensions were thawed, used, and discarded. By determining the infectious titer in eggs of suspensions of adapted and unadapted lines of influenza virus, it was possible, by appropriate dilution, to prepare inocula containing approximately equal concentrations of virus of both lines. Dilutions of the suspensions were considered to be equivalent if the infectious titers of the suspensions in eggs agreed within 0.5 log.

Titrations of Egg Infectivity.—Serial 10-fold dilutions of virus suspensions were made in 10 per cent normal horse serum–saline containing 600 units of penicillin and 1.25 mg. of streptomycin per ml. A volume of 0.2 ml. of the desired dilution of virus was inoculated into the allantoic cavity of fertile chicken eggs incubated at 38°C. for 9 or 10 days prior to inoculation. Four eggs were used for each dilution. The inoculated eggs were incubated, prior to harvest, at 35°C., for 48 hours when the Rhodes strain of virus was employed and for 72 hours when the Warner strain was used. Allantoic fluid from each egg was tested in a pattern test for agglutination of chicken red cells (2) and the 50 per cent infectivity titer (EID_{50}) was calculated (3). Titers are expressed as the reciprocal of the dilutions employed.

Rate of Multiplication of Virus in Mouse Lung.—A volume of 0.05 ml. of a suitable dilution of virus suspension was inoculated intranasally into each of 60 lightly anesthetized young albino mice. The mice used in each experiment were of the same breed and were approximately 3 to 4 weeks old. Groups of mice were inoculated with dilutions of unadapted or adapted lines of virus prepared so as to contain an equal concentration of virus. Simultaneously the EID_{50} of the inoculum was determined in eggs. Ten mice from each group were sacrificed at 0, 6, 12, 24, 48, and 72 hour intervals after inoculation, the lungs removed, sealed in glass, frozen, and stored as described above. Subsequently, a 10 per cent suspension was prepared of the lungs which had been removed and stored at each time interval, and the EID_{50} of each suspension was determined.

Solutions.—Throughout this paper saline refers to 0.15 M NaCl solution buffered at pH 7.2 with 0.01 M phosphate. Normal horse serum was inactivated at 56°C. for 30 minutes prior to use.

EXPERIMENTAL

Rate of Multiplication in the Mouse Lung of Unadapted and Adapted Lines of Influenza Virus, Type B.—Two experiments were conducted employing each line of the Warner strain of influenza virus. Suspensions of an unadapted line containing log 7.7 EID_{50} per ml. and of an adapted line containing log 7.4 EID_{50} per ml. were diluted 1000-fold and used as inocula. The experiments were not carried out simultaneously. The results of these experiments are recorded in Table I and Fig. 1. These data demonstrate that the growth characteristics in the mouse lung of the adapted and unadapted line of virus are strikingly different. At 6 hours after inoculation with the adapted line, the virus content of the lungs was similar to that observed immediately after inoculation. In the interval between 6 and 48 hours, there was a marked increase in the infectious titer but after 48 hours no further significant increase was observed. In contrast, 6 hours after inoculation with the unadapted line of virus, there was a marked decrease in the virus content of the lungs. The infectious titer then increased and reached a level at 12 hours approximately equal to that

observed immediately after inoculation. Between 12 and 48 hours the virus content of the lungs increased progressively but thereafter no further increase was detected. The failure of the unadapted line to reach at 12 hours an in-
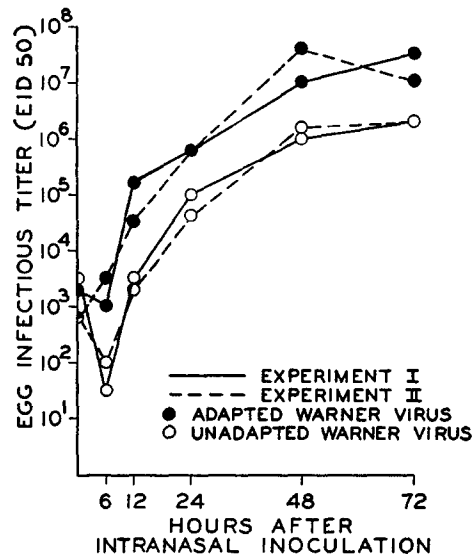

FIG. 1

TABLE I

Rate of Multiplication of Adapted and Unadapted Lines of a Type B Strain (Warner) of Influenza Virus in Mouse Lung

Experiment No.	Influenza virus, Type B Warner strain	Inocula			EID$_{50}$ titer at intervals after intranasal inoculation					
		EID$_{50}$ per ml.	Dilution	Volume	0 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.
I	Unadapted	7.7	10^{-3}	0.05	3.5	1.5	3.5	5.0	6.0	6.3
	Adapted	7.4	10^{-3}	0.05	3.3	3.0	5.2	5.8	7.0	7.5
II	Unadapted	7.7	10^{-3}	0.05	2.8	2.0	3.3	4.6	6.2	6.3
	Adapted	7.4	10^{-3}	0.05	2.8	3.5	4.5	5.8	7.6	7.0

fectious titer strikingly different from that observed immediately after inoculation, will be referred to hereafter as the lag phase of growth of the unadapted line. As will be shown, the decrease in infectious titer at 6 hours was not as marked following inoculation of an unadapted line of Type A-prime influenza virus. A comparison of the growth curves of both lines of virus in Fig. 1 demonstrates that the growth curve of the unadapted line is distin-

guished from that of the adapted line by the lag phase in growth of the former, as well as by the failure of the unadapted line to reach the same maximal titer at 48 hours. The slopes of both curves after 6 hours are indistinguishable, which indicate that the rates of multiplication of both lines of virus are similar during this period.

It will be recalled that the adapted line of virus was prepared and stored as a 10 per cent suspension of mouse lungs in normal horse serum–saline, while the unadapted line was prepared and stored as a 10 per cent suspension of allantoic fluid in normal horse serum. That the lag phase of growth exhibited by the unadapted line is an inherent property of the virus rather than an artifact due to a difference in the medium in which both lines of virus were suspended and stored is indicated by the following considerations. Both lines of virus were diluted 1000-fold prior to inoculation, so that the major non-viral component of the inocula was 10 per cent horse serum–saline. During storage, the infectivity of the two lines was not impaired and at 0 time mouse lungs inoculated with equal concentrations of each line of virus contained approximately the same number of egg infectious doses. Moreover, in the experiments reported by Wang (1), in which freshly prepared virus suspensions were used as inocula, the lag phase of the unadapted line is also evident. Finally, when similar experiments were carried out with the unadapted line that had been passaged once in mice, so that the inoculum was prepared from mouse lung and stored in a manner identical with that used for the adapted line, the lag phase in growth was not eliminated.

Effect of the Concentration of Virus upon the Growth Curves of Unadapted and Adapted Lines of Influenza Virus, Type B.—It has been shown by Taylor (4) that the shape of the growth curve in the mouse lung of an adapted line of influenza virus may be influenced by the concentration of virus in the inoculum. If the lag phase in growth of an unadapted line of virus is an inherent property of the virus particle, it would be expected that although the shape of the growth curve of unadapted virus might be altered, the lag phase would not be eliminated when mice were inoculated with larger amounts of virus. To test this hypothesis, similar experiments were carried out using 10-fold dilutions of the unadapted line, containing log 7.9 EID_{50} per ml., and of the adapted line of the Warner strain, containing log 7.4 EID_{50} per ml. These experiments were conducted simultaneously. The results of a typical experiment are recorded in Table II and Fig. 2. It is evident that when the concentration of adapted virus in the inoculum is increased approximately 100-fold over that used in the experiments of the preceding section, the slope of the growth curve is unchanged in the first 24 hours, but, thereafter, the rate of multiplication of virus is greatly retarded. An increase of 100-fold in the concentration of unadapted virus in the inoculum does not eliminate the lag phase but there appears to be a suppression of growth so that the maximum infec-

tious titer does not exceed that observed immediately after inoculation. The mechanism of this limiting effect of concentrated unadapted virus upon multiplication is obscure. It will be discussed subsequently. Whatever the mechanism may be, the effect is to increase the disparity in maximal infectious titers of virus attained by the adapted and the unadapted lines.
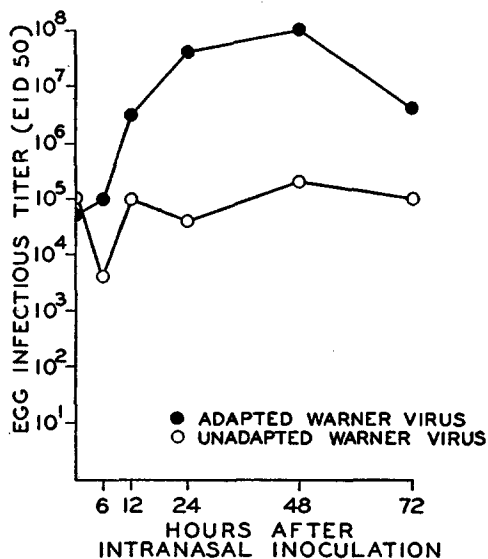

FIG. 2

TABLE II

The Influence of Increased Dosage of Virus upon the Growth Curves of a Type B Strain (Warner) of Influenza Virus in Mouse Lung

Influenza virus, Type B Warner strain	Inocula			EID₅₀ titer at intervals after intranasal inoculation					
	EID₅₀ per ml.	Dilution	Volume	0 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.
Unadapted	7.9	10⁻¹	0.05	5.0	3.6	5.0	4.6	5.3	5.0
Adapted	7.4	10⁻¹	0.05	4.7	5.0	6.5	7.6	8.0	6.6

Rate of Multiplication in the Mouse Lung of Unadapted and Adapted Lines of Influenza Virus, Type A.—It was of interest to determine whether the differences in growth characteristics of adapted and unadapted lines of virus were peculiarities of the strain of Type B influenza virus studied, or were also exhibited by a Type A strain. Accordingly, similar experiments were performed employing suspensions of an unadapted line of the Rhodes strain containing 9.3 EID₅₀ per ml. and of an adapted line containing 7.7 EID₅₀ per ml. Separate

groups of mice were inoculated simultaneously with approximately equal amounts of each line of virus. Two experiments were carried out. The results of these experiments are recorded in Table III and Fig. 3. Multiplication of
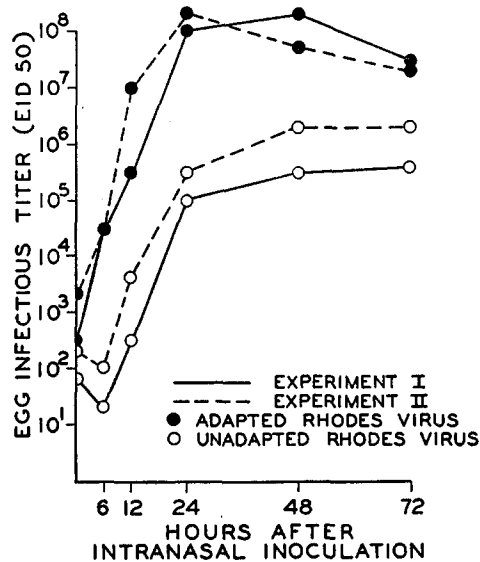

FIG. 3

TABLE III

Rate of Multiplication of Adapted and Unadapted Lines of a Type A-Prime Strain (Rhodes) of Influenza Virus in Mouse Lung

Experiment No.	Influenza virus, Type A Rhodes strain	Inocula			EID_{50} titer at intervals after intranasal inoculation					
		EID_{50} per ml.	Dilution	Volume	0 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.
I	Unadapted	9.3	$10^{-4.5}$	0.05	1.8	1.3	2.5	5.0	5.5	5.6
	Adapted	7.9	10^{-3}	0.05	2.5	4.5	5.5	8.0	8.3	7.5
II	Unadapted	8.7	$10^{-4.5}$	0.05	2.3	2.0	3.6	5.5	6.3	6.3
	Adapted	7.7	10^{-3}	0.05	3.3	4.5	7.0	8.3	7.7	7.3

the adapted line was detectable in 6 hours, and the virus content of the lungs increased progressively with time to reach a maximal infectious titer at 24 hours. In contrast, no increase in virus content of the lungs was observed 6 hours following inoculation with the unadapted line, and at 12 hours the net increase was slight compared with that shown by the adapted line. A comparison of the slopes of the growth curves of both lines from the 6th to the 24th hour demonstrates that they are essentially similar, which indicates that

the rates of multiplication of both lines of virus during this period were similar. The failure of the unadapted line to reach the same maximal infectious titer as the adapted line is also evident. A comparison of the growth curves recorded in Fig. 3 and Fig. 1 demonstrates that following inoculation with an A-prime strain, the infectious titer rose earlier and the slope of the growth curve was steeper than when a Type B strain was used for inoculation. These findings may be related to the observations of Henle (5) that strains of Type A influenza virus showed an earlier rise in infectious titer and a higher yield of virus in eggs than strains of Type B influenza virus. The experiments reported in this section demonstrate that the differences in growth curves of unadapted and adapted lines of influenza virus are not peculiar to the strain of Type B virus studied but are shared by a strain of Type A virus as well.

DISCUSSION

It was previously reported by Wang (1) that the rate of multiplication of an adapted line of influenza virus was greater than that of an unadapted line of the same strain (Rhodes) when both lines were inoculated into mice. Further investigation of the rate of multiplication of influenza virus in mice has shown that there are two principal differences between the growth curves of adapted and unadapted lines of virus. They are the lag phase of growth peculiar to the unadapted line, and the failure of the unadapted line to reach as high an infectious titer as the adapted line. In addition, it has been shown that the shape of the growth curve is dependent not only upon the line of virus but also upon the concentration and strain of virus used for inoculation.

An explanation of the observation that little or no virus multiplication is demonstrable during the lag phase of growth of the unadapted line, must await an understanding of the fate of the virus during this period. It is possible that only a small proportion of the inoculum has the capacity to multiply in the mouse lung and that the lag phase is the resultant of two processes; one being the inactivation of most of the virus given, and the other, the multiplication of the surviving proportion of the inoculum. It is also possible that nearly all of the virus survives and multiplies but that during the lag phase, a longer period of time is required for the unadapted line to overcome a natural resistance of the host or to organize and utilize the materials and energy required to initiate viral synthesis. Finally, it is possible that in the process of multiplication the unadapted virus may temporarily become, during the lag phase, an incomplete or non-infectious particle (6). Experimental data, at present, do not seem to favor any of the hypotheses presented.

Nevertheless, it is evident that the events occurring during the lag phase may be of great importance. It is possible that during this phase a natural selection occurs of those variants in the inoculum for which the mouse lung affords the most suitable medium for growth. That this selection may be a gradual process which is not completed in a single mouse passage is indicated

by the fact that the lag phase was demonstrated with an unadapted line passaged twice in mice. On the other hand, it is possible that in subsequent passages a variant type of virus may appear abruptly, the rapid growth of which during the first 12 hours may eliminate the lag phase in a single passage. When dilute inocula were used, a disparity between the rates of multiplication of adapted and unadapted lines was noted only during the lag phase. If a selection of variants in the inoculum were to occur as a result of a difference in the rate of multiplication of these variants, the lag phase would appear to afford the greatest opportunity for such a selection to take place. Whether adaptation and the disappearance of the lag phase occur simultaneously in subsequent passages of an unadapted line has not been investigated.

No satisfactory explanation can be given for the observation that the maximal infectious titer reached by the unadapted line was less than that attained by the adapted line. It is possible that the suppression of multiplication noted at 24 or 48 hours after inoculation of small doses of both lines of virus results from a response to a stimulation of the host's defense mechanisms. This reponse would inhibit the unadapted line of virus at a lower infectious titer, since multiplication by this line was retarded during the lag phase. However, when large doses of virus were given, a suppression of the infectious titer of the unadapted line is noted at 12 hours but multiplication by the adapted line is not similarly affected. To explain these results a dual hypothesis would be required; namely, that the larger dose of virus evoked a more vigorous response of the host's defense mechanisms and that this response was more effective against the unadapted line. On the other hand, the suppression of multiplication of the unadapted line when large doses of virus are given, may result from "auto interference" due to a blockade effect of the non-surviving portion of the inoculum. Finally, it is also possible that more energy and host materials are required by the unadapted line for viral synthesis and that the failure of the unadapted line to reach the same maximal infectious titer achieved by the adapted line is due to a depletion of these requirements. In the absence of experimental data, a choice of explanations must be deferred. Whatever the explanation may prove to be of the differences in the growth curves of adapted and unadapted lines of virus, these experiments indicate that the adapted lines of virus are more efficient parasites. Multiplication by the adapted line begins earlier and the adapted line attains a higher infectious titer than does the unadapted line. Moreover, the maximal infectious titer reached is less influenced by the dose of virus inoculated. These characteristics are those which would be expected of highly virulent epidemic strains of influenza virus.

SUMMARY

A comparison of the growth curves of adapted and unadapted lines of the same strain of Type A or Type B influenza virus in mice has demonstrated

that the unadapted lines of virus exhibit a lag phase of growth lasting approximately 12 hours during which little or no multiplication of virus can be demonstrated. Multiplication by the adapted line begins earlier and may be evident as soon as 6 hours after inoculation. Following the lag phase, the rates of multiplication of both lines of virus were indistinguishable when dilute inocula were used. The maximal infectious titer of the unadapted line was lower than that of the adapted line of the same strain and this disparity was increased following inoculation with large doses of virus. The shape and slope of the growth curves of influenza virus in mice were shown to be determined by the line, strain, and concentration of virus used for inoculation. The significance of these findings has been discussed.

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