GROWTH CURVES OF INFLUENZA VIRUS BASED ON HEMAGGLUTINATION TITERS IN INDIVIDUAL EMBRYONATED EGGS*

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Examination of growth curves, which reflect changes in quantity of the products of viral multiplication over a period of time, offers a promising approach to further understanding of the phenomenon of viral reproduction.

Henle *et al.*[4-6] have described growth curves based upon the titers of pooled allantoic fluids from groups of eggs sacrificed at various intervals of time after inoculation of influenza virus. They have demonstrated the step-like nature of the rises in titer by means of "one-step" curves, which they obtained by utilizing the interfering action of non-infectious virus injected subsequent to the infectious inoculum. Blumenthal *et al.*[1] have recently described a multiple-step growth curve based on the infectivity titers of a series of pooled fluids. Hoyle[7] has studied curves based upon the titers not only of pooled fluids, but also of serial samples from individual eggs. Growth curves in individual eggs, which may be obtained relatively easily by the technique described by Green and Freymann,[3] have an advantage in revealing significant characteristics which may be obscured when pooled fluids are used. Individual curves have been used recently by McClelland and van Rooyen[8] as a means of studying the effect of specific agents on viral multiplication.

The present paper presents growth curves of the PR8 strain of influenza A virus based on serial titrations of hemagglutinin in the allantoic fluids of single infected eggs. These data confirm the findings of previous observers regarding the step-like nature of the curves and regarding an effect of the size of viral inoculum upon the lag period before appearance of detectable hemagglutinin. In addition, there are described a multiple step curve, an effect of age of egg upon the lag period, and some unusual variations of the "normal" curves.

It is realized, of course, that titers of hemagglutinating capacity may not be directly proportional to the amount of infectious virus present in tissues or fluids of the host, and that these curves are but one reflection of the reproductive phenomenon.

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Methods

Embryonated hens' eggs were inoculated with 0.1 cc. of 10-fold dilutions of the PR8 strain of influenza A virus. Dilutions were made in 0.1 molar phosphate buffer having a pH of 7.0. Serial aspirations of 0.2 cc. of allantoic fluid were performed; the interval between the inoculation of virus and the first withdrawal of fluid, and between subsequent withdrawals, varied with the purpose of the experiment. The technique used for aspiration has been described previously.[3] The eggs were incubated at 37° C. Prior to each aspiration, the eggs were candled; those eggs in which no movement could be detected were considered dead and were discarded. Allantoic fluids from embryos which died were cultured aerobically on blood agar plates. Eggs yielding bloody or yolky fluids were discarded. The fluid obtained at each aspiration was titrated for hemagglutinating ability by a modified Salk technique.[11] Allantoic fluid (0.2 cc.) was added to 0.2 cc. of 0.85 per cent saline solution, and serial 2-fold dilutions were made in saline in 0.2 cc. amounts. A single 1.0 cc. pipette was used for each set of dilutions.

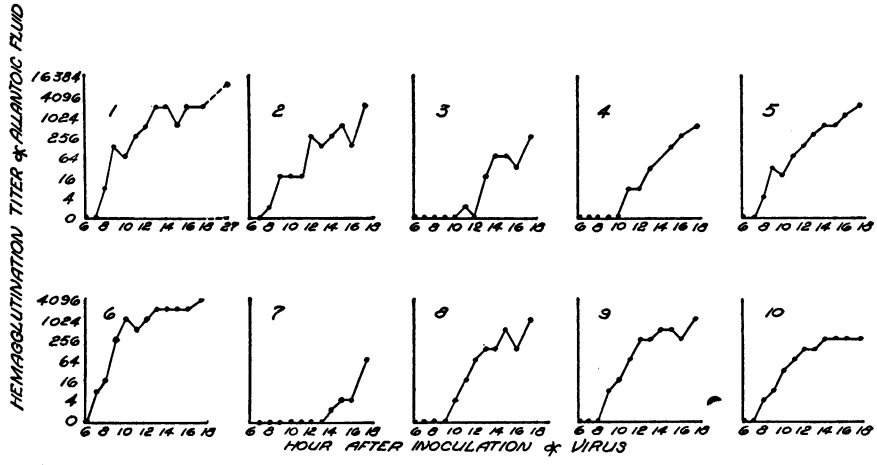

FIG. 1. Typical growth curves of PR8 strain of influenza A virus, based on hourly hemagglutination titrations of allantoic fluids, from individual 12-day eggs inoculated with $10^{6.5}$ EID_{50}. Note individual variations and tendency towards stepwise increases in titer.

To each tube was then added 0.1 cc. of a 1 per cent suspension of chicken red blood cells in saline. Readings were made after one hour at room temperature, and the last tube in which a well-formed (3+) agglutination pattern was visible was taken as the end point. The reciprocal of the original dilution of allantoic fluid in this tube was called the hemagglutination titer.

Results

Contour of typical growth curves. Hourly aspirations of allantoic fluid were performed in several experiments in order to determine the characteristics of the curves. Frequently the smooth rises in titer of hemagglutinin appeared to be interrupted by one or two plateaus or actual drops in titer, which were followed in turn by secondary or tertiary rises in titer. A regular relationship was suggested between the times of inoculation of virus and the times of onset of the secondary and the tertiary rises. Figure 1

shows the results of one experiment, in which a group of 12-day eggs received inocula of a 10^{-1} dilution of virus having an EID_{50} of $10^{-7.5}$. Eggs which survived less than 4 hours after appearance of hemagglutinin were discarded. The primary rises in hemagglutination titer appear to start at widely variable times: between the 6th and 11th hours after inoculation of virus in curves No. 1-6, between the 8th and 14th hours in curves No. 7-9, and between the 7th and 8th hours in curve No. 10. Suggestive secondary rises in titer appear to start between 10 and 13 hours after inoculation in curves No. 1-6 and between 16 and 17.5 hours after inoculation in curves No. 7-9. Tertiary rises in titer appear to start between 15 and 17.5 hours after inoculation in curves No. 1-3. With one exception (egg No. 10), the secondary or tertiary rises appear either between 10 and 13 or between 15 and 17.5 hours after inoculation of virus.

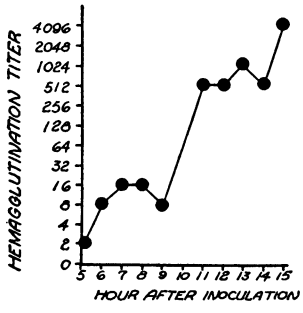

FIG. 2. Multiple step growth curve in a 10-day embryo inoculated with $10^{6.5}$ EID_{50}.

In another experiment, using the same inoculum, but 10-day eggs instead of 12-day eggs, 14 of 18 eggs produced curves which showed plateaus followed by secondary rises in hemagglutination titer; secondary rises started, on the average, between 9.5 and 10.5 hours after inoculation. In the curves from the remaining 4 eggs no such step phenomenon was demonstrated. In 4 of the 14 curves which showed the usual early plateaus there were also well-defined second plateaus or drops in titer, followed by third and final rapid rises which started between 2 and 6 hours after the second rises. One of these curves is presented in Figure 2.

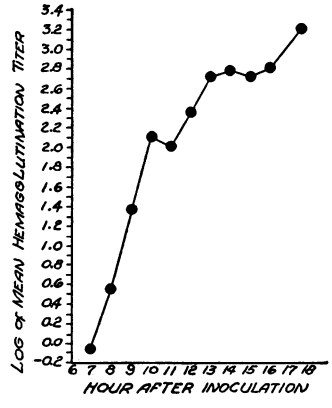

FIG. 3. Composite of curves presented in Figure 1. Each point represents the log of the arithmetic mean of 10 individual titers at a given hour after inoculation. No hemagglutinin was detected in any egg at 6 hours.

In many of the curves described above, a plateau is suggested by a two-fold deviation of titer from the smooth curve. However, with the method used, probably only a four-fold or greater difference between single titers is significant. A composite curve is presented in Figure 3 which is based upon the curves depicted in Figure 1. Each point represents the log of the arithmetic mean of individual titers from 10 eggs. Two plateaus interrupt the smooth rise in titer. These are followed by rapid secondary and tertiary rises which start, respectively, at about 10 and 15 to 16 hours.

An unexpectedly long delay in the appearance of hemagglutinin occurred in a few eggs scattered through several experiments. The curves from these eggs are presented in Figure 4. The rate of multiplication of virus appears slower in these than in other eggs, but data sufficient to establish this are not available. This phenomenon appears unrelated to the size of the inoculum.

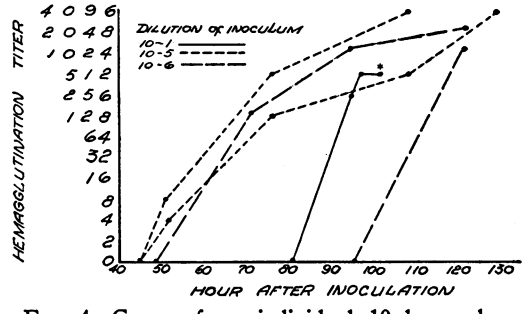

FIG. 4. Curves from individual 10-day embryos demonstrating unusually long delays in appearance of hemagglutinin. Inocula consisted of 0.1 cc. of various dilutions of virus having an EID_{50} of approximately $10^{-7.5}$.

* Test of this fluid for infectivity was positive.

Effect of dose of virus on growth curves. Decreasing the dose of virus prolongs the apparent lag period before the appearance of detectable hemagglutinin in the allantoic fluid. This is illustrated in Table 1 by the results of a typical experiment. In this experiment, fluid was aspirated every two hours, and eggs which survived less than 8 hours after the appearance of hemagglutinin were discarded. The virus used for inoculation was titered simultaneously and found to have an EID_{50} of $10^{-7.5}$. With the maximum dose of virus used in this experiment, $10^{6.5}$ EID_{50}, the time of

TABLE 1

EFFECT OF DOSE OF VIRUS ON INTERVAL BETWEEN INOCULATION AND APPEARANCE OF HEMAGGLUTININ

EID_{50} of virus inoculated		$10^{6.5}$	$10^{5.5}$	$10^{4.5}$	$10^{3.5}$
Hour of detection of hemagglutinin in allantoic fluid:	Average	8.4	12.5	16.3	23.0
	Range	8–10	10–14	14–18	21–25
Number of eggs		10	8	8	6

10-day eggs were inoculated with various amounts of virus. Fluids were aspirated every 2 hours.

appearance of hemagglutinin averaged 8.4 hours. In another experiment, an inoculum containing $10^{8.0}$ EID_{50} of virus was used. In this instance the time of appearance of hemagglutinin in 23 10-day eggs averaged 6.6 hours, with a range of 5 to 8 hours, after inoculation of virus.

Experiments in which small doses of virus were used repeatedly showed more variable results than those in which the doses were larger. This was true with respect both to the interval between inoculation of virus and appearance of hemagglutinin, and to the contour of the growth curve.

Calculations of rates of increase of hemagglutination titers have suggested that the rate may vary with the amount of virus inoculated; however, more data are needed to establish the validity of this observation.

Effect of age of embryos on growth curves. An experiment in which 10-day eggs and 12-day eggs were simultaneously infected with 10^8 EID_{50} per egg revealed that the period between inoculation and appearance of hemagglutinin was approximately 1.5 hours longer in 12-day eggs than in 10-day eggs. In other experiments it appeared that a comparable lag occurred in times of onset of secondary and tertiary rises in titer. The contours of curves obtained in 10 and 12-day eggs were similar.

Comments on technique. An experiment was performed to ascertain that the hemagglutination titer of a small sample of allantoic fluid obtained by needle and syringe was representative of the fluid in the entire sac. Five groups of 10 eggs each were inoculated with a 10^{-2} dilution of virus (about $10^{5.5}$ EID_{50} per egg.) After 13, 24, 48, 72, and 96 hours, two samples of fluid were obtained from each egg in a group, first by aspirating with the needle, then by collecting as much fluid as possible through the air sac with a 10 cc. pipette. The eggs were not chilled, and care was taken to avoid bleeding. There were no significant differences between the hemagglutination titers of the two samples obtained by different methods from the same eggs.

In order to observe the effect on the titer of virus of repeated aspirations of allantoic fluid, some experiments included control groups of 10 eggs which were aspirated for the first and only time at 8, 12, or 18 hours after inoculation of virus. The titers of these control samples did not differ significantly from the titers of samples obtained at the same time from eggs traumatized by 3 to 10 previous withdrawals of fluid.

The mortality rate among embryos subjected to repeated aspirations of fluid varied with the dose of virus, the number of aspirations, and the age of the embryo. As observed by Miller,[9] the mortality was slightly greater when larger doses of virus were used. Regardless of the dose of virus, the frequency of withdrawals of fluid, or the age of the embryo, very few embryos died until after the fourth aspiration. With each subsequent aspiration the mortality rate increased. Twelve-day embryos appeared more hardy than 10-day embryos; about 70 per cent of the former as opposed to about 10 per cent of the latter survived 10 aspirations. The incidence of various technical complications, any one of which disqualified the embryo from an experiment, is indicated in Table 2.

Discussion

Growth curves of influenza virus based on repeated hemagglutination titers in individual eggs demonstrate a rise in titer which may be interrupted by one or two well-defined steps. The times of occurrence of the steps tend to bear a relationship to the time of inoculation of virus and to each other.

It may be postulated that the plateaus represent pauses between "bursts" of release of virus from infected cells; each release causes a sudden rise in titer and is associated with infection of more cells and the onset of another reproductive cycle. Occasional curves show an apparent decrease in the hemagglutination titer during the plateau phase; this may reflect adsorption of the released particles onto new cells.

Measurement of the interval between the terminations of two plateaus (the onsets of secondary and tertiary rises) may provide a means of estimating the length of a reproductive cycle. Application of this measurement to the data presented above indicates a cycle length of 4 to 5 hours. Henle has already suggested that the length of the reproductive cycle of this virus is 5 to 6 hours. His estimate was based on the duration of the interval be-

TABLE 2

COMPLICATIONS ENCOUNTERED DURING THE FIRST 6 ASPIRATIONS IN A GROUP OF 10-DAY EGGS INOCULATED WITH VARIOUS AMOUNTS OF VIRUS

Technical complications	*No. of eggs*
None	63
Death of embryo	20
Bloody fluid aspirated	7
Yolky fluid aspirated	6
Fluid unobtainable	5
Bacterial contamination	4
Total	105

Fluids were aspirated every 2 hours.

tween the inoculation of virus and the appearance of a rise in the level of the infectivity titer of the allantoic fluid.[5]

The interval between inoculation and the rise in the infective property of the fluid is reported to remain constant despite large variations in the size of the infecting dose of virus.[5] The length of the interval between inoculation and the appearance of detectable hemagglutinin, however, bears an inverse relationship to the size of the dose of virus. Larger doses of virus apparently cause earlier accumulation of an amount of hemagglutinin sufficient to be detectable by the Salk test. With the use of doses of virus as large as $10^{9.6}$ EID_{50}, Henle has demonstrated a reduction of this apparent lag period to 4 hours.[4]

Blumenthal, Pinkerton, Greiff, *et al.*[1, 8, 10] have described a curve based on infectivity titers in which suggestive plateaus appear 8 to 10, 14 to 16, and 18 to 22 hours after inoculation. After the 22d hour they have also demonstrated wide fluctuations in the infectivity and hemagglutination titers of the virus and in the oxygen consumption of the embryo. They suggest that

these fluctuations are related to the periodic accumulation and degradation of inactive virus particles which inhibit multiplication of virus, and to variations in the quantity of a viral "toxin" which affects the respiration of the embryo.

The longer lag phase in 12-day eggs than in 10-day eggs inoculated with the same amount of virus may be related to a greater dilution of virus by the greater quantity of fluid in the older eggs.

Examination of curves from individual embryos of the same age inoculated with the same dose of virus reveals that there are often considerable differences in length of the lag period and in contour. It seems likely that these differences are dependent more upon factors inherent in the individual eggs than upon minor variations in techniques, and that they are examples of biological variation in the host, a phenomenon so commonly seen in other laboratory animals. The variations occur more frequently when small inocula are used than when large inocula are used. A possible explanation for the fact that some curves do not show clear-cut evidence of steps is that a number of reproductive cycles overlap, with consequent poor co-ordination of "bursts" of infected cells. The fact that curves without steps are more frequently seen when small inocula are used supports this view. The occasional eggs in which unusually long lag periods occur are particularly interesting. Fluid was aspirated from one infected egg 25 times and contained no demonstrable hemagglutinin until over 97 hours after inoculation.

Summary

1. A selection of growth curves based on serial hemagglutination titers of allantoic fluids from individual eggs infected with the PR8 strain of influenza A virus is presented. A rapid rise in titer appears to be interrupted in many curves by a single plateau, and in occasional curves by two plateaus. The interval between terminations of successive plateaus usually approximates 4 to 5 hours.

2. Decreasing the dose of virus results in a prolongation of the time before appearance of detectable hemagglutinin in the allantoic fluid and in an increase in the variability of this lag period and of the contour of the growth curve.

3. A prolongation of the lag period, without marked change in the contour of the curves, appears when 12-day rather than 10-day eggs are used.

4. Some aspects of the technique used are discussed.

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