STUDIES ON HOST-VIRUS INTERACTIONS IN THE CHICK EMBRYO–INFLUENZA VIRUS SYSTEM*

II. The Propagation of Virus in Conjunction with the Host Cells

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In previous studies on the mechanism of infection of the entodermal layer of the allantoic sac of the developing chick embryo by influenza viruses, a somewhat indirect approach has been employed. It has been possible to deduce certain facts from observation of changes in the infectivity and hemagglutinating activity of allantoic fluids bathing the infected tissues. Thus it has been found that only about 70 per cent of the virus injected into the allantoic sac becomes adsorbed, as demonstrated by the difference between the quantity of virus injected and that found free in the allantoic fluid in the first few hours after infection (1). The titer of the non-adsorbed virus in the allantoic fluid remains constant for different periods of time depending on the type of virus injected. The constant periods in case of strains of influenza A virus extended over 5 to 6 hours, those of the influenza B strains over 8 to 10 hours (2, 3). At the end of these periods, during which it is presumed that the agents are propagating in association with the host cells, the titer suddenly increases as newly formed virus is liberated, terminating the first cycle. The released virus will immediately initiate a second cycle as part of it is adsorbed onto some of the residual non-infected host cells. If adsorption is prevented by the presence of irradiated interfering heterologous virus, injected during the constant periods, all the released virus remains free in the allantoic fluid. Consequently, information has been gained as to the amount of virus liberated into the allantoic fluid as a result of adsorption of one ID_{50} of seed virus (2, 3). The injection of homologous irradiated virus during the constant periods, however, prevented or delayed the appearance of virus in the allantoic fluid either through inhibition of formation or of liberation of virus.

It is obvious that much could be learned about the infectious process if one could follow the course of infection within the allantoic membrane during the time corresponding to the constant periods in the allantoic fluid. It is the aim of this and the subsequent papers of this series to summarize studies on the development of virus activities in the allantoic tissue.

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Materials and Methods

Most of the pertinent technics for the preparation of materials and the experimental procedures have been discussed in previous papers (1, 2). It suffices, therefore, to outline briefly the general procedure of the growth curve experiments to be reported.

An adequate number of 12-day-old chick embryos was injected at t_0 time with 0.2 ml. of suitably diluted allantoic fluid containing either the PR8 strain of influenza A or the Lee strain of influenza B virus. In some experiments or experimental groups, this was the only injection given. In others, a second injection of 0.5 ml. of homologous or heterologous virus inactivated by ultraviolet irradiation was administered one-half hour after the infecting dose. These latter preparations were obtained by sedimenting the virus from allantoic fluid by high speed centrifugation at 20,000 R.P.M. for 20 minutes, and resuspending the sediment in one-eighth volume of the supernatant fluid. The concentrates were dialyzed against 20 volumes of buffered saline solution and irradiated by ultraviolet light for a sufficient length of time, as determined by preliminary tests, to destroy infectivity but to leave the interfering property largely intact (4). As a rule, 0.5 ml. of these preparations diluted at least 1:27 prevented propagation of subsequently injected active virus (about 10^6 ID_{50}) to such an extent as to prevent formation of measurable amounts of hemagglutinins. In earlier experiments, the materials were injected into the allantoic cavity through the air space, in later tests, through holes drilled into the shell at the side of the eggs a few millimeters below the limit of this space, a separate hole being used for each injection. An additional hole was made over the air sac for the relief of pressure. Care was taken to inoculate through areas of chorioallantoic membrane free of blood vessels. After each injection, the corresponding hole was sealed with nail polish. By this technic, flooding of the shell membrane at the air sac end caused by backflow of fluid through the punctures was avoided, thus preventing the occasional deaths of embryos due to this occurrence. The injections were performed at intervals in order to allow for the harvest of the individual groups of eggs at the precise time planned. The trays of eggs were returned to the incubator immediately after each injection to minimize cooling of the eggs. Harvests were made at hourly intervals, using five to six embryos per group. First, aliquots of the allantoic fluids were removed by needle and syringe and pooled according to groups, then the remainder was poured off and the total volume measured in some of the groups of eggs. Thereafter, the contents of the eggs were discarded except for the allantoic membranes which adhered to the shell. They were washed twice *in situ* with buffered saline solution before removal from the shells, and rinsed twice more with saline solution in Petri dishes. The fluids were kept at 4°C. until titrations could be made. The membranes were kept at −15°C. for a few days. They were thawed usually 1 day prior to the titrations, emulsified in ice cold Waring blendors for 3 minutes, using 5 ml. per membrane of buffered saline solution which contained 100 units of penicillin and 100 μg. of streptomycin per ml. The suspensions were clarified by centrifugation at 2000 R.P.M. for 20 minutes and kept at 4°C. until titrations were made. The titer remained constant at this temperature for at least 1 week. Titrations for infectivity were made as described (1), using five 10- or 11-day-old chick embryos per dilution in case of the experimental preparations, and ten per dilution for the titration of the seed virus. The 50 per cent infectivity end-points were calculated according to Reed and Muench. The values recorded in the figures are expressed as ID_{50}/ml. of allantoic fluid or membrane suspension.

EXPERIMENTAL

The first set of experiments to be presented was devised to assay the virus content of the allantoic membranes following infection with either the PR8 strain of influenza A or the Lee strain of influenza B, respectively. The period

under study corresponded to the constant period, as far as the infectivity of the allantoic fluids was concerned. As can be seen in Fig. 1, the virus titer, in agreement with previous reports (2), remained constant in the allantoic fluids for 6 hours in the case of the PR8 strain, and for 9 hours in that of the Lee virus. On the other hand, titration of the suspensions of allantoic membranes collected from the same eggs at the same time intervals showed that constant levels of activity were encountered only during the first 4 hours with PR8 and for the first 6 hours with the Lee strain. Thereafter, the virus content of the tissues increased rather sharply so that the titer exceeded that of

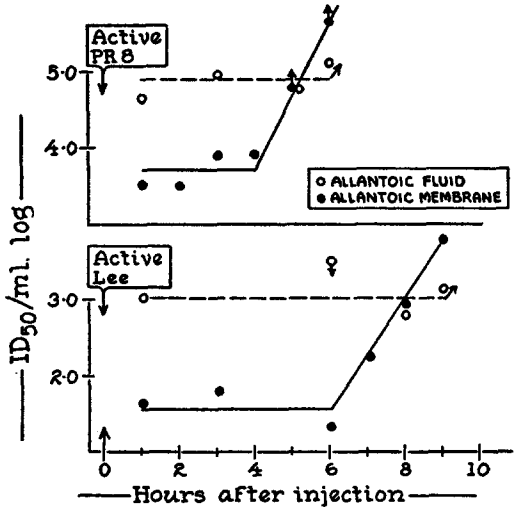

FIG. 1. Comparison of infectivity of allantoic fluids and membranes in the first 6 and 9 hours after infection with PR8 and Lee virus, respectively.

the corresponding allantoic fluids. Since the average yield of allantoic fluid at this stage of embryonic development amounts to between 8 and 10 ml., whereas the membranes were suspended in only 5 ml. of saline solution, the difference between the total amount of virus found during the constant periods in the allantoic fluids, on the one hand, and in the membranes, on the other, was of the order of log 1.4 to 1.7 in favor of the fluid. This finding emphasized again the previously demonstrated discrepancy between the amount of seed virus found in the tissues and that calculated to be adsorbed (1). The amount of non-adsorbed virus in the allantoic fluid corresponded to about 30 per cent; that found in the tissue, to only about 1 to 2 per cent of the seed, instead of the expected 70 per cent.

The effect of inactivated, interfering virus of the homologous or heterologous type upon the propagation of the active agent in conjunction with the host cells

is shown in Fig. 2. The curves shown represent the combined results of three experiments conducted with the PR8 strain, using approximately 10^4 ID_{50} for infection. The upper part of the figure records the data obtained with the allantoic fluids which agreed with the observations previously published (2); *i.e.*, a typical one-step growth curve in the case where heterologous irradiated virus was injected subsequent to infection; no release of virus from the infected

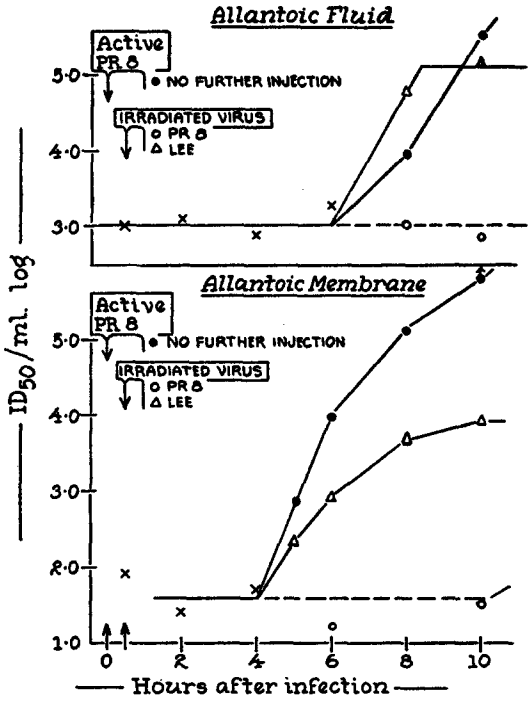

FIG. 2. The effect of injection of homologous and heterologous irradiated virus, following infection, upon infectivity curves in allantoic fluids and membranes. The crosses denote mean titers during the constant periods.

cells into the allantoic fluid in case homologous irradiated virus was used; and a somewhat slower rise in infectivity in the controls which, however, continued at an increased rate beyond the period shown in the figure. The titrations of the membranes derived from the embryos not treated with irradiated virus showed a constant period of 4 hours, followed by a sharp rise in activity. The incline of the curve fell off gradually as the time of incubation increased, but it continued to rise after the 10th hour of incubation in one of the experiments carried over a 24 hour period. If heterologous irradiated virus (Lee) was injected one-half hour after infection, the constant period in the membranes extended, likewise, over 4 hours, but the subsequent rise in infectivity was less

steep than that observed in the control curve. The divergence between the two curves became more pronounced as time proceeded, and there was no significant further increase in titer after the 10th hour of incubation. The third curve represents the results of membrane titrations after secondary injection of homologous irradiated virus, and shows that virus production was markedly suppressed. There was no rise in infectivity during the 10 hour period shown in the figure, but, thereafter, in the one experiment extending over 24 hours, a definite rise in titer occurred.

A similar experiment employing 10^4 ID_{50} of the Lee strain as the infecting agent instead of the PR8 virus gave similar results except for the more prolonged constant periods expected from the data shown in Fig. 1. In other experiments it became apparent that the dose of active virus used was of some importance. Using 10^6 ID_{50} of PR8 virus, results were obtained which agreed in principle with those reported above with 10^4 ID_{50}, except that suppression of propagation by homologous irradiated virus was less pronounced, and a distinct rise in infectivity became measurable in the membranes about 8 hours after infection. Little effect of the homologous inhibitor was noted when injected subsequent to infection with about 10^9 ID_{50}.

These experiments revealed a number of facts. First, the heterologous irradiated virus, which had been used for the demonstration of one-step growth curves (2, 3), is not without effect upon the propagation of active virus in the tissues of the allantoic sac. The difference in the infectivity curve of this series as compared to that of the controls is not merely the result of the prevention of additional adsorption of virus freshly released from the tissues in the case of the eggs treated with the heterologous irradiated virus. As can be seen in the figure, liberation of new virus into the allantoic fluid became measurable only after the 6th hour of incubation, whereas the difference in the amounts of active virus in the membranes was apparent as soon as the titers began to rise. The irradiated heterologous virus is able then to exert some inhibitory effect upon virus propagation in the cells after infection has taken place.

Second, not all the virus formed in the membrane is released into the allantoic fluid. When heterologous irradiated virus is injected one-half hour after infection, it is assumed that only those cells infected during that first one-half hour produce virus, and that all the other cells are rendered resistant through interference. It might be expected, then, that at the end of the intracellular growth period, the titer of the membrane would decrease, since all the cells have either just liberated virus or are resistant to the uptake of virus. However, as is evident from Fig. 2, this is not the case, but the virus curve levels off gradually and the titer remains constant thereafter. This indicates that some of the produced virus is apparently still attached to the cells of the allantoic membrane.

Finally, the experiment shows that the effect of the homologous irradiated

virus is directed toward a delay in multiplication and, usually, a reduction in the amount of virus produced, and not toward prevention of the release from the cells of virus that might have multiplied, a differentiation which could not be made by considering the data of the allantoic fluid titrations (2). The degree of inhibition depends on the size of the original inoculum of active virus; the smaller the infecting dose, the more pronounced the inhibition.

This third point raised a new question. As had been shown in a previous report (Fig. 9, reference 2), the injection of homologous irradiated virus at increasingly delayed intervals after infection with the active agent decreased successively the degree of inhibition, as measured by the liberation of increasingly larger amounts of virus into the allantoic fluid at the end of the proper constant period. For example, if, in the cited experiment, the homologous inhibitor was injected 1 hour after infection with the active Lee virus, practically no rise in virus titer was noted in the allantoic fluid at the end of the 9 hour constant period. With injection of the inhibitor 2, 3, or 4 hours after infection, the amount of virus released increased gradually, but when given after a 6 hour interval, the resulting curve corresponded to that observed following secondary injection of heterologous irradiated virus. If the titers obtained in the allantoic fluid at 10 or 12 hours after infection with the PR8 or Lee strains, respectively, are plotted against the time after infection, when the injection of homologous irradiated virus was given, the points fall reasonably well on a straight line (Fig. 3) connecting the base, which represents the titer of residual non-adsorbed seed virus, with the top line which represents the titer obtained after injection of heterologous irradiated virus.

Inspection of Fig. 3 shows then that propagation of virus may be interrupted any time up to 3 hours after infection with PR8, and up to slightly more than 4 hours after infection with the Lee strain. The figure also seems to suggest that whatever quantity of virus has been produced up to the time of interruption is liberated after the proper constant period. However, during these same periods, (see the curves presented in Figs. 1 and 2), no increase in active virus is demonstrable in the membranes. These findings seem to indicate that viral production occurs in at least two major stages. Conceivably during the first phase, immature, non-infectious forms or building blocks of virus are produced; this development can be interrupted by the homologous inhibitor within certain time limits. In phase 2, these non-infectious forms or building blocks are converted or combined into fully active virus, and this transformation cannot be interrupted by the homologous inhibitor, so that whatever amount of this immature product has been formed up to the time of interruption is subsequently demonstrable as active virus. The data imply, too, that the first stage of virus formation begins shortly after infection of the cells and is completed by the 4th to 6th hour thereafter, depending on the type of virus used. Fully active virus begins to appear only at the end of the first phase.

DISCUSSION

The studies on the propagation of influenza virus in the allantoic membrane have shown that a certain amount of the active seed virus can be demonstrated in suspensions of the tissue immediately after adsorption of the agent (1). The titer of this virus remains constant for about 4 and 6 hours after infection with the PR8 and Lee strains, respectively. Thereafter, a rapid increase in

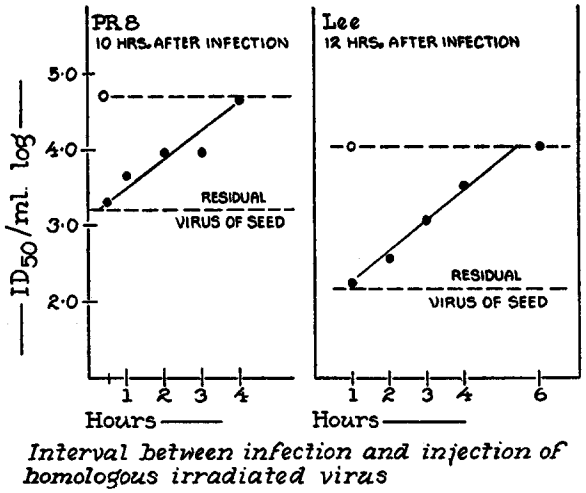

FIG. 3. The height of the virus titer in the allantoic fluid 10 to 12 hours after infection with PR8 and Lee virus, respectively, as influenced by subsequent injection of homologous irradiated virus at increasing time intervals.

infectivity is noted; *i. e.*, 2 to 3 hours before release of any virus into the allantoic fluids can be detected. Injection of irradiated heterologous virus subsequent to infection exerts a slight inhibitory effect on virus production in the tissues, whereas homologous virus may entirely prevent or delay this propagation. The slight inhibitory effect of the heterologous irradiated virus may be comparable to the "depressor effect" described by Delbrück (5), who found in studies on interference between serologically unrelated bacterial viruses that the excluded agent may decrease the yield of the infecting virus.

The quantitative aspects of virus production in the influenza virus–chick embryo system have been considered in a previous report (2). Calculations were presented concerning the yield of active virus in allantoic fluid derived from adsorption of one ID_{50} of seed virus in the first infectious cycle (adsorption-propagation-liberation). This value amounted to about 60 ID_{50} in the average of five experiments with the PR8 strain, and to about 35 in a similar number of tests with the Lee strain. Corresponding figures were obtained

with other strains of influenza A and B virus (3). These calculations now require some comment.

The degree of adsorption was estimated by subtracting the amount of seed virus left free in the allantoic fluid from the total amount injected. As has been pointed out in the preceding paper of this series (1), only between 1 and 5 per cent of the virus thus calculated to be adsorbed can actually be demonstrated in the allantoic tissue. If this small quantity of active virus were the "infecting agent" and the remainder of the seed "wasted," then the yield of virus per ID_{50} of seed would be 20 to 100 times the amount mentioned above; *i. e.*, 1200 to 6000 ID_{50} in the case of PR8, and 700 to 3500 ID_{50} in the case of Lee virus, quantities which conceivably still could result from the infection of one host cell. However, as will be shown in the subsequent paper of this series (6), a large part of the adsorbed active seed virus remains accessible to the action of neutralizing antibodies injected into the allantoic sac following infection without markedly affecting multiplication of virus in conjunction with the host cells. This indicates strongly, that the active seed virus found in the suspensions of the tissues collected during the constant periods may represent "superficially adsorbed" seed virus, and that the non-demonstrable part of the adsorbed virus is probably the one involved in the process of propagation. If this assumption is correct, the values given for the yield of virus per ID_{50} of seed virus would increase only very slightly.

A second point has to be considered in this connection. The calculations under discussion are based on one-step growth curve experiments in which heterologous irradiated virus was injected subsequent to infection. In case the infected eggs are not treated with irradiated virus, multiplication of the agent proceeds in the tissues at a rapid rate, but the liberated virus will spread to other susceptible cells either by continuity or *via* the allantoic fluid thus largely escaping detection and initiating the second infectious cycle before all the virus produced in the first has been released. It is obvious that no accurate information may be gained under these conditions as to the quantity of virus produced as the result of one infectious cycle. On the other hand, the secondary injection of heterologous irradiated virus inhibits the initiation of a second infectious cycle by preventing extensive adsorption of the liberated virus onto the remaining susceptible host cells and by induction of the interference phenomenon. However, this injection is not without effect upon multiplication of the virus in the first cycle, in that the curve of viral increase in the tissues is definitely lower than that found in controls not treated with heterologous irradiated virus. Thus, the yield of virus per ID_{50} of seed virus is not optimal under these conditions.

The tests with the heterologous irradiated virus also showed that not all of the virus produced is actually released into the allantoic fluid, but that part of it remains attached to the tissues. However, this quantity of virus amounts

to only a fraction of that found in the fluid at the end of the infectious cycle and, therefore, would not alter substantially the calculations discussed.

Although the quantitative aspects of virus multiplication cannot be clarified further at the present time the experiments presented may offer some insight into the mechanism of viral propagation. The data would seem to warrant the assumption that propagation of virus occurs in two major phases. Presumably in the first, non-infectious material is built, production of which is completed at about the end of the second third of the growth period in conjunction with the host tissues. Conversion into fully active virus appears to begin only then. In this regard it is of interest to note that Gard and von Magnus (7) observed in allantoic fluids infected with influenza A virus, which were obtained under certain conditions, a component differing from the fully active virus which they considered a precursor of the virus. The suggested multiphase development of influenza virus would seem to be in agreement with certain observations made in the field of bacterial viruses. By exposure of infected bacteria to radiations and subsequent assay of the remaining "infectious centers," Latarjet (8) has shown that inactivation during the first third of the intracellular growth period proceeds according to single hit curves, but that thereafter, the rate of inactivation corresponds to that of multiple hit curves. This indicates that the increase in virus material begins only in about the second third of the period. It also was found that resistance to ultraviolet and x-radiation becomes increasingly stronger during the second phase of the intracellular period, presumably by the accumulation of newly produced material which adsorbs part of the radiation (9). Chemical analyses reported by Cohen (10) showed that synthesis of virus protein begins in the bacteria immediately after infection, whereas the development of desoxyribosenucleic acid can be detected only after about one-third of the period has elapsed. The curve of nucleic acid production runs parallel to that of active virus content of the bacteria, but precedes it by a definite time interval (11).

SUMMARY

Experiments have been reported on the propagation of influenza viruses in the allantoic membrane of the developing chick embryo during the first infectious cycle.

After adsorption of the seed virus onto the host cells, only a small percentage of it remains demonstrable by infectivity titrations. This amount remains constant for 4 hours in the case of infection with PR8 virus, and for 6 hours in that of infection with Lee virus. Thereafter, a sharp rise in infectivity occurs 2 to 3 hours before liberation of the new generations of active virus into the allantoic fluid can be detected.

Injection of homologous virus, inactivated by ultraviolet irradiation, following infection prevents or delays the production of virus in the tissues,

depending to some extent upon the number of ID_{50} of active virus used as inoculum. The smaller the dose, the more pronounced the inhibitory effect.

With increasing delay in the injection of the inhibitor, progressively more virus is produced and liberated 6 and 9 hours after infection with PR8 and Lee virus, respectively. Thus, production of virus may be interrupted by the homologous inhibitor when given up to 3 hours after infection with PR8, and up to $4\frac{1}{2}$ hours after infection with Lee virus. Since no increase in infectivity can be detected during these 3 and $4\frac{1}{2}$ hour periods in the tissues, it is suggested that influenza virus propagates in at least two major stages: first, non-infectious, immature virus material is produced which, subsequently, is converted into the fully active agent. Presumably the first step can be interrupted by the homologous inhibitor, while the second cannot.

Heterologous irradiated virus, injected after infection of the tissue, exerts only a slight inhibitory effect on the production of virus.

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