FACTORS INFLUENCING VARIOLA VIRUS GROWTH ON THE CHORIOALLANTOIC MEMBRANE OF EMBRYONATED EGGS

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The propagation of variola virus on the chlorioallantoic membrane of embryonated eggs was first reported by Torres and Teixeira (1935). Application of this finding was made by Lazarus et al. (1937), Buddingh (1938) and Markham and Bozalis (1939) who utilized embryonated eggs as a diagnostic medium. Nelson (1940, 1943), described the development and stability of variola virus in egg passage but gave only limited information regarding its multiplication on the chorioallantoic membrane. The paucity of data characterizing the propagation of variola virus prompted an investigation of its growth pattern on the chorioallantoic membrane of embryonated eggs and certain factors which influence viral multiplication.

MATERIALS AND METHODS

Virus seed preparation. The Yamada strain of variola virus obtained through the courtesy of Dr. Joseph E. Smadel, Army Medical Service Graduate School, Washington, D. C., was used in this study. It was isolated from a Japanese patient with a moderately severe case of smallpox and when received was in the 2nd chorioallantoic passage. Additional virus passages were made by chorioallantoic membrane inoculation of 11- to 12-day white Leghorn embryonated eggs. On the 6th passage, eggs were inoculated with approximately 5×10^3 infectious units in a quantity of 0.05 ml and incubated at 35 C for 48 hr. A virus pool of these infected membranes was prepared by making a 20 per cent suspension in heart infusion broth, pH 7.3, emulsified in a Waring Blendor for 3 min and centrifuged at 2000 rpm for 10 min in an angle head centrifuge. The supernatant liquid was stored in sealed vials in an electrically operated freezer at −60 C. This virus suspension was used in the experimental studies reported.

Virus assay procedure. In all experimental studies, the pooled membranes were treated in precisely the same manner as described for the preparation of virus pools. Virus titrations of these pools were carried out as follows: Serial tenfold dilutions ranging from undiluted to 10^{-6} were made in heart infusion broth which contained 500 units of penicillin and 100 μg of streptomycin per ml. Eggs were prepared for chorioallantoic membrane inoculation according to the method of Hahon et al. (1957). Appropriate dilutions in a volume of 0.05 ml were inoculated on the chorioallantoic membrane of 11- to 12-day embryonated eggs. Seven to 8 eggs were inoculated per dilution and incubated at 35 C for 72 hr. The harvested chorioallantoic membranes were floated in petri dishes containing formal saline and examined for pocks with the aid of an illuminator (Hahon and Ratner, 1957).

The number of infectious units per ml of a virus suspension was determined by averaging the number of pocks per membrane, multiplying by 20 (for conversion to a ml basis) and by the dilution factor. The membranes employed to determine the concentration of infectious units were those which contained between 0 and 100 pocks. The accuracy of titrations was found to vary ±0.3 log about the mean at the 95 per cent probability level.

Growth curve studies. The viral growth on the chorioallantoic membrane was estimated at different incubation temperatures and with varying doses of inoculum in a volume of 0.05 ml. Eggs were routinely incubated at 35 C unless indicated otherwise. Three to 5 chorioallantoic membranes were harvested at different intervals of time after inoculation to measure viral multiplication. Membranes harvested in the first 8 hr after inoculation were washed 2 or 3 times in heart infusion broth. It was shown by Scott et al. (1953) that the presence of residual viral inoculum interfered with the determination of the actual amount of virus in membranes up to 8 hr after inoculation. The membranes were pooled and stored at −60 C. At the completion of a

growth experiment, they were made into 20 per cent suspensions and assayed for viral content.

A similar procedure was employed in studies to determine the viral content of the chorioallantoic membrane as a result of varying the volume of inoculum and the age of eggs.

Virus distribution study. Groups of embryonated eggs 11 days of age were inoculated with 5×10^3 infectious units in a volume of 0.05 ml by one of four routes (chorioallantoic membrane, allantoic, amniotic, or yolk sac) and incubated at 35 C for 48 hr. The following components were then harvested from each inoculated egg group: chorioallantoic membrane, embryo, yolk sac, yolk fluid, albumin, allantoic fluid, and amniotic fluid. The harvested parts from 3 to 5 infected eggs were pooled. Membranes and embryos were thoroughly rinsed in sterile heart infusion broth to reduce virus contamination of tissues from surrounding fluids. Tissues and albumin were made into 10 per cent suspensions for titration purposes while egg fluids were considered as undiluted. The various components were assayed for virus. Several normal 13-day embryonated eggs, comparable in age to the virus-inoculated eggs after incubation, were selected at random and carefully dissected for the removal of embryo, membranes, fluids, and albumin. The wet weight in grams of these components was determined, and a mean value was obtained for each tissue or fluid. The total virus content of each tested component from infected eggs was calculated by multiplying the infectious units per ml or gram by the normal mean weight of each component.

RESULTS

Growth curves at different incubation temperatures. Results plotted in figure 1 revealed that the growth of variola in the chorioallantoic membrane varied sharply with the temperature. It was evident that 35 C was more conducive for viral multiplication than at other temperatures tested. This incubation temperature resulted in a short, ill-defined latent period which was noted prior to viral multiplication. The peak growth level was reached at approximately 46 hr. At 37 C, an extended latent phase was observed which persisted for 41 hr after inoculation. Maximum viral growth occurred in 49 hr and was 1.9 log lower in titer than the peak level found at 35 C. A 5-hr latent period occurred at 39 C followed by a period of limited viral propa-
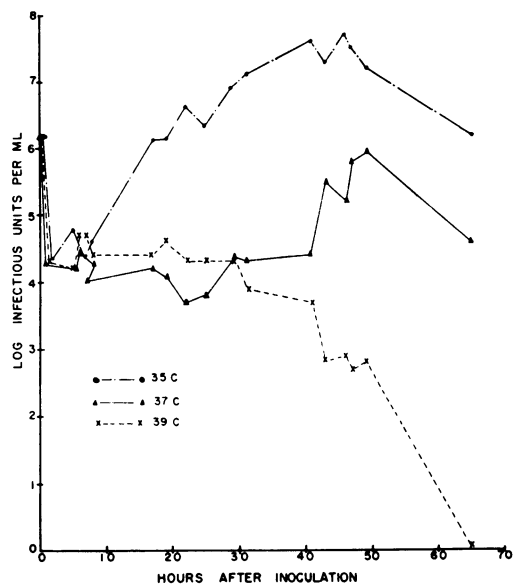

Figure 1. Growth of variola virus on the chorioallantoic membrane of embryonated eggs incubated at different temperatures.
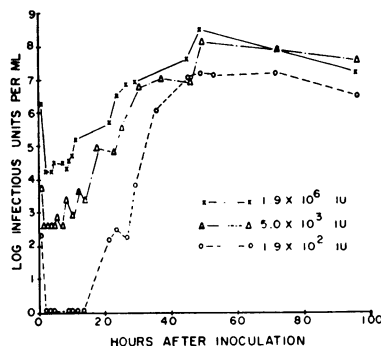

Figure 2. Growth of variola virus on the chorioallantoic membrane of embryonated eggs with varied doses of inoculum.

gation during the ensuing 25 hr. The level of multiplication was only slightly above that found in the latent period. It then progressively declined until viral infectivity could no longer be demonstrated 65 hr after inoculation.

Growth curves with different doses of inoculum. The length of the latent periods noted in the 3 multiplication curves (figure 2) varied inversely with the strength of the inoculum. A latent period (eclipse type) of 11 hr duration occurred with the smallest dose, 1.9×10^2 infectious units. It was a period in which no viral activity could be

detected. The peak growth level was reached in 48 hr irrespective of the virus quantity initially inoculated. However, the two larger inocula 1.9×10^6 and 5×10^3 infectious units, gave peak virus titers which were higher than that found with 1.9×10^2 infectious units of inoculum. The maximum rate of virus growth was independent of the virus quantity inoculated. The rate of increase is plotted against time in figure 3. Experimental points were plotted during the period of the highest rate of virus multiplication but not after limiting concentrations of virus were attained. Points with all 3 inocula formed straight lines having almost identical slopes. During this period, virus increased approximately 6000-fold per 10 hr.
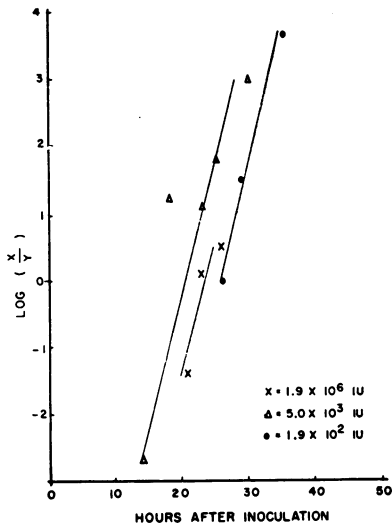

Figure 3. Linear relationship between amount of virus recovered (x) divided by quantities of variola virus inoculated (y) during the period of maximum virus growth.

TABLE 1

Variola virus content of chorioallantoic membrane in 48 hr with different volumes of inoculum

Volume*	Content of Chorioallantoic Membrane
ml	*IU†/ml*
0.05	1.2×10^8
0.10	2.1×10^8
0.25	3.9×10^8
0.50	2.6×10^8

 * Each volume regardless of amount contained 5.0×10^4 infectious units.
 † IU = Infectious units.

TABLE 2

Variola virus content of chorioallantoic membrane in 48 hr with different doses of inoculum

Dosage	Content of Chorioallantoic Membrane	Increase
IU/0.05 ml*	*IU/ml*	*log₁₀*
5.5×10^6	2.6×10^8	1.7
5.5×10^5	1.8×10^8	2.5
5.5×10^4	2.1×10^8	3.6
5.5×10^3	1.8×10^7	3.5
5.5×10^2	3.6×10^6	3.7
5.5×10^1	5.6×10^5	4.0
5.5×10^0	3.0×10^4	3.8

 * IU = Infectious units.

TABLE 3

Variola virus content of chorioallantoic membrane from embryonated eggs of different ages 48 hr after inoculation with 5.5×10^4 infectious units

Age of Eggs	Content of Chorioallantoic Membrane
days	*IU*/ml*
6	1.7×10^6
7	8.6×10^5
8	7.0×10^6
9	1.0×10^8
10	1.3×10^8
11	1.5×10^8
12	2.1×10^8
13	3.9×10^8
14	1.4×10^7
15	5.8×10^6

 * IU = Infectious units.

Effect of varying the volume and dose of inoculum on viral content of chorioallantoic membrane. The effect of increased inoculum volume on the virus content of membranes was investigated because it was conceivable that 0.05 ml was an insufficient quantity for disseminating virus to susceptible chorioallantoic membrane cells. Virus was diluted, therefore, so that the number of infectious units did not vary with different injected volumes. Results in table 1 indicate that the smallest volume, 0.05 ml, was capable of carrying virus to susceptible cells. Considering the variation inherent in titration, there was no apparent difference in the viral content of membranes inoculated with the varying volumes.

In the growth curve experiment (figure 2), the peak viral content of the chorioallantoic
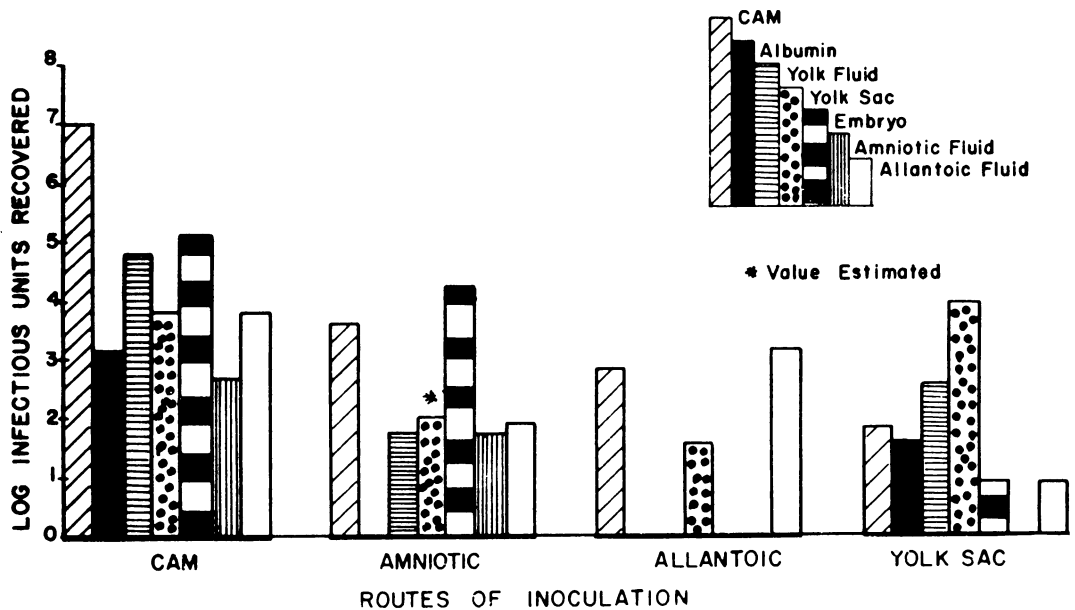
Figure 4. Distribution of variola virus in 13-day embryonated eggs after inoculation of 5 × 10³ infectious units by various routes.

membrane was influenced by the dose of inoculum. Because the doses tested were limited, it was of interest to test a more encompassing range. The results given in table 2 show that the virus content of the chorioallantoic membrane was greatest with the 3 highest injected doses and progressively lower with those below 5.5 × 10⁴ infectious units. The inoculation of 5.5 × 10⁴ infectious units was the only dose that resulted in maximum virus content of the chorioallantoic membrane with a high rate of increase. Below this inoculum, a high rate of increase in virus occurred, but the peak virus content of membranes decreased directly proportional to the dose.

Virus content of chorioallantoic membrane in embryonated eggs of different ages. As shown in table 3, the age of eggs was an additional factor which influenced the maximum virus content of the chorioallantoic membrane. The quantity of virus was highest in embryonated eggs inoculated 9 to 13 days of age and correspondingly lower in eggs younger and older than this age span. The slight differences between virus quantities found in membranes in the 9- to 13-day age group were approximately within the parameters of titration variability.

Distribution of virus in the embryonated egg. Virus concentration from the various components of eggs inoculated by 4 different routes is given in figure 4. In those eggs inoculated by the chorioallantoic membrane route, the highest virus concentration was obtained from the chorioallantoic membrane and, in descending order, from the embryo, yolk sac, and other parts of the egg. The virus was disseminated to all the tested components. The total virus recovered, 1 × 10⁷ infectious units, was indicative of multiplication since it was significantly higher than the injected quantity of 5 × 10³ infectious units. After amniotic inoculation, the highest concentration of virus was found in the embryo, with the chorioallantoic membrane containing the next lower amount. Viral multiplication occurred after amniotic inoculation because the total infectious units recovered, 1.9 × 10⁴, was higher than the initial dose. Very little dissemination of virus was noted after allantoic inoculation with no evidence of multiplication; virus recovery, 1.7 × 10³ infectious units, was of a lower order than the amount injected. A moderate distribution was found after yolk sac inoculation with the greatest virus quantity appearing in the yolk sac. At best, only limited propagation occurred after this route of injection. The total virus recovered, 9 × 10³ infectious units, was only slightly above the injected quantity. The evidence supports the conclusion that the greatest distribution occurred

after chorioallantoic membrane inoculation with chorioallantoic membrane tissue providing the best growth medium.

DISCUSSION

Evidence was presented to show that an incubation temperature of 35 C favored the multiplication of variola virus on the chorioallantoic membrane of embryonated eggs, while higher temperatures were found to be less optimal, resulting in limited viral growth at 37 C and almost complete inhibition at 39 C. Although lower incubation temperatures below 35 C were not tested, Nelson (1940) reported that variola virus was maintained at temperatures as low as 28 C, but the cellular response on the chorioallantoic membrane was retarded. It would be of interest to ascertain whether the findings obtained with variola virus with respect to incubation temperature is applicable to the growth of other pock-inducing viruses.

The growth curve of variola was in some aspects similar to that reported by Scott et al. (1953) with herpes, and Forsyth et al. (1954) with vaccinia. The latent periods for each virus varied inversely with the strength of inoculum. The peak growth levels for variola and herpes were attained in 48 hr regardless of viral dose but for vaccinia the length of time required to reach maximum growth was found to vary. For variola and herpes, the incremental rate of growth appeared to be the same irrespective of the injected dose. However, individual rates of increase between viruses differed. These rates were, $10^{3.8}$-fold per 10 hr for variola, 10^{6}-fold per 10 hr for herpes, and 10^{3}-fold per 12 hr for vaccinia. The different rates may be an intrinsic growth characteristic of each virus or a function of the particular experimental conditions.

Varying the volume of variola inoculum did not notably affect the viral content of membranes when the infectious units were constant. Overman and Tamm (1956), investigating variation in pock counts with different volumes of vaccinia inoculum, found that 0.05 ml gave slightly lower counts than the expected figure. However, statistical analysis of the data by these investigators revealed that the difference was not significant.

The dissemination of variola virus in the embryonated egg was greatest after chorioallantoic membrane inoculation. Like vaccinia

(Buddingh, 1936), variola apparently had the ability to spread from the chorioallantoic membrane lesion by way of the blood stream to the embryo. With the exception of the innoculated chorioallantoic membrane, the highest variola virus concentration was found in the embryo (figure 4). An interesting observation recorded by Buddingh was the presence of vaccinal pocks on embryos of eggs incubated for 20 days. However, extended incubation periods of embryonated eggs inoculated on the chorioallantoic membrane with variola did not reveal pocks on embryos.

SUMMARY

The growth of variola virus on the chorioallantoic membrane of embryonated eggs was in many respects similar to other pock viruses. The latent period varied inversely with the strength of inoculum and the peak growth level was attained in 48 hr regardless of viral dose. The rate of maximum viral growth, $10^{3.8}$-fold per 10 hr, was independent of the virus quantity inoculated.

A 35 C incubation temperature was more favorable for viral growth on the chorioallantoic membrane than 37 or 39 C. Varying the dose of inoculum markedly influenced the viral content of membranes; however, no apparent difference was noted when the volume of inoculum was changed. Optimal virus growth occurred in embryonated eggs inoculated 9 to 13 days of age.

Dissemination of virus was greatest throughout the embryonated egg after chorioallantoic membrane inoculation. Limited viral growth and distribution occurred after amniotic, yolk sac, and allantoic injection.

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