THE INFLUENCE OF AGE OF HOST AND TEMPERATURE OF INCUBATION ON INFECTION OF THE CHICK EMBRYO WITH VESICULAR STOMATITIS VIRUS

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(Received for publication, April 10, 1943)

Experimental infections with vesicular stomatitis virus (VSV) illustrate in an interesting way how complicated and obscure can be the pathogenesis of a central nervous system (CNS) infection after peripheral inoculation. VSV produces a rapidly fatal encephalitis in mice after intracerebral inoculation, the susceptibility of young and adult mice being approximately equal. After peripheral inoculation (intranasal, intramuscular, intraperitoneal), however, adult mice require at least 10,000 times more virus than mice under 15 days of age inoculated by the same route (1, 2).

After intranasal inoculation of mice of all ages the virus usually reaches the anterior rhinencephalon in about 2 days. The fate of the virus from then on depends on the age of the host. In young mice it progresses and kills on about the 5th day after inoculation. In adult mice, on the other hand, it is arrested in the anterior rhinencephalon and the animal remains well. The guinea pig acquires a similar resistance as it grows older (3).

The age resistance of the mouse to VSV is by no means an exception in the pathology of virus infections. Young mice are more susceptible than adult mice to infections with most neurotropic viruses. Such evidence exists for yellow fever virus (4), Eastern equine encephalomyelitis (5, 6), herpes (7), rabies (8), and St. Louis encephalitis (9).

In these experimental infections evidence for age resistance seems conclusive. It is believed that a similar age resistance exists to some naturally occurring infections, but there the possibilities that older individuals may have been in contact with the agent previously and become immunized, or that opportunities for contagion are not equal, complicate the problem. Higher incidence among younger individuals in naturally occurring infections is therefore no proof in itself that a natural resistance has developed with age.

It is of course not certain that the same developments are responsible for the increased resistance of adult animals to all the CNS infections mentioned, but two facts seem to indicate that a similar mechanism might be involved: The age resistance is more marked when the virus is inoculated peripherally in at least four of the infections (VSV, equine encephalomyelitis, St. Louis

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encephalitis, and yellow fever). In the work with rabies such a difference was not noticeable, but in that case a highly susceptible inbred strain of mice was used (8). The second fact is that in cases where both an ordinary unmodified strain and a fixed or a less invasive strain were employed (rabies, equine encephalomyelitis), the age resistance was much more marked in infections with the fixed strain.

In the course of studies on the pathogenesis of VSV in the mouse it became desirable to have a convenient method of titrating virus activity. Early attempts to use the developing chick embryo for this purpose proved unsuccessful, as many of the embryos survived inoculations of large quantities of virus. 10 day embryos were used and the temperature of incubation was 39.5°C. Similar results had been reported previously by Burnet and Galloway (10).

It was of interest to know whether the resistance of one 10 day old embryo to amounts of virus thousands of times greater than was necessary to kill another embryo of the same age might be an "age-resistance," similar to that shown by other experimental animals. It was found that the virus killed 7 day old embryos without exception under the same conditions. The problem of age resistance as manifested in the chick embryo was then investigated further and the results are presented here. It was soon realized that besides the age of the embryo the temperature of incubation was of importance and had to be taken into account.

A few observations on the effect of age of embryos on the pathogenesis of virus infections in the egg have been reported in the literature. Embryos younger than 10 days do not develop marked lesions on the membrane when inoculated with herpes virus (11). Embryos over 16 days old seem to have acquired an increased resistance to the infection. Enders and Pearson (12) found no definite difference in the susceptibility to influenza virus between 15 and 19 day embryos, as measured by survival of virus when the eggs were incubated at 37°C. Pseudorabies virus produced confluent lesions and extensive ulcers on 12 day old membranes, discrete pocks on 15 day old, and no lesions on 18 day old membranes (13). CNS lesions similarly changed from extensive hemorrhage and destruction in 12 day old embryos to more moderate inflammatory changes in 15 day old embryos. Cox (14) reported that 5 day old embryos are best suited for growing rickettsiae by the yolk sac route. Younger embryos died too readily from non-specific causes and 8 to 9 day old embryos yielded lower titers of infectivity.

As concerns the effect of the temperature of incubation on virus infections in the chick embryo, several authors have reported differences in the course of the disease when different temperatures were used.

Membrane lesions: In ectromelia and influenza infections, pocks developed much better at 36–37°C. than at 38.5° or 39.5°C. (12 day embryos) (15, 16). With vaccinia and variola only minor lesions developed on the membrane at 28°C. in contrast to 37°C. where marked lesions were found. In spite of this the virus increased normally at the low temperature (17).

Death of embryo: Ectromelia virus usually killed 12 day embryos in 4 to 5 days at 36–37°C. Embryos incubated at 39.5°C. usually survived (15). Egg-adapted influenza virus in 12 day embryos seemed to be more virulent at 36–37°C. than at 38.5° although direct comparison was not made (16).

Amount of virus in embryo: Influenza virus could be demonstrated in 15 day embryos after 3 days' incubation at 37°C. but not after 5 days. When the eggs were incubated at 41°C. it could not be found after 2 days (12).

Cox (14) obtained markedly better yield of rickettsiae in eggs inoculated by the yolk sac route when the eggs were incubated at 35°C. than when incubated at 39°C.

To sum up: The age of the embryonal host and temperature of incubation have an effect on development of membrane lesions that varies from one virus to another. An optimal age of host as well as an optimal temperature seems to exist for each virus infection. Lower temperatures of incubation increase the proportions of fatal infections with ectromelia and probably with egg-adapted influenza viruses. Data on the amount of virus formed at different temperatures in embryos at different ages are not available except in the excellent work of Cox (14), in which an optimum for growth of rickettsiae as concerns both temperature of incubation and age of host was established.

It was decided to try to investigate the pathogenesis of the VSV infection in the embryo by following up the quantitative changes in the virus during the course of the disease both in the relatively resistant 10 day old embryo and in the relatively susceptible 7 day embryo. Embryos of both ages were studied at two different temperatures of incubation, 39–40° and 35–36°C. The fate of the embryo under these different conditions was also studied.

Material and Methods

The New Jersey strain of VSV was obtained through the courtesy of Dr. P. K. Olitsky in the form of mouse brain (107th mouse passage). The virus was passed twice in mouse brains and from then on in the chick embryo. It has now been through 60 passages in the embryo and still maintains its full virulence for the mouse brain. No signs of a change in virulence for the embryo have been noted.

To control the identity of the egg-passaged virus, guinea pig immune serum was prepared against the 2nd mouse brain passage of virus. This serum neutralized about $10^{3.5}$ M.L.D. virus from the 35th egg passage. In the experiments reported here the 40th to 60th egg passages were used.

The eggs were prepared by cutting a square piece out of the shell and spreading melted paraffin over the area. When the shell membrane was cut with a knife and the shell piece removed, the egg contents settled slowly and left the chorioallantoic membrane exposed (18). After inoculation the opening was covered with Scotch tape.

For passing the virus, 7 day embryos were inoculated on the chorioallantoic membrane with one drop of virus suspension. The opening was covered with Scotch tape and the eggs were incubated for about 20 hours. The dead embryos were harvested, ground, and suspended in a saline solution buffered with phosphates to about pH 7.4

to make a 10 per cent suspension by weight. This suspension was run at about 4000 R.P.M. in an angle centrifuge for 10 minutes. The supernatant was kept in the refrigerator until used. The activity titer usually remained unchanged for at least 5 days and some virus activity remained for many weeks.

The rate of increase of virus in the embryos was followed by titrating them for virus content at varying intervals after inoculation on the membrane. Eggs intended for testing were prepared (cut and paraffined) and put at the desired temperature 24 hours before inoculation. The inoculation was done at room temperature but was performed as quickly as possible so that the eggs should not be cooled.

The eggs containing the embryos to be tested were wiped with cotton soaked in 5 per cent phenol solution. After the egg shell over the artificial air space had been removed with scissors and the chorioallantoic membrane exposed, it was wiped with a cotton swab soaked in phenol. The membrane was then wrapped around the swab by rotating the stick and was pulled up from one side. The chorionic membrane then separated from the underlying yolk sac and was thrown to one side. The amnion with the embryo was thus exposed and the embryo could be lifted out with a forceps without any danger of contamination from the chorioallantoic membrane.

Three or four embryos were used in each pool for titration unless otherwise stated.

Titrations.—The embryos to be tested were ground thoroughly in a glass grinder with buffer saline to make a 10 per cent suspension by weight. This suspension was run at 4000 R.P.M. in an angle centrifuge for 10 minutes. If titrations could not be carried out immediately the supernatant, which was called dilution 10^{-1}, was kept on ice during the intervening hours. Tenfold dilutions were then made in cold buffer saline and inoculated. Five 7 day eggs were used for each dilution.

The eggs were incubated at 35–36°C. and observed daily for 3 days. Embryos receiving 10 M.L.D. or more usually died within 24 hours; those receiving less died within 48 hours. The titration endpoint was determined according to the method of Reed and Muench (19). The titrations by this method have proved very satisfactory, due to the high susceptibility of the 7 day embryos.

Fate of the Virus

The rate of increase of virus was determined in embryos inoculated when 7 and 10 days old. The rate of increase was investigated for both age groups when incubated at 35–36°C. and at 39–40°C. Figs. 1 and 2 show the rate of increase of virus in 7 day embryos at these temperatures.

The virus was detected in the embryo about 2 hours after inoculation on the membrane, and from that point on increased rapidly. At 35–36°C. it reached the highest titer (50 per cent mortality endpoint), 10^{-8} to $10^{-8.5}$, in approximately 16 to 18 hours and about that time the embryos died. At 39–40°C. the virus did not go higher than 10^{-5} to 10^{-6}, which was about 1 per cent of the amount of virus found at the lower temperature. In spite of this the embryos died much earlier at the higher temperature, or about the 12th hour. It may be that cause and effect should be reversed: the titer did not go higher because the eggs were already dying.

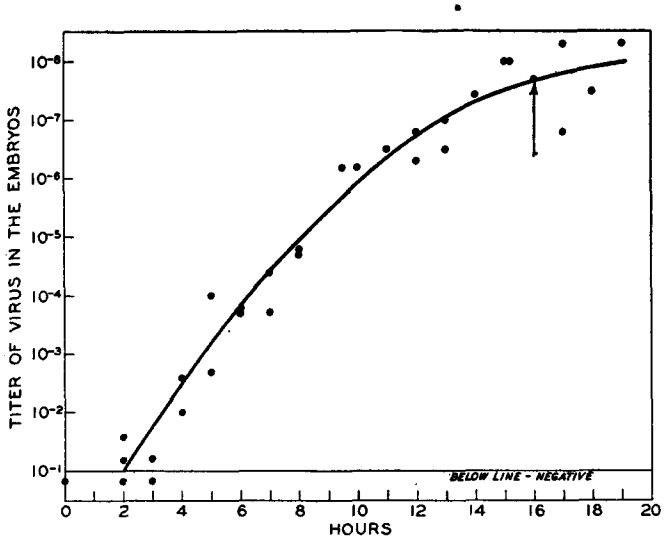

FIG. 1. Rate of increase of VSV in the 7 day embryo when incubated at 35–36°C. Three or 4 embryos were pooled for each titration. The arrow indicates the approximate time of death.

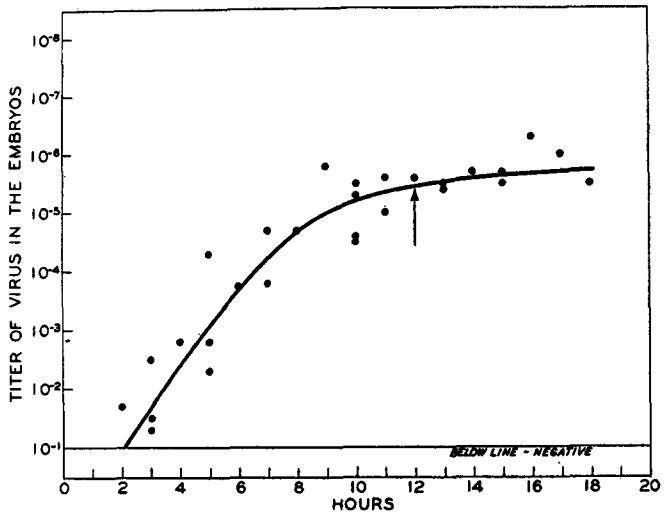

FIG. 2. Rate of increase of VSV in the 7 day embryo when incubated at 39–40°C. Three or 4 embryos were pooled for each titration. The arrow indicates the approximate time of death.

Figs. 3 and 4 show the course of events when 10 day instead of 7 day embryos were used.

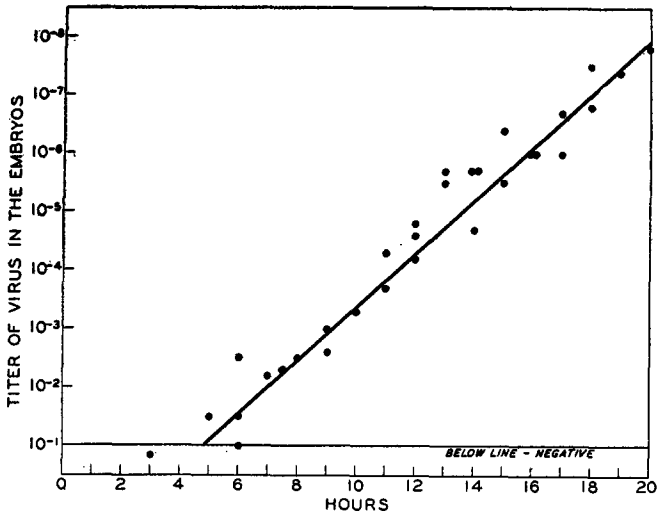

FIG. 3. Rate of increase of VSV in the 10 day embryo when incubated at 35–36°C. Three or 4 embryos were pooled for each titration.

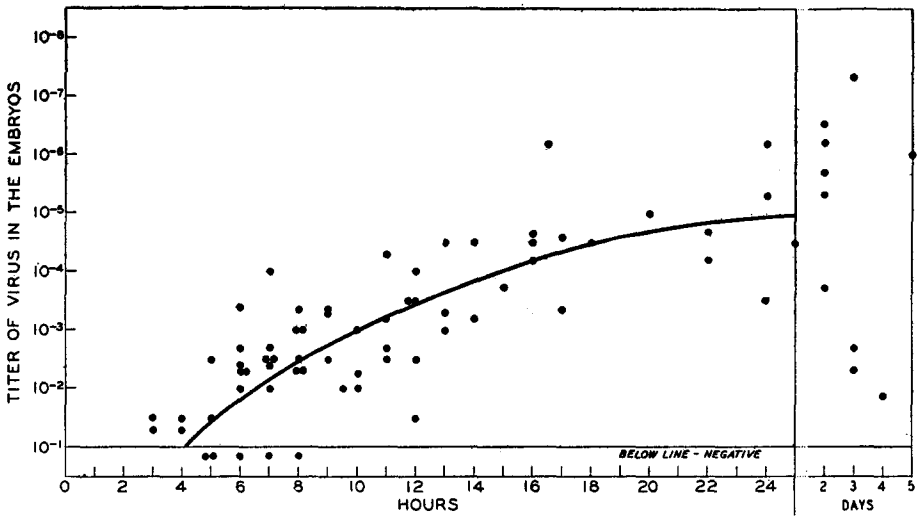

FIG. 4. Rate of increase of VSV in the 10 day embryo when incubated at 39–40°C. Single embryos were used for the titrations.

The time elapsing before virus could be detected in the embryo (4 to 5 hours) was at least twice as long as in the 7 day embryo. In 10 day embryos kept at 35–36°C. it increased at approximately the same rate as in the 7 day embryo after it reached the embryo and attained a high titer (10^{-7} to 10^{-8}) in about 18 to 20 hours, at about which time the embryos died.

As will be shown later, a majority of 10 day embryos kept at the higher temperature survived the infection. Because of that fact it was desirable to test single embryos rather than pools which would have obscured the individual variation. As was to be expected, the results in this series were rather heterogeneous because of the effective resistance of many of the 10 day embryos at 39–40°C. It was found that virus appeared in the embryo at approximately the same hour as it did when incubation at 35–36°C. was employed. The titer rose fairly rapidly but never became very high even in embryos that died, and virus persisted for many days in surviving embryos.

TABLE I

Fatal Infections and Time of Death of Chick Embryos 7 and 10 Days Old Inoculated with about 10^6 Lethal Doses of Vesicular Stomatitis Virus and Incubated at 35–36°C. and 39–40°C.

No. of		Age of embryo	Temperature of incubation	No. of dead embryos on days after inoculation					No. of survivors
Experiments	Eggs			1	2	3	4	5	
		days	°C.						
13	115	10	39–40	18 (42%)	11 (26%)	6 (15%)	7 (16%)	1 (2%)	72 (63%)
7	61	10	35–36	31 (51%)	22 (36%)	8 (13%)			0
4	20	7	39–40	20 (100%)					0
10	100	7	35–36	100 (100%)					0

Fate of the Host

Table I shows the fate of the embryos. The inoculum contained about 10^6 times the amount of virus necessary to kill a 7 day embryo. The only survivors were among the 10 day embryos incubated at the higher temperature, the mortality in that case being only 37 per cent. The 10 day embryos that died had a longer average period of survival than the 7 day embryos, which always died within 20 hours. The 7 day embryos died in about 12 hours when kept at 39–40°C. and in about 16 hours when kept at 35–36°C. This is not shown in the table.

Embryos which died from the infection were hemorrhagic and had a uniform dark purplish color. They were therefore rather easily recognized. Small whitish pocks were commonly seen on the chorioallantoic membrane of 10 day embryos incubated at 39–40°C. They appeared on the 2nd or 3rd day and have not been observed in the 7 day or 10 day embryos at the lower temperature.

DISCUSSION

The chick embryo comes closer to being a universal host for animal viruses than any other organism. Its relatively high susceptibility is supposedly due to the immaturity of its tissues, as the adult chicken is completely refractory to several of the viruses that may infect the embryo. This suggests that it might be profitable to use very immature tissues—very young embryos—in cases where maximum susceptibility is desired. In some instances the 10 or 12 day old embryos, which are generally used for virus work, are still highly susceptible (Eastern equine encephalomyelitis); in others they are not. It is well known that the rate of differentiation and maturation is quickest in the very young embryo and decreases with age. One day in the young is probably equivalent in that respect to several days later. The different anatomical features and physiological functions appear successively. The circulation is established, macrophages and leucocytes appear, blood proteins develop, and fundamental metabolic changes take place. These processes, although known to happen, are only imperfectly understood, especially the chemical ones, but the 15 day old and 5 day old embryos are indeed on a quite different level of differentiation and maturation. It would therefore seem indicated *a priori* to try very young embryos as hosts for viruses whose virulence for the 10 or 12 day embryo is low and where a more susceptible host is desired either for titration purposes or to yield virus in high titer.

In the work presented here only a narrow age range has been tested, but a high degree of resistance was found to develop between the 7th and 10th days. When it was first observed that the infection in 10 day embryos was much more severe at 35–36°C. than at 39–40°C. it could not be decided whether the higher temperature exerted its protective effect by influencing primarily the virus or the host. In a case like that, one must conclude cautiously because the effects on the host or the virus cannot be observed separately. What is observed is a dynamic relationship between the two systems, each of which is only imperfectly known. With this reservation in mind it seems reasonable to conclude as follows:—

The higher temperature is not unfavorable to the progression of the virus as such, as seen in the 7 day embryo which it kills even earlier than at the lower temperature. The rate of increase of virus for the first 8 hours or so is apparently not influenced by the higher temperature as would be expected if the virus were directly affected.

Ten day embryos are more resistant than 7 day, even when kept at the lower temperature, although all die within 3 days. When kept at the higher temperature, however, they show a high degree of resistance, *i.e.*, in these embryos the higher temperature has a marked protective influence.

From this it must be concluded that the embryo develops between the 7th

and 10th days a potential resistance much more effective at the higher temperature, although able to prolong the average incubation period even at 35–36°C.

These relatively moderate temperatures on both sides were chosen because they are within the limits used as routine for the cultivation of viruses. More extreme changes might give greater differences in the susceptibility of the embryo. The mechanism of the resistance is unknown. It may be pointed out that according to Pickering and Gladstone (20), few if any serum globulins are present before the 12th day of age, so antibodies could hardly be involved. Further, it is now becoming clear that resistance to virus infections and neutralizing antibodies in the circulation may develop independently (21–24). The findings reported here are probably one more instance of unexplained cellular resistance to a virus.

Whether the 10 day embryos that survive do so because of an active immunization, *i.e.*, whether the resistance increases during the infection under the influence of the virus, cannot be decided; but the relatively consistent increase of virus during the early hours compared with the irregular increase after that, might indicate such a process.

SUMMARY

Chick embryos after 7 days of incubation were found to be much more susceptible to infection with vesicular stomatitis virus than were 10 day embryos. They had a 100 per cent mortality and were very suitable for titrations of the virus. The rate of increase of virus in 7 and 10 day embryos was studied. Two different temperatures of incubation were employed, 35–36°C. and 39–40°C., and the growth curves for the virus under the different conditions are presented. 10 day embryos were highly resistant and at 39–40°C. more than half of them survived. At the lower temperature of incubation, 35–36°C., all 10 day embryos died, but they survived much longer than did 7 day embryos.

In the 7 day embryos death occurred after about 12 hours at 39–40°C. and after about 16 hours at 35–36°C., or earlier at the higher temperature.

In embryos of both ages the virus titer reached at the higher temperature was only about 1 per cent of that reached at 35–36°C., even in those that died.

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