THE COURSE OF EXPERIMENTAL INFECTION OF THE CHICK EMBRYO WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

By FREDERIK B. BANG, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

(Received for publication, December 21, 1942)

One of the first experimental virus infections of the chorioallantoic membrane of chick embryos to be studied was that of equine encephalomyelitis (1). Subsequently the embryo itself was found to contain large quantities of virus (2), and infected embryos have since been used as sources of virus for vaccines (3), for a complement fixation antigen (4), and for studies on concentrated suspensions of the virus (5). Scant attention, however, has been given to host-parasite relationships in this experimental infection; nor has the rate of growth of the virus in the embryo, the effect of temperature, the distribution of the virus in the embryo, or the influence of the age of the embryo on these relationships been fully investigated. Such studies on a variety of experimental virus infections of the embryo host will yield a more complete knowledge of the behavior of the viruses and may ultimately serve as a more accurate basis for virus classification.

Quantitative studies on viruses depend on the accuracy of the measuring devices. The chick embryo itself may be used for titrations by the determination of the point at which 50 per cent of 10 day embryos die. This is the 50 per cent end-point method of titration (6). We wish to report data on the growth and behavior of the virus in the embryo as measured by this method. These findings will be correlated with the histological changes in the embryo itself.

Material and Methods

Both the Eastern and Western strains of equine encephalomyelitis virus were used. They had been carried by monthly passage through guinea pig brains from 1933 to 1938 when they were dried and stored in a refrigerator. Material for use was taken from guinea pig brains infected by this stored virus.

Eggs from the Institute flock of Rhode Island Red chickens were used throughout most of the year, although occasional lots of eggs were obtained from a nearby flock of White Leghorns. No difference in susceptibility to these viruses has been noted.

The eggs were opened by making a window over the chorioallantoic membrane and allowing the membrane to recede (7). They were inoculated with a 1/20 saline dilution of the glycerinated suspension of guinea pig brain, and the window was covered with Scotch tape. Suspensions of embryos, 20 per cent by weight, were made by grinding the embryo in a Waring blendor in cold saline buffered with phos-

phate. Such suspensions were centrifuged in the Swedish angle centrifuge at about 3000 R.P.M. for 15 minutes before titration of the supernatant.

All titrations were carried out in eggs incubated 10 or 11 days and subsequently incubated at 37°C. 5 embryos were used for each dilution and were kept under observation for 3 days although results were usually complete in 36 to 48 hours. All obviously contaminated embryos were discarded, but routine smears or cultures were not undertaken.

RESULTS

It was first necessary to develop an easy method of titration and define the limits of its accuracy. This was done by inoculating serial tenfold dilutions

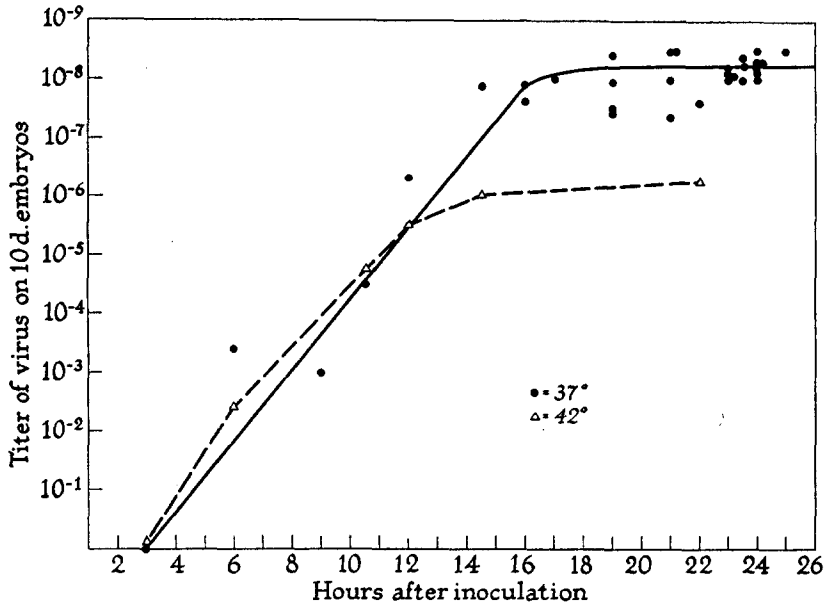

FIG. 1. Multiplication of the virus of Eastern equine encephalomyelitis in the 10 day chick embryo.

of the virus on 10 to 11 day chorioallantoic membranes and calculating the 50 per cent mortality end-point (6). It was soon found that the titer of the supernatants from embryos incubated for 23 to 24 hours at 37°C. regularly fell between $10^{-8.0}$ and $10^{-8.5}$ (Fig. 1). This would yield a median value of $10^{-8.25 \pm .25}$. The accuracy of this was further tested by running simultaneously a duplicate series of titrations of the same suspension. Table I shows that the titer may differ as much as $10^{-0.6}$ but that the average difference is 0.4. This agrees with the suggestive findings shown in Fig. 1 and also demonstrates that variation in the method of titration from sample to sample is entirely sufficient to account for the spread of the points at 23 to 24 hours' incubation. Greater accuracy could presumably be obtained by smaller dilutions and more embryos.

Multiplication of Virus in the Embryo

The curves shown in Fig. 1 were obtained by inoculating 6 or more 10 day embryos on the chorioallantoic membrane, incubating at 36–37°C., then titrating a 20 per cent suspension of all the embryos. Such a method might be expected to show a lag in multiplication, representing the time necessary for the virus to take hold on the membrane and then gain entrance to the embryo. Multiplication of the virus then rapidly progresses into the logarithmic phase of growth, attaining the maximum at, or a little before, the onset of generalized destruction of the embryo. It is impossible to fix this latter point accurately, for the individual embryos in one lot all similarly inoculated vary in the time of death. With the destruction of the embryo, virus multiplication abruptly

TABLE I

Duplicate Titrations on the Same Sample of Virus

Source and strain of virus	50 per cent mortality of		Difference
	1st dilution series	2nd dilution series	
Eastern strain, embryo suspension, 2 days old	$10^{-7.5}$	$10^{-8.0}$	+0.5
Eastern strain, embryos incubated 25 hrs.	$10^{-7.3}$	$10^{-7.6}$	+0.3
Western strain, embryos incubated 24 hrs.	$10^{-7.4}$	$10^{-6.8}$	−0.6
Eastern strain, allantoic fluid	$10^{-6.8}$	$10^{-7.0*}$	+0.2
Western strain, incubated	$10^{-6.7}$	$10^{-7.2*}$	+0.5
Eastern strain	$10^{-7.8}$	$10^{-7.6*}$	−0.2
Same strain, 1 day later	$10^{-7.5}$	$10^{-7.0*}$	−0.5
Average difference			0.4

* This titration was made on the same sample of virus after the addition of chicken red blood cells which were then thrown down in the centrifuge. Titrations were done on the supernatant.

slows down and remains approximately stationary until 24 or more hours after inoculation, when the embryo begins to disintegrate and the titer decreases.

Pathology of the Embryo Infection[1]

The viruses of Eastern and Western equine encephalomyelitis have a typical predilection for the central nervous system of man and of the horse, but in a great variety of animals they fail under natural conditions to penetrate the central nervous system (8, 9). Experimental virus infections of the chick embryo have demonstrated selective tissue destruction by other viruses (10–13). In this study the central nervous system of embryos infected with equine encephalomyelitis was investigated by sectioning embryos at regular intervals after

[1] The pathological studies herein reported were for the most part done while the author was National Research Council Fellow in the Department of Pathology, Vanderbilt University Medical School.

inoculating the membrane with a dilute solution of virus. Dilution allows for a longer incubation period and might afford a chance for the demonstration of tissue specificity.

10 day embryos were inoculated on the membrane as usual with a 10^{-7} dilution of Eastern and a 10^{-3} dilution of Western chick embryo suspension and then incubated at 36–37°C. The membranes and embryos were fixed in Zenker's solution plus 10 per cent acetic acid at intervals of 18, 22, 24, 25, 31, and 41 hours for the Eastern, and 18, 22, 25, and 31 hours for the Western virus. Sections were stained with hematoxylin and eosin. Older embryos, when still alive, were selected from other experiments for section. Dead embryos were never used.

Membranes infected with both strains of the virus soon show vascular collapse, thrombosis, and hemorrhage. A further characteristic finding is the extensive phagocytosis of the red blood cells surrounding a hemorrhage. The similarity of this with the early lesions of the swine influenza virus on the membrane (13) suggested that the red cells might be covered with virus, as they are in swine influenza (14), and thus act as foreign bodies. A series of titrations done after 1 to 2 hours of standing with suspensions of chick red blood cells failed to demonstrate any absorption of the virus on the cells (Table I).

Study of the embryo itself may reveal at first a few scattered hemorrhages and thromboses, indicating that the virus first destroys vascular endothelium. After about 24 to 25 hours' incubation (20 hours if inoculated with concentrated guinea pig brain), the whole embryo shows widespread hemorrhage, thrombosis, and tissue destruction. No tissue shows any special susceptibility; kidney, liver, lung, and nervous tissue all seem to disintegrate simultaneously. The muscle tissues do not show the same immediate destruction, but this probably does not indicate lack of involvement. We have been unable to find the intranuclear inclusions described for the embryo infection (15)) even with the help of special stains.

Distribution of Virus in the Egg

Table II shows that large amounts of virus are obtainable from all parts of the egg but that the embryo usually contains most of it.

Effect of Age of Embryo

Viruses are characterized by variable host ranges, which indicates varying abilities on the part of the parasite to adapt itself to different host cells. Such varying adaptabilities may also be reflected in the reaction of different ages of embryos to the same virus. The viruses of Eastern and Western equine encephalomyelitis show a characteristic gradual decrease in their ability to infect embryos of increasing age (Fig. 2). These curves were obtained by

TABLE II

Distribution of the Virus of Eastern Equine Encephalomyelitis Following Membranal Inoculation

Age of embryo	Incubation period	Virus in:*				
		20 per cent suspension of embryo	Allantoic fluid	Amniotic fluid	20 per cent suspension of membrane	20 per cent suspension of yolk sac
days	*hrs.*					
10	22	8.0	7.0	7.2	8.3	5.8
11	22	8.6	8.3	6.0	7.5	6.5
11	22	—‡	7.7	7.9	7.7	6.9
10		8.3	8.0			
10	23	8.3	6.6			
10	23	8.0	7.3			
Average.....		8.2	7.5			

* All figures are logarithms of the 50 per cent mortality end-point.

‡ Titration unsatisfactory.

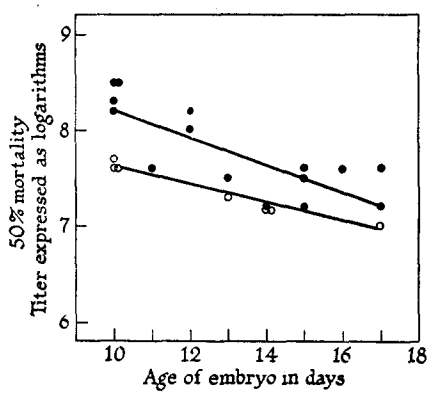

FIG. 2

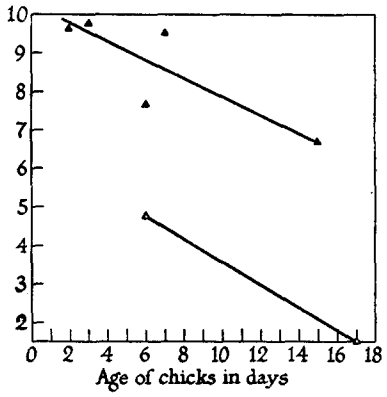

FIG. 3

FIG. 2. The effect of increasing age on the susceptibility of embryos to Eastern and Western strains of equine encephalomyelitis. Upper line represents Eastern strain, lower represents Western strain.

FIG. 3. The effect of increasing age on the susceptibility of chicks to Eastern and Western strains of equine encephalomyelitis. Upper line represents Eastern strain, lower represents Western strain.

inoculating serial tenfold dilutions of a fresh 20 per cent 10 day embryo suspension onto the chorioallantoic membranes of embryos of varying ages. The 50 per cent mortality end-point was then obtained in the usual way and was plotted against the age of the embryo when inoculated. With increasing

resistance on the part of the embryo, death was delayed and the period of observation was therefore extended to 4 days.

It is apparent that the chorioallantoic membrane gradually becomes more resistant to both varieties of the encephalomyelitis virus. Since there is no sudden change at any one point, the gradual effect of maturing tissue may account for this, rather than other physiological changes which take place more suddenly in the embryo (16).

We have not included any points beyond 17 days, for thereafter the natural mortality of embryos at hatching time makes the 50 per cent end-point inaccurate. Suffice it to say that the embryos are still highly susceptible, so that inoculation just before hatching may even be followed by hatching and subsequent death of the chick due to virus infection.

Fig. 3 shows the susceptibility of hatched chicks following subcutaneous inoculation, and the increased resistance with age. It is interesting that the embryo is less susceptible following the membranal inoculation than is the chick when inoculated subcutaneously with the same amount of virus. This probably does not indicate any increased susceptibility on the part of the hatched chick but is rather due to the increased availability of cells when the virus is inoculated subcutaneously.

The difference in susceptibility of the chick to the Eastern and Western viruses is greater than that of the chick embryo, and this probably accounts for the frequent belief that the chick is susceptible to the Eastern but not to the Western variety (17, 18). This would be particularly true when guinea pig brain with relatively small amounts of virus is used for inoculum. Our strain of Western virus is also more neurotropic than field strains, since it has been carried by guinea pig brain passage for 5 years.

Histological study of older embryos reveals the same hemorrhagic destruction seen in the 10 day embryo and also fails to demonstrate tissue selectivity. Hatched chicks do not show this widespread hemorrhage. If large doses of virus are given subcutaneously, the chick dies within the first day or two with symptoms of encephalitis; and sections of the brain show scattered foci of destruction, in which polymorphonuclears are absent. If smaller amounts, 10 to 100 minimum lethal embryo doses, are given subcutaneously, the chick survives up to 5 or 6 days, fails to develop symptoms of encephalitis, but becomes drowsy, droops, and dies slowly without characteristic symptoms. Sections of these chicks show the remarkable myocardial destruction described by Tyzzer and Sellards (19) besides the encephalomyelitis.

Part, if not all, of the increased resistance of the hatched chick to the viruses of influenza (20) and pseudorabies (12) may be related to the increase in body temperature. This was studied for the Eastern virus by following its growth in embryos incubated at 42°C. (Fig. 1). It demonstrates that the virus will grow at this higher temperature; but since embryos frequently die from the high temperature, no interpretation of the shape of the curve is legitimate.

DISCUSSION

The growth curve illustrates several interesting points. It emphasizes that multiplication of the virus depends on the presence of active living cells, and agrees with the finding that in tissue culture the virus also grows best in active cultures (21). This is in contrast to the pleuropneumonia-like organisms and the coccobacilliform bodies which grow best on recently killed embryos (22, 23).

The growth curve for the virus is very much like that of the increase of extracellular bacteriophage in a bacterial culture (24). The reaction is also like the kinetics of the bacteriophage reaction in that the maximum titer is obtained at or just before the complete lysis of the bacteria. The cell destruction shown by histological sections of embryos inoculated with the virus of encephalitis is also complete and occurs almost simultaneously in all cells and tissues. In the case of bacteriophage, it has been possible to measure not only the extracellular, but also the total amount of phage present in a culture. When this is done the initial lag period disappears and the whole curve more nearly approaches a straight line or that of a simple autocatalytic reaction. It is not possible to measure the total amount (extra- or intracellular) of the virus in chick embryos with our present methods, since virus may well be thrown down with intact fragments of cells during preliminary centrifugation. It is also well to remember that the curve for bacterial growth is similar to that of the growth of the encephalitis virus, so that no definite conclusions can be based on the shape of the curve alone (25).

SUMMARY

The titration curve for the virus of Eastern equine encephalomyelitis inoculated into the 10 day old chick embryo shows that the maximum increase in virus content continues until shortly before the generalized destruction of the embryo is apparent. This is followed by a stationary phase.

Histological studies of infected embryos fail to demonstrate selective tissue destruction, and titrations show the virus to be distributed throughout the egg, although concentrated in the embryo.

The chorioallantoic membrane gradually becomes increasingly resistant with age to both the Eastern and Western viruses. Increased resistance with age is also apparent in the hatched chick.

These findings are based on the use of the chick embryo itself as the test animal to determine the 50 per cent mortality end-point. The limits of accuracy of this method are defined.

BIBLIOGRAPHY

1. Higbie, E., and Howitt, B., *J. Bact.*, 1935, **29**, 399.
2. TenBroeck, C., quoted in Beard, Beard, and Finkelstein (3).
3. Beard, J. W., Beard, D., and Finkelstein, H., *J. Immunol.*, 1940, **38**, 117.
4. Mohler, W. M., *J. Am. Vet. Med. Assn.*, 1939, **94**, 39.

5. Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36,** 771. Taylor, A. R., Sharp, D. G., Finkelstein, H., Beard, D., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43,** 648.
6. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
7. Goodpasture, E. W., and Buddingh, G. J., *Am. J. Hyg.*, 1935, **21,** 319.
8. TenBroeck, C., *Arch. Path.*, 1938, **25,** 759; *Proc. 3rd Internat. Cong. Microbiol.*, New York, 1940, 300.
9. Hammon, W. McD., Lundy, H. W., Gray, J. A., Evans, F. C., Bang, F., and Izumi, E. M., *J. Immunol.*, 1942, **44,** 75.
10. Buddingh, G. J., *J. Exp. Med.*, 1936, **63,** 227.
11. Burnet, F. M., *Brit. J. Exp. Path.*, 1940, **21,** 147.
12. Bang, F. B., *J. Exp. Med.*, 1942, **76,** 263.
13. Bang, F. B., *J. Exp. Med.*, 1943, **77,** 7.
14. Hirst, G. K., *J. Exp. Med.*, 1942, **76,** 195.
15. Covell, W. P., *Proc. Soc. Exp. Biol. and Med.*, 1934–35, **32,** 51.
16. Needham, J., Chemical embryology, London, Cambridge University Press, 1931.
17. Olitsky, P. K., Cox, H. R., and Syverton, J. T., *J. Exp. Med.*, 1934, **59,** 159.
18. Levine, N. D., and Graham, R., *Vet. Med.*, 1942, **37,** 116.
19. Tyzzer, E. E., and Sellards, A. W., *Am. J. Hyg.*, Section B, 1941, **33,** 69.
20. Enders, J. F., and Pearson, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48,** 143.
21. Zinsser, H., and Schoenbach, E., *J. Exp. Med.*, 1937, **66,** 207.
22. Swift, H. F., *J. Exp. Med.*, 1941, **74,** 557.
23. Nelson, J. B., *J. Exp. Med.*, 1936, **64,** 749.
24. Krueger, A. P., and Northrop, J. H., *J. Gen. Physiol.*, 1930–31, **14,** 223.
25. Chesney, A. M., *J. Exp. Med.*, 1916, **24,** 387.