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Studies on the Neurotropic Virus of Horse-sickness. VI.—Propagation in the Developing Chick Embryo.

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THE introduction into general practice of the neurotropic virus method of immunization of horses and mules against horsesickness has necessitated the annual production of sufficient virus for the treatment of approximately 40,000 animals. The entire supply of virus has been obtained by propagating it in the brains of mice by the methods detailed in a previous publication (Alexander, Neitz and du Toit 1937). The present vaccine contains 5 antigenically different strains of virus, each of which is propagated separately and two of which are present only in low titre in the brains of moribund mice. Bearing this in mind, together with the fact that large numbers of mice are required for research into many essential problems connected with immunity and vaccine production, it is apparent that any interference with the regular supply of mice is a most serious matter. Consequently, apart from the scientific value attached to the discovery of a number of other methods for the production of large supplies of virus, a substitute for mice has become of extreme practical importance.

Initially, it was believed that the susceptible guinea pig with its larger central nervous system would be the animal of choice, but when it was found that an emulsion prepared from a single guinea pig brain invariably contained no more virus than an equal volume of emulsion prepared from one mouse brain, the use of guinea-pigs was abandoned. Similar observations were made in rats; rabbits, being insusceptible to the virus, were of no value whatever.

A cursory investigation of the possibility of using the recognized tissue culture method of virus cultivation namely, cultivation in an appropriate modification of the Maitland medium was carried out. It was apparent at an early stage that, in spite of definite though somewhat irregular multiplication, the method was of no value for the production of a high titre virus emulsion.

Finally, the work of Woodruff and Goodpasture (1931) and Goodpasture and his collaborators (1932) with vaccinia prompted the use of the developing chick embryo or its membranes as a suitable culture medium, more particularly because a number of investigators

has used this technique not only for the propagation of many different viruses, but also have adapted modifications to the study of problems such as immunity production and virus identification. The results of the cultivation of neurotropic horsesickness virus in the developing chick embryo form the basis of this communication.

No attempt is made to review the relevant literature on this subject since this has been dealt with adequately in recent publications by Burnet (1936).

Technique.—A slight modification of Burnet's (1936) technique is employed with the object of simplifying the procedure as far as possible with an eye to mass production of virus.

Eggs are obtained from mated pens of young hens maintained at the Institute. An ordinary electrically controlled chicken incubator running at 103° F. (39·4° C.) is used for incubation, and before the fresh eggs are added every day no precautions were taken other than to wash them carefully but thoroughly in clean water. On the third or fourth day of incubation the eggs are candled in the usual way and all "infertiles" and "dead-in-shells" discarded. For seeding purposes eggs containing 8-9 day old embryos are used. If younger embryos are used there is a danger of damaging the poorly developed vascular membranes during the manipulations, resulting in a high percentage of early deaths of the embryos; alternately, if older embryos are used the fluff and feathers become a source of great inconvenience when harvesting the virus.

The selected egg is washed with 96 per cent. alcohol and the position of the air space, the embryo and the membranes noted by transillumination. A 250 candle power "Pointolite" lamp in a dark room is well suited to this purpose, and it was soon apparent that the entire operation of seeding may be carried out most conveniently in the beam from this lamp. By means of a dental drill* fitted with a No. 2 Solila burr a hole $\pm 0{\cdot}5$ mm. in diameter) is bored through the shell and outer shell membrane into the air space. It is advisable to drill this hole first as it gives the operator a good idea of the density and thickness of the egg shell, properties which were found to vary within very wide limits. A second hole of the same size is then drilled at a point about the centre of the long axis of the egg, over the position of maximum development of the chorio-allantoic membrane. This hole must penetrate the shell only and should leave the shell membrane intact. With the point of a fine sterile needle the exposed shell membrane is pricked and a minute triangular flap detached from the underlying vascular membrane using a picking rather than a piercing movement. The air is evacuated from the air space by means of a rubber teat. This results in the formation of an artificial air space usually about 2-2·5 cm. in diameter by 3 mm. in greatest depth bounded below by the chorio-allantoic membrane and above by the shell membrane which

* A dental drill was constructed locally from an ordinary Singer sewing machine motor with variable speed foot control, the dental handpiece being attached to a 3-ft. length of motor-car speedometer flexible drive which in turn was welded on to the motor spindle.

remains in apposition to the pierced shell. The point of a fine needle fitted to a syringe is inserted into the artificial air space and the inoculum ejected on to the membrane. Both holes are sealed with melted paraffin wax, and the egg is placed in an incubator at the required temperature.

For optimum results certain precautions are necessary. Care should be taken to drill the hole, through which the inoculation is to be carried out, at a point between the anastomotic branches of the bloodvessels and not directly over a large vessel which might be punctured and cause considerable haemorrhage. Should the chorio-allantoic membrane be pierced, an artificial air space will not result from evacuation of the true air space, but a bubble will be formed within the albumen and naturally the inoculum will not be deposited upon the membrane. This may be controlled by observing by trans-illumination that during evacuation a widening shadow is formed starting from the point of entry of air and that simultaneously the clearly visible blood vessels disappear over this area from their position beneath the shell. An air bubble within the albumen is recognized by being easily movable and by retaining its dorsal position on rotating the egg. All the air must be sucked out of the air space so as to bring the outer and inner shell membrane together or else it will be found that, after a few days incubation, there is a tendency for the artificial air space to disappear wholly or partially, and for the true air space to reform; such an occurrence greatly hampers the subsequent removal of the membrane in a sterile manner. During incubation the eggs should be immobilized by placing them upon a wire screen of suitable mesh or upon cotton wool so as to prevent rolling and the detachment of too large a portion of foetal membrane from the egg shell. Throughout the operation reasonable aseptic precautions only are necessary. For instance it has been found adequate to clean and disinfect the dental burr each time merely by dipping it into alcohol, and no advantage was gained from flaming the eggs or washing them in strong disinfectant.

The above described operation may sound somewhat tedious, but actually this is not the case. In actual practice a technician with average " hands " will be able to drill down to the shell membrane by sense of feel alone with great rapidity and, after a little experience will be able to seed and seal off eggs at the rate of approximately one per minute with an embryo mortality rate of not more than 5 per cent.

When it is desired to harvest the membrane or embryo the shell in the neighbourhood of the point of inoculation is cracked by tapping with a blunt instrument. The shell is detached carefully with forceps leaving the shell membrane exposed and practically intact over an area which will depend in size upon the age and development of the embryo. The shell membrane is torn off, the chorio-allantoic membrane grasped with forceps and the requisite portion snipped off with the scissors. The embryo is grasped by the neck with forceps taking care not to rupture the yolk sac, lifted out and the umbilical vessels severed. Aseptic precautions are used throughout.

In the present study the volume of inoculum was 0·15 c.c of a 5 per cent. emulsion of the infective material in 10 per cent. normal horse serum-saline. Incubation was carried out at 37·5° C. In a few experiments an incubation temperature of 32° C. was tried without any apparent advantage; no work was done at temperatures higher than 37·5° C. In the great majority of cases sub-inoculation was carried out on the fourth day after seeding.

As embryo brain, is considered that portion of the head which remains after snipping off the beak, and enucleating the eyes; the term brain therefore is not strictly accurate but the arbitrary procedure obviated the necessity of removing the soft, pulpy brain from the relatively small amount of cartilaginous skull. Emulsions are made by mincing the material as finely as possible with scissors and grinding it up with a roughened glass rod without sand before adding the diluent. Then the emulsion is forcibly sucked into and ejected from a syringe until the solid particles are finely disintegrated. In the later experiments emulsions were frozen and thawed rapidly to facilitate disintegration, a procedure which appeared to increase the titre slightly. The supernatant fluid obtained after centrifugation at 3,000 revs. per min. for 15 minutes is titrated by the injection of decimal dilutions in 0·05 c.c. amounts intracerebrally into mice and the figures given represent dilutions of the original infective material.

The strain of virus used was that known as 20449 which was in its 181st intracerebral passage in mice. No work has been carried out with other virus strains.

After a few preliminary trials it was apparent that the greatest multiplication of virus took place in the brain of the embryo. It was decided to passage the virus in series through eggs for several generations and then to determine the site and rate of multiplication. The results obtained through 17 passages are shown in tabular form in Table 1.

A consideration of the results brings out the following points:—

1. The virus was maintained by serial passage through the brains of chick embryos for 17 generations when the experiment was discontinued voluntarily.

2. Undoubted multiplication of virus occurred. Had no multiplication taken place the titre in successive generations would have decreased progressively so that the infectivity for mice would certainly have been lost by the fifth generation.

3. The virus titre attained on the fourth day was fairly constant at a level only slightly below that of the original mouse brain emulsion.

4. In one instance only (egg 1, generation 8) was no virus detectable in the embryo on the fourth day. The reason is quite obscure but it probably represents a single instance of faulty technique of inoculation.

TABLE I.

Multiplication of Neurotropic Virus in the Brain of Chick Embryos.

Generation.	Date of Seeding.	Date of Harvesting.	Time of incubation days.	Egg No.	Virus Titration. 1/20.	1/100	1/1,000.	1/10,000.	1/100,000.
—	—	—	—	—	‡ 3,4	5,6	6,7	7,0	00
1	10.3.36	13.3.36	3	1	4,5	4,0	5,5	0,0	x 0
				2	4,5	5,6	6,0	0,0	x 0
		14.3.36	4	*3	x 3	4,5	3,5	5,6	5,6
				4	4,5	4,6	3,4	0,0	3,0
		15.3.36	5	5	4,4	4,5	5,0	0,0	0,0
2	14.3.36	18.3.36	4	*1	5,5	5,5	5,0	0,0	0,0
				2	5,5	5,5	5,0	x 3	0,0
		19.3.36	5	3	4,4	5,6	5,0	6,0	0,0
3	19.3.36	23.3.36	4	*1	7,0	6,6	0,0	0,0	0,0
				2	5,5	5,0	5,5	7,0	0,0
4	23.3.36	26.3.36	3	1	4,4	4,5	4,4	0,0	0,0
		27.3.36	4	*2	5,5	3,4	5,5	5,5	0,0
				3	3,4	4,5	5,0	3,0	0,0
		28.3.36	5	4	4,4	4,4	4,0	4,0	0,0
5	27.3.36	31.3.36	4	*1	4,5	5,6	0,0	0,0	0,0
				2	5,5	5,6	0,0	0,0	0,0
6	31.3.36	4.4.36	4	*1	4,4	4,4	5,5	6,0	0,0
				2	4,4	4,5	5,5	6,0	0,0
7	4.4.36	8.4.36	4	*1	5,5	5,5	5,0	6,0	x 0
				2	5,0	5,5	6,0	5,0	0,0
8	10.4.36	14.4.36	4	*1	0,0	0,0	0,0	0,0	0,0
				2	6,6	6,6	6,0	0,0	0,0
9	16.4.36	20.4.36	4	*1	5,5	5,6	7,0	0,0	0,0
				2	5,0	5,5	5,5	0,0	0,0
10	8.6.36	9.6.36	1	1	0,0	0,0	0,0	0,0	0,0
		10.6.36	2	2	0,0	0,0	0,0	0,0	0,0
		11·6·36	3	*3	5,5	5,5	0,0	—	—
11	12.6.36	15.6.36	3	1	4,6	5,7	6,6	—	—
		16.6.36	4	*2	5,0	5,5	4,6	—	—
		17.6.36	5	3	4,5	4,7	0,0	6,0	—
		18.6.36	6	4	4,4	5,5	0,0	0,0	—
12	16.6.36	20.6.36	4	*1	4,4	4,4	5,6	4,5	—
				2	4,5	x 5	5,5	0,0	—
13	22.6.36	26.6.36	4	*1	5,5	5,0	5,0	0,0	—
				2	4,4	4,5	5,0	0,0	—
14	26.6.36	29.6.36	3	1	5,6	6,6	6,6	6,0	0,0
		30.6.36	4	2	5,6	5,0	0,0	x 0	0,0
		1.7.36	5	*3	4,5	5,5	5,5	6,0	6,0
15	6.7.36	10.7.36	4	*1	4,5	5,5	5,7	7,0	0,0
				2	4,5	4,4	4,5	7,0	0,0
16	10.7.36	14.7.36	4	1	5,5	5,5	5,5	0,0	—
17	14.7.36	19.7.36	5	1	4,7	4,6	5,6	0,0	0,0

NOTE.—In the virus titrations the numeral = day after the injection on which the mouse died, *l* = survival, *x* = death from traumatic injury.

* Indicates inoculum used for seeding the next generation. ‡ Titration of mouse brain emulsion used as original inoculum.

Concurrently with this experiment an attempt was made to determine the site of greatest multiplication of virus, and the optimum time of incubation for maximum yield. Embryos were divided into three portions:—

(*a*) Membrane—a portion of the chorio-allantoic membrane usually about 2 cm. square selected from the site of inoculation.

(*b*) Head—as before.

(*c*) Body—the entire embryo minus the head.

Details of the experiments together with the results are given in Table II. In each case a 5 per cent. emulsion was made as before and dilutions are in terms of the material harvested.

Results.

A consideration of the results brings out the following points:—

1. *Membrane.*—It is not possible to determine whether multiplication of the virus took place on the chorio-allantoic membrane since the virus demonstrated may represent simply survival of the inoculum; if multiplication did take place it was slight. From the twenty-fourth hour there was a steady decrease in the titre of the membrane so that from the third to the eighth day of incubation no more than a trace could be demonstrated.

2. Body.—In the body, twenty-four hours after seeding, no virus was detectable. Virus had made its appearance by the forty-eighth hour, and persisted, if only in traces, up to the seventh day. It is worthy of note that as the virus content of the membrane decreased so did that of the body rise, but the titre was always low. This suggests that the virus demonstrated at the forty-eighth hour represented that which had been absorbed from the membrane, that little or no multiplication took place in the body outside the spinal cord and nerves which were not separated from the parenchymatous organs or the skin.

3. *Head.*—The site of the greatest multiplication was in the brain of the embryo. Virus had made its appearance by the forty-eighth hour in greater concentration than in either the membrane or body. The titre increased rapidly to reach a maximum on the fourth to fifth day after which there was a gradual but steady decline until only traces were demonstrable on the eighth day of incubation.

The Effect of the Virus on the Embryo.

At no time was any gross pathological lesion on the membrane observed; usually there was noticed slight oedema and opacity particularly at the twenty-fourth hour but these changes were never more marked than in control eggs which had been seeded with normal saline. The formation of discrete pocks or opaque areas was never observed. The parenchymatous organs appeared normal. In the brain, particularly on the fourth and fifth day, slight haemorrhagic infiltration in the form of minute red dots was noted.

TABLE II.

Virus content of Membrane, Body and Head of Chick Embryos.

Time of Incubation.	Virus Titration.										
	Membrane.			Body.			Head.				
	1/20.	1/100.	1/1,000.	1/20.	1/100.	1/1,000.	1/20.	1/100.	1/1,000.	1/10,000.	1/100.000.
24 hours.....	5,5	6,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	—	—
	5,0	5,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	—	—
	5,5	6,7	0,0	0,0	x 0	0,0	0,0	0,0	0,0	—	—
48 hours.....	0,0	0,0	0,0	5,6	x 0	0,0	0,0	0,0	0,0	—	—
	4,6	0,0	0,0	4,5	5,0	6,0	5,5	5,5	5,5	—	—
	6,6	6,6	0,0	5,0	0,0	0,0	6,6	6,6	x 0	—	—
3 days.....	8,0	0,0	0,0	5,0	5,0	0,0	5,5	5,5	0,0	—	—
	0,0	0,0	0,0	5,6	0,0	0,0	4,6	5,7	6,6	—	—
	0,0	0,0	0,0	6,6	0,0	0,0	5,6	6,6	6,6	6,0	0,0
	0,0	0,0	0,0	0,0	x 0	0,0	4,5	4,5	0,0	0,0	0,0
	6,0	0,0	0,0	6,7	7,0	0,0	5,7	6,0	0,0	0,0	0,0
4 days.....	4,0	0,0	0,0	x 0	0,0	0,0	5,0	5,5	4,6	—	—
	0,0	0,0	—	0,0	0,0	—	5,6	5,0	0,0	x 0	0,0
	0,0	0,0	0,0	0,0	0,0	0,0	4,5	4,4	4,5	7,0	0,0
	0,0	0,0	0,0	7,7	0,0	0,0	4,5	5,5	5,7	7,0	0,0
5 days.....	0,0	0,0	—	6,0	0,0	—	4,5	4,7	0,0	6,0	—
	0,0	0,0	—	6,0	0,0	—	4,5	4,5	5,5	6,0	6,0
	0,0	0,0	—	0,0	0,0	0,0	4,7	4,6	5,6	0,0	0,0
6 days.....	0,0	0,0	—	5,0	0,0	—	4,4	5,5	0,0	0,0	0,0
	0,0	0,0	0,0	6,7	6,0	0,0	6,5	6,6	6,0	0,0	0,0
7 days.....	5,0	0,0	—	0,0	0,0	—	4,4	5,0	0,0	0,0	0,0
	6,0	0,0	—	5,6	0,0	0,0	5,5	5,6	6,0	0,0	0,0
8 days.....	0,0	0,0	—	0,0	0,0	0,0	6,0	0,0	0,0	0,0	0,0
	0,0	0,0	—	0,0	0,0	—	5,6	0,0	0,0	0,0	0,0

Up to the fifth day of incubation the mortality rate of the embryos did not exceed 4 per cent. This may be considered normal when it is remembered that the temperature of incubation was 37·5°, that the eggs were never cooled off or aired, and that owing to the presence of the artificial air space it was not possible to turn them. From the fifth day onwards the mortality rate increased steadily and this explains the omission of the virus titrations of embryos older than 16-17 days. Actually, however, no fewer than 7 embryos developed to maturity and hatched, the chicks in every case being deformed to a greater or lesser extent but all lived for more than 48 hours. No virus could be demonstrated in the brain of any chick 48 hours after hatching.

An attempt was made to compare the hatchability of eggs inoculated with horsesickness virus and controls seeded with sterile saline all being maintained under the same conditions. It was soon apparent that the mortality rate in both sets from the seventeenth day onwards was so high that significant figures could be obtained only after a statistical survey of numbers of fertile eggs greater than were available.

No histological examination of any organs was undertaken.

Conclusions.

From the experiments which have been described it may be concluded that the developing chick embryo constitutes a medium entirely suited to the propagation of neurotropic horsesickness virus. In the embryo the virus retains its essential neurotropism, multiplication occurring chiefly, if not exclusively, in the brain. The degree of multiplication in the chorio-allantoic membrane and in the body of the embryo, if it occurs at all, is negligible. The titre of the virus in the brain reaches a peak on the fourth or fifth day of incubation, after which there is a steady decrease so that by the eighth day only residual traces are detectable. Virus has not been found to multiply or persist in the chick after hatching.

These conclusions apply to only a single strain of virus (that known as 20449 which was used in its 181st passage in mice). It is not anticipated that the site of multiplication will differ in the case of other virus strains but it is possible that there will be some variation in the rate of multiplication and the maximum titre.

The virus appears to have no special lethal effect upon the embryo and after maximum development " dies " at a rate considerably faster than it does in a serum saline emulsion preserved at the same temperature (37·5° C.).

Discussion.

Before entering into a discussion of the interesting results obtained in this series of experiments it is necessary to state that three attempts were made to propagate a field pantropic strain of virus in a similar manner. Detailed protocols are omitted because of the entirely negative results obtained. Virus in the form of infective defibrinated blood haemolyzed by freezing and thawing,

and clarified by centrifugation at 3,000 r.p.m. was seeded on to 9-day embryos in the usual manner. After incubation for four or five days, 3 embryos were harvested, divided into membrane, body and head, and a pooled 5 per cent. emulsion of each portion seeded separately on to other embryos. After three passages emulsions were injected separately into susceptible horses. No reactions were produced. This finding cannot be taken to imply definitely that pantropic virus will not multiply in the chick embryo, since the number of trials is too small, but it does indicate that a strain will be adapted only with difficulty.

It now becomes necessary to direct attention to the phenomenon of a virus, the original effect of which has been modified by intracerebral passage through mice, retaining its acquired neurotropism on propagation in the relatively poorly differentiated brain cells of the chick embryo, the mature chicken in turn being quite insusceptible to infection. Further, the multiplication of the virus proceeds rapidly to a point which apparently represents the saturation point of the host cells and then disintegration and death of the virus commences without having had any specific lethal effect. In other words, the findings represent an experimental symbiosis between a virus and a particular type of cell.

The fact that the virus has no lethal effect upon the embryo, and produces no easily detectable lesion on the membrane or in any of the organs, makes the use of the chick embryo quite unsuitable for the study of problems connected with immunity by the orthodox methods (Burnet, Keogh and Lush 1937). On the other hand, the chick embryo remains suitable for virus propagation in large quantities.

On numerous occasions 5 per cent. saline emulsions prepared from the brains of mice moribund after intracerebral infection of neurotropic virus strain 20,449 have been titrated and it is quite remarkable with what consistency ·05 c.c. of a one-ten-thousandth dilution constitutes 1 M.L.D. The weight of the brain of a mouse of the age used is approximately 0·45 grams which means that 100 c.c. of 5 per cent. emulsion can be obtained from about 12 mice. The " brain " of a chick embryo incubated from 12-14 days under the conditions described was found to weigh from 1·2-1·9 grams which means that 100 c.c. of virus emulsion of the same titre could be obtained from 3 or 4 embryos. This brings the propagation of attenuated virus for vaccine production in the embryo within practical bounds although it must be pointed out the work involved in seeding and harvesting 4 eggs is considerably greater than injecting and subsequently removing the brains from 12 mice. On the other hand, it is probable that provision could be made for sufficient fertile eggs to be available throughout the year whereas the incidence of a disease such as ectromelia, ring worm or mouse typhoid can wipe out an entire mouse stock. Were it not for the fact that the present routine horsesickness vaccine contains 5 strains of virus thus necessitating the use of at least 20 embryos for each batch of vaccine a change from mice to eggs would be indicated. In the present circumstances the egg method should be kept in reserve to replace the use of mice in case of dire necessity.

In the meantime there remains a considerable amount of essential work to be done e.g. it is necessary to determine whether all virus strains will grow in embryos as easily as strain 20449, what is the optimum period of incubation and the virus titre of all other strains, whether the degree of attenuation of the neurotropic virus remains unaltered for horses, and whether the antigenic response in horses remains the same. This work is proceeding and will be reported on in due course.

Summary.

1. The technique for seeding eggs for the propagation of neurotropic horsesickness virus in the developing chick embryo is described in detail.

2. It is found that the virus retains its neurotropic character and multiplies in the brain of the embryo; little if any multiplication takes place on the chorio-allantoic membrane or in the body of the embryo.

3. The titre of the virus reaches a peak after 4-5 days incubation at 37° C. after which there is a progressive fall in virus content; after 8 days only traces of virus are detectable.

4. The virus produces no macroscopically detectable lesion on the membrane and only slight heamorrhagic infiltration in the brain.

5. The virus exerts no specific lethal action on the embryo. Several chicks have hatched and were found to retain no virus in the brain.

6. The embryo mortality ratio amongst seeded eggs during the first 5 days of incubation does not exceed 4 per cent.

7. The possibility of using the chick embryo method for the propagation of virus for vaccine production is discussed.

Addendum.

The continuation of this work on the propagation of neurotropic horsesickness virus was discontinued for approximately a year. An attempt was then made by my colleague, Dr. J. H. Mason, to cultivate two other neurotropic attenuated strains in chick embryos but without success. In the light of the above report which actually constituted the third separate adaptation to chick embryos these failures appeared so remarkable that parallel control experiments were run by me using strain 449 in its 246th mouse passage. The virus was lost by the third egg passage. The work was then repeated using the original strain 449 generation 181 that had been stored in a refrigerator at 4° C. and was still fully virulent for mice. Again no multiplication could be demonstrated and the virus died out by the fourth egg passage. Eggs from the same source but naturally from different birds were used and it is believed that the identical technique was followed but the reason for the consistent failure remains obscure.

Bearing in mind the ease with which the virus had been cultivated for 17 passages these quite unexpected findings are the present subject of close investigation. It would appear that adaptation to the chick embryo is not the simple matter that the above work had lead us to believe.

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