Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 23, Numbers 1 and 2, March, 1948.

Printed in the Union of South Africa by the
Government Printer, Pretoria.

Preliminary Note on the Cultivation of Green's Distemperoid Virus in Fertile Hen Eggs.

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USING infected dog blood as his source of virus, Mitscherlich (1938) attempted to propagate distemper virus in developing hen eggs. He found that the virus was still active after 6 days incubation on the chorio-allantoic membrane but failed to survive subinoculation into other eggs. Plumber (1939) using ferret-adapted virus was able to maintain infectivity for 5 and 6 generations respectively in two series of chorio-allantoic passages, but the virus was lost on further subinoculation. Beveridge and Burnet (1946) state that in their laboratory various routes of inoculation were tried without any indication that the virus multiplied.

Green (1939) reported that after 54 serial passages through ferrets, canine distemper virus was so modified that while retaining high virulence for ferrets, it had become avirulent for dogs. This modified virus which he termed " disteimperoid virus ", was believed to be suitable for use as a vaccine for dogs. The object of this preliminary note is to show that Green's modified distemperoid virus can be cultivated readily in developing hen eggs. No opinion can be expressed as yet, as to the possibility of using the egg-propagated virus for immunization purposes.

METHODS AND MATERIALS.

The eggs used were obtained from a flock of bacillary white diarrhoea and fowl typhoid-free White Leghorns maintained at this institute. After 8 days preliminary incubation in a Jamesway forced draught incubator at 100° F. the eggs were delivered to the laboratory. The further handling of the eggs has been described in detail by Alexander (in press).

All injections were made onto the chorio-allantoic membrane by Alexander's (1938) modification of Burnet's technique. To serve as a source of virus an ampoule of " Canine Distemper Vaccine, Ferret origin, Green method " was obtained. The ampoule carried the serial number 57AX4. The dried material was reconstituted with 2 c.c. diluent and 1 c.c. of the mixture was injected intraperitoneally into a ferret. On the 4th day after injection the temperature of the ferret was markedly elevated and on the following morning the ferret was killed and its spleen removed. The spleen was emulsified in 40 c.c. of horseflesh infusion broth in a Waring Blendor, centrifuged at 3,000 r.p.m. for 30 minutes in a Clay Adams angle-head centrifuge and the supernatant fluid was used for inoculation in 0·2 c.c. amounts onto the chorio-allantoic membranes of eighteen embryos. Further incubation was carried on in a Jamesway forced draught incubator at 35° C.

Propagation in Eggs.

On the day after injection 15 of the 18 embryos were found dead and examination of smear preparations showed that in the majority numerous contaminating bacteria were present. The remaining 3 embryos were alive on the 4th day when they were opened with the usual aseptic technique. The only apparent deviation from normal was a slight thickening and oedema of the membrane. The membranes were harvested, emulsified in mincer tubes (Alexander, in press) in about 10 c.c. of broth and transferred to the chorio-allantoic membranes of other 8 day old embryos. Sterility tests in broth showed the absence of contaminating bacteria and routine serial passage has been carried on from that time. The culture is now in its 30th passage.

The temperature of incubation of the injected eggs at all times has been 35° C. Subinoculation has always been made with fresh chorio- allantoic membrane emulsion injected in 0·2 c.c. amounts onto the membranes of 8 day old embryos. The interval between transfers has almost invariably been 4 days but on one or two occasions it was found convenient to subinoculate on either the 3rd or the 5th day.

Up to about the 8th generation no marked macroscopic changes in the eggs were noticed other than a slight oedema of the chorio-allantoic membranes confined to that portion on which the inoculum had been deposited. From the 9th generation many membranes were found to be markedly thickened, moist and showed numerous minute light gray foci which tended to coalesce. From the 14th generation the area of membrane thickening became progressively more extensive and has regularly involved the portion immediately below the true air sac. A few embryos have died but the deaths have been irregular and could not be correlated in any way with virus multiplication or progressive adaptation of the virus to its new environment.

Bacterial sterility has been rigidly controlled throughout the course of the work. One or two cultures became contaminated but these were detected easily and were discarded.

Animal Inoculation.

A. *Ferrets.*

It is a routine practise in this laboratory not to subinoculate a serial culture back to the original susceptible host until the 3rd subculture so as to reduce to a minimum the possibility of simple carry over of virus. This procedure was followed in this case.

Generation 3.—Several membranes of the third passage were pooled, minced without the addition of any diluent, and 1 c.c. of the supernatant fluid obtained by angle centrifugation was injected intraperitoneally into a ferret. A marked febrile reaction commenced on the fourth day after injection and the ferret was moribund on the tenth day.

Generation 5.—Similar membrane material was injected intraperitoneally into a ferret, which commenced a febrile reaction on the third day and was found dead in its cage in the morning two days later.

Generation 8.—After an incubation period of four days the reaction commenced and the ferret was destroyed *in extremis* on the 11th day.

Generation 14.—The oedematous membranes of four eggs were harvested, minced without any diluent, and decimal dilutions in broth up to 10^{-6} were prepared from the supernatant fluid obtained after angle centrifugation. Owing to the urgent necessity of conserving the small supply of available ferrets a single ferret was taken for each dilution. One c.c. of the various dilutions was given intraperitoneally with the results indicated below:—

Undiluted.—A marked febrile reaction commenced on the third day; temperatures up to 106·6° F. were recorded; animal died on the 8th day.

10^{-2}. No fever produced but animal died on 5th day.

10^{-4}. No febrile reaction was produced but typical symptoms developed and the animal was killed when moribund on the 12th day after injection.

10^{-5}. No fever was produced; animal died on 6th day.

10^{-6}. No reaction and ferret survived.

Generation 16.—From four eggs on the 4th day of incubation in this subculture the extra-embryonic fluids, the chorio-allantoic membranes and the embryos were pooled separately. The tissue material was disintegrated by mincing as before and centrifuged. Serial decimal dilutions of each were then prepared for intraperitoneal injection into ferrets in 1 c.c. amounts as shown below:—

Embryos.

10^{-1}. A marked febrile reaction resulted after 3 days, with temperatures up to 105° F. Animal killed *in extremis* on the 9th day.

10^{-3}. Reacted 10 days later with temperatures up to 105° F. Killed on 13th day.

10^{-4}. No reaction.

Extra Embryonic Fluids.

10^{-1}. Reacted on 3rd day with temperatures up to 105·5° F.; died on the 10th day.

10^{-3}. Reacted after 14 days with temperatures up to 104·5° F.; was very sick on the 20th day and was killed.

10^{-5}. No reaction.

10^{-6}. No reaction.

Chorio-allantoic Membranes.

10^{-1}. Reacted on 3rd day with temperatures up to 106·6° F.; was very sick on the 9th day and was killed.

10^{-3}. Reacted on 3rd day with temperatures up to 106·2° F., and was killed on the 11th day.

10^{-5}. No reaction.

10^{-6}. No reaction.

It will be noticed that in this experiment reacting ferrets were destroyed as soon as the reaction could be diagnosed with complete certainty. This procedure in no way invalidates the results but was adapted to minimise the risk of accidental infection of those animals that received subinfective doses of virus.

Results.

The chorio-allantoic membranes, the extra embryonic fluids and the embryos all contained virus possibly in decreasing concentrations in that order. In no case was a dilution of 10^{-5} infective. Whereas the 10^{-3} dilution of membrane produced a reaction after an incubation period of only 3 days, indicating a fairly high concentration of virus, the incubation period of the reaction produced by the dilution of embryo and fluid was significantly prolonged. Only embryo diluted 10^{-4} was injected and that proved non-infective.

Generation 21.—Undiluted membrane material of the 21st subculture was injected into the survivors in the above experiment on the 20th day. All reacted after an interval of 2 days with temperatures up to 107·6° F.

The Disease in Ferrets.

The incubation period in ferrets injected into the peritoneal cavity varied between 2 and 14 days depending on the concentration of the suspension injected.

Three types of reactions were observed. In the peracute form there was usually no rise in temperature but the animal became very sick, would not eat and the fur round the perineum was soiled. After 2 or 3 days the temperature dropped so low that it did not register on the ordinary clinical thermometer and the animal was dead by the following day.

In the acute form there was a marked thermal reaction up to 107·5° F. The temperature remained high about 6 days and then collapsed suddenly shortly before death. These animals showed some soiling of the perineal region and their lips were usually somewhat swollen and red.

In the sub-acute form there was usually a marked thermal reaction with temperatures up to 106° F. The temperature remained high for 2 or 3 days, would return to normal and again rise. After 14 days the animals were in a pitiful state; their eye-lids and lips were encrusted and they would not eat. There was, however, only slight emaciation.

Post mortem examination showed very little change in the internal organs. In some the spleen was enlarged, and in some there were signs of a cystitis.

B. *Dogs.*

No facilities whatever were available either for obtaining or breeding distemper-free dogs. Therefore it was necessary to obtain the assistance of colleagues to inject what dogs they were able to obtain and to keep what records they could. Their willing co-operation is gratefully acknowledged even though the scope of the work was limited.

In every instance the material, either undiluted or diluted 10^{-1} in saline immediately before use, was injected in 1 c.c. amounts subcutaneously on the same day as harvested. The following results were obtained.

Generation 6.—Four Daschshund cross-bred pups two months of age were injected with infected membranes from the 6th generation. They showed no apparent reaction.

Generation 11.—A six months old Ridge-back and a 5 months old Mastiff cross were injected with material from the 11th generation. They showed no apparent reaction.

Generation 15.—An Irish setter pup, 5 months of age and a Pointer pup 2 months of age were injected with membranes from the 15th generation. The Irish setter reacted four days after injection with a temperature of 104° F. The temperature on the 6th day was 105·8° F., and it then slowly returned to normal but from the 8th day the dog showed eye lesions and on the 11th day pustules were found on the insides of its thighs. This dog recovered in spite of a concurrent attack of biliary diagnosed and treated on the 15th day.

The Pointer pup had a temperature of 104·4° F. on the day after injection. The temperature rapidly returned to normal, but rose again on the 6th day to 103° F.; it remained more or less at this level until the 12th day after injection when the temperature collapsed and the animal died. This pup showed pustules from the 7th day after injection and eye lesions from the 9th day. Shortly before death it showed marked nervous symptoms.

Generation 16.—One year old Mastiff cross, a three year old Fox-terrier cross, a 10 months old Doberman and an 8 months old Mastiff were injected with material from the 16th generation. The last two animals mentioned showed no reactions. The Fox-terrier was slightly sick on the 7th, 8th and 9th days after injection but recovered rapidly. The year-old Mastiff cross, however, was very sick from the 5th to the 9th day. It would not eat and vomited frequently. It then recovered completely.

Generation 17.—Two aged Daschshund bitches and the 3 months old pup of one of them were injected with material from the 17th generation. None showed any apparent reaction.

Identification of the Virus.

1. An ampoule of canine anti-distemper serum prepared by Messrs. Burroughs Wellcome, England, labelled Q1946N with an expiry date 1 May 1949 was obtained for an *in vitro* neutralization test. It contained 0·35 per cent. cresol as preservative. Five c.c. of this serum was mixed with 1 c.c. of a 10^{-1} dilution of infected membrane from the 21st egg generation. As a control 1 c.c. of the same membrane dilution was added to 5 c.c. of broth diluent and both were allowed to stand at room temperature for 30 minutes, before injection intraperitoneally into 2 ferrets (6 c.c. each).

The ferret which received the serum virus mixture showed no clinical reaction and when challenged with undiluted membrane suspension three weeks later was found to be fully susceptible. The control ferret which received the virus-broth mixture reacted after an incubation period of three days, showed the usual clinical syndrome, and was killed on the 8th day.

2. In an endeavour to develop a rapid *in vitro* technique for the quantitative estimation of the virus titre of various emulsions a fairly detailed study was made of the possible application of the well known Hirst (1941) chick red cell agglutination phenomenon. Numerous experiments were made with different pools of chick and guinea pig red cells in concentrations of 1 per cent. It is merely necessary to say that quite irregular partial agglutination

was observed only in the presence of high concentrations of virus emulsion (10^{-1}). To serve as a control of technique, formalised egg-propagated influenza type A virus was obtained from the South African Institute for Medical Research (Dr. J. H. Gear), to whom our indebtedness is acknowledged. The haemaglutination titres obtained corresponded closely with those indicated by Dr. Gear.

3. The supernatant fluid from membranes in the 16th generation was instilled into the nostrils of 12 anaesthetised white mice. Ten days later the mice were still healthy and were killed. Their lungs were pooled and the supernatant fluid after angle centrifugation was instilled into other mice. The process was repeated seven days later. No lung lesions were produced.

From the clinical picture of the disease produced in ferrets and dogs, and the apparently specific *in vitro* neutralisation by canine anti-distemper serum it is believed that the virus which was propagated over 30 generations is that of distemper. Chance air borne infection of the cultures with influenza virus is excluded by the failure to infect mice by the intra-nasal route and the failure to demonstrate chick red cell agglutination. Contamination with other viruses being cultivated in the laboratory, such as horsesickness, bluetongue, fowl and pigeon pox can be excluded with confidence. Since no other strain of distemper virus is being investigated or could possibly have been a source of contamination it is concluded that Green's Distemperoid Virus (Ferret origin) has been propagated by serial passage in fertile eggs.

Summary.

Green's distemper vaccine virus has been propagated 30 generations in developing hen eggs.

Injections were made onto the chorio-allantoic membranes of eggs that had received a preliminary incubation of 8 days. Re-incubation was done at 35° C., and passage of the membranes was made at aproximately 4 day intervals.

The egg-adapted strain produced markedly oedematous changes with some necrosis in the membranes, but only occasionally killed the embryo.

The titre of infected chorio-allantoic membranes, measured by ferret injection was between 10^{-3} and 10^{-5}. That of the embryos and extra-embryonic fluids of the same eggs was 10^{-3}.

The reactions produced by the egg-adapted strain when injected into ferrets were similar to those produced by the parent strain.

Five c.c. of anti-distemper serum neutralized the egg-cultured virus.

Thirteen dogs were inoculated with suspensions of chorio-allantoic membranes. In nine there was no reaction. In one there was a slight reaction, but in three the reactions were very severe and one died after showing typical distemper symptoms.

The egg-adapted virus did not agglutinate chicken or guinea pig red cells and failed to infect mice when instilled intra-nasally.

ACKNOWLEDGMENTS.

I wish to thank Dr. R. A. Alexander for help and guidance in this work; Drs. A. D. Thomas, D. Coles and W Neitz for the observations on the dogs and the Misses Smith, Erasmus and De Jager for technical assistance.

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