Infection, Haemorrhage and Death of Chick Embryos after inoculation of Herpes Simplex Virus Type 2 on to the Chorioallantoic Membrane

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SUMMARY

When the chorioallantoic membrane of the embryonated hen's egg was inoculated with herpes simplex virus type 2 strains, an infection resulted which spread rapidly throughout the egg. Haemorrhage and death occurred in the embryo, and haemorrhage was also observed in the chorioallantoic membrane. Virus was recovered from the infected chorioallantoic membrane, allantoic fluid, amniotic fluid and selected organs of the embryo. In contrast, similar inoculation of herpes simplex virus type 1 strains gave no haemorrhage in chorioallantoic membrane or embryos, embryos did not die and virus was recovered only from the inoculated chorioallantoic membrane. Inoculation of either type into the allantoic cavity did not result in spread of virus to the embryo. Prolonged adaptation of herpes simplex viruses to growth in eggs eventually resulted in spread of type 1 virus from the chorioallantoic membrane to other regions of the egg and there were also marginal increases in virulence of type 2 virus.

It is generally recognized that there are two types of herpes simplex virus (HSV), which can be differentiated by various tests, one of which is the appearance of the pocks they produce on inoculation on to the chorioallantoic membrane (CAM) of fertile hen's eggs (Hutfield, 1967; Parker & Banatvala, 1967; Nahmias et al. 1968). Preliminary observations in this laboratory indicated another difference between type 1 and type 2 strains in pathogenicity for the whole egg. This was explored in detail with four strains of each type, of which three were established laboratory strains and one was a fresh isolate. Type 1 laboratory strains were HFEM, HIL, WAL and the fresh isolate was 22101; type 2 laboratory strains were LOV, PAR, 17152, and the fresh isolate was 9889. An outline study was also made with a further 21 HSV strains; nine strains were type 1 and twelve were type 2. Of the nine type 1 strains, five had had several passages in tissue culture and four were fresh isolates; of the twelve type 2 strains five had had several passages in tissue culture and seven were fresh isolates. All strains were typed by pock characteristics on the CAM and appearance of c.p.e. in tissue culture; laboratory strains were also studied by the temperature marker test (Longson, 1971; Ratcliffe, 1971). Five of the eight strains used in the detailed study were supplied by Professor P. Wildy (Birmingham University) and had been further typed by the kinetic neutralization test (Rawls et al. 1968). Infectivities of the eight strains studied in detail were assayed in human embryo lung cell cultures using the 50% end-point dilution method (Reed & Muench, 1938), titres varied from $2.1 \times 10^6$ to $4.7 \times 10^7$ TCD$_{50}$ per ml for type 1 strains and from $2.1 \times 10^5$ to $3.2 \times 10^7$ TCD$_{50}$ per ml for type 2 strains.

Fertile Leghorn hens' eggs were incubated at 36 °C and after 11 days 0.1 ml undiluted stock virus pool was inoculated on to the CAM which was exposed using the false air sac technique. Great care was taken to ensure that during the drilling of the eggs the membranes remained intact and any eggs showing signs of bleeding were discarded. Harvests of allantoic
### Table 1. Spread of types 1 and 2 herpes simplex viruses in the egg

<table>
<thead>
<tr>
<th>Days of harvest</th>
<th>HSV type 1 strains</th>
<th>HSV type 2 strains</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hr~EM 22101</td>
<td>HIL WAL LOV PAR 17152 9889</td>
</tr>
<tr>
<td>CAM + + + + + + + + + + + + + + + + + + + + +</td>
<td></td>
<td></td>
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<tr>
<td>Fluids</td>
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<tr>
<td>Allantoic</td>
<td>- - - - + + + + + + + + + + + + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>Amniotic</td>
<td>- - - - + + + + + + + + + + + + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>Embryo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>- - - - - + + + + + + + + + + + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>Gut</td>
<td>- - - - - + + + + + + + + + + + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>- - - - - + + + + + + + + + + + + + + + + +</td>
<td></td>
</tr>
</tbody>
</table>

+ = herpes virus recovered from harvests as indicated by pocks formed on the CAMs of eggs inoculated with harvests.

- = herpes virus not recovered from harvests, no pocks formed on CAMs of eggs inoculated with harvests.

Fluid, CAM, amniotic fluid, heart, gut and brain were made 1, 2, 3 and 6 days post-inoculation, taking the following precautions to prevent cross-contamination. A syringe with a fine gauge needle (26G) was inserted 4 to 5 mm into the blunt end of the egg, and allantoic fluid was withdrawn slowly. After carefully cutting away the dropped region of the CAM with the shell still attached, the amniotic sac was lifted with blunt forceps and the amniotic fluid was removed with a syringe. The embryo was removed from the amniotic sac by carefully cutting the amniotic membranes so as to prevent contact between the embryo and allantoic fluid. The embryo was washed free of amniotic fluid in three changes of 150 ml normal saline, and cut longitudinally to expose the heart which was removed together with the heart blood. Samples of liver and intestine were removed at this stage. The brain was aspirated using a syringe with a coarse gauge needle (18G), which was inserted into the back of the head between the eyes. The shell and CAM removed earlier were separated to harvest the CAM.

Allantoic cavity inoculation was performed by inserting a fine gauge needle under the shell and directly inoculating the allantoic fluid with 0.1 ml undiluted stock virus pool. Allantoic fluid and brain were harvested as described above.

Harvests were stored at -70 °C. Virus in the harvests was demonstrated by inoculation of harvests on to the CAMs of further eggs, and observation of the CAM for development of typical herpes pocks within 3 days. Allantoic and amniotic fluids were inoculated undiluted, heart and gut material were inoculated as 40% (v/v) suspensions in penicillin/streptomycin broth saline (PSBS), brain was inoculated as a 20% (v/v) suspension in PSBS and CAM was homogenized and inoculated at a 10^-3 dilution in distilled water.

Each strain produced pocks on the CAM typical of the type to which the strain belonged, i.e. type 1 pocks were less than 0.5 mm in diam. and type 2 pocks were greater than 1 mm after 3 days. Type 2 always caused a haemorrhagic condition of the CAM. No haemorrhage was seen after inoculation of the CAM with type 1 strains. Three days after CAM inoculation with type 2 strains, the embryos were also haemorrhagic and often dead. Neither death nor haemorrhage of embryos occurred when CAMs were inoculated with type 1 strains even after prolonged incubation.
Type I viruses were recovered only from harvests of CAM. All other regions of the egg harvested after inoculation of type I viruses on to the CAM failed to yield pocks on further passage in eggs, indicating that HSV type I had not spread beyond the CAM. Results were identical for all type I strains on all days tested. (Table I).

In contrast, all type 2 strains tested spread rapidly from the CAM to other regions of the egg, appearing in the allantoic fluid 1 or 2 days after CAM inoculation, in the amniotic fluid 2 days after inoculation and in the embryo harvests 2 or 3 days after inoculation. All selected parts of the embryo yielded virus. The results were identical for all type 2 strains tested (Table I). Similar results were obtained when HSV type 2 virus strain 9889 (fresh isolate) was diluted $10^{-1}$ to $10^{-4}$ although progressive dilution resulted in delayed spread to the embryo. When even higher dilutions of the type 2 strain were inoculated on the CAM, so that inocula contained only 5 to 20 pock forming units, no haemorrhage of the CAMs was observed, and no virus was recovered from the embryo. Under these conditions, embryos hatched normally. In contrast spread of type I virus beyond the CAM was never demonstrated even using the stock virus pools undiluted. The titres of type I virus in these pools were comparable with the titres of the type 2 virus pools.

Herpes simplex virus type 1 strain 22101 and HSV type 2 strain LOV were examined after 12 and 24 serial egg passages. The nature and rate of spread were unaffected with either strain after 12 egg passages, but after 24 egg passages the type 2 strain spread more rapidly to the organs of the embryo, appearing in the allantoic fluid and brain only 1 day after CAM inoculation. The type I strain spread into the allantoic fluid after 4 days but did not spread to the brain of the embryo.

Following inoculation of either type I or type 2 virus into the allantoic fluid, no virus was recovered from the embryo brain. The amount of virus recovered from the allantoic fluid declined with time.

Further studies with 21 other strains showed that all 12 type 2 strains spread from the CAM to the allantoic fluid within 3 days, whilst none of the 9 type I strains had spread beyond the CAM 3 days after inoculation.

These results show that type 2 herpes simplex viruses spread rapidly from the inoculated CAM to other regions of the egg including the embryo, whereas type I viruses were restricted to the inoculation site. The rate of spread of type 2 virus was related to the concentration of infectious virus in the inoculum. Following inoculation with small concentrations of virus spread was delayed and with even smaller amounts the CAM was not haemorrhagic and no virus was recovered from the embryo which, apparently unaffected, hatched normally. Inoculation of HSV type 1 in concentrations equal to or exceeding those of type 2 did not induce spread beyond the CAM, indicating that restriction of type 1 virus to the inoculation site was truly type specific and not concentration dependent.

There was evidence that frequent serial passages in eggs somewhat increased the spreading potential of the type 1 strain. After 24 egg passages the type 1 strain had spread into the allantoic fluid 4 days after CAM inoculation, although embryos were not infected even 6 days post-inoculation. Type 2 virus at the 24th egg passage had become even more pathogenic for the CAM, causing greater haemorrhage and necrosis. It was also more rapidly pathogenic for the embryo, and the rate of spread to the allantoic cavity was also increased.

As observed by Nahmias et al. (1968), HSV type 2 infection of the CAM produced lesions involving all three layers of the membrane, that is ectoderm, mesoderm and endoderm, whilst HSV type 1 lesions were restricted to the outer ectoderm layer. It was interesting that in this study type 2 HSV apparently passed through the CAM, perhaps infecting all three germ layers on route, to reach the allantoic fluid. It could be recovered from the fluid in
large quantities for at least 6 days, but virus inoculated directly into the allantoic fluid declined in concentration. This suggests that the cells lining the allantoic cavity are either insusceptible, or are infected only with difficulty from the allantoic fluid side of the membrane. Since virus inoculated into the allantoic fluid did not spread to the embryo it seems likely that after inoculation of HSV type 2 on to the CAM, virus infection of the embryo is not via the allantoic fluid, but is due to virus penetration of the blood vessels lying in the mesoderm layer of the CAM. Spread of virus to the embryo was always associated with haemorrhage of the CAM. When a small dose of HSV type 2 was inoculated onto the CAM few pocks were produced, the CAM was not haemorrhagic, and there was little if any spread of virus to the embryo. It is possible that spread was prevented because the few pocks formed were remote from blood vessels. Anderson (1940) using the HF strain and Wildy & Holden (1954) using the multiple egg passaged derivative strain HFE observed death of embryos. These observations were made prior to dividing HSV into two types and a comparative study of HF strains obtained from several sources revealed that some cultures were type 1 while others were type 2 (Dowdle et al. 1967).

Anderson (1940) showed that vascular spread of herpes virus within the egg was possible by demonstrating herpes virus intra-nuclear inclusions in the endodermal cells lining the embryonic blood vessels and by isolating virus from the allantoic artery 2 days after inoculation of the CAM.

The reason for the restriction of type 1 strains to the inoculation site on the CAM compared with the rapid spread of type 2 strains to other regions of the egg can at present only be assumed to be due to the relatively superficial nature of the primary CAM lesion. It appears that this property of invasiveness is reproducible and type specific, although caution is necessary in interpreting results observed with strains which have had multiple egg passages. Recovery of infectious virus from the allantoic fluid three days after inoculation of a herpes strain onto the carefully preserved intact CAM, taken together with the appearance and size of the CAM lesions produced, could prove a useful additional test in the differentiation of type 1 from type 2 herpes simplex viruses.

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REFERENCES


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