Investigation of Plaque Formation in Chick Embryo Cells as a Biological Marker for Distinguishing Herpes Virus Type 2 from Type 1

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SUMMARY

Difference in plaque-forming ability in chick embryo cells was shown to be a reliable marker for differentiating herpes virus types 1 and 2 when original or low-passaged isolates of the viruses were used. Type 2 viruses produced large plaques at titres equal to those obtained in rabbit kidney cells, while type 1 viruses failed to produce plaques or produced small plaques at a low plaquing efficiency. Naturally occurring populations of type 1 virus were found to contain a small proportion of virus which produced plaques in chick embryo cells, and this proportion increased with passage of the virus in tissue culture. The plaques produced by type 1 virus in chick embryo cells were morphologically distinct from the plaques produced by type 2 virus. The type 1 variants which plaqued in chick embryo cells could be plaque-purified, and the progeny viruses maintained antigenic and biological similarities to the parent type 1 virus except for the relative resistance to the DNA inhibitor ara-A.

INTRODUCTION

The antigenic composition of herpes viruses isolated from different anatomical sites has been shown to differ sufficiently to warrant division of the viruses into two types (Dowdle et al. 1967; Plummer, 1964). Type 2 viruses have been isolated primarily from genital lesions and type 1 viruses have been isolated from non-genital lesions. The two types of viruses appear to have different modes of transmission. The densities of the nucleic acids of the two types of virus differ (Goodheart, Plummer & Waner, 1968) and a number of other biological differences have been described (Munk & Donner, 1963; Figueroa & Rawls, 1969; Nahmias & Dowdle, 1968). The validity of grouping herpes virus isolates into two types has been questioned by the isolation of viruses from genital or non-genital sites which do not conform to the prototype virus in certain biological markers (Amstey & Balduzzi, 1970; Terni & Roizman, 1970). The present study was undertaken to examine more closely one biological marker, the ability of the type 2 virus to form plaques in primary chick embryo cells, which has been reported to distinguish between the two types of virus (Figueroa & Rawls, 1969).

METHODS

Tissue culture. Primary rabbit kidney cell cultures and primary chick embryo cell cultures were prepared as described previously (Figueroa & Rawls, 1969). Human embryonic kidney cells were obtained commercially and used after 1 to 3 subculturings. Maintenance medium for all cell cultures consisted of Eagle's medium supplemented with 2% foetal bovine serum,
antibiotics (penicillin 100 units/ml and streptomycin 100 μg./ml.) and 1·5 g./l. of NaHCO₃. Rabbit cell cultures were used when a complete cell monolayer had formed, while chick embryo cell monolayers were routinely used 2 to 3 days after planting.

**Viruses and virus assays.** Viruses were obtained from herpetic lesions by swabbing the ulcerations with a cotton-tipped swab or by aspirating the vesicle fluid with a 25 gauge needle attached to a tuberculin syringe. The samples were placed in maintenance medium and stored at −70°. The plaque-forming ability of these viruses was examined directly or was examined after 1 to 2 passages of the viruses in rabbit kidney cell or human embryonic kidney cell cultures. Viruses were assayed in monolayers of cells grown in 60 mm. plastic Petri dishes. An inoculum of 0·2 ml. was added to the cell monolayers and the viruses permitted to adsorb for 2 hr at room temperature. Chick embryo cell monolayers were covered with Eagle's medium containing 2% methyl-cellulose (Rapp, 1963) and rabbit kidney cell monolayers were covered with Eagle's medium solidified with 1·5% Bacto-agar and containing protamine sulphate (Wallis & Melnick, 1968). Plaques were counted after 4 days of incubation at 37° and the virus concentrations were expressed as plaque-forming units/ml (p.f.u./ml.). Virus stocks were prepared by inoculating cell cultures and incubating the cultures until 75 to 100% of the cells showed cytopathic effect (CPE). The viruses were freed from the cells by three cycles of freezing and thawing. The cell debris was removed by centrifugation and the supernatant fluid was ampouled and stored at −70°. Plaque purification of virus isolates was achieved by aspirating the agar and cells over plaques using sterile Pasteur pipettes and passing the material aspirated in rabbit kidney cells. The number of virus particles present and the proportion with envelopes were assayed in the electron microscope by methods previously described (McCombs, Benyesh-Melnick & Brunschwig, 1966).

**Virus neutralization.** Virus stocks were compared using the kinetics of neutralization. The preparation of antiserum in rabbits and the methods of performing the test have been previously described (Rawls et al. 1968).

**Inhibitor studies.** 5-iodo-2'-deoxyuridine (IDU) was obtained from Nutritional Biochemicals Corporation. 9-β-D-arabinofuranosyladenine (ara-A) was kindly supplied by Parke Davis & Co. The inhibitors were dissolved in Eagle's medium. Stock solutions of 1000 μg./ml. of IDU and 500 μg./ml. of ara-A were prepared and stored at −20°. The effect of the inhibitors was determined by inoculating monolayers of rabbit kidney cells with several concentrations of virus. After 2 hr of adsorption, the monolayers were covered with 3 ml. of agar overlay containing various concentrations of the inhibitors. A second overlay containing the inhibitors was added after 2 days of incubation at 37° and plaques were counted 1 to 2 days later. Virus titres in the presence of the inhibitors were compared with titres obtained in cultures to which the inhibitors had not been added, and the surviving virus expressed as a fraction. The inhibitors were also evaluated by determining the concentration of inhibitor incorporated into the overlay medium which would reduce the surviving virus to 30%. The 70% reduction dosage was determined by inoculating cell monolayers with approximately 100 p.f.u. of virus and adding agar overlay medium containing various concentrations of inhibitor, as described above. The surviving virus was plotted against inhibitor concentration and the 70% reduction concentration extrapolated. In addition, the sensitivity of viruses to the inhibitors was evaluated by saturating antibiotic discs with the inhibitors (Herrmann et al. 1960).
RESULTS

Differentiation of herpes virus type 2 from type 1 by plaque formation in chick embryo cells

Using a limited number of isolates, Figueroa & RaMs (1969) reported that herpes virus type 1 did not produce plaques in primary chick embryo cells. The replication of type 1 viruses in chick embryo cells was characterized by a prolonged eclipse period and the production of low quantities of infectious virus. In contrast, the type 2 isolates tested replicated well in chick embryo cells and formed plaques in this cell system. Additional isolates of virus which had been shown by neutralization kinetics tests to be antigenically type 1 or type 2 were, therefore, examined for plaque formation in rabbit kidney and chick embryo cells. Forty isolates with properties characteristic of type 2 herpes virus were examined. The sites of isolation of these strains included genitalia (34 strains), tissues of infants with neonatal herpes (2), hand lesions (2), and knee and buttock (1 strain each). All the isolates tested were found to form distinct plaques of about 2-3 mm. diameter in chick embryo cells at titres equal to those obtained when the viruses were simultaneously titrated in rabbit kidney cells.

Twenty-five type 1 strains of herpes virus isolated from patients with primary or recurrent oral lesions were examined for their ability to plaque in chick embryo cells. These tests were performed by simultaneously assaying stocks of passaged virus or original clinical specimens in chick embryo cells and rabbit kidney cells. In contrast to the type 2 isolates, type 1 isolates either formed no plaques in chick embryo cells or formed small plaques of about 1-3 mm. diameter or smaller, when plated at a high virus multiplicity.

Small plaques developed in chick embryo cells inoculated with low dilutions of most specimens. In the original specimen or low-passaged stocks, the frequency of occurrence of variants which formed plaques in chick embryo cells ranged from 1/290 to less than 1/10⁶ p.f.u. as determined in rabbit kidney cells. There was no correlation between the proportion or size of plaques produced in chick embryo cells by virus in the original specimens and the type of infection, primary or recurrent.

It was noted, however, that stocks of virus strains which had been passaged many times in tissue culture contained a large percentage of virus variants capable of forming plaques in chick embryo cells (36 to 62%). Three isolates were passaged 25 times in rabbit kidney cells, and one of the isolates (Pa) was also passaged 25 times in human embryonic kidney cells. The proportion of the virus capable of forming plaques in chick embryo cells was determined every five passages. There was a gradual increase in the proportion of virus capable of plaquing in chick embryo cells with passage of the viruses through either rabbit kidney cells or human embryonic kidney cells; by the 25th passage, 12 to 33% of the virus in the stocks plaqued in chick embryo cells.

Isolation and growth characteristics of type 1 variants capable of producing plaques in chick embryo cells

To study the properties of the type 1 viruses, attempts were made to isolate and plaque-purify both the type 1 viruses capable of producing plaques in chick embryo cells (ch⁺ virus) and the viruses which produced plaques only in rabbit kidney cells (ch⁻ virus). Ch⁺ virus was plaque-purified by picking 1-3 mm. plaques from monolayers of chick embryo cells which had been covered with an agar overlay. The viruses were plaque-purified three times and final stocks were made in rabbit kidney cells. These ch⁺ viruses were found to breed true and grew well in chick embryo cells. When assayed in chick embryo cells, the ch⁺ viruses produced 1-3 mm. plaques at titres which ranged from 18 to 75% of the titres produced in rabbit kidney cells.
Isolates of ch⁻ viruses were made by picking plaques from plates of rabbit kidney cells which had been inoculated with high dilutions of clinical specimens of oral lesions. Several plaques were chosen from each isolate and grown in rabbit kidney cells. The isolates were plaque purified 1 to 3 times. Final stocks were made in rabbit kidney cells. Despite plaque purification, many of the stocks of purified isolates contained a small amount of virus which formed plaques in chick embryo cells. Only those isolates which showed no evidence of replication or plaque formation in chick embryo cells were used as ch⁻ virus in subsequent studies.

The growth characteristics in chick embryo cells of the type 1 plaque-purified ch⁻, ch⁺ and wild-type strains were compared: five strains of each were tested. Representative growth curves are shown in Fig. 1. The ch⁺ viruses were found to replicate well in chick embryo cells. They had a latent period of about 6 hr and maximum titres of infectious virus were found at 24 hr. The wild-type viruses had a prolonged latent period and did not reach peak titres until 48 hr. The ch⁻ strain, which formed no detectable plaques in chick embryo cells, failed to replicate in these cells, as shown by the lack of increase in infectious virus in chick embryo cells when assayed in rabbit kidney cells.

Biological and antigenic characterization of type 1 ch⁺ viruses

The biological and antigenic properties of the type 1 ch⁺ viruses were compared to those of the wild-type 1 and type 2 strains (Table 1). Neutralization kinetics tests using antisera prepared in rabbits against wild-type 1 virus showed the type 1 ch⁺ virus to be antigenically similar to the type 1 strains and clearly different from the type 2 strains. The thermal inactivation rate of the ch⁺ viruses and the proportion of enveloped particles present in these virus stocks when grown in rabbit kidney cells also resembled the type 1 viruses and differed from the type 2 viruses. Wild-type 1 virus and the ch⁺ virus formed small pocks on the
chorioallantoic membranes of eggs, while the type 2 virus formed large pocks. No pock formation by type 1 ch− strains could be detected on the chorioallantoic membrane of eggs.

Origin of the ch+ virus

The increasing proportion of ch+ viruses upon multiple passage of type 1 strains in rabbit kidney cells or human embryonic kidney cells suggests a selective growth advantage of the ch+ viruses in these cells. Alternatively, the increasing frequency of the ch+ viruses could be accounted for by the interference of the ch+ viruses with the growth of the ch− viruses, by different rates of adsorption or release of the viruses, or by a high mutation rate of the ch− viruses to ch+ viruses. The replication of ch+ and ch− viruses was examined in rabbit kidney cells. The latent period was found to be identical for both ch+ and ch− viruses, and a mean yield of 320 p.f.u./cell was observed for both viruses at peak titres of the growth cycle. No apparent selective growth advantage for the ch+ viruses was found.

Table 1. Comparison of properties of type I ch+ viruses to wild-type I and type 2 viruses

<table>
<thead>
<tr>
<th></th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetics of neutralization with antiserum prepared against type I virus. (K value)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type I Ch+</td>
</tr>
<tr>
<td></td>
<td>9.2 ± 2.4</td>
</tr>
<tr>
<td>Inactivation at 37° (log. loss/hr)</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Per cent of enveloped particles of virus grown in rabbit kidney cells</td>
<td>49.3 ± 5.8</td>
</tr>
<tr>
<td>Pock size on chorioallantoic membrane</td>
<td>Small</td>
</tr>
</tbody>
</table>

When cells were double infected with ch+ and ch− viruses, there was a reciprocal interference with virus production by both viruses. Rabbit kidney cells inoculated with ch− virus 1 hr before inoculation with ch+ virus produced 85% less ch+ virus than cells infected only with ch+ virus. If the cells were inoculated initially with ch+ virus and then with ch− virus, 47% less ch− virus was produced. These data suggest that interference with ch− virus replication by ch+ virus does not account for the appearance of ch+ viruses, and that the appearance of the ch+ viruses is due to a high mutation rate of ch− virus to ch+ virus, or to differences in virus adsorption or release.

Effect of DNA inhibitors on the formation of herpes virus plaques in rabbit kidney cells

The DNA inhibitor 5-iodo-2'-deoxyuridine (IDU) has been shown to inhibit the replication of herpes virus in tissue culture (Herrmann et al. 1960; Smith, 1963; Lowry & Rawls, 1969; Person, Sheridan & Herrmann, 1970). There are reports that herpes virus type 2 is less sensitive to the inhibitor than type 1 virus (Nahmias & Dowdle, 1968). Therefore, the sensitivity of the viruses to IDU was compared. The effect of various concentrations of IDU on the formation of plaques of type 1 ch+ and ch− and type 2 viruses is shown in Fig. 2. The type 1 ch+ and ch− viruses were found to be much more sensitive to the inhibitor than the type 2 strains. With 25 μg./ml. of IDU, there was a 230-fold decrease in the titres of the type 1 viruses, while the type 2 viruses showed about a 50% reduction in titre.

The sensitivity of the different viruses to IDU was also compared by determining the dose (μg./ml.) of IDU which would reduce the number of plaques to 30% of the controls (PRD 70). A clear-cut difference between the type 1 and type 2 viruses was again observed (Table 2). Similar results were obtained when an antibiotic disc saturated with a solution containing 1000 μg./ml. of IDU were placed on the agar overlay of plates inoculated with
sufficient virus to produce confluent plaques. The sensitivity of the viruses to the inhibitor was determined by measuring the diameter of the area of plaque inhibition (Herrmann et al. 1960). As can be seen in Table 3, the ch+ and ch− strains were more sensitive to IDU than the type 2 strains.

The sensitivity of the viruses to another DNA inhibitor, 9-β-D-arabinofuranosyladenine (ara-A), was also compared. Using the inhibitor-overlay method, 3 strains each of type 1 ch+, type 1 ch−, and type 2 viruses were tested. The mean values of these results are plotted in Fig. 3. Type 1 ch− viruses were found to be the most sensitive, while type 1 ch+ and type 2 viruses were found to be relatively resistant to ara-A.

![Fig. 2](image)

**Fig. 2.** Inhibition of type 1 ch+, type 1 ch−, and type 2 virus plaques by 5-iodo-β'-deoxyuridine.

![Fig. 3](image)

**Fig. 3.** Inhibition of type 1 ch+, type 1 ch− and type 2 virus plaques by arabinofuranosyladenine.

![Table 2](image)

**Table 2. Determination of 70% plaque reduction dose* of IDU and ara-A**

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>No. of strains tested</th>
<th>PRD 70 (μg.IDU/ml)†</th>
<th>PRD 70 (μg.ara-A/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 ch+</td>
<td>7</td>
<td>3.9 (2.3–5.5)</td>
<td>20.3 (14.6–24.0)</td>
</tr>
<tr>
<td>Type 1 wild-type</td>
<td>12</td>
<td>5.6 (4.0–8.0)</td>
<td>12.2 (9.7–15.3)</td>
</tr>
<tr>
<td>Type 1 ch−</td>
<td>2</td>
<td>2.0 (2.0)</td>
<td>7.9 (6.5–9.3)</td>
</tr>
<tr>
<td>Type 2</td>
<td>12</td>
<td>34.4 (28.8–43.8)</td>
<td>13.4 (10.4–17.7)</td>
</tr>
</tbody>
</table>

* That concentration of inhibitor which reduces the plaque number to 30% of the control.
† Mean values with range of values in parentheses.

To eliminate the possibility that the relative resistance of ch+ viruses at high concentrations of inhibitor was due to resistant mutants which represented a small fraction of the population two approaches were used. The PRD 70 values, which measure the sensitivity of the major portion of the population of the various viruses, were compared. Also, plaques were picked from the plates containing 100 μg./ml. of ara-A; stocks were made from this virus, and the sensitivity of the progeny viruses was determined. These stocks were found to have the same sensitivity as the original parent virus. The results of PRD 70 tests are shown in Table 2.
Biological marker for herpes virus types 1 and 2

The type I ch+ viruses were once again found to be the most resistant, and the type I ch− viruses most sensitive. Comparison of the strains by the antibiotic disc method also showed the ch+ viruses to be the most resistant and the ch− viruses the least (Table 3).

Table 3. Determination of area of plaque inhibition with antibiotic disc saturated with IDU or ara-A

<table>
<thead>
<tr>
<th>Virus strains</th>
<th>1000 µg/ml. IDU</th>
<th>500 µg/ml. ara-A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean diameter of area of inhibition (cm.)</td>
<td>Mean diameter of area of inhibition (cm.)</td>
</tr>
<tr>
<td>Type 2a</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Type 2b</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Type 2c</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Type 1 Ch+a</td>
<td>2.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Type 1 Ch+b</td>
<td>2.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Type 1 Ch+c</td>
<td>4.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Type 1 Ch-a</td>
<td>5.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Type 1 Ch-b</td>
<td>3.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Type 1 Ch-c</td>
<td>3.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

DISCUSSION

Previous reports have established that the two antigenic types of herpes virus differ in their mode of transmission, thermal stability, neurovirulence, pock formation on the chorioallantoic membrane of eggs, replication in rabbit kidney cells, nature of cytopathic effect and nucleic acid composition (Nahmias & Dowdle, 1968). However, the magnitude of variation of these biological properties within each virus type has not been adequately studied. One suggested biological marker was the ability of the viruses to form plaques in primary chick embryo cells; type 2 viruses readily formed plaques in these cells, while type I viruses failed to do so (Figueroa & Rawls, 1969). The testing of 40 additional genital isolates and 25 oral isolates in the present study clearly establishes plaque formation in chick embryo cells as a distinguishing biological property for herpes viruses which are antigenically type 2. Herpes viruses isolated from oral lesions and classified antigenically as type I, when assayed simultaneously at comparable dilutions in chick embryo cells and rabbit kidney cells, failed to form plaques in chick embryo cells. It was found, however, that the population of viruses in oral lesions contained a small fraction of virus capable of forming plaques in chick embryo cells. In the early passages of the isolates, this fraction varied from 10^-3 to 10^-6. The plaques formed by the type I viruses were distinctly smaller than the plaques formed by type 2 viruses. Type I virus strains which were passaged numerous times in rabbit kidney cells or human embryonic kidney cells were found to contain a far greater proportion of virus capable of forming plaques in chick embryo cells, and it appears that the non-plaquing viruses may readily mutate in tissue culture to viruses capable of plaquing.

Reports of type I viruses which form plaques in chick embryo cells have appeared in the literature (Waterson, 1958; Taniguchi & Yoshino, 1964). Variations in plaque size in chick embryo cells has been noted in different strains and sublines of strains of type I viruses. These observations were made using viruses which had been passaged many times in tissue culture or which had been egg-adapted. Replication of type I viruses in chick embryo cells or eggs selects for the variants capable of replicating in chick cells.

Although a proportion of the type I viruses may acquire the ability to plaque in chick embryo cells, this is not accompanied by changes in antigenic markers nor in a number of other biological markers. Tests of neutralization kinetics demonstrated that the viruses
capable of plaquing in chick embryo cells remained antigenically similar to the parental type I viruses. Thermal stability, particle/infectivity ratio and percentage of enveloped particles of viruses grown in rabbit kidney cells were similar to type I viruses and unlike type 2 viruses. The viruses capable of plaquing in chick embryo cells also maintained the same sensitivity to IDU as the parent type I virus. These observations indicate that type I viruses may vary in certain characteristic biological markers and may even resemble the type 2 viruses in certain markers, but still retain the antigenic and other biological properties of type 1.

The ability of the type I variants to replicate in chick embryo cells was found to be accompanied by a relative resistance of these viruses to the DNA inhibitor ara-A when tested in rabbit kidney cells. Since type 2 viruses do not have this same resistance to ara-A as the type I variants, resistance to ara-A does not necessarily correlate with the ability to replicate in chick embryo cells, but may indicate a difference in the enzymes involved in DNA synthesis. Other studies (to be published) indicate that the parental type I virus fails to replicate in chick embryo cells because virus DNA is not synthesized; therefore, a difference in the enzymes of DNA synthesis could account for the ability of the type I variants to replicate in chick embryo cells.

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