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A Chick-Embyo Cell Microtest for Typing of *Herpesvirus Hominis*[1] (38531)

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(Introduced by J. T. Grayston)

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Characteristics of plaque formation in cell culture of primary chick embryo (PCE), have been found useful as a biological marker in differentiating *Herpesvirus hominis* (HVH) oral type 1 from genital type 2 strains (1, 2). Type 1 strains appear to or have been reported to produce small plaques and to have lower efficiency of plaque formation than type 2 strains in PCE cell culture. We describe a simple microtest for differentiation of HVH types based on the apparent differential sensitivity of PCE cell culture in microplates. The type of cytopathic effect (CPE) and the efficiency with which the virus infects the PCE monolayer are used as criteria for typing. The results of microtyping in PCE are compared with kinetic neutralization (K) and immunofluorescence (IF) typing.

Materials and Methods. Tissue Culture. Ten per cent fetal calf serum in Eagle's minimal essential medium (MEM) with 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 50 μg/ml of mycostatin, 6.6 mM sodium bicarbonate and 2.0 mM L-glutamine was used throughout.

Primary chick embryo cells were obtained from minced 9- to 10-day old embryos by dispersion with 0.2% trypsin. Strains of golden Syrian hamster embryo cells (HE) and rabbit kidney cells (RK), originated in our laboratories. Human diploid fetal tonsil (FT) fibroblasts, were kindly supplied by Dr. Berttina Wentworth, Seattle. The passage numbers were as follows: HE 15-17, RK 1-3; and FT 15-25.

Viruses. Herpesvirus hominis strains used in these experiments included four that were of known type and 106 clinical isolates from Taipei and Seattle. The reference strains were: UW-168 (3) and KOS (4), type 1; UW-268(3) and 196-2(4), type 2. The UW-168 and UW-268 strains had been passed approximately 50 times in HEp-2, HeLa and FT cells. Of the 106 clinical isolates, 59 were genital in origin; 57 were from the cervix of patients attending venereal disease clinics, and two were urinary isolates. The nongenital isolates included, 45 from throat swabs (from a study of respiratory infection), one from typical herpes labialis, and one from a brain specimen obtained at autopsy. The previously untyped herpes strains were in the 2nd through 10th passage at the time of typing, initially isolated in FT, HEp-2 or primary monkey kidney cells and passed in FT and/or HEp-2 cells. Virus pools for all strains were prepared in HE, RK or FT cells and have titers ranging from $10^{4.0}$ to $10^{7.0}$ $TCID_{50}$/ml as assayed in a microtest system using HE or FT cells. Two of the untyped clinical isolates, NHS-17 and 23, were passed an additional nine times in RK and then either ten times in FT (NHS-17) or 21 times in HE cells (NHS-23).

Virus microtyping. Serial tenfold dilutions of the stock virus to be typed were made in MEM. Diluted virus (0.05 ml) was delivered to two wells per dilution in each of two

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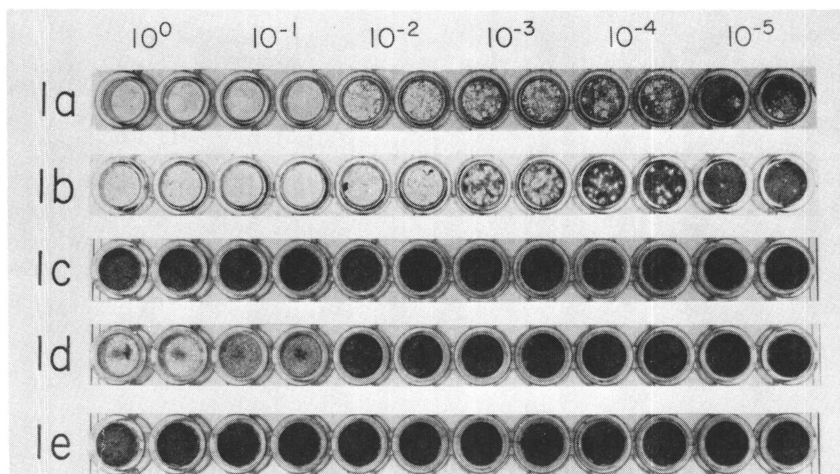

FIG. 1 a–e. Crystal violet staining of microtest wells. From left to right, each row shows a virus strain with each virus dilution (10^0–10^{-5}) occupying two wells. Absence of staining indicates CPE: (a) Wells with HE; all HVH virus strains showed CPE; this type is designated "complete" CPE; (b) Wells with PCE; only certain HVH strains including the known type 2 strains showed "complete" CPE similar to (a); (c) Wells with PCE; some HVH strains showed no CPE despite demonstrated infectivity in HE. These strains are designated "no" CPE strains; (d) Wells with PCE; certain HVH strains showed a limited effect on the cell sheet. These strains are designated "incomplete" CPE strains; (e) Uninoculated wells with PCE cells only.

Linbro microtest plates. One set of wells was then supplied with 1.5×10^5 freshly prepared PCE cells per well in 0.10 ml of MEM. The other set of wells received 3×10^4 of FT or HE cells per well. Two negative control wells received the same number of PCE, HE or FT cells without virus. The microplate was finally covered with a sterile plastic lid and incubated at 36°C in a humidified, 2.5% CO_2 atmosphere for 3–4 days. At the end of the experiment the cells were fixed with formalin and stained with 0.5% crystal violet. Either with unaided vision or the aid of a stereomicroscope (both techniques are found satisfactory so long as both sets of wells from any particular test virus strain were evaluated similarly by one or the other method). Wells were scored negative or positive for cytolysis based on either the obvious presence of CPE or the reduced intensity of the staining as compared to uninoculated controls. These results were used to calculate virus titer in $TCID_{50}$.

Immunofluorescence (IF). Sixty-four clinical isolates were typed by an indirect immunofluorescence technique using type 1 and type 2 guinea pig anti-sera and anti-guinea pig globulin rabbit conjugate.

Kinetic neutralization (K). Twenty-eight isolates which had been previously typed by kinetic neutralization (5) were also typed in the microtest and the results compared with the data kindly supplied by Dr. Berttina B. Wentworth.

Results. Virus microtyping. In HE or FT cells, all 110 strains tested produced characteristic CPE in monolayers consisting of irregular, patchy, plaquelike lesions and holes or total cell sheet effacement (Fig. 1a). This type of CPE is referred to as *complete type*.

In PCE cells, however, only those strains eventually classified as type 2, including the reference type 2, UW-268 and 196-2 strains, produced *complete type* CPE (Fig. 1b). The remaining virus strains, including the known type 1, UW-168 and KOS strains, produced either no CPE or a limited, *incomplete type* of CPE (Fig. 1c and d). PCE monolayers with *incomplete* CPE were never totally destroyed, even in the presence of up to 10^6 $TCID_{50}$ of virus per well. (Total cytolysis of monolayer was generally evident with type 2 strains having 10^1–10^2 $TCID_{50}$ of virus per well.) Here the CPE was characterized by a diffuse uniform thinning of the cell mono-

layer without any holes or disruption in the cell sheet. With type 1 infection a thinned out, nondisrupted layer of cells always covered the entire well surface.

Strains that showed *complete* CPE (the type 2 strains) invariably titered within $10^{1.5}$ $TCID_{50}$ of each other in PCE, and HE or FT cells. Strains showing *incomplete* CPE (type 1 strains) titered $10^{2.0}$–$10^{7.0}$ $TCID_{50}$ less in PCE than in HE or FT cells.

TABLE I. RESULT OF PRIMARY CHICK EMBRYO (PCE) MICROTYPING OF 4 KNOWN AND 106 UNKNOWN HVH STRAINS.

Strains	Source	No. strains tested	Results of PCE typing	
			Type 1	Type 2
Known:				
UW-168, KOS (Type 1)	Oral	2	2	0
UW-268, 196-2 (Type 2)	Genital	2	0	2
Unknown:	Oral	47	46	1
	Genital	59	6	53

To summarize, HVH type 1 and 2 strains showed two distinguishing characteristics in PCE in microtest plates: Type 1 strains caused either *incomplete* CPE or no CPE in contrast to *complete* CPE caused by type 2 strains. Type 1 strains titered more than $10^{2.0}$ $TCID_{50}$ lower in PCE than in HE or FT cells; type 2 strains titered within $10^{1.5}$ $TCID_{50}$ of each other in PCE and in FT or HE.

Based on these two sets of criteria the 4 known reference and 106 untyped clinical strains were readily separable into types 1 and 2 (Table I). For example, both reference type 2 strains and the unknown UW-14, 25 and 30 strains showed *complete* CPE in PCE cells and had low HE vs. PCE titer differences of $10^{1.5}$ $TCID_{50}$ or less (Table II). The type 1 reference strains and the unknown UW-4, 8, and 15 strains that exhibited either no CPE or the *incomplete* CPE in PCE titered $10^{3.0}$ $TCID_{50}$ higher in HE than PCE.

TABLE II. EFFECT OF HERPESVIRUS STRAINS ON HAMSTER EMBRYO (HE) AND PRIMARY CHICK EMBRYO (PCE) CELLS

Virus	Source	Assay Cell	CPE	$TCID_{50}$ titer/ml	PCE Type
		Known Type			
UW-168	Oral	HE	C[a]	$2 \times 10^{6.5}$	1
		PCE	IC[b]	$2 \times 10^{2.0}$	
KOS	Oral	HE	C	$2 \times 10^{6.0}$	1
		PCE	No[c]	0	
UW-268	Genital	HE	C	$2 \times 10^{4.0}$	2
		PCE	C	$2 \times 10^{3.0}$	
196-2	Genital	HE	C	$2 \times 10^{5.5}$	2
		PCE	C	$2 \times 10^{4.0}$	
		Unknown Type			
UW-4	Genital	HE	C	$2 \times 10^{4.5}$	
		PCE	No	0	1
UW-8	Oral	HE	C	$2 \times 10^{4.5}$	1
		PCE	IC	$2 \times 10^{1.0}$	
UW-14	Genital	HE	C	$2 \times 10^{4.5}$	2
		PCE	C	$2 \times 10^{4.5}$	
UW-15	Oral	HE	C	$2 \times 10^{6.0}$	1
		PCE	IC	$2 \times 10^{2.5}$	
UW-25	Genital	HE	C	$2 \times 10^{6.5}$	2
		PCE	C	$2 \times 10^{5.5}$	
UW-30	Oral	HE	C	$2 \times 10^{4.5}$	2
		PCE	C	$2 \times 10^{4.5}$	

[a] C—"complete."
[b] IC—"incomplete."
[c] No—No CPE.

TABLE III. MICRO PCE CELL TYPING COMPARED WITH TYPING BY KINETIC NEUTRALIZATION (K) AND IMMUNOFLUORESCENCE (IF).

	Strains typed by other methods		PCE typing results		No. agreeing/ No. tested	% Correlation
			I	*II*		
K-	Type I	22	21	1	27/28	96
	Type II	6	0	6		
IF-	Type I	40	38	2	60/64	94
	Type II	24	2	22		

Comparing typing in the micro PCE vs HE (or FT) cell system with the other typing methods (Table III), a 96% correlation (27/28) between the PCE and Kinetic Neutralization (K) tests and 94% correlation (60/64) between the PCE and Immunofluorescence (IF) tests was seen. A genital isolate typed as type 2 by PCE and type 1 by K was classified as type 2 by IF. Two of the 4 PCE typings that were not in agreement with IF, were in agreement with K. The remaining viruses which showed divergent results in the PCE and IF systems were not tested by K.

Discussion. In the macro procedure previously employed the original or low passage isolates must be used to avoid an increase of type 1 variants which will form plaques upon tissue culture passage (even in non-chick embryo cells). Additionally, difference in plaque sizes, upon which the distinction between types 1 and 2 may also depend is found to be as small as 1 mm (2). The relative plaque sizes can obviously be affected by the assaying conditions such as thickness of cell monolayer and duration of assay and this makes the second criterion a much less constant feature.

In contrast to the macro test, the usefulness of the present microtest was found to be unaffected by the high tissue culture passages of the virus strain. UW-168 strain in a passage above 50 and other type 1 strains, NHS 17 and NHS 23 carried up to passage 39 were tested. They were all readily identified as type 1 strains. Both the low and high passage NHS-17 and 23, (at a titer of 10^6 $TCID_{50}$/ml) showed a titer difference between HE vs PCE of $10^{4.0}$ $TCID_{50}$. Both high passage strains, however, exhibited more pronounced *incomplete* CPE, with a greater cell thinning, in wells containing the two highest concentrations of virus. The results of testing by this method were also highly reproducible since they were little affected by a wide range of PCE cells used per test well. All strains were readily differentiated using 10^4–10^6 cells per well, but 1.2–1.5 $\times$ 10^5 cells per well produced the most suitable monolayer for assay.

In the PCE microtest only certain type 1 virus strains with high multiplicities produced partial CPE. This apparent difference among the type 1 strains was due presumably to the varying proportion of "plaque forming" and "nonplaque forming" variants reportedly present in type 1 virus strains. (2) In wells with incomplete CPE, the "plaque forming" variants were assumed to be responsible for the monolayer thinning. In the present test the fraction of such "plaque forming" variants varied from 10^{-2} to 10^{-6} which is similar to what had been reported with the macro assaying procedure. To test the "plaque forming" hypothesis the chick cell-adapted type 1 KOS, US-168, UW-5, 8 and 24 strains were run. Starting at the 3rd PCE passage for UW-168 and the 8th PCE passage for UW-5, 8 and 24, the infectivity of these chick cell adapted type 1 strains in PCE improved greatly and became much like the type 2 strains. In wells with high concentrations of adapted virus, extensive cell sheet destruction (up to 100%) was seen and there was little titer difference between PCE and HE. In wells with lower virus concentrations plaque-like lesions indistinguishable from those of type 2 strains were present.

In the present series two thirds of type 1 strains produced no CPE in the microplate PCE system. The remaining third showed an incomplete CPE in high titer wells and a nondisruptive thinning of the monolayer. Despite the elimination of overlay medium in the present test system the "plaque forming" variants of type 1 virus appear unable to produce a more extensive CPE through secondary infection. To further investigate

this phenomenon, wells were inoculated with the type 1 reference strains, and 48 hr later were infected with 200 $TCID_{50}$ of UW-268, a type 2 reference strain. In previously uninoculated wells, or those having a small type 1 infecting dose the cell sheet was totally destroyed within 96 hr. In wells which had been infected with a high concentration of type 1 virus (10–100 $TCID_{50}$/cell) the monolayer was totally or partially preserved. It appeared that cells surviving infection by type 1 virus became resistant to normally cytopathogenic type 2 virus strains and therefore resistant to the "plaque forming" variants of type 1 virus as well. Whether this resistance involved direct interference by prior infection with variants which failed to produce plaques or an interferon phenomenon is not known. If the type 1 inoculum was preheated at 56° for 15 min to destroy the infectivity of HVH, the protective effect was abolished. The latter is cogent to the direct inference phenomenon hypothesized by Roizman (6).

Summary. Oral type 1 and genital type 2 *Herpesvirus hominis* (HVH) strains demonstrate distinctive biological properties in primary chick embryo cells (PCE) cultivated in microtest plates. With this procedure four reference strains of known types and 106 clinical isolates were differentiated as type 1 or 2. The type 1 strains showed low efficiency of infection and either no cytopathic effect (CPE) or only an incomplete CPE characterized by uniform thinning of the cell sheet in test wells. Type 2 strains had a high efficiency of infection and with CPE characterized by patchy plaque-like lesions readily distinguished from CPE of type 1 strains.

A 96% correlation (27/28) between the PCE microtyping and kinetic neutralization tests and a 94% correlation (60/64) between the PCE microtyping and immunofluorescence test was obtained.

The microplate PCE test is a simple, clear-cut, and reliable procedure for the typing of HVH.

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