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The Use of Temperature Sensitivity and Selective Cell Culture Systems for Differentiation of Herpes Simplex Virus Types 1 and 2 in a Clinical Laboratory[1] (39757)

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The clinical and epidemiological importance of infections caused by herpes simplex virus (HSV) or *Herpesvirus hominis* has gained attention in recent years due to reports associating HSV type 2 with cervical cancer (1, 2). Today the isolation of HSV in a clinical laboratory is relatively simple. However, routine serological typing of HSV isolates can be complicated by antigenic cross reaction between HSV types 1 and 2(3).

Several biological markers for differentiating HSV types 1 and 2 have been described. These include differences in pock size when virus was inoculated onto chorioallantoic membrane (4), ability to form plaques in chick embryo cell cultures (5, 6), and, more recently, variations in sensitivity of HSV to heparin in cell culture (7). In addition, growth of viruses at different temperatures has provided a useful tool for differentiating virulent and avirulent poliovirus strains (8), poxviruses (9), and, more recently, HSV types (10). Therefore, a combination of temperature sensitivity together with microplaque formation in chick embryo cell monolayers was employed in this study for differentiating the two HSV types in order to assess their applications for routine use in a diagnostic virology laboratory. Epidemiological evidence based on clinical histories and virus isolation was used to ascertain the pattern of circulation of the two types of HSV in a young adult population.

Materials and methods. Cell cultures. Chick embryo (CE) cell cultures. Cell suspensions were prepared with trypsin (0.25%) from 9- to 10-day-old chick embryos by the standard method (11). Cell pellets were suspended at a 1:400 dilution in Hanks' balanced salt solution containing 0.5% lactalbumin hydrolysate and 2% calf serum. The cell suspension was seeded into Linbro microtest plates, 1 ml/well (16-mm diameter), and incubated in the presence of 5% CO_2 at 35° for 2–3 days, at which time the cell monolayer was confluent.

Primary rabbit kidney (RK) cell cultures. Kidney tissues were obtained from 1- to 2-month-old rabbits and cell suspensions were prepared as described above. The human kidney (HK) growth medium consisting of Hanks' balanced salt solution, 0.5% lactalbumin hydrolysate, 1% medium 199, and 10% calf serum was used for seeding. Cells were seeded in culture tubes or bottles and incubated at 35° for 6–7 days, at which time confluent monolayers were ready for virus assay.

Virus strains. Prototype strains of HSV type 1 (strain McIntyre) and type 2 (strain MS), obtained from the American Type Culture Collection, were used as controls. New HSV strains obtained from patients attending a dermatology clinic were isolated in RK cell cultures by the Virology Laboratory at the West Haven Veterans Administration Hospital. A few strains that previously had been isolated in Hep-2 or HeLa cells and subcultured in RK cells were included in the study for comparison.

Virus assay. Virus stocks were prepared in RK cell cultures for all isolates. Virus assays were performed simultaneously in CE and RK cells. Serial 10-fold dilutions of each virus stock were prepared. Aliquots (0.1 ml) were inoculated into either Linbro plates containing CE cells, 2 wells/dilution,

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or into RK cell culture tubes, 4 tubes/dilution. After 1 hr adsorption at 35°, 1 ml of maintenance medium, containing Earle's balanced salt solution and 2% calf serum, was added. The CE cell culture plates were incubated at 35° in a CO_2 incubator and the RK cell culture tubes were incubated at 35 or 40° in standard incubators. Cytopathic effects (CPE) in tube cultures and microplaques in Linbro plates were recorded. For microplaque counts in the CE cultures, the cell monolayers were fixed in methanol 3 days after inoculation and stained with 10% crystal violet as previously described (6).

Identification of viral types was confirmed by neutralization and/or immunofluorescence tests.[4] Antisera to HSV-1 and HSV-2 were purchased commercially. Antiserum to HSV-1 had a titer of 1:160 against the homologous virus and a titer of 1:40 against the heterologous strain. Antiserum to HSV-2 had an equivocal titer against both viruses. Neutralization tests were performed in tube cultures of RK cells. A virus suspension containing 100 $TCID_{50}$ was mixed with anti-HSV-1 serum dilutions of 1:40 and 1:160. The mixtures were incubated at room temperature for 1 hr. Each mixture was inoculated into RK cell cultures. The degree of inhibition of CPE in cell cultures inoculated with the virus–serum mixtures was compared to control cultures inoculated with the virus alone.

Patient population. Patients from a prepaid health plan with herpetiform lesions were specially examined in dermatology clinics. The diagnosis of herpetic infection was confirmed by clinical history and appearance of the lesions. Patients were carefully questioned regarding previous herpetic infections or exposure to family members, close associates, or sexual partners with herpetic lesions. Specimens for viral cultures were obtained from all patients. When possible, samples were also obtained from the contacts from whom the proposit were thought to have received the virus. Specimens were taken frequently from patients with recurrent lesions, especially when infections developed at different sites of the body. All specimens were taken with a sterile cotton swab and placed directly into a RK cell culture tube. All inoculated cultures were delivered to the virology laboratory for incubation at 35°. Laboratory personnel performing the virus isolation and identification tests had no information about the patients, the source or site of infection, or the epidemiology. After the viruses were isolated and typed, the laboratory information was correlated with the clinical history and epidemiology. The present paper reports data pertinent to certain aspects of the epidemiologic studies.

Results. Virus isolation and identification. With the exception of a few early isolates, i.e., NYU78, 160, and 189 which were isolated either in Hep-2 or HeLa cells, all other isolates were obtained in primary RK cell cultures (Table I). On most occasions the RK cultures inoculated with the clinical specimens were held at room temperature for 24–48 h before delivery to the virology laboratory. Typical herpesvirus-induced CPE was evident 1–2 days after incubation at 35°, when the concentration of virus in the original specimens was high. Since most human viruses do not grow in primary rabbit cells, the possible presence of HSV could be inferred as soon as distinct CPE was visible in the RK cells. Forty new isolates were tested for microplaque formation in CE cells, as well as for temperature sensitivity as described in the following paragraphs. Final identification was performed by neutralization test in RK cultures using anti-HSV-1 sera at titers of 1:160 and 1:40; the former was used for identification of HSV-1 and the latter for HSV-2. Several isolates were identified by immunofluorescence test in Dr. Nahmias' laboratory where conjugated monospecific antisera were available.

Microplaque formation in chick embryo cell cultures. A total of 40 HSV isolates were tested in CE cell cultures. Among these, 13 strains produced distinct microplaques in 3 days and were presumptively typed as HSV-2 and subsequently confirmed by serologic typing. A representative sample is illustrated in Fig. 1 (top two rows). These results were consistent and reproducible regardless of the source of isolation and the cell type in which the virus was originally isolated. Newly isolated HSV-1 failed to

[4] Immunofluorescence tests were performed by Dr. A. Nahmias' laboratory, Emory University, Department of Pediatrics, Atlanta, Ga.

TABLE I. PHYSICAL, BIOLOGICAL, AND SEROLOGICAL PROPERTIES OF HSV TYPES 1 AND 2.

Virus strain	Source of isolates	Infectivity titers[a] log $TCID_{50}$/0.1 ml in rabbit kidney cells			Infectivity titers[a] log PFU/0.1 ml in chick embryo cells at 35°	Serotyping[b]
		35°	40°	Log reduction at 40°		
New isolates						
NYU78	Brain	5.5	5.2	0.3	–	1*
189	Finger	4.5	3.8	0.7	–	1*
227	Throat	6.0	4.3	1.7	–	1
361	Mouth washing	5.2	4.5	0.7	–	1
489	Lip	3.0	3.0	0.0	–	1
752	Eye	5.0	5.2	−0.2	–	1
793	Vagina	4.0	2.0	2.0	–	1*
794	Mouth	4.0	2.5	1.5	–	1*
875	Mouth	3.0	3.2	−0.2	–	1
878	Soft palate	3.2	2.0	1.2	–	1
884	Lip	2.5	1.0	1.5	–	1
895	Lip	4.5	2.5	2.0	–	1
899	Lip	4.5	3.5	1.0	–	1
903	Lip	6.0	4.3	1.7	–	1
914	Hand	4.0	3.0	1.0	–	1
915	Penis	3.0	3.0	0.0	–	1
937	Penis	5.0	4.2	0.8	–	1
950	Neck	4.5	2.8	1.7	–	1
1003	Chin	4.5	3.5	1.0	–	1
1018	Lip	5.0	4.0	1.0	–	1
1033	Cheek	5.0	3.3	1.7	–	1
1042	Lip	6.0	4.3	1.7	–	ND[c]
1049	Tongue	5.5	4.5	1.0	–	1
1062	Lip	5.5	5.0	0.5	–	1
1091B	Popiteal fossa	4.5	2.8	1.7	–	1
1122A	Penis	5.5	5.5	0.0	–	1
1122B	Penis	5.5	5.2	0.3	–	ND
New isolates						
160	Penis	3.2	1.2	2.0	4.8	2*
857	Hand	2.0	<0.1[d]	2.0	2.9	2*
880A	Mouth	4.0	2.0	2.0	3.5	2*
880B	Penis	3.0	1.0	2.0	3.5	2*
881	Vagina	3.8	1.0	2.8	4.3	2*
892	Vagina	3.8	1.0	2.8	4.3	2
896	Knee	3.8	2.2	1.6	4.2	2
920	Hand	4.5	2.0	2.5	2.3	2
1034	Buttock	4.2	2.0	2.2	5.2	2
1091A	Penis	3.6	1.0	2.6	2.6	2
1105	Penis	4.0	2.7	1.3	1.6	2
1116	Unknown	3.0	1.2	1.8	2.5	2
1145	Finger	5.4	4.6[e]	1.0	5.3	2
Prototype						
HSV-1	Brain	6.0	5.5	0.5	6.0[e]	1
HSV-2	Brain	5.5	5.2	0.3	4.8	2

[a] Readings were made 3–4 days postinoculation.
[b] By neutralization test confirmed by immunofluorescence test as indicated by *.
[c] ND, Not done.
[d] No evidence of virus-induced CPE when undiluted virus suspension was used.
[e] Minute plaques.

show any characteristic microplaques in CE cells (Fig. 1, bottom two rows). However, prototype HSV-1, which has undergone extensive laboratory manipulation, produced minute plaques in CE cells. Table I lists examples of the absence of growth of HSV-1 in CE cell cultures in contrast to HSV-2, from which infectivity titers were easily obtainable by the microplaque techniques in cell cultures.

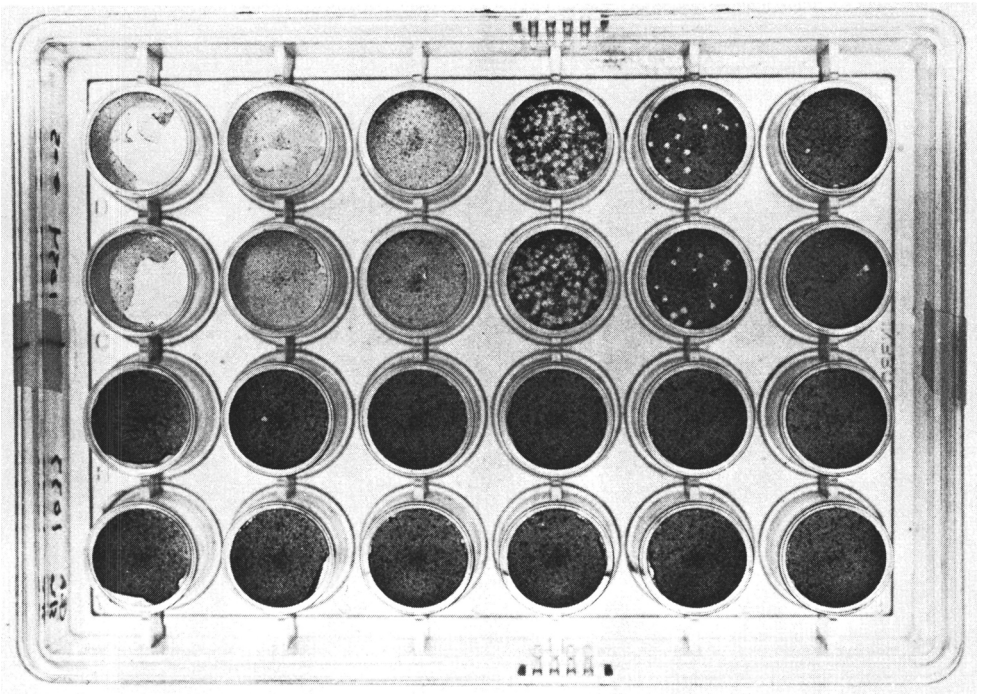

FIG. 1. Microplaques produced by HSV-2(1034, top two rows) in CE fibroblast cell cultures 3 days postinoculation. From left to right virus dilutions were: undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. HSV-1(1033, bottom two rows) showed no evidence of the virus infection after inoculation with similar serial 10-fold dilutions of the virus suspension containing 10^5 $TCID_{50}$ virus.

Temperature sensitivity tests in RK cell cultures. Evidence of distinct differences in sensitivity to temperature were observed when serial 10-fold dilutions of both HSV-1 and HSV-2 were incubated at 40° (Table I). Most of the HSV-1 strains produced CPE at 40° 3–4 days after incubation, although the titers were somewhat lower at 40° than at 35°. HSV-2 strains showed retarded growth, and a reduction of greater than 1.5 log $TCID_{50}$ was noted when they were incubated at the higher temperature, with the exception of prototype HSV-2 which has been passaged many times in the laboratory.

Epidemiological studies. It is well known that active lesions caused by herpes simplex are contagious to the host himself by autoinoculation or to another contact. Examples are illustrated in Table II. Patient 1 was a nurse, working in a gynecology–obstetrics clinic, who had recurrent vaginal infections caused by HSV-2. After a year of such infections, she developed recurrent lesions on her hands and the genital lesions disappeared. HSV-2 was isolated from her hand. We presumed the hand lesions was autoinoculated, although the patient could have been exposed by her occupation to another exogenous source of virus. Since she worked in a gynecology clinic, most probably this would be a genital infection. In this case, she exemplifies transmission of HSV-2 to a nongenital site.

Patient 2, a young man, had herpes on the face and penis which developed several days after oral–genital and genital–genital contact with a girl who had an active vaginal infection. Herpesvirus was isolated from both the boy's face and penis and the girl's vagina. All viruses isolated were HSV-2.

Patient 3, a young man with mild eczema, had a lip lesion which spread to his trunk and penis (eczema herpeticum). All lesions contained HSV-1.

For patients 4 through 9, the source of infection was determined by history. The virus isolated from each patient's lesion was typed. Patient 4 had recurrent lip infections over several years. A new source developed on the thigh extending onto the labia ma-

TABLE II. EPIDEMIOLOGIC STUDIES OF HSV TYPES 1 AND 2 INFECTION.

Patient number	Source of infection	Primary site of infection	Serotype of virus from primary site	Secondary site	Serotype of virus from secondary site
1	Self	Vagina	HSV-2	Hand	HSV-2
2	Sexual contact	Vagina	HSV-2	Face and penis	HSV-2
3	Self	Lip	HSV-1	Generalized penis, trunk	HSV-1
4	Self	Lip	NA[a]	Inner surface of thigh	HSV-1
5	Self	Penis	NA	Popliteal fossa	HSV-2
6	Self	Lip	NA	Hand	HSV-1
7	Sexual contact	Penis	NA	Hand	HSV-2
8	Sexual contact	Penis	NA	Buttock	HSV-2
9	Sexual contact	Vagina	NA	Thumb	HSV-2

[a] NA, not available.

jora. The blisters on the thigh were found on several occasions to contain HSV-1 virus. Patient 5 had recurrent penile lesions until he developed a large confluent bulla covering one entire popliteal fossa. HSV-2 was found in the blisters. Patients 6 and 7 recalled touching active herpetic lesions on the mouth and genitalia, respectively, transmitting the virus to their hands. Patient 8 developed a type 2 lesion on the buttocks following exposure to an active penile lesion, whereas patient 9 developed a lesion on the thumb following exposure to an active vaginal lesion.

Discussion. The availability of simplified procedures for the biologic differentiation of HSV types 1 and 2 is of great value to the diagnostic virology laboratory where large numbers of isolates are obtained. Differentiation by neutralization test is very time-consuming and laborious. Serologic tests such as immunofluorescence (12), immunoperoxidase (13), and radioimmunoassay (14) have been used with success for distinguishing between HSV types 1 and 2 by research laboratories where monospecific, high titer antisera were available. However, since commercially prepared antisera often show extensive cross reaction to the heterologous virus, difficulties can be encountered when using the latter for differentiating these two virus types. Furthermore, the presence of IgG receptors on cells infected with both HSV types has been reported (R. N. Feorino *et al.*, ASM abstract 1976, p. 248) and nonspecific fluorescent staining due to these receptors has been observed in HSV-infected cells, rendering this technique impractical for differentiating HSV types 1 and 2. Thus, biologic markers, such as those described in the present paper, can provide a basis for rapid differentiation between HSV-1 and HSV-2 in clinical laboratories where tissue culture materials and equipment are routinely available.

The present study has shown that utilization of the combination of a biological marker (formation of plaques by HSV type 2 in CE cells) and a temperature marker (resistance of HSV type 1 at 40°) provides a rapid, convenient, and economical method for distinguishing HSV type 1 from type 2, and can be used routinely in a clinical laboratory. However, the results obtained with the temperature markers were not as clear-cut as those of the chick embryo cell cultures. Therefore, the temperature markers should be used as an adjunct procedure for confirming virus type and not as the sole method for differentiating the two.

Chick embryo cell cultures have been used for differentiation of herpes simplex virus types 1 and 2 by several investigators (5–7). The procedures employed varied from laboratory to laboratory. We found that the chick embryo microtest described by Yang *et al.* (6) without any overlay medium is superior to other methods previously described. As demonstrated in the present study, none of the newly isolated HSV type 1 strains produced plaques in CE cells. On the other hand, all freshly isolated HSV type 2 strains were capable of producing microplaques in chick embryos in 2–3 days at 35°. Thus the plaque formation in chick embryo cells in combination with the temperature sensitivity test permits a reliable typing procedure for the differentiation of new isolates of HSV types 1 and 2. These procedures are simple and can be easily ap-

plied in any diagnostic laboratory. Differences between new virus isolates, when compared with the prototype strains, may be the result of selection of viral mutants following repeated laboratory passage (3). Since the prototype HSV strains used in the present study have undergone considerable laboratory manipulation both before and after arrival in our laboratory, this may account for the capability of prototype HSV-1 to produce plaques in chick embryo cells as well as the capacity of prototype HSV-2 to replicate well at 40° (Table I, bottom line).

The patients included in Table II clearly illustrate the point that HSV-1 and HSV-2 virus can be inoculated into a secondary site anywhere on the body. Although the data presented by patients 4–9 is somewhat less reliable without firm culture evidence from the primary site of infection, the historical information appeared to be sufficient to support the data obtained from the secondary sites.

Typing of herpesviruses has become important because of the possible role of HSV-2 in the induction of squamous cell carcinoma of the uterine cervix. Women with recurrent HSV-2 infections on the genitalia should have frequent pap smears. However, there is some serologic evidence, using serum antibodies to nonvirion antigens, that squamous cell carcinoma of the head and neck could be related to recurrent herpetic lesions of the mouth (15). The data did not eliminate the possibility that these patients had an aberrant HSV-2 infection on their face. Whether HSV-2 could be carcinogenic in sites other than the cervix is not known. It is very clear that any type of HSV can infect any part of the body (16, 17). Although a careful review of a patient's medical history can alert the physician to the source of infection, it may be valuable to type HSV isolates from all infections, especially since the method employed is relatively simple and economical.

Summary. A simple technique for differentiation of herpes simplex virus type 1 from type 2, using growth variations in tissue culture and temperature sensitivity is described. All HSV-2 isolates were capable of producing distinct microplaques in chick embryo cells while the newly isolated HSV-1 strains failed to do so. Newly isolated HSV-1 strains showed similar or slightly lower titers in rabbit kidney when incubated at 35 or 40°. In contrast, the HSV-2 isolates generally showed distinctly lower titers or delayed CPE at 40°. Virus typings by selective growth in cell culture and temperature markers were confirmed by neutralization and/or immunofluorescence tests. Since both types of HSV can be isolated from any site of the body, the availability of a simple laboratory technique for distinguishing between the two provides physicians and diagnostic virology laboratories with a means for rapid and accurate diagnosis of herpetic infections.

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