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Use of Biological Characteristics to Type *Herpesvirus hominis* Types 1 and 2 in Diagnostic Laboratories

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Fifty clinical isolates of *Herpesvirus hominis* were typed by pock size on chicken embryo chorioallantoic membranes, sensitivity to heparin, and plaque formation on chicken embryo cell cultures. Of 19 isolates that were typed serologically, there was 100% concordance with respect to plaque formation. Pock size was incorrect in one instance and indeterminate in another. Heparin sensitivity was incorrect for one. Of 31 strains that were only biologically characterized, there were four for which heparin sensitivity did not agree with the other two characteristics. Of the three biological tests, the ability to form plaques in chick cell monolayer was the most reliable indicator type.

Strains of *Herpesvirus hominis* (HVH) can be differentiated antigenically into two groups. Dowdle et al. (1) showed that type 1 is primarily associated with nongenital infections and that type 2 is usually associated with genital infections.

In addition to antigenic differences, many investigators have described biological characteristics which further differentiate HVH types 1 and 2. However, as Nahmias and Dowdle (9) pointed out, most of the work has been done with high-passage laboratory strains rather than low-passage clinical isolates. Repeated passage of HVH isolates tends to diminish the distinctive properties of the two types by means of selection and mutation (9).

Certain of the biological tests might be more amenable to routine diagnostic virology procedures than are the involved serological tests used to determine antigenic differences. Some techniques, such as buoyant density determinations (3), infectivity titers in different cell cultures (12), and inoculation of HVH into animals such as mice (7), are research techniques requiring special equipment or facilities or an excessive amount of supplies for a diagnostic laboratory. The same criticism applies to the method of Ratcliffe (13), who, using Vero cells, found that type 2 strains were markedly inhibited at temperatures greater than 39 C, whereas type 1 strains replicated at 39.8 and 40.3 C. Other techniques, such as determination of cytopathic effect (CPE) on a variety of cell cultures (2), may be rather subjective.

For the present study, 50 unknown clinical isolates, either the original isolation material or that passed one to three additional times, were tested by three biological methods: pock size on the chicken embryo chorioallantoic membrane, heparin sensitivity, and plaque size on chicken embryo cell cultures. Nineteen of the isolates were then submitted to a reference laboratory for serological typing.

MATERIALS AND METHODS

Virus. Fifty clinical isolates were obtained from the Virology Laboratory of Presbyterian-University Hospital, Pittsburgh, Pa. The viruses were isolated in human embryonic lung (WI-38, Microbiological Associates) cell cultures and identified by neutralization with rabbit antiserum against an undifferentiated HVH strain. The following information was known about each viral isolate: patient, site of infection, and passage history. All virus stocks used in this research were either culture fluids from the original isolation or harvests obtained after one to three additional passages (see Table 1). Virus samples were stored at −50 to −60 C. Not all virus samples were titrated. However, the infectivity titers in any of the systems described ranged from 10^4 to 10^6 infectious doses per 0.1 ml. All isolates were coded so that clinical information, particularly knowledge of source of the isolate, could not influence evaluation of results.

Pock size on the chorioallantoic membrane of chicken embryos. The chorioallantoic membranes of 10- to 11-day-old chicken embryos were inoculated with 0.1 ml of 10^{-3} and 10^{-4} virus dilutions using the false-air sac technique (4, 10). Embryos were incubated at 36 to 37 C for 3 to 4 days. The membranes were harvested and placed in petri dishes containing 10% formalin. Pocks tended to group together along the blood vessels of the membrane, even at higher dilutions. The diameters of about 10 distinctly separated pocks were measured with an ocular microme-

TABLE 1. *Source and biological and serological characteristics of HVH type 1 and 2 isolates*

Isolate no.	Source of isolate	Passage no.	Pock size[a]	Heparin sensitivity	Plaque formation	Biological type[b]	Serological type[c]
100	Penis	1	Intermediate	−	+	2	2
101	Female labia	2	Large	−	+	2	2
102	Mouth	1	Small	+	−	1	1
103	Throat	1	Small	+	−	1	1
104	Brain biopsy	1	Small	+	−	1	1
105	Lip	1	Small	+	−	1	1
106	Throat	1	Small	+	±	1	
107	Palate	1	Small	+	−	1	
108	Lip	1	Small	+	−	1	
109	Buttocks	1	Large	−	+	2	2
110	Vagina	1	Large	−	+	2	2
111	Female genital	1	Large	−	+	2	
112	Mouth	1	Small	+	−	1	1
113	Cervix	1	Large	−	+	2	
114	Forehead	1	Small	+	−	1	
115	Mouth	1	Small	+	−	1	
116	Female labia	3	Small	−	+	2	2
117	Groin	1	Small	−	−	1	1
118	Neck	1	Small	+	−	1	
119	Face	1	Small	+	−	1	
120	Chin	1	Small	+	−	1	
121	Vulva	1	Large	+	+	2	
122	Arm	2	Large	−	+	2	
123	Palate	1	Small	+	−	1	
124	Face	1	Small	+	−	1	
125	Eye	1	Small	+	−	1	
126	Cervix	1	Small	−	−	1	
127	Lip	1	Small	+	−	1	1
128	Throat	1	Small	+	−	1	
129	Mouth	1	Small	+	−	1	
130	Mouth	1	Small	+	−	1	
131	Female labia	1	Large	−	+	2	
132	Throat	1	Small	+	−	1	
133	Arm	1	Small	+	−	1	1
134	Throat	1	Small	+	−	1	
135	Lip	1	Small	+	−	1	
136	Vulva	1	Large	−	+	2	
137	Skin, neonatal	1	Small	+	−	1	1
138	Vulva	1	Small	+	−	1	
139	Urethra	1	Large	−	+	2	2
140	Female labia	1	Large	+	+	2	
141	Thumb	1	Small	+	−	1	
142	Cervix	1	Large	−	+	2	2
143	Penis	1	Large	−	+	2	2
144	Eyelid	1	Small	+	−	1	1
145	Throat	1	Large	+	+	2	
146	Sputum	1	Small	+	−	1	
147	Brain biopsy	1	Small	+	−	1	1
148	Lip	1	Small	+	−	1	
149	Mouth	1	Small	+	−	1	

[a] On chorioallantoic membrane.

[b] Type indicated by at least two of three biological characteristics. The italicized isolate numbers indicate those in which one characteristic was at variance with the other two.

[c] Type determined in A. Nahmias' laboratory. Type 1 strains produce small pocks, are heparin sensitive (+), and do not produce plaques on chicken embryo cell cultures. Type 2 strains produce large pocks, are heparin resistant (−), and do produce plaques.

ter mounted on a light microscope, and a mean was computed.

Nahmias et al. (10) divided pock sizes into two groups on the basis of the following critera. "Small pocks," produced primarily by type 1 strains, had a mean diameter of less than 0.5 mm and the diameter of any one pock did not exceed 1 mm. "Large pocks," produced by type 2, averaged greater than 0.5 mm in

diameter and many exceeded 1 mm in diameter.

Heparin sensitivity. Heparin (100 U/ml; USP) was added to fresh culture fluids of either HEP-2 or WI-38 tube cell cultures depending upon availability of cultures. Plummer et al. (12) inoculated 1,000 plaque-forming units in rabbit kidney tube cultures containing heparin. We added 0.1 ml of a 1:5 dilution of each virus sample to two cultures after a 1-h incubation of the cells with heparin. The cultures were incubated at 37 C and observed daily for CPE. Two cultures without heparin were also inoculated with each virus to compare the progression of CPE.

Sensitivity to heparin was demonstrated by a delay in the appearance of CPE. A ratio was constructed from visual estimates of the degree of CPE from tube culture with and without heparin (1+, 2+, 3+, and 4+ represent <50, 50, 75, and 100% CPE, respectively). The ratio was taken on the day that the control tubes without heparin showed 4+ CPE. Therefore, ratios were 0/4, 1/4, 2/4, 3/4, or 4/4. Viruses with ratios 0/4 or 1/4 were considered to be sensitive to heparin, whereas ratios of 2/4, 3/4, or 4/4 represented viruses insensitive to heparin.

Plaque size on chicken embryo cell cultures. Three-day-old chicken embryo cell cultures (6) planted in six-welled plastic plates were inoculated with 0.1 ml of 10^{-1}, 10^{-2}, and 10^{-3} virus dilutions. After 1 h at 37 C for adsorption, a medium 199 overlay containing 1.6 g of tragacanth gum per 100 ml was added. The cultures were incubated at 37 C in a 5% humidified CO_2 atmosphere for 4 days. The overlay was removed, and the monolayers were fixed and stained with a vital gentian violet stain (0.5% gentian violet, 0.9% sodium chloride, 5% of a 37% stock solution of formalin, 52.6% of a 95% stock solution of ethyl alcohol, and 41% double-distilled water). All plaques were measured with an ocular micrometer in plates where there were 10 plaques or less. The mean was recorded.

Antigenic typing. Andre Nahmias typed 19 of the HVH isolates by immunofluorescent methods without knowledge of the results of our biological tests. With conjugated anti-type 1 serum and anti-type 2 serum, HVH isolates were typed as type 1 if infected cells fluoresced 3 to 4+ with both anti-type 1 and 2 sera. Type 2 isolates demonstrated fluorescent staining only with anti-type 2 serum (8).

RESULTS

Pock size. All of the HVH isolates except for no. 100 were readily divided into small (type 1) or large (type 2) pock groups (Table 1). Isolate 100 produced pocks with an average diameter of 0.60 mm, and none exceeded 1.00 mm. Since these measurements do not coincide with the criteria for "large" or "small," they were labeled "intermediate." Another isolate (no. 116) produced small pocks when, by the other two biological characteristics and by serology, it was a type 2 virus. Presumably this result was erroneous.

Heparin sensitivity. To ascertain the appropriate amount of heparin to be used, a titration of the heparin was done against a constant amount of virus. A 1:5 dilution of isolates 156 from a brain biopsy was added to cell cultures containing 50, 100, 150, 200, 250, and 300 U of heparin per ml. After 3 days of incubation, the CPE in tubes without heparin was 4+, whereas the CPE in all the tubes with various heparin concentrations was 1+, giving 1/4 ratios and indicating inhibition by heparin. Isolate 157 from a penis lesion was treated in the same fashion with the following results: when the CPE in the tube culture without heparin was 4+, the CPE in the tubes with heparin was also 4+, giving 4/4 ratios. This suggested a type 2 isolate that could not be inhibited by any of the heparin concentrations. In light of the above uniform results, the concentration selected was 100 U/ml (12). The sensitivity of the HVH isolates to heparin is also shown in Table 1. Of the three biological tests, this one was the most discordant. In five instances it disagreed with the other tests, including one isolate that was typed serologically.

Plaque size. Figueroa and Rawls (2) found that genital strains of HVH formed plaques 0.50 mm in diameter in chicken embryo cell cultures, whereas oral strains did not form any discernable plaques. Using the same techniques, i.e., a methyl cellulose over-lay and 4 days of incubation, Lowry et al. (5) found that type 1 isolates formed no plaques or small ones less than 1.3 mm, whereas type 2 plaques were about 2.3 mm. According to our results (Table 1), plaque formation was concordant with at least two other biological characteristics in all cases, and with serological typing when it was done. Plaques in chicken embryo cell cultures had average diameters ranging from 0.53 to 0.81 mm. Isolate 106 produced plaques averaging only 0.27 mm, which was distinctly smaller and perhaps compatible with type 1 (5) although it is designated indeterminate (±) in the table.

DISCUSSION

Recognizing the clinical and epidemiological importance of ascertaining the type of HVH isolates, the diagnostic virology laboratory must seek a relatively easy and rapid technique for doing this. Methods relying upon biological rather than antigenic properties of HVH appear to fit the need.

According to the criteria set forth for each of the three biological methods, a type 1 isolate would be expected to form small-sized pocks on chicken embryo chorioallantoic membranes, to be sensitive to heparin, and not to form plaques on chicken embryo fibroblasts. On the other hand, a type 2 would be "large pock, insensitive to heparin, plaque (+)" (2, 10, 12). Generally,

the 50 herpes isolates demonstrated rather consistent biological properties. If two of three biological characteristics were used to determine antigenic type, there was perfect correlation of biological typing and of serological typing when it was done.

Only 3 of the 19 specimens tested serologically showed discrepancies in one of the three biological characteristics. Specimen no. 100 was "intermediate, insensitive, plaque (+)"; no. 116 was "small, insensitive, plaque (+)"; and no. 117 was "small, insensitive, plaque (−)". Thus, specimens no. 100 and 116 differed by pock size results and 117 differed by heparin sensitivity from what was expected from their serological type. The plaque formation characteristic of these three isolates corresponded to serological typing. There was 1 strain of 50 in which the plaque test gave an equivocal result (no. 106). Even there, the plaques formed were distinctly smaller and therefore would be distinguished from the usually larger plaques formed by type 2. The strain was a type 1 by other criteria.

Type 1 isolates are generally associated with nongenital sites of infection, whereas type 2 isolates are from genital sites (1). Exceptions do occur. No. 137, an isolate from a neonate, may have been expected to be type 2 but was type 1 by all biological and serological tests. One isolate (no. 122) from an arm and one (no. 145) from a throat demonstrated type 2 properties. Two others, no. 126 from a cervix and no. 138 from the vulva, had type 1 properties. Of these isolates, the heparin test was discordant for no. 145 and 126. Thus, the division of types cannot be absolutely predicted from the site of isolation (11). Admittedly, it is possible, although not likely, that biological typing was in error, especially for the later two specimens.

Of the three biological tests, plaquing was the most reliable. Unfortunately it also requires the most time. Chicken cell cultures are not routinely used in diagnostic laboratories. However, they are very easy to prepare, and there is no need for unusual equipment or supplies. Plaquing methods are feasible and economical once established.

Since eggs are readily available and inoculation on chorioallantoic membranes is a relatively simple procedure, pock size studies might be used routinely. One drawback is the time required for eggs to age properly.

Probably the most expeditious technique for a diagnostic laboratory from the point of view of ease of performance is the heparin sensitivity test. The procedure is simple, the materials are readily obtained, and the time required for the test is short. Unfortunately, this method was discordant for five of the virus strains, and in one instance the result did not agree with the serological typing. This method therefore cannot be recommended for the diagnostic laboratory without further evaluation.

We conclude that, for a diagnostic laboratory, the plaque test alone may suffice to distinguish type 1 from type 2, but the pock test or the heparin test alone is not sufficient. Known type 1 and type 2 control strains should be run with any test.

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