Typing of *Herpesvirus hominis* Strains by a Direct Immunofluorescent Technique[1] (34221)

A. J. NAHMIAS,[2] W. T. CHIANG,[3] I. DEL BUONO, AND A. DUFFEY
(Introduced by M. Tager)

Departments of Pediatrics and Preventive Medicine, Emory University School of Medicine, Atlanta, Georgia and Department of Preventive Medicine, University of Washington School of Medicine, Seattle, Washington

Herpesvirus hominis (HVH) can be differentiated into at least two antigenic types which have different biological and epidemiological characteristics (1). Several serological tests, such as the microneutralization (2) and the kinetic neutralization (3) tests, have been used for antigenic differentiation of the 2 HVH types. Differentiation of HVH isolates by an immunofluorescent (FA) test would offer several advantages for basic and clinical studies. This report presents the methodology and results obtained with a direct FA test used to type HVH isolates from 191 clinical specimens, 95 of which had been tested previously by a microneutralization test (1).

Materials and Methods. Virus strains. Initial experiments were done using the MS (type 2) and VR_3 (type 1) HVH strains (2). Ninety-five isolates previously assayed by the microneutralization test (1) were also tested by the direct FA technique. In addition, 96 HVH isolates from different sites were tested by the FA technique only. Viruses had been isolated in primary rabbit kidney tissue culture cells, had received one to four passages in these cells and had a titer of 10^5–$10^{6.5}$ TCD_{50}/ml.

Preparation of infected cells for FA test. Monolayers of HEp-2 tissue culture cells were prepared using Eagle's MEM containing 10% calf serum. One ml of undiluted virus to be tested was added to a 6-oz HEp-2 bottle. After incubation of the infected monolayer at 36° for 48 hr, the medium was removed and the cell sheet washed 2X with Hanks' BSS. After trypsinization, the cells were resuspended in 10 ml of Eagle's MEM with 2% calf serum. The infected cells were then washed 3X in this medium without serum by centrifugation at 1000 rpm for 10 min. After the third wash, the pellet was resuspended in 1 ml of Eagle's MEM without serum and a drop of 1% bovine albumin added. The cells were mixed and one drop placed on each of eight spots on a microscopic slide. The cells were then air-dried and fixed in prechilled acetone for 15 min at —20°. Control uninfected HEp-2 cells were prepared in a similar way. After fixation, cells were either used immediately or stored at —70° for later staining.

Preparation of fluorescein-labeled antisera to type 1 and type 2 HVH. The preparation of high-titered type 1 and type 2 HVH antisera in rabbits using three intramuscular and intraperitoneal inoculations of VR_3 (type 1) or MS (type 2) strains grown in primary rabbit kidney tissue culture has been previously described (2, 4). The anti-type 1 serum had a neutralization titer of 1:160 to type 1 and 1:40 to type 2; the anti-type 2 serum had a titer of 1:80 to type 1 and 1:160 to type 2. The sera were conjugated with fluorescein isothiocyanate (5) and stored at —20°. Prior to use, the conjugated antisera were absorbed twice (for 1 hr each at 4°)

[1] Supported by CC-00159 from the National Communicable Disease Center and a Contract DADA 17-68-C8141 from the Office of the Surgeon General.

[2] A. Nahmias is recipient of a Research Career Development Award IK-A1-18637 from the USPHS.

[3] Present address: Dept. of Obstetrics and Gynecology, National Taiwan University Hospital, Taipee, Taiwan.

TABLE I. Intensity of Fluorescence Observed on Application of Varying Dilutions of Fluorescein Conjugated Anti-Type 1 or Anti-Type 2 Sera to Infected Type 1 HVH, Infected Type 2 HVH, and Noninfected HEp-2 Cells.

	Dilution of conjugated serum	Type 1 infected HEp-2 cells	Type 2 infected HEp-2 cells	Noninfected HEp-2 cells
Fluorescein-conjugated anti-type 1 serum	1:2	3–4+[a]	2–3+	0
	1:4	3–4+	0–1+	0
	1:8	3–4+	0	0
	1:16	2–3+	0	0
	1:32	2–3+	0	0
Fluorescein-conjugated anti-type 2 serum	1:2	3–4+	3–4+	0
	1:4	3–4+	3–4+	0
	1:8	3–4+	1–2+	0
	1:16	2–3+	1+	0
	1:32	1+	0	0

[a] Intensity of fluorescence.

with mouse liver powder-BBL-(50 mg per 1 ml of conjugate) and filtered through a 0.45 μ Millipore filter. The filtrate was absorbed with a HEp-2 cell pellet for 1 hr at room temperature and overnight in a shaker at 4°. Preparation of the HEp-2 cell pellets was similar to that previously described for infected cells; pellets were frozen at —70° and thawed at 37° three times before use for absorption of the conjugate. One drop of rhodamine-conjugated normal rabbit serum was added to 1 ml of the working dilution of the adsorbed conjugate prior to use. Absorbed conjugates were not used for FA staining for more than 3 days after preparation.

Determining working titer of FA conjugates. Conjugates to type 1 and type 2 HVH were diluted 1:2 to 1:32 in phosphate-buffered saline (PBS) pH 7.2. One drop of each dilution was added to previously prepared noninfected cells and cells infected with type 1 (VR_3 strain) or type 2 (MS strain) HVH. The slides were incubated in a moist chamber at room temperature for 20 min, washed with PBS for four/10 min periods and air-dried. They were then mounted in glycerol buffer and examined with a Leitz OrthoLux microscope using a UGI primary filter and GG13 secondary filter.

The specificity of the reactions was ascertained using an inhibition test with unconjugated rabbit anti-type 1 and anti-type 2 HVH and conjugated normal rabbit serum, as well as with controls consisting of noninfected cells.

Results obtained with several tests to ascertain working dilutions are summarized in Table I. A dilution of 1:8 of conjugated anti-type 1 serum reacted only with type 1 infected cells. In addition, many of the cells showed not only perinuclear fluorescence, but also granules of varying sizes in the nucleus and near the nuclear membrane (Fig. 1). A dilution of 1:4 of conjugated anti-type 2 serum reacted with both type 1 and type 2 infected cells, while higher dilutions of this conjugate gave less intense fluorescence with type 2 than with type 1 infected cells. Type 1 infected cells stained with the type 2 conjugate showed similar perinuclear fluorescence and granules as with conjugated anti-type 1 serum (Fig. 1). However, type 2 infected cells stained with conjugated anti-type 2 serum did not have any coarse granular fluorescence, showing in addition to perinuclear staining, homogeneous nuclear fluorescence (Fig. 2). For testing of HVH isolates reported here, the type 1 conjugate was therefore used at 1:8 dilution and type 2 conjugate at 1:4 dilution. Viruses were identified as to type as follows: type 1 HVH, if positive (3–4+) staining was observed with both conjugated anti-type 1 and anti-type 2 conjugates, with fluorescent coarse granules also

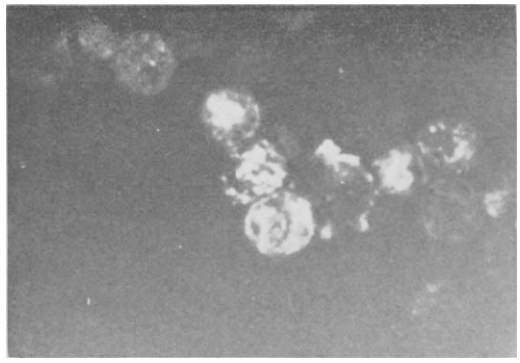

FIG. 1. Perinuclear fluorescence and fluorescent granules observed in type 1 HEp-2 infected cells with fluorescein-conjugated anti-type 1 rabbit serum. (Similar staining is observed in type 1 infected cells with fluorescent-conjugated anti-type 2 rabbit serum).

seen; type 2 HVH, if positive staining was found only with anti-type 2 conjugate. Every test included known type 1 and type 2 infected and uninfected cells which served to control the potency and specificity of the reagents on the day of their use. FA tests were done on unknown HVH isolates without prior knowledge as to type(in cases where a previous microneutralization typing test had been done), or to site or origin of the isolates.

Results. Forty-seven HVH isolates previously found by the microneutralization (MN) test to be type 1 were also found to belong to this type by the FA test (Table II: MN-FA column). Similarly, 48 isolates, typed as 2 by the MN test, were found to be of that type by the FA technique. There was thus complete agreement between the MN and FA tests.

In view of these results, 96 HVH isolates from a variety of sites and clinical conditions were typed by FA only (Table II: FA column). The HVH type of these isolates compared favorably with previous experience on relation of site of origin to isolates typed by the MN test. For instance, all HVH strains which had been isolated from mouth, lips, eyes, spinal fluid, or brain in patients outside the newborn age group when tested by FA only were type 1, as had been previously found with strains tested by the MN test. On the other hand, all but one of 69 genital isolates (with no concurrent mouth HVH isolated) tested by FA only were found to be type 2 HVH, in comparison to 91 out of 94 previously tested by MN only.

The overall perspective of our current experience with typing of 322 HVH isolates is also presented in Table II.

Discussion. Although various biological markers (reviewed in 1)—such as pock size on chorioallantoic membrane, tissue culture cytopathic effect, and mouse virulence—can be used to differentiate type 1 from type 2 HVH strains, exceptions have been noted with such methods, mostly resulting from virus passage in various host systems. Similarly, there are several exceptions in the association of one HVH type with site of viral recovery, *e.g.*, 8 of 171 genital isolates were type 1 (Table II).

A direct FA test for the differentiation of HVH strains is an additional means to those serological tests—microneutralization (2), plaque reduction (3, 6), and kinetic neutralization (3, 7)—currently in use for typing. Once the conjugated anti-type 1 and type 2 sera are prepared and the conditions of their use ascertained, the FA test offers advantages in rapidity and ease of performance for laboratories equipped for this technique. In addition, the FA technique offers the possibility of not only identifying HVH directly from clinical specimens but also of obtaining concurrent typing.

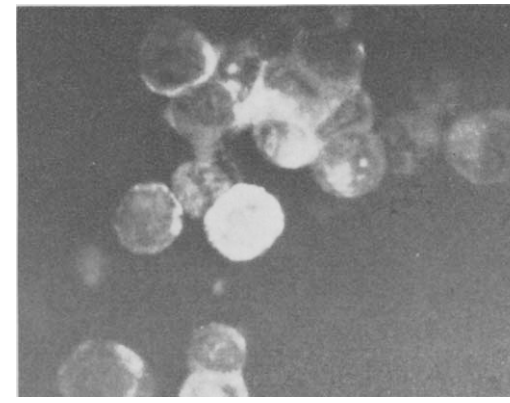

FIG. 2. Perinuclear and nuclear fluorescence observed in type 2 HEp-2 infected cells with fluorescein-conjugated anti-type 2 rabbit serum.

TABLE II. HVH Type as Assayed by Microneutralization (MN) Test, Immunofluorescent (FA) Test, or Both MN and FA Tests—Relation to Type of Clinical Involvement and Site of Isolation.

Type of clinical involvement	Site of isolation	Type 1				Type 2			
		MN	MN–FA	FA	Total	MN	MN–FA	FA	Total
Children and adults									
Genital tract									
Male	Penis					11	9	19	39
Female	Vulva		3	1	4	28	10	26	64
	Cervix, vagina					26	10[a]	23	59
Genital tract and mouth	Penis and mouth		1		1				
	Vulva or cervix and mouth		2	1	3		1		1
Gingivostomatitis or asymptomatic	Mouth	16	7	4	27				
Herpes labialis	Lip	5	9	2	16				
Dermatitis	Face or trunk	8	2	3	13				
	Thighs, buttocks					7	3	1	11
	Hands or arms			1	1	1	1	1	3
Eczema herpeticum	Skin	2	1		3				
Eye	Conjunctiva, cornea	5	7	2	14				
Meningoencephalitis	Spinal fluid, brain	17	10	8	35		1[b]		1
Generalized herpes	Visceral organs		2		2				
Newborns									
Generalized, encephalitis, Keratitis, dermatitis	Visceral organs, brain, eye, skin		3	0	3	4	14	4	22
Total		53	47	22	122	77	48	74	200

[a] One female patient with simultaneous herpes cervicitis (type 2) and herpes labialis (type 1).

[b] MS strain from brain of patient with multiple sclerosis (12).

The pattern of reactivity of the conjugated anti-type 1 and type 2 sera follows those previously found with microneutralization (2) and complement-fixation (3, 8) tests; *i.e.*, antibodies to type 1 HVH react better with type 1 than type 2 strains, whereas type 2 antibodies react almost equally to the homologous or heterologous type. Type differentiation could thus be accomplished by using appropriate dilutions of conjugated anti-type 1 serum (Table I). Another difference was the finding of fluorescent coarse granules in cells infected with type 1 using conjugated sera of either type; these granules were not observed in type 2 infected cells. Similar morphological features have been observed by us with type 1 and type 2 conjugated sera from rabbits infected in the cornea and by using other host cells (green monkey kidney, BHK-21, primary rabbit kidney) infected with type 1 or type 2 virus. The finding of similar fluorescent granules has been previously noted by several other workers using different methods for preparing conjugates to presumably type 1 strains (9–11). Studies are in progress to define the nature of the morphological staining differences observed which might assist in defining the common and different antigens in the two HVH types.

Summary. A direct immunofluorescent (FA) test was used to facilitate typing of *Herpesvirus hominis* (HVH) strains. Using specific dilutions of fluorescein-conjugated rabbit anti-type 1 and anti-type 2 sera, HVH strains grown in HEp-2 cells could be typed as 1 if they reacted with both conjugated sera and as type 2 if they reacted only with anti-type 2 sera. In addition, a difference in staining between type 1 and type 2 infected cells was observed: type 1 infected cells showed perinuclear staining and fluorescent granules with either conjugated serum; type 2 infected cells showed no coarse granules and had both perinuclear and nuclear staining.

There was total agreement between results using the FA technique and those obtained previously by a microneutralization (MN) test in the typing of 95 HVH isolates. When an additional 96 HVH isolates were tested by the FA technique only, the type found correlated well with site of isolation. Current experience with 322 HVH isolates typed by the MN test only, FA test only or by both tests provides further perspective on the relation between HVH type, site of isolation, clinical manifestation, and age of the infected individual.

1. Nahmias, A. and Dowdle, W., Progr. Med. Virol. **10**, 110 (1968).

2. Dowdle, W. R., Nahmias, A. J., Harwell, R. W. and Pauls, F. P., J. Immunol. **99**, 974 (1967).

3. Schneweis, K. E., Z. Immunitaets Forsch. **124**, 24 (1962).

4. Nahmias, A., Dowdle, W., Kramer, J., Luce, C., and Mansour, C., J. Immunol. **102**, 956 (1969).

5. Riggs, J. L., Seiwald, R. J., Burckhalter, J. Y., Downs, C. M., and Metcalf, T. G., Am. J. Pathol. **34**, 1081 (1958).

6. Plummer, G., Brit. J. Exptl. Pathol. **45**, 135 (1964).

7. Rawls, W. E., Tompkins, W. A. F., Riguero, M. E., and Melnick, J. L., Science **161**, 1255 (1968).

8. Nahmias, A., Dowdle, W., Naib, Z., Josey, W., and Luce, C., Pediatrics **42**, 659 (1968).

9. Munk, K. and Fischer, H., Arch. Ges. Virusforsch. **15**, 539 (1965).

10. Roizman, B., Spring, S. B., and Roane, P. R., J. Virol. Kyoto **1**, 181 (1967).

11. Geder, L., Lehel, F., Jeney, E., and Gonezol, E., Acta Microbiol. Acad. Sci. Hung. **15**, 161 (1968).

12. Gudnadottir, M., Helgadottir, H., and Bjarnason, O., Exptl. Neurol. **9**, 85 (1964).

Received June 5, 1969. P.S.E.B.M., 1969, Vol. 132.