TECHNIQUES FOR TYPING *HERPESVIRUS HOMINIS* ANTIBODY: A COMPARISON OF INHIBITION OF PEROXIDASE-LABELLED ANTIBODY STAINING WITH INHIBITION OF INDIRECT HAEMAGGLUTINATION AND WITH MICRONEUTRALISATION

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OWING to the reported association of *Herpesvirus hominis* (HVH) type 2 with cervical cancer, interest in typing HVH antibody in human sera has been growing (Rawls *et al.*, 1968; Naib, *et al.* 1969; Adam *et al.*, 1973; Melnick *et al.*, 1976). At present, the most reliable method for HVH antibody typing is probably the quantal microneutralisation (MN) test introduced by Pauls and Dowdle (1967) and recently simplified by Stalder, Oxman and Herrmann (1975). This method, however, is relatively cumbersome and time-consuming. Consequently, new and more rapid methods for typing HVH antibody have been sought. Inhibition of the indirect haemagglutination (IHA) reaction has proved a satisfactory alternative to the MN test (Bernstein and Stewart, 1971; Back and Schmidt, 1974), as has an indirect fluorescent-antibody technique (IFA) for detecting antibodies to surface antigens of cells infected with HVH type 1 (HVH-1) and type 2 (HVH-2) (Smith *et al.*, 1972*b*). The reliability of neutralisation tests for typing HVH antibody has been questioned recently and a complement fixation (CF) test with type-specific antigens has been proposed for detection of type-specific antibodies in human sera (Skinner, Hartley and Whitney, 1976). Radioimmunoassay has been shown to be very sensitive for HVH antibody typing (Forghani, Schmidt and Lennette, 1975).

This report describes the typing of HVH antibodies in sera from patients with HVH-1 and HVH-2 infections by a new technique based on the inhibition of peroxidase-labelled antibody staining (PLAS). The results are compared with those of the IHA inhibition and MN tests.

MATERIALS AND METHODS

Virus strains, cell cultures and antigen preparation. The McIntyre strain of HVH-1 (titre $10^{7.5}$ TCD₅₀ per ml) and the MS strain of HVH-2 ($10^{6.5}$ TCD₅₀ per ml) were initially obtained from the American Type Culture Collection (Rockville, Md, USA). Viruses were propagated in fibroblast cell cultures (MA-184) derived from infant foreskin and supplied by Microbiological Associates, Bethesda, Md, USA. Growth medium consisted of Eagle's minimal essential medium (MEM) in Hanks' balanced salt solution (BSS) supplemented with

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10% foetal calf serum; maintenance medium consisted of Eagle's MEM in Earle's BSS with 2% foetal calf serum. HVH type 1 and 2 antigens were prepared as described by Bernstein and Stewart (1971).

Reference human sera. Sera from two cases of primary HVH-1 and two cases of primary HVH-2 infection were used as HVH-1 and HVH-2 reference sera. Diagnosis of primary infection was based upon (1) absence in pre-infection sera of HVH antibody detectable by the CF, MN and IHA tests, and presence of specific HVH-1 or HVH-2 antibody in sera taken a few weeks or months after the onset of infection, (2) detection of HVH-specific IgM antibody in the same post-infection sera for up to 3 months by means of the IHA test performed on sucrose density-gradient fractions, and (3) isolation of HVH virus of the type indicated by IHA-inhibition (Bernstein and Stewart, 1971) and MN (Stalder *et al.*, 1975) tests on convalescent sera. The antibody titre to human cytomegalovirus (CMV) and varicella-zoster (V-Z) virus was for all sera less than 8 by CF and less than 16 by IHA.

Test based on the inhibition of PLAS. Ethanol-fixed MA-184 cell cultures infected with HVH-1 and HVH-2 in disposable microtest culture plates (Falcon Plastics, Oxnard, Calif., USA) have been used to develop an indirect peroxidase-labelled antibody (PLA) test for HVH-1 and HVH-2 antibody. The basic procedure has already been described in relation to CMV antibody determination (Gerna, McCloud and Chambers, 1976a). Goat antihuman-IgG antibody (Electro-Nucleonics, Bethesda, Md, USA) was coupled to horseradish peroxidase (type VI, Sigma Chem. Co., St Louis, Mo., USA) by the glutaraldehyde method (Avrameas and Ternynck, 1971). The optimal dilution of the conjugate (1 in 160) was determined by chequer-board titration against reference HVH-1 and HVH-2 human sera to give specific staining of homologous virus-infected cells without non-specific staining of uninfected cells. Control procedures for specificity and cross-reactivity with CMV and V-Z virus were performed as described by Gerna *et al.* (1976a and b).

Serial two-fold dilutions of test sera, starting with a 1 in 16 dilution, were layered in 0.1 ml volumes on monolayers of tissue culture in microplate wells and incubated at 37°C for 30 min. After three washings with phosphate-buffered saline (PBS), the cell monolayers were covered with the optimal dilution of the conjugate, incubated at 37°C for 30 min. and then stained for enzymatic activity. The test was read with an inverted microscope. Negative, positive HVH-1 and positive HVH-2 reference sera were tested in each run. The end-point titres were determined by considering as negative the first serum dilution with the same amount of staining as the known negative serum at a dilution of 1 in 16.

When a titre was determined both on cells infected with HVH-1 and HVH-2, the dilution of each serum containing 10 PLA antibody units to HVH-1 and that containing 10 PLA antibody units to HVH-2 were absorbed at room temperature with equal amounts of the optimal dilution of HVH-1 antigen and HVH-2 antigen. Optimal dilution of the antigens was considered to be the highest dilution giving complete inhibition of 10 PLA homotypic antibody units. Mixtures were then tested on HVH-1-infected and HVH-2-infected cells.

TABLE I

Examination of sera from patients with HVH-1 infection, HVH-2 infection or "dual" HVH infection by the method based on inhibition of peroxidase-labelled antibody staining (PLAS)

Serum contained antibody to the viruses	Results given by PLAS on cells infected with HVH-1 virus after absorption of serum with		Results given by PLAS on cells infected with HVH-2 virus after absorption of serum with	
	HVH-1	HVH-2	HVH-1	HVH-2
HVH-1	—	+	—	—
HVH-2	—	—	+	—
HVH-1 and HVH-2	—	+	+	—

HVH-antibody staining activity was inhibited by homotypic but not heterotypic antigen, according to patterns shown in table 1.

Microneutralisation test. The MN test was performed according to the method of Stalder *et al.* (1975) except that BS-C-1 cells were used instead of diploid human-embryo fibroblast cultures, and human sera from primary HVH-1 and HVH-2 infections instead of rabbit antisera. The growth medium used for diluting sera and viruses and for preparing the cell suspension consisted of Medium 199 in Earle's BSS supplemented with 5% foetal calf serum and 1% of a 20% solution of dextrose. Fifty per cent end points for serum neutralisation titrations were calculated as described by Reed and Muench (1938). Titres were transformed into pN values by the formula: $pN = \log \log \text{virus neutralised} - \log \text{final serum end point} + \log \text{test volume}$ (Fazekas de St. Groth, 1961) or in corrected titres according to the formula $t_c = t + 0.5 (c - a - 2)$ (Stalder *et al.*, 1975). For any serum, ΔpN denoted the difference pN against HVH-1 - pN against HVH-2. The difference between corresponding corrected titres was denoted by Δt_c . A series of experiments in which virus input ($\log_{10}$ TCD₅₀) was within the range 1.20-2.87 indicated an approximate linear relationship between serum neutralisation titres and virus input. Under experimental conditions, the slope of the linear equation expressing this relationship ranged from 0.34 to 0.68 (mean $\pm$ SD, 0.49 ± 0.14). This resembled the data reported by Stalder *et al.* (1975) who used diploid human cells.

Indirect haemagglutination and indirect haemagglutination inhibition test. The IHA and IHA inhibition methods for HVH antibody typing (Bernstein and Stewart, 1971) were carried out with sensitising and inhibiting HVH-1 and HVH-2 antigens produced in MA-184 cell cultures. Typing of human sera was achieved by examining the ability of the optimal dilution of HVH-1 and HVH-2 antigen to inhibit the IHA reaction between eight units of HVH-1 antibody and HVH-1 sensitised erythrocytes, or eight units of HVH-2 antibody and HVH-2 sensitised cells. Typing was based upon complete inhibition of IHA antibody to one virus type, but not the other.

Human sera examined. The following four groups of human sera were tested: 61 sera from individuals with recurrent HVH-1 oral infection; 15 sera from women with recent HVH-2 genital infection and no previously detectable HVH-1 antibody; sera from three cases of recent HVH-2 genital infection and remote HVH-1 infection; sera from four cases of HVH-2 genital infection of more than a years' duration in patients whose sera contained HVH-1 antibody.

Herpesvirus hominis isolation and typing. All HVH isolations were made in human embryonic kidney, WI-38 and African green-monkey kidney cell cultures (Flow Laboratories, Rockville, Md, USA). HVH isolates were typed by MN (Stalder *et al.*, 1975) and IHA inhibition (Bernstein and Stewart, 1971; Back and Schmidt, 1974).

RESULTS

Controls for specificity of the PLA test

Specificity was demonstrated by (1) disappearance of the titre of reference sera after absorption with homologous virus-infected cells or cell-free virus, (2) lack of cross-reactions with CMV and V-Z virus when reference sera were used at a dilution ≥ 1 in 16, and (3) lack of endogenous peroxidase activity in infected as well as uninfected cells.

Typing of HVH-1 and HVH-2 reference human sera by MN, IHA inhibition and PLAS inhibition methods

To assess the specificity of the three methods 10 sera from two cases of HVH-1 primary infection and 10 sera from two cases of HVH-2 primary

TABLE II

Examination of serum mixtures containing different proportions of HVH-1 and HVH-2 antibody by means of three tests based on microneutralisation (MN), inhibition of indirect haemagglutination (IHA) and inhibition of peroxidase-labelled antibody staining (PLAS)*

Each millilitre of serum mixture contained the stated HVH antiserum in a volume of (ml)		Results of						Types of HVH antibody detected by the		
HVH-1	HVH-2	MN test expressed as		inhibition† test for IHA antibody to		inhibition† test for peroxidase-labelled antibody to		MN test	IHA inhibition test	PLAS inhibition test
		ΔpN	Δt_c	HVH-1	HVH-2	HVH-1	HVH-2			
0.0	1.0	-0.09	-0.27	+	-	+	-	2	2	2
0.2	0.8	+0.18	+0.18	±	-	±	-	1, 2	2	2
0.4	0.6	+0.34	+0.34	-	-	-	-	1, 2	1, 2	1, 2
0.6	0.4	+0.49	+0.49	-	-	-	-	1, 2	1, 2	1, 2
0.8	0.2	+0.63	+0.63	-	±	-	±	1	1	1
1.0	0.0	+0.75	+0.70	-	+	-	+	1	1	1

* One serum, taken from a case of primary HVH-1 infection had HVH-1 titres of $10^{2.09}$ by MN, 1024 by IHA and 512 by PLAS; the other serum, taken from a case of primary HVH-2 infection had HVH-2 titres of $10^{2.06}$ by MN, 2048 by IHA and 1024 by PLAS.

† - = Inhibition; + and ± = no inhibition.

infection were used. Their neutralising capacity for HVH-1 and HVH-2 was determined as well as the Δt_c and ΔpN values. As shown in the figure, the Δt_c values of all the sera associated with HVH-1 primary infection fell within the range +0.6 to 1.2, whereas the Δt_c values of all the sera associated with HVH-2 primary infection were within the range 0 to -0.5. No intermediate Δt_c values between 0 and +0.6 were encountered. In differentiating the two HVH antibody types, Δt_c and ΔpN values were both satisfactory. The criteria for HVH antibody specificity proposed by Dowdle *et al.* (1967) as a result of observations on ΔpN values and rabbit antisera, and confirmed by Stalder *et al.* (1975) with Δt_c values and rabbit antisera, have been reconfirmed with Δt_c values and human sera.

The IHA inhibition and PLAS inhibition methods showed that the 10 sera from HVH-1 primary infections contained only HVH-1 antibody and the 10 sera from HVH-2 primary infections contained only HVH-2 antibody.

Sensitivity of the PLAS inhibition, IHA inhibition and MN methods

A serum from a case of HVH-1 primary infection and a serum from a case of HVH-2 primary infection were mixed in different proportions (table II). The two sera possessed comparable titres to homotypic virus and any difference between the results of the three tests was within the limits of a single doubling dilution. When the volumes of the two sera in a mixture differed only slightly, both types of antibody were readily detected, i.e., the mixture was shown to contain "dual" or "intermediate" antibody. When HVH-1 and HVH-2 sera were mixed in the proportion 2 : 8, only the MN test was capable of

FIGURE.—Typing of *Herpesvirus hominis* (HVH) antibody by the microneutralisation test. Distribution of Δt_c values for 83 sera from patients infected with HVH-1 (■) or HVH-2 (□) virus (above). Distribution of Δt_c values for 10 sera taken at different times from two patients with primary HVH-1 (■) infection and 10 sera from two patients with primary HVH-2 (□) infection (below).

demonstrating antibody against HVH-1 as well as against HVH-2. When this proportion was reversed, no method detected HVH-2 antibody. It seems reasonable to conclude that small amounts of antibody to one HVH type mixed with large amounts of antibody to the other type, cannot be reliably detected.

HVH antibody typing in four different groups of sera from patients with active HVH-1 and HVH-2 infection

The sera from 61 patients with recurrent *herpes labialis* were shown by all three methods to contain only HVH-1 antibody. In no patient was there a history of herpetic genital infection. The sera from 15 women with recent HVH-2 genital infection but no previous history of HVH infection or antibody, were shown by all three methods to contain only HVH-2 antibody. The sera from three women with HVH-2 genital infection of less than 3 months' duration, and with remote HVH-1 infection, were shown by all three methods to contain HVH-1 antibody but not HVH-2 antibody. The sera containing HVH-1

antibody, taken from four patients with recurrent HVH-2 genital infection of more than a year's duration, were shown by the MN test to contain both types of antibody ("intermediate" or "dual" antibody); similar results were given by the PLAS inhibition and IHA inhibition methods in respect of three of the four patients, but in the fourth patient HVH-1 antibody, although present, could not be detected. The figure shows the distribution of Δt_c values for the 83 sera, as obtained in the MN test.

DISCUSSION

At present, HVH antibody typing is more easily accomplished by the IHA inhibition test than by various types of neutralisation test (Back and Schmidt, 1974). The PLAS inhibition test, like the IHA inhibition test, is simple and rapid and the specificity and sensitivity of the two techniques appear to be closely comparable.

The MN test, introduced by Pauls and Dowdle (1967), and simplified by Stalder *et al.* (1975), was modified in our experiments by substituting BS-C-1 cells for secondary rabbit kidney or diploid human-embryo fibroblast cultures. The use of comparable cells in each test eliminates a source of variation. With BS-C-1 cells, the range of Δt_c values of reference human sera consistently met the criteria proposed by Dowdle *et al.* (1967). The MN, PLAS inhibition and IHA inhibition tests gave identical results for all sera containing only monotypic HVH antibody. With sera containing both HVH-1 antibody and HVH-2 antibody, the results of the three tests were usually identical, but there was evidence that the MN test might be the most sensitive method under certain conditions, e.g., when the amount of HVH-2 antibody greatly exceeded that of HVH-1 antibody.

With regard to the reported association of HVH-2 with cervical cancer, the slightly higher sensitivity of the MN test in detecting "dual" antibody would seem of little significance because in adults HVH-2 genital infection usually follows a remote HVH-1 infection (Nahmias *et al.*, 1970). As shown in this study, an initial HVH-2 antibody response may be detected with equal ease by all three methods, but may be masked for a few months by HVH-1 antibody. Similar results have been reported by others (Nahmias *et al.*, 1970). It has been suggested that in dual infections the antigenic relationship between the two HVH types might result in the stimulation of heterotypic antibody against the first infecting virus, but that formation of antibody against the second virus is minimal or delayed (Smith *et al.*, 1972a). However, in two early HVH-2 infections following a remote HVH-1 infection (Forghani *et al.*, 1975) HVH-2 antibody has been detected by radioimmunoassay.

Reservations concerning the reliability of neutralisation tests for typing HVH antibody in hyperimmune animal antisera (Skinner *et al.*, 1976), have not been supported by our experiments with human reference sera. Forghani *et al.* (1975) found that radioimmunoassay, the MN test and the IHA inhibition test all gave similar results in the typing of HVH antibodies.

In conclusion, we believe that the MN test remains the most sensitive test for detecting "dual" HVH antibody in human sera. However, the PLAS

inhibition technique should prove to be a valuable tool for epidemiological studies and a satisfactory alternative to the IHA inhibition test.

SUMMARY

A new technique based upon the inhibition of the peroxidase-labelled antibody staining (PLAS) has been used to type *Herpesvirus hominis* (HVH) antibodies in four groups of human sera taken from patients with one or both types of HVH infection and in mixtures of different proportions of type 1 and type 2 antisera. The results were compared with those of the microneutralisation (MN) test and the indirect haemagglutination (IHA) inhibition test. The sensitivity and specificity of the three methods were identical for sera containing only one type of HVH antibody. The MN test was slightly more sensitive than the other tests for detecting small amounts of HVH-1 antibody mixed with large amounts of HVH-2 antibody. Nevertheless, the PLAS inhibition technique was far more rapid and it would seem a satisfactory alternative to the IHA inhibition test for HVH antibody typing.

REFERENCES

- ADAM, E., KAUFMAN, R. H., MELNICK, J. L., LEVY, A. H. AND RAWLS, W. E. 1973. Sero-epidemiologic studies of herpesvirus type 2 and carcinoma of the cervix. IV. Dysplasia and carcinoma in situ. *Am. J. Epidemi.*, **98**, 77.
- AVRAMEAS, S. AND TERNYNCK, T. 1971. Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochemistry*, **8**, 1175.
- BACK, A. F. AND SCHMIDT, N. J. 1974. Typing *Herpesvirus hominis* antibodies and isolates by inhibition of the indirect hemagglutination reaction. *Appl. Microbiol.*, **28**, 400.
- BERNSTEIN, M. T. AND STEWART, J. A. 1971. Method for typing antisera to *Herpesvirus hominis* by indirect hemagglutination inhibition. *Appl. Microbiol.*, **21**, 680.
- DOWDLE, W. R., NAHMIAS, A. J., HARWELL, R. W. AND PAULS, F. P. 1967. Association of antigenic type of *Herpesvirus hominis* with site of viral recovery. *J. Immun.*, **99**, 974.
- FAZEKAS DE ST. GROTH, S. 1961. Evaluation of quantal neutralization tests. *Nature, Lond.*, **191**, 891.
- FORGHANI, B., SCHMIDT, N. J. AND LENNETTE, E. H. 1975. Solid-phase radioimmunoassay for typing herpes simplex viral antibodies in human sera. *J. clin. Microbiol.*, **2**, 410.
- GERNA, G., MCCLOUD, C. J. AND CHAMBERS, R. W. 1976a. Immunoperoxidase technique for detection of antibodies to human cytomegalovirus. *J. clin. Microbiol.*, **3**, 364.
- GERNA, G., VASQUEZ, A., MCCLOUD, C. J. AND CHAMBERS, R. W. 1976b. The immunoperoxidase technique for rapid human cytomegalovirus identification. *Archs Virol.*, **50**, 311.
- MELNICK, J. L., COURTNEY, R. J., POWELL, K. L., SCHAFFER, P. A., BENYESH-MELNICK, M., DREESMAN, G. R., ANZAI, T. AND ADAM, E. 1976. Studies on herpes simplex virus and cancer. *Cancer Res.*, **36**, 845.
- NAHMIAS, A. J., JOSEY, W. E., NAIB, Z. M., LUCE, C. F. AND DUFFEY, A. 1970. Antibodies to *Herpesvirus hominis* types 1 and 2 in humans. I. Patients with genital herpetic infections. *Am. J. Epidemi.*, **91**, 539.
- NAIB, Z. M., NAHMIAS, A. J., JOSEY, W. E. AND KRAMER, J. H. 1969. Genital herpetic infection associated with cervical dysplasia and carcinoma. *Cancer, Philad.*, **23**, 940.
- PAULS, F. P. AND DOWDLE, W. R. 1967. A serologic study of *Herpesvirus hominis* strains by microneutralization tests. *J. Immun.*, **98**, 941.
- RAWLS, W. E., TOMPKINS, W. A. F., FIGUEROA, M. E. AND MELNICK, J. L. 1968. Herpes virus type 2: association with carcinoma of the cervix. *Science, N. Y.*, **161**, 1255.

- REED, L. J. AND MUENCH, H. 1938. A simple method of estimating fifty per cent end points. *Am. J. Hyg.*, **27**, 493.
- SKINNER, G. R. B., HARTLEY, C. AND WHITNEY, J. E. 1976. Detection of type-specific antibody to herpes simplex virus type 1 and 2 in human sera by complement-fixation tests. *Archs Virol.*, **50**, 323.
- SMITH, J. W., ADAM, E., MELNICK, J. L. AND RAWLS, W. E. 1972*a*. Use of the ^{11}Cr release test to demonstrate patterns of antibody response in humans to herpes virus types 1 and 2. *J. Immun.*, **109**, 554.
- SMITH, J. W., LOWRY, S. P., MELNICK, J. L. AND RAWLS, W. E. 1972*b*. Antibodies to surface antigens of herpesvirus type 1- and type 2-infected cells among women with cervical cancer and control women. *Infect. Immun.*, **5**, 305.
- STALDER, H., OXMAN, M. N. AND HERRMANN, K. L. 1975. Herpes simplex virus micro-neutralization: a simplification of the test. *J. inf. Dis.*, **131**, 423.