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Typing of Isolates of Herpes Simplex Virus by Mixed Agglutination (38039)

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The characteristics of herpes simplex virus (HSV) type 1 surface antigen(s) appearing on infected cells have been studied in our laboratory by the method of mixed agglutination (MA) (1). Since it has been demonstrated by membrane immunofluorescence (2, 3) that type specificity may be shown for surface antigens, our studies were extended to characterization of HSV type 2 surface antigen and the use of MA for typing HSV isolates. The results of this investigation are the subject of this communication.

Materials and Methods. Cell cultures. BIRK, a continuous line derived from rabbit kidney cells, and HEp-2, were used for detection of HSV surface antigen (1). Cells were grown in Eagle's basal medium (BME) in a base of Hank's balanced salt solution supplemented with 10% fetal calf serum, penicillin (200 units/ml) and streptomycin (200 μg/ml). After virus inoculation, cultures were maintained in BME in a base of Earle's saline (sodium bicarbonate 1.8 mg/ml), 3% fetal calf serum, and antibiotics. BGM, a continuous line derived from African green monkey kidney cells, was used for assay of infectivity (4). Cells were grown in Eagle's minimal essential medium (MEM) in Earle's saline and maintained in MEM with 3% fetal calf serum following virus inoculation.

Viruses. Herpes simplex virus (HSV) type 1 strains were: MacIntyre VR#3 (1), F and MP (refer Results) supplied by Dr. B. Roizman, University of Chicago, Chicago, Ill., and HF propagated in this laboratory. Type 2 strains were: MS (1), and 69-375 from the Erie County Virology Laboratory. Isolates of HSV were supplied by the Erie County Virology Laboratory. Laboratory strains were plaque-purified twice in BGM and passaged once in BIRK. Most HSV isolates were employed after 1–3 passages in BIRK or HEp-2.

Sera. Rabbit antisera to HSV type 1 (VR#3), #284, and type 2 (MS), #274 have been previously described (1).

Absorption of sera. Sera were absorbed with BGM cells infected with either type 1 or type 2. Cells were infected with approximately 0.1–0.3 PFU/cell and incubated at 36° for 24–48 hr. Infected cells were collected by scraping, and after washing with phosphate buffered saline (PBS) pH 7.2, a cell pack was obtained by centrifugation at 2,000 rpm for 15 min. One ml of a 1:20 dilution of antiserum was mixed with approximately 5×10^7 packed infected cells in a volume of approximately 0.1 ml and incubated at 36° for 2 hr followed by further incubation overnight at 5°. The serum was removed following centrifugation and absorption was repeated a second time. The absorbed serum was heated at 56° for 20 min to inactivate any infectious virus.

Mixed agglutination (MA). The method used has been reported in detail elsewhere (1, 5). The attachment of rabbit anti-HSV antibodies to infected cell cultures was revealed by the indicator system. In the present experiments the indicator cell suspension was prepared with sheep erythrocytes (SRBC) + rabbit anti-SRBC serum + sheep anti-rabbit globulin serum, Grand Island Biological Company, Grand Island, N. Y. The sheep anti-rabbit globulin serum served as a bridge between the rabbit anti-HSV antibodies and the indicator RBC. The

mixed agglutination test was read by examination of the cultures for adherence of the indicator cells through the cell monolayers using the low power of the microscope. Positive reactions were graded from 1+ to 4+ depending on the degree of adherence. For titration of sera, the endpoint was determined as the highest dilution of serum yielding a 2+ or stronger MA reaction.

Typing of HSV isolates by MA. Three BIRK or HEp-2 cultures were inoculated with the HSV isolate to produce a moderate cytopathic effect following incubation for 16–20 hr at 36°. Infected cultures were treated with 1% formalin in PBS for 30 min at 36° as described in the Results section. After washing twice with PBS, 1 culture was treated with a 1:3000 dilution of monotypic anti-HSV type 1 serum prepared by absorption and a second culture received 1:3000 dilution of monotypic anti-HSV type 2 serum (see Results). The remaining culture served as control. Following incubation for 90 min at 25° cultures were washed twice with PBS and the indicator cells were added. The isolate could be typed by the presence of a positive MA reaction in the culture tube treated with the appropriate serum.

Neutralization. HSV neutralizing antibody titers were determined by an 80% plaque reduction method as previously reported (6). Kinetics of neutralization were studied using procedures reported by McBride (7) and Geder and Skinner (2) with slight modifications. The neutralization rate constant k of each strain was calculated for the anti-type 1 (k_1) or anti-type 2 (k_2) serum used in this study. The serum was diluted 1:10, warmed to 36° for 10 min, and mixed with an equal volume of virus diluted to contain approximately 3×10^5 PFU. Following incubation for 2 min at 36°, 0.2 ml of the mixture was removed and diluted 1:100 in LH (1) maintained in an ice bath to prevent further neutralization. The mixture was then assayed for infectious HSV. The k value was calculated by the formula:

$$k = \frac{2.3 \log (V_o/V_t)}{Ct}$$

where C = concentration of antiserum, t = time in min, V_o = original virus infectivity, and V_t = surviving infectivity at time t (2).

Chemical treatment of surface antigen. Treatment of HSV-infected HEp-2 cells with formalin, periodate, 2,4-dinitrophenol (DNP), acetone, and ethanol was as described previously (1).

Adherence of sensitized SRBC. The pro-

TABLE I. Production of HSV Monotypic Antisera for Mixed Agglutination by Absorption with Cells Infected with Heterologous Type.

	Antibody Titer			
	MA[a]		Neutralization[b]	
Serum	Type 1 (VR#3)	Type 2 (MS)	Type 1 (VR#3)	Type 2 (MS)
Anti-type 1				
Unabsorbed	5.0	3.5	2560	320
Absorbed with[c]				
Type 1 cells	<2.0	<2.0	<20	<20
Type 2 cells	4.5	<2.0	320	<20
Anti-type 2				
Unabsorbed	4.5	5.0	80	1280
Absorbed with				
Type 1 cells	<2.0	5.0	<20	160
Type 2 cells	<2.0	<2.0	<20	<20

[a] Mixed agglutination ($\log_{10}$).

[b] Reciprocal of highest dilution of serum yielding 80% plaque reduction.

[c] Rabbit antiserum was absorbed with BGM cells infected with either type 1 or type 2 (refer *Materials and Methods*).

cedure for adherence of SRBC treated with rabbit anti-SRBC serum to cell cultures infected with HSV, as originally reported by Watkins (8), was used as previously reported (1).

Results. As shown in Table I, specific monotypic antiserum reactive with surface antigen produced on cells infected by type 1 or type 2 could be prepared by absorption with cells infected with the heterologous type. The absorbed sera were also monotypic in the plaque neutralization test.

Occasionally, adherence of indicator cells occurred in the absence of serum and treatment with 1% formalin in PBS obliterated this nonspecific adherence. Cell cultures were treated with 1% formalin in MA tests used for typing of isolates and this method was employed in the experiments described below.

Laboratory strains of type 1 and type 2 were tested as well as clinical isolates. The results of typing by the MA test were compared to the kinetic neutralization test (Table II). Laboratory strains and isolates could be readily typed by the MA test and the results were compatible with the kinetic neutralization test in which type 1 strains had a high k_1/k_2 ratio (>1.0) and type 2 strains had a low ratio (<1.0). The MA results were also in agreement with the typing of laboratory strains as reported in other studies (2, 9, 10). MP was classified as intermediate by Roizman *et al.* (11) and type 1 by MA and kinetic neutralization in the present study.

TABLE III. Typing of HSV Isolates by Mixed Agglutination.

Site	Number of isolates		
	Type 1	Type 2	Total
Genital-rectal	4	15	19
Oral	53	1[a]	54
Dermal	6	2	8
Ocular	6	0	6
Other[b]	3	6	9
Total	72	24	96

[a] Patient 71–849 see Table V.
[b] Various tissues, see Tables IV and V.

As shown in Table III, a total of 96 isolates were typed by the MA test; 72 were identified as type 1 and 24 as type 2. Of 54 isolates from the oral cavity, 53 were type 1, whereas, 15 of 19 isolates from the genital tract were type 2. Typing of isolates recovered from post mortem cases is tabulated in Table IV. The majority of isolates from neonatal cases were identified as type 2. A number of cases were available in

TABLE II. Typing of HSV Strains by Mixed Agglutination and Kinetic Neutralization.

HSV strains	Mixed agglutination			Kinetic neutralization			
	Anti-type 1	Anti-type 2	Type	k_1	k_2	k_1/k_2	Type
Laboratory							
VR#3	+	–	1	5.02	0.48	10.43	1
HF	+	–	1	8.68	1.02	8.50	1
F	+	–	1	4.33	0.28	15.44	1
MP	+	–	1	6.67	1.36	4.91	1
MS	–	+	2	2.76	7.05	0.39	2
69–375	–	+	2	3.14	6.33	0.50	2
Isolate							
70-642	+	–	1	10.4	0.83	12.6	1
71-351	+	–	1	7.80	0.35	22.3	1
71-536	+	–	1	5.66	0.78	7.25	1
71-1141	+	–	1	9.65	1.35	7.13	1
71-183	–	+	2	2.96	15.5	0.19	2
71-849	–	+	2	0.74	8.40	0.09	2
71-921	–	+	2	2.99	7.13	0.42	2

TABLE IV. Typing of HSV Isolates from Post Mortem Cases by Mixed Agglutination.

Patient	Specimen	Type 1	Type 2
Neonatal			
70-480	Lung	X	
68-359	Lung		X
69-91	Skin ?		X
69-280	Liver		X
72-1283	Brain, Lung, Lesion (pre-mortem)		X
Adult			
71-536	Brain	X	

which multiple isolates were obtained from different sites (Table V). In no instance were 2 types isolated from the same person. Type 1 was recovered from oral infections or infections of other sites and type 2 was recovered when genital infections were apparent.

Surface antigen produced on cells infected by type 2 strains was stable to heat at 56°, formalin, periodate, and DNP, but was not detected after treatment with acetone or ethanol in agreement with results obtained for type 1 (1). It was found previously (1) that for VR#3 strain of type 1, adsorption of sensitized SRBC was inhibited following treatment with formalin or DNP, which distinguished this reaction from MA. In further experiments, adsorption of SRBC was also inhibited by periodate. However, treatment with DNP or periodate did not affect adsorption of sensitized SRBC on cell cultures infected with other type 1 or type 2 strains and the difference was apparently restricted to VR#3 strain.

Discussion. In the present study using MA, cross-reactions were obtained with type 1 and type 2 and specificity was demonstrated only following absorption of sera. In typing HSV strains by membrane immunofluorescence (2, 3) it was also necessary to absorb the sera. Antisera could be typed by absorption as determined in the indirect hemagglutination-inhibition test (12). Absorption of serum with cells infected with the heterologous type for MA also affected the neutralization test but apparently not to the same degree (Table I). These results might be a consequence of the sensitivity of the 2 methods or may indicate that the envelope antigens of HSV virions are not completely equivalent to surface antigens which appear on the plasma membrane of infected cells as suggested by Falke *et al.* (13).

Many procedures have been employed to distinguish HSV types and several have been adapted for rapid identification of isolates (2, 3). The MA test is useful for iden-

TABLE V. Typing of HSV Isolated from Multiple Sites by Mixed Agglutination.

Patient No.		Type 1	Type 2
70-1017	Lesion, Throat (NT) [a]	X	
71-1088	Finger lesion, Eye swab	X	
73-818	Conjunctiva swab, Lip swab (NT)	X	
73-1272	Brain biopsy, Throat, Lesion	X	
73-1323	Throat, Lesion	X	
71-183	Genital lesion, Face lesion (NT)		X
71-921	Skin lesion, Vaginal lesion (NT)		X
71-849	Skin swab, Throat, Rectal		X

[a] NT—Not tested.

tification of HSV isolates since the cell cultures are directly employed and this can even be done (unpublished data) following primary isolation from the specimen. The clinical and epidemiological significance of HSV types has recently been discussed (14).

Summary. Cross-reactions were obtained between HSV type 1 and type 2 rabbit antisera using the mixed agglutination (MA) test for detection of HSV surface antigen on infected cells. Monotypic sera could be prepared by appropriate absorption with cells infected with the heterologous type. Using monospecific sera, HSV isolates were typed by MA. Results obtained by MA revealed solid agreement with kinetic neutralization test. A total of 96 isolates were typed and the association with the clinical situation was as might have been expected. The characteristics of surface antigen produced by HSV type 2 were found to be similar to those described in previous studies from this laboratory for HSV type 1.

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