Herpesvirus hominis Types I and II: A Specific Microindirect Hemagglutination Test (34554)

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Recent findings demonstrate that strains of *Herpesvirus hominis* (HVH) can be grouped into two antigenic types designated as Type I or Type II (1, 2). Various techniques such as chorioallantoic membrane plaquing (3), kinetic neutralization (4), quantal microneutralization (1), and complement fixation (CF) (5) have been used to determine the antigenic type of isolates and to differentiate type-specific antisera. The tissue culture procedures required for the plaquing and neutralization tests are quite cumbersome, slow, and expensive. The CF test does not appear to be as sensitive as the quantal microneutralization test in detecting prior infection with both HVH types (5).

A macroindirect hemagglutination test for herpesvirus was described by Scott and *et al.* in 1957 (6). We could find no other publications relating to this method and no reports of its application to type-specific antibody studies of HVH. A micromodification of the original test was made in our laboratory and a new tissue culture line was used to prepare the viral antigens for both Type I and Type II strains of herpesvirus. The present report describes the indirect hemagglutination test (IHA) for detection of strain specific herpesvirus antibody and compares this with the quantal microneutralization and standard CF tests.

Materials and Methods. Virus strains. The HVH strains VR_3 (Type I) and MS (Type II) were obtained from Dr. Andre J. Nahmias, Emory University, Atlanta, Georgia, at passage level 25 and passed an additional 10 times in our laboratory. These virus strains were used for the microneutralization test and for the production of viral antigens used in the IHA method. Herpes simplex antigen (MacIntyre strain), for standard CF testing was obtained from Microbiological Associates, Bethesda, Maryland.

CF test. Tests were performed utilizing the microtechnique (7) with two exact units of complement and four units of antigen.

Tissue culture. Both strains of HVH were propagated in MA-196, a new human skin line (Microbiological Associates, Bethesda, Maryland). Cell culture growth medium consisted of Eagles' minimal essential medium (MEM) with 10% fetal calf serum. MEM with 1% fetal calf serum was used for culture maintenance. All media contained penicillin (100 units/ml) and streptomycin (100 μg/ml).

Primary rabbit kidney (PRK) cell cultures (MicroTest II tissue culture plates, Baltimore Biological Laboratories, Cockeysville, Maryland) were used for the microneutralization tests with the same growth and maintenance medium as above.

Microneutralization test. The quantal microcrossneutralization test of Pauls and Dowdle (1) for determining the amount of antibody to the two strains of HVH was modified and performed as previously described (8). After the neutralization titers were determined by the Reed-Muench method, the pN values (loglog of virus neutralized — log of final serum endpoint + log of test volume) were calculated for each specific antiserum (1, 9). This calculation expresses the neutralizing potency of an antiserum to the particular strain of herpesvirus by correcting for the final dilution of serum and virus. Using established criteria (10), a pN difference between Type I and Type II of less than + 0.05

was considered to be a Type 2 response and a pN difference of + 0.05 or greater was considered to be a Type 1 response.

Antigen preparation. The MA-196 cell cultures were grown to confluency in 32-oz (surface area 135 cm^2) glass bottles. After removal of growth medium, the cell cultures were inoculated with 1 ml of Type I (titer of $10^{7.5}TCID_{50}$/ml on PRK) or Type II (titer of $10^{6.0}TCID_{50}$/ml on PRK) HVH seed virus. Virus absorption was allowed to occur for 30 min at room temperature, then maintenance medium (40 ml per bottle) was added and the bottles incubated at 37°. Cultures were observed daily until a 3 – 4+ cytopathic effect was obtained, which occurred within a 4-day period. The cells were then scraped from the bottle with a rubber spatula and the suspension centrifuged at 600*g* for 10 min in a refrigerated (4°) centrifuge. Supernatant fluids were removed, and the packed cells were resuspended in phosphate buffered saline (PBS) (pH 7.2) to give a 10 % cell suspension. The resuspended cells were repetitively frozen at —90° and thawed three times. After the third thaw, cellular debris was removed by centrifugation at 600*g* for 10 min at 4° and the clear supernatant fluid was used as the antigen preparation.

Sera. Fourteen pairs of human sera which demonstrated seroconversion for HVH with the standard CF test were coded by our statistical personnel and returned to the laboratory to be tested. The sera were initially screened for herpes antibody by macroneutralization in PRK using a 1:4 dilution of serum in MEM with 2 % fetal calf serum (11). Two-fold dilutions of inactivated sera, starting at a 1:10 dilution, were used for the microneutralization test with 100 $TCID_{50}$ of either Type I or Type II HVH. Heat inactivated (56°, 30 min) normal rabbit serum (NRS) diluted to a 2 % solution in PBS was used as diluent for the IHA test. The initial dilution of sera tested was 1:8 with IHA method.

Indirect hemagglutination test. The test used was a micromodification of the macrohemagglutination technique of Scott *et al.* (6). Sheep erythrocytes (3 % suspension) were washed three times in PBS and then treated with an equal volume of freshly prepared tannic acid (Fischer Scientific Co., Fairlawn, N. J., Lot #773722) diluted 1:80,000 in PBS. The suspension was incubated at 37° in a water bath for 15 min, then centrifuged at 600*g* for 10 min. After washing once in one volume of PBS, the tanned cells were sedimented by centrifugation (600 *g*, 10 min), the supernatant fluid was discarded, and the cells resuspended to a 3 % concentration in saline.

Cells were then sensitized with a previously determined dilution of antigen by mixing equal volumes of cells (3 %) and antigen in saline (pH 6.4), and the mixture was allowed to stand at room temperature for 15 min. The suspension was centrifuged at 600*g* for 10 min (4°) to pack the cells and then washed in two volumes of 1 % NRS. Cells were then centrifuged and resuspended in 1 % NRS. The dilution of antigen-sensitized cells used in the test was determined by chessboard titration using two-fold dilutions of antigen against an immune serum previously typed by the microneutralization test. Antigens prepared as described above were satisfactory when employed in a dilution of 1:4. This dilution was selected since further increase in antigen dilution did not appreciably change the titer of the control immune serum.

Testing was performed with the micromethod utilizing spiral loops and V-bottom, soft, plastic plates (Cooke Engineering Co., Alexandria, Va.), as previously described (7). Equal volumes (0.025 ml) were used for each reagent. After making two-fold serum dilutions in 2 % NRS, 0.025 ml of 2 % NRS and 0.025 ml of sensitized 1 % sheep erythrocytes were added to each well. Plates were then sealed with cellophane tape, shaken by hand and read after incubation at room temperature for 1½ hr.

A standard antiserum was included in all tests. Controls consisted of serum diluent with sensitized and antigen-free tanned red cells. Titers were taken as the highest dilution of serum which gave a 3 + agglutination

on a scale of 0 to 4 +.

Results. Attempts to produce antigen by growing the two types of HVH on a number of other cell lines usually failed because of a lack of production of hemagglutinating antigen or the antigen produced gave irregular results. In our hands, the MA-196 cell lines proved to be the best tissue for antigen production since high titers and specificity were demonstrated with all reference sera. Human sera with and without CF antibody for herpes zoster and cytomegalovirus, and with no antibody to *Herpesvirus hominis,* as tested with the microneutralization method, were used to detect possible cross-reactions with the IHA test. No cross-reactions were found. No high speed centrifugation or further purification was needed to produce satisfactory antigens. Testing of original culture fluids did not demonstrate sufficient nor usable antigen.

To determine the sensitivity of the herpes IHA test for the serological determination of Types I and II herpesvirus antibody, acute and convalescent sera from 14 patients with standard CF seroconversions to herpes were selected. The sera were then coded and tested with the microneutralization test and the IHA techniques. Results of the serological tests are summarized in Table I.

In general, the IHA titers were 4–8 fold higher than those obtained by microneutralization. No neutralizing or IHA antibody was detected in the acute serum of seven patients (#1, 2, 3, 6, 8, 9, 13), whereas six of these patients demonstrated a trace CF reaction at a 1:8 dilution. In three acute specimens (#5, 7,12), the IHA test was positive in low titers and negative by microneutralization at a 1:10 dilution. Two of these specimens demonstrated trace CF reactions.

Type I HVH infection was clearly demonstrated by microneutralization of nine pairs of sera (#1, 3, 4, 5, 6, 7, 10, 12, 13) and possibly one other (#8). The pattern of response of the IHA definitely confirmed these results in 8 (#1, 3, 4, 5, 6, 7, 12, 13) and probably a ninth (#10). In one case (#8), a borderline intermediate - Type I infection by microneutralization appeared to have a probable Type II response by IHA. The paired sera of patient #2 showed a CF seroconversion and slight IHA rise, but no neutralizing antibody was detected in either specimen at a 1:10 dilution. A CF rise was also shown in one case (#11), but no evidence for infection could be demonstrated by neutralization or IHA testing.

Two Type II HVH responses were demonstrated by microneutralization (#9, 14); the neutralization and IHA tests showed a parallel response to both strains of HVH in patient #9. In the other case, serum was not available to evaluate the convalescent specimen by IHA.

Discussion. In an effort to obtain the flexibility and ease of performance of a rapid serological test for type-specific herpesvirus antibody, the indirect hemagglutination technique was investigated using a microserological system. The data presented in this report indicate that the type-specificity of the HVH antibody response obtained by the IHA test correlates quite well with data obtained with microneutralization. When no neutralizing antibody was found, IHA antibody was absent or very low in all cases tested. Furthermore, Type I HVH infection (as determined by microneutralization) was detected by the IHA test, even in the presence of preexisting antibody. In only one case (#8) was an HVH antibody-type discrepancy possibly evident between the IHA and microneutralization tests. The technical difficulties of performing microneutralization at very low titers of serum are considerable because of the cytotoxicity and foreign material found in some serum specimens. The significance of this one discrepancy is questionable since there is only a two-fold difference in the IHA titers to the HVH types. A later convalescent serum might allow for sufficient antibody differentiation to discriminate a specific type-response by both tests.

In five cases (#2, 3, 4, 6, 12), there was a definite rise to only one type of HVH with the IHA test. In these cases, there was no cross-reacting response by this method, whereas there generally was such a response by the microneutralization test. With one patient (#9), titers obtained with the IHA test were identical, thus no definitive type response could be determined. Neutralization

TABLE I. Serological studies on *Herpesvirus hominis* type I and II infections.

	Serum, acute (A) or convalescent (C)	Complement fixation titer[a]	Microneutralization test: Neutralizing antibody titers[a] Type I	Microneutralization test: Neutralizing antibody titers[a] Type II	Microneutralization test: pN difference, Type I—Type II	Indirect herpes hemagglutination titers: Type I	Indirect herpes hemagglutination titers: Type II
1.	A	T[b]	<10	<10	—	<8	<8
	C	16	550	56	+0.9290	4096	1024
2.	A	T	<10	<10	—	<8	<8
	C	16	<10	<10	—	16	<8
3.	A	T	<10	<10	—	<8	<8
	C	64	17	2	+0.5898	64	<8
4.	A	T	40	10	+0.4653	256	256
	C	16	1145	95	+1.0128	>8196	256
5.	A	T	<10	<10	—	8	<8
	C	32	80	10	+0.8358	512	16
6.	A	T	<10	<10	—	<8	<8
	C	32	13	2	+0.6824	16	8
7.	A	T	<10	<10	—	16	8
	C	32	844	113	+0.7159	>1024	128
8.	A	T	<10	<10	—	<8	<8
	C	32	10	2	+0.5624	16	32
9.	A	T	<10	<10	—	<8	<8
	C	64	67	61	−0.1263	256	256
10.	A	T	378	24	+1.0375	512	64
	C	16	761	44	+1.1749	8192	2048
11.	A	<8	95	20	+0.6098	1024	256
	C	32	177	26	+0.6817	1024	256
12.	A	<8	<10	<10	—	8	<8
	C	16	211	10	+1.1575	512	<8
13.	A	<8	<10	<10	—	<8	<8
	C	32	394	26	+1.0075	256	16
14.	A	T	758	10	+1.7127	>1024	128
	C	32	1808	287	+0.6630	QNS	QNS

[a] Reciprocal of titer dilution.

[b] T = Trace reaction at 1:8 dilution.

titers on this patient were also very close; however, the pN difference indicated a Type II response. It would appear, therefore, that this type of response with the IHA (a parallel antibody rise in both Type I and Type II) is also indicative of a Type II infection.

The present findings suggest that the IHA test, performed as described, is comparable in accuracy and as sensitive a diagnostic method as the quantal microneutralization test. Repeat serum titrations (using two new lots of Type I and Type II antigens) were reproducible within two-fold of the original determination. Furthermore, the IHA test is rapid, more easily performed, and more economical than the standard microneutralization tissue culture method. Since the levels of antibody detected by the IHA test are slightly higher, separation of cross-reacting antibodies should be easier to evaluate. With this technique

the effects of recurrent episodes of herpesvirus infection on the levels of type-specific antibody should be rapidly demonstrated.

The relatively simple procedure developed for preparation of type-specific hemagglutinating antigens demonstrates the added potential usefulness of applying the method for the classification of herpesvirus isolates.

The IHA test was found to be very stable with excellent persistence of agglutination. Readings can be made after 1½ hr at room temperature or overnight at 4°. The test patterns did not change after 5 days of storage at 4°. This flexibility and stability will enable laboratories to perform large scale testing without loss of accuracy. The ease of performance should make this technique a valuable tool for the detection of Type I and Type II herpesvirus infection.

Summary. A microindirect hemagglutination test (IHA) for the determination of type-specific antibody to *Herpesvirus hominis* (Types I and II) was developed and evaluated in comparison to the quantitative microneutralization procedure. Paired human sera from 14 patients with complement-fixing antibody rises to herpesvirus were tested with all three methods. The IHA test, as described, appears to be rapid, simple and valuable technique for a detection of Type I and Type II herpesvirus infections and may be useful for classifying herpesvirus isolates.

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