Type-Specific Surface Antigens of Cells Infected with Herpes Simplex Virus (1 and 2)[1] (35824)

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Application of immunodiffusion and inhibition-passive hemagglutination techniques to the study of antigens of type 1 and type 2 herpes simplex virus (HSV) has revealed that, in addition to common antigens, there are also specific type antigens for each HSV type (1). On the basis of these findings, a working formula was developed: type 1 HSV = AC; type 2 HSV = BC; where A, B, or C may comprise more than one antigen. It was of interest to determine if the same antigenic pattern could be detected on the surface membrane of type 1 or type 2 HSV-infected cells by application of membrane fluorescence (MF) techniques.

This report describes results of both direct and indirect membrane fluorescence techniques, as applied to tissue culture cells infected with type 1 or type 2 HSV, which provide further corroboration for the presence of specific antigens for each of the two HSV types.

Materials and Methods. Virus strains. Most of the experiments were done with the MS (type 2) and VR_3 (type 1) HSV strains (2). For certain experiments, recent isolates were also used: type 1 HSV-Tyler (cornea); type 2 HSV-Curtis (female genitalia).

Preparation of infected cells for MF test. Monolayers of HEp-2 tissue culture cells were prepared using Eagle's MEM containing 10% calf serum. Type 1 or type 2 HSV was added to a 32-oz bottle to yield a multiplicity of infection of approximately 5–10:1. When 3–4+ cytopathic effect was demonstrated after incubation at 36° (usually 15–18 hr for type 2 and 30–36 hours for type 1 HSV), the infected cells were beaded off and washed 3 times in PBS (pH 7.5) by centrifugation at 1000 rpm for 10 min. After the final wash, the pellet was resuspended in 10–15 ml of PBS depending on cell concentration. Noninfected HEp-2, human fibroblast or BHK-21 cells, prepared in a similar manner, were also used as controls.

Membrane fluorescence techniques. a. Direct tests. The preparation of fluorescein-conjugated type 1 and type 2 rabbit antisera, and sorption of the conjugated sera with 2 mouse liver powder sorptions (1 hr each at 4°) and HEp-2 pellet overnight has been described earlier (3). To aliquots of type 1, type 2, or noninfected HEp-2 pellets, several dilutions of the conjugated antisera were first used to ascertain the titer (Table I). Thereafter, undiluted type 1 conjugated serum or 1:2 dilution of type 2 conjugated serum was used for most experiments. The cells were exposed to the conjugate for 30 min at 37°, then washed 3 times with PBS (pH 7.5), and resuspended in PBS with 50% glycerine. Controls included fluorescein-conjugated normal rabbit serum.

b. Absorption procedures. To prepare specific anti-1 or anti-2 conjugated sera, type 1 or type 2 fluorescein conjugates were absorbed with homologous or heterologous HSV-infected HEp-2 cells, or with noninfected HEp-2 cells, as follows: to a pellet of HSV-infected live cells, prepared as described above, an equal volume of conjugated antisera was added, incubated in a rotor at 36° for 1 hr, and the supernate, after centrifugation at 2000 rpm for 10 min, used for testing

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TABLE I. Membrane Fluorescence Obtained by the Direct Method Using Varying Dilutions of Fluorescein-Conjugated Antitype 1 or Antitype 2 Sera to Infected Type 1 HSV, Infected Type 2 HSV, and Noninfected HEp-2 Cells.

	Dilution of conjugated serum	Infected HEp-2 cells				Noninfected HEp-2 cells
		Type 1		Type 2		
		%[a]	—[b]	%[a]	—[b]	
Fluorescein-conjugated antitype 1 serum	Undiluted	98	3–4+	82	2–3+	0
	1:2	92	3–4+	56	2–3+	0
	1:4	90	3+	24	1–3+	0
	1:8	78	2–3+	1	1+	0
	1:16	20	1+	0		0
Fluorescein-conjugated antitype 2 serum	Undiluted	82	2–3+	65	2–3+	0
	1:2	66	2–3+	43	2–3+	0
	1:4	32	2+	14	2+	0
	1:8	20	1+	5	1+	0
	1:16	5	±	0		0

[a] Average from several experiments of percentage of cells demonstrating membrane fluorescence.

[b] Intensity of fluorescence.

with type 1, type 2, or noninfected live HEp-2 cells. To prepare cells which demonstrated only specific antigens, type 1 infected HEp-2 cells were exposed for 90 min in the rotor at 36° with undiluted human serum containing type 2 antibodies, as determined previously by a microneutralization technique (4). Similarly, type 2 infected cells were exposed to human serum containing anti-1 antibodies. The cells were sedimented, washed 2× with PBS, and stained with nonsorbed conjugated antitype 1 or antitype 2 sera.

c. Indirect tests. Sera from rabbits either hyperimmunized (2) or infected via the cornea or brain (5), and human sera previously assayed for HSV type antibodies (4), were used as follows: a 1:2 dilution of rabbit sera or a 1:4 dilution of human sera was applied to type 1, type 2, or noninfected HEp-2 live cells, incubated for 30 min at 36° and washed 2 times with PBS; fluorescein-conjugated goat antirabbit gamma globulin serum (Antibodies Inc. Labs)* or fluorescein-conjugated antihuman IgG serum (Hyland Labs) was then applied to the appropriate serum–cell system for 30 min at 36° and washed 3 times with PBS (pH 7.5); an equal volume of glycerine was then added to the cell pellet. A fluorescein-conjugated goat antihuman IgM (obtained from C. Reimer) was used in a similar way using sera of newborns found earlier (6) to possess HSV antibodies in the IgM serum fraction. Sera of individuals with primary herpetic infections, as well as genital secretions of women with type 2 HSV infection, were also tested in a similar manner with fluorescein-conjugated antihuman IgA serum (Hyland Labs). Fluorescein-conjugated antisera used were sorbed with two mouse liver powder and one HEp-2 cell sorptions, as described above for the antitype 2 conjugates. Rabbit sera and human sera and secretions, which contained no HSV antibodies, were also used for control purposes.

In order to compare results obtained with the MF technique using live cells, similar preparations were also tested with acetone-fixed cells, obtained as previously described (3).

d. Examination of cell preparations for fluorescence. One drop of the suspended cells of the various preparations described above was placed on a slide and mounted with a cover slip. The cells were examined, either immediately or within 2 days (cells saved at 4°), with a Leitz Ortholux microscope using a BG-12 primary filter and UG-1 secondary filter.

Results. Determining titers of FA con-

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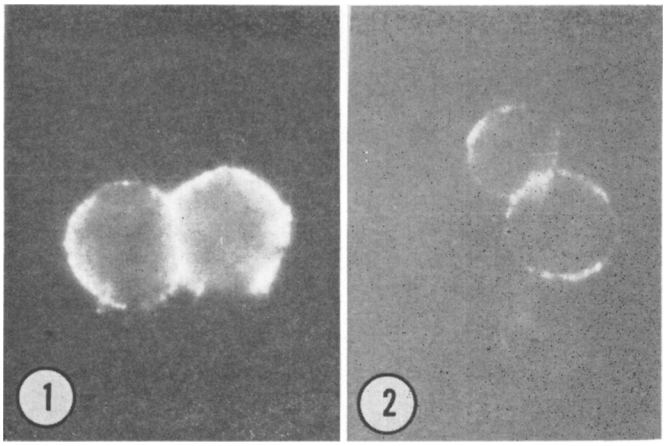

FIG. 1. Membrane fluorescence (complete rim) observed in type 1-infected cells with fluorescein-conjugated antitype 1 rabbit (similar fluorescence is observed with antitype 2 conjugated serum).

FIG. 2. Membrane fluorescence (partial rim) observed in type 2-infected cells with fluorescein-conjugated antitype 2 rabbit serum (similar fluorescence is observed with antitype 1 conjugated serum).

jugates. Table I presents a summary of results obtained from several experiments aimed at determining the titers of antitype 1 and antitype 2 fluorescent antibody (FA) conjugates to use for the detection of membrane fluorescence. The membrane fluorescence noted in type 1-infected cells exposed to the less dilute FA conjugates to either HSV type consisted most commonly of complete (Fig. 1) or partial rims; less frequently, the fluorescence detected consisted of several spots on the cell membrane. In case of type 2-infected cells exposed to either FA conjugate, there were less complete rims and about equal numbers of cells with partial (Fig. 2) or spotty membrane fluorescence. No fluorescence was detected with noninfected HEp-2, human fibroblast, or BHK-21 cells, or when a fluorescein-conjugated normal rabbit serum was applied to type 1 or type 2 HEp-2-infected cells.

As found previously with acetone-fixed cells (3), antitype 1 FA conjugates reacted better (2–4-fold higher dilutions) with type 1-infected cells than with type 2-infected cells. On the other hand, similar again to experience with the fixed cell techniques (3), antitype 1 FA conjugates reacted slightly better with type 2-infected cells than did antitype 2 conjugates.

Sorption of anti-1 and anti-2 conjugates with homologous or heterologous HEp-2-infected and noninfected cells. In order to determine whether specific type 1 or type 2 antigens could be detected on the HSV-infected cell membranes, anti-1 and anti-2 conjugates were sorbed with live HEp-2 cells infected with homologous or heterologous viruses. Sorption with noninfected HEp-2 cells was also done. Initially, pellets of live cells from two HEp-2 monolayers (32-oz bottles) were used and found to reduce cross-reactivity only partially. When pellets from five HEp-2-infected monolayer bottles were used for sorption of 1.0 ml of the conjugates, results, as noted in Table II, were obtained. Table II summarizes observations on 4 experiments, using VR_3 and MS strains as the type 1 and type 2 viruses, and one experiment, using "Tyler" and "Curtis" strains as the type 1 and type 2 viruses. Note that a small reduction in reactivity was found, after sorption with noninfected HEp-2 cells, and complete reduction of reactivity, when homologous virus-infected cells were used for sorption. Of particular interest was the finding that cross-reactivity with the heterologous virus type could be abolished when the FA conjugate of one type was sorbed with the heterologous HSV type; reactivity with the

TABLE II. Effect of Sorption of Fluorescein-Conjugated Antitype 1 or Antitype 2 Sera with Noninfected HEp-2 Cells and Type 1- or Type 2-Infected HEp-2 Cells.

	Infected HEp-2 cells (%)[a]		Noninfected HEp-2 cells
	Type 1	Type 2	
Fluorescein-conjugated antitype 1 serum			
1. Nonsorbed	90[a]	76	0
2. Sorbed with			
a. Type 1 cells	0	0	0
b. Type 2 cells	75	0	0
c. Noninfected cells	70	55	0
Fluorescein-conjugated antitype 2 serum			
1. Nonsorbed	80	75	0
2. Sorbed with			
a. Type 1 cells	0	58	0
b. Type 2 cells	0	0	0
c. Noninfected cells	61	48	0

[a] Average from several experiments of percentage of cells demonstrating membrane fluorescence.

homologous HSV type was retained, however.

When FA conjugates sorbed in the above way were applied on acetone-fixed virus infected cells, perinuclear fluorescence (3) could still be detected with the heterologous type. However, cross-reactivity with the heterologous HSV type could be removed if a 1:2 or 1:4 dilution of the conjugate sorbed with the heterologous virus type was used. It has been found previously (3), as well as in experiments summarized in Table I, that nonsorbed FA conjugates react with fixed cells at dilutions 4–8 times greater than with live cells.

Preparation of HSV-infected HEp-2 cells with type-specific membrane antigens. In order to obtain HSV-infected cells, which demonstrate only type-specific membrane antigens, type 1 HEp-2-infected cells were sorbed with undiluted human type 2 serum; similarly, type 2-infected cells were sorbed with undiluted human type 1 serum. Results, presented in Table III, show that cells infected with one type virus, after sorption with heterologous serum, retained their abili-

TABLE III. Effect of Sorption of Type 1 and Type 2 HEp-2 Infected Cells with Heterologous Antisera on Testing with Fluorescein-Conjugated Antitype 1 and Antitype 2 Sera.

	Cells (%) with positive membrane fluorescence[a] when tested with:	
	Fluorescein-conjugated antitype 1 serum	Fluorescein-conjugated antitype 2 serum
Type 1 HEp-2 infected cells		
a. Before sorption	78	75
b. After sorption with heterologous serum	75	0
Type 2 HEp-2 infected cells		
a. Before sorption	30	40
b. After sorption with heterologous serum	0	38

[a] Intensity of fluorescence 2–4+.

ty to react with the homologous conjugated antiserum and lost their ability to react with the heterologous FA conjugate.

Indirect MF tests. It was of interest, as regards the possibility of performing HSV antibody typing by the membrane fluorescence technic, to determine if HSV antibodies in rabbits and humans could be demonstrated by an indirect MF test. In addition, the possibility of detecting HSV antibodies in specific serum immunoglobulins (IgG, IgA, and IgM) or genital secretions was also sought. No titrations of sera were done to determine differences in titers with the two HSV types (studies in progress).

Sera (1:2 dilutions) of seven rabbits with type 1 HSV neutralizing antibodies and of seven rabbits with type 2 antibodies reacted with both type 1- and type 2-infected cells by the indirect MF test. Sera of 11 rabbits which lacked HSV neutralizing antibodies failed to give a positive MF reaction.

Sixteen human sera were tested by the indirect MF reaction using antihuman IgG serum. Six sera had been typed (4) as having type 2 antibodies, four as having type 1 antibodies, two as having dual (type 1 + type 2) antibodies, and four had been previously found to lack HSV neutralizing antibodies. All the human sera (1:4 to 1:8 dilution) with HSV antibodies gave positive MF reactions with either HSV type 1 or type 2 cells, but not with noninfected cells. The four human sera without HSV antibodies failed to react in the indirect MF test with HSV-infected cells.

Sera (1:4 dilution) of five infants with neonatal herpes, which contained IgM HSV antibodies as detected earlier with fixed cells (6), gave positive indirect MF reactions with both infected cell types, when tested with antihuman IgM conjugated serum. Two human sera, lacking IgM HSV antibodies, failed to react in this test.

Sera (1:2 dilution) from six patients with primary type 2 HSV infections, obtained 1–4 weeks after onset, were found to react by the indirect MF technique, when tested with type 1- or type 2-infected cells and antihuman IgA conjugated serum. Sera of two individuals lacking HSV antibodies failed to react. Genital secretions, obtained from two women in the convalescent stage of type 2 HSV infection and from two other women with no HSV serum neutralizing antibodies, were also tested with HSV-infected cells and the antihuman IgA conjugated serum. Both secretions of women with genital herpes gave positive reactions, whereas secretions from the two control women failed to react.

Discussion. The major aims of this study were to confirm by another technique the proposition that there were type-specific antigens, as well as common antigens, for HSV type 1 and HSV type 2. Prior results with neutralization and complement-fixation tests (4, 7) had suggested that type 1 HSV might have specific antigens, but that type 2 HSV might not. More recent results with immunodiffusion and inhibition-passive hemagglutination tests have pointed strongly to the concept that there were type-specific antigens for both HSV types (1). However, these techniques used primarily soluble viral materials. The results presented here (Tables II & III) demonstrate the presence on the cell membrane of infected cells of type-specific, as well as common, antigens. Preliminary experiments also suggest that neutralizing activity correlates with membrane fluorescence reactivity. Thus, it has been found that anti-HSV conjugated sera, when sorbed with the heterologous virus-infected cells, lose both their neutralizing activity and membrane fluorescence reactivity when tested with the heterologous virus type. On the other hand, both serological activities are retained with the homologous HSV type. Further work is in progress to relate specific membrane fluorescence activity to complement-fixing and passive hemagglutination reactions.

It is not surprising that membrane fluorescence could be detected in HSV-infected cells, since MF has been detected with other herpesviruses, particularly EB and Marek's viruses (8, 9), and several other DNA and RNA viruses (10–13). Several reports have also pointed to a change in the cell surface membranes after infection with HSV. A variety of techniques have been used for this purpose, including complement-assisted HSV-infected cell lysis using sera containing HSV

antibodies (14), hemadsorption of HSV-infected cells with sheep erythrocyte–antisheep erythrocyte serum (15), and mixed hemadsorption (16). Keller *et al.* (17) have also found a difference in the glycoproteins of the membranes of cells infected with HSV type 1 or type 2. Moreover, Watkins (15) has shown that membrane changes can occur without viral DNA synthesis, a finding confirmed by us in preliminary experiments with the MF test, using iododeoxyuridine or cytosine arabinoside, which block viral synthesis but not MF reactivity.

The ability to make HSV-infected cells "type specific" after sorption with heterologous serum (Table III), and to use the indirect FA techniques to demonstrate HSV antibodies in rabbit or human sera, should permit the development of a specific antibody type differentiation test, presently under study. Such a serological test is badly needed for serological surveys, *e.g.*, type 2 antibody frequency in various populations, including women with cervical cancer (4, 18), since current techniques do not use specific antigens. In addition, the localization of HSV antibodies in specific immunoglobulin fractions (IgG, IgA, and IgM) by the indirect MF technique has provided a handle to determine specific HSV antibody responses in primary and recurrent HSV infections. It also appears possible to run a similar test for the detection of HSV antibodies in genital secretions (IgA).

Already in use in our laboratories is the application of conjugated anti-HSV sera, made type-specific by sorption with heterologous HSV type-infected cells, to the typing of HSV isolates in fixed cells, as well as to the detection and direct typing of HSV from clinical specimens (19). Heretofore, using the direct immunofluorescent technique and fixed cells, HSV was termed type 2 if it reacted with only anti-2 conjugate at a particular dilution, and termed type 1, if it reacted with both antitype conjugates (3). It has now been possible to obtain type-specific reactions with each of the HSV type FA conjugates; recent experience with this new approach has proved successful in typing over 50 HSV isolates or clinical specimens.

Summary. Direct and indirect immunofluorescent (FA) techniques were used to demonstrate membrane fluorescence (MF) in herpes simplex virus (HSV) type 1- and type 2-infected cells. Sorption procedures of antisera with heterologous virus-infected cells, or of HSV-infected cells with heterologous sera, enabled the preparation of fluorescein-conjugated anti-HSV sera specific for each of the two HSV types, as well as of HSV-infected cells containing type-specific membrane antigens. Using the indirect FA technique, it has been possible to demonstrate HSV antibodies in the IgG, IgA, or IgM serum fractions, and to detect HSV IgA antibodies in the genital secretions of women with recent HSV genital infections.

The ability to prepare fluorescein-conjugated sera specific for type 1 and type 2 HSV has permitted more specific typing of HSV isolates or clinical specimens from patients with HSV infections. Similarly, the preparation of HSV-infected cells with type-specific membrane antigens permits more finite serological typing of type 1 and type 2 antibodies in human sera or secretions. In addition, the results obtained provide further support for the working formula for the antigens of HSV: type 1 HSV = AC, type 2 HSV = BC, where A, B, or C may comprise more than one antigen.

Footnote added in press: Since submission of this paper, we have learned that Geder *et al.*, (J. Gen. Virol, in press) have also employed membrane fluorescence technics and sorption with heterologous infected cells with essentially similar results.

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