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Complement-Fixing Antigens of Herpes Simplex Virus Types 1 and 2: Reactivity of Capsid, Envelope, and Soluble Antigens

MARY LANE MARTIN, ERSKINE L. PALMER, AND ROBERT E. KISSLING
Center for Disease Control, Atlanta, Georgia 30333

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Capsid, envelope, and nonvirion-associated soluble components of type 1 and type 2 herpes simplex virus (HSV) were obtained from infected monolayer cell cultures and used as complement fixation (CF) antigens. Capsids were prepared by treatment of cells with the nonionic detergent Nonidet-P40, envelope material by treatment of virions with ether and high *p*H, and soluble components were obtained from culture fluids of untreated cells. Serological studies with experimental anti-herpesvirus sera indicate that these serotypes share cross-reacting envelope, capsid, and soluble antigens with each other and with herpesvirus B but not with varicella virus. In addition, animals immunized with crude HSV preparations contain high levels of CF antibody (1:32 to 1:64) to soluble antigens, whereas sera from humans who have experienced natural infection contain low levels of antibody ($\leq$1:8) to this antigen. Further testing with reference, capsid, and envelope antigens indicates that antibody levels to reference and capsid antigens are about the same in sera from healthy humans, whereas antibody to the envelope is decidedly lower in these sera. Herpes convalescent-phase sera contain higher levels of antibody to reference and envelope antigens than to capsid antigen.

Preliminary studies in this laboratory indicated that cell cultures infected with either herpes simplex virus (HSV) strain VR #3 (type 1) or MS (type 2) produced two complement-fixing (CF) antigens. One was associated with the virus and the other with a soluble viral product present in cell-free culture fluids. Sera of animals immunized with crude virus preparations had high levels of CF antibody to both antigens, whereas sera from humans who had experienced natural infection had low levels of antibody to soluble antigens and high levels to virus-associated antigen. The latter was true although the virus-associated antigens of VR #3 contained large numbers of both enveloped and nonenveloped particles; similar preparations of MS contained predominantly nonenveloped particles. In addition, other data have indicated that HSV nonenveloped particles (capsids) are weakly antigenic (9). These findings suggest that sera of animals immunized with crude virus material and humans with a history of herpetic infection may have different CF antibody levels to separate HSV-specific components or products.

In this study, the preparation and CF activity of type 1 and type 2 herpes soluble products, particles deenveloped by detergent treatment (capsid antigen), and envelope material are reported. The antigenic relationship between these subviral components, as demonstrated by CF in reactions with human anti-HSV sera and experimental antisera to HSV, varicella virus (VV), and herpesvirus B (HVB), is described.

MATERIALS AND METHODS

Virus. The VR #3 strain of type 1 (12) and the MS strain of type 2 (4) HSV were used to prepare HSV antigens and antisera. HVB was obtained from Robert Hull, Eli Lilly & Co., Indianapolis, Ind. VV CF antigen was prepared as described previously (5).

Virus propagation. HSV strains were grown in primary rabbit kidney (PRK) or continuous African green monkey (VERO) monolayer cultures in 32-oz (approximately 1 liter) glass prescription bottles. Culture medium consisted of a modified (6) Eagle's essential medium with 10% calf serum. The same medium with or without 2% serum was used to propagate virus. Monolayer cultures were inoculated with a multiplicity of 5 to 10 and incubated for 48 hr at 35 C. HVB was propagated only in PRK monolayer cultures.

Capsid HSV CF antigens. Capsid CF antigens of strains VR #3 and MS consisted of preparations of particles from which the viral envelope was dissociated by detergent treatment. Maintenance media were decanted from infected cultures, and the cells were

scraped into 10 ml of 2.5% Nonidet P-40 (NP-40) in distilled water per culture. Cells were sonically treated for 10 min in 2-oz (approximately 60 ml) Boston round bottles with a 10-kc Raytheon sonic oscillator and then incubated for 1 hr at 37 C. The material was then filtered through Nalgene membrane filters of 0.45 and 0.20 μm porosity. Deenveloped particles in the final filtrate were freed of NP-40 and dissociated envelope components by layering over a 30% (w/v) sucrose cushion and centrifuging for 45 min at 35,000 rev/min in a Spinco SW40 rotor. The pellet resulting from this centrifugation contained the partially purified HSV deenveloped particles used in these studies. Control antigens were prepared from uninfected cell cultures treated the same as infected cultures. Before use, each preparation was monitored by electron microscopy for the presence of enveloped particles.

Envelope HSV CF antigen. Envelope CF antigens of strains VR ✻3 and MS consisted of viral material extracted with ether and high *p*H. Infected cells were scraped into the maintenance media, and the suspension was sonically treated for 10 min as described above. The sonicate was centrifuged for 1 hr at 30,000 rev/min in a no. 30 Spinco rotor. The pellet, which contained virions, from this centrifugation was resuspended in 3 ml of distilled water per culture and treated with an equal volume of diethyl ether at 4 C for 30 min. Ether was removed by bubbling nitrogen through the treated suspension. The ether-treated virus preparation was then dialyzed at 4 C for 24 hr against 0.85% NaCl buffered at *p*H 10.5 with 0.05 M glycine-NaOH. The dialyzed material was layered onto a 35% sucrose cushion and centrifuged for 1 hr at 30,000 rev/min in an SW40 Spinco rotor to separate the envelope material from capsids or capsids with envelope remnants. The supernatant fluid from this centrifugation contained envelope material. It was collected and dialyzed against Veronal buffer before use in CF tests.

Soluble HSV CF antigen. Soluble CF antigens of strains VR ✻3 and MS were prepared from the fluid phase of virus propagated in serum-free maintenance media. The media were decanted and centrifuged at 30,000 rev/min for 1 hr in a no. 30 Spinco rotor. The supernatant fluid from this centrifugation was concentrated approximately 25-fold by pervaporation, and then dialyzed at 4 C against Veronal buffer, *p*H 7.3. The dialysate was further purified by layering it onto a 10 to 30% (w/v) sucrose gradient and centrifuging it at 25,000 rev/min for 14 hr in an SW40 Spinco rotor. The soluble CF antigen remained in fractions at the top of the gradient. These were collected and dialyzed against Veronal buffer.

Sera. Antisera to HSV capsid CF antigens were prepared in guinea pigs by immunization with antigen prepared from infected PRK cells. They were injected intramuscularly with 1 ml of antigen derived from three monolayer cultures mixed with an equal amount of Freund's incomplete adjuvant. After the initial injection, two weekly injections of 1 ml of antigen in saline were given. The animals were bled 10 days after the final injection.

Antisera to enveloped HSV were prepared in guinea pigs by immunization with strains VR ✻3 and MS which were partially purified by equilibrium centrifugation in CsCl density gradients and rate/zonal centrifugation in sucrose gradients. Control antisera were prepared by using uninfected tissue culture treated the same as virus-infected cultures. The immunization schedule was the same as that used in preparing antisera to capsid antigen.

HVB antisera were prepared in New Zealand White

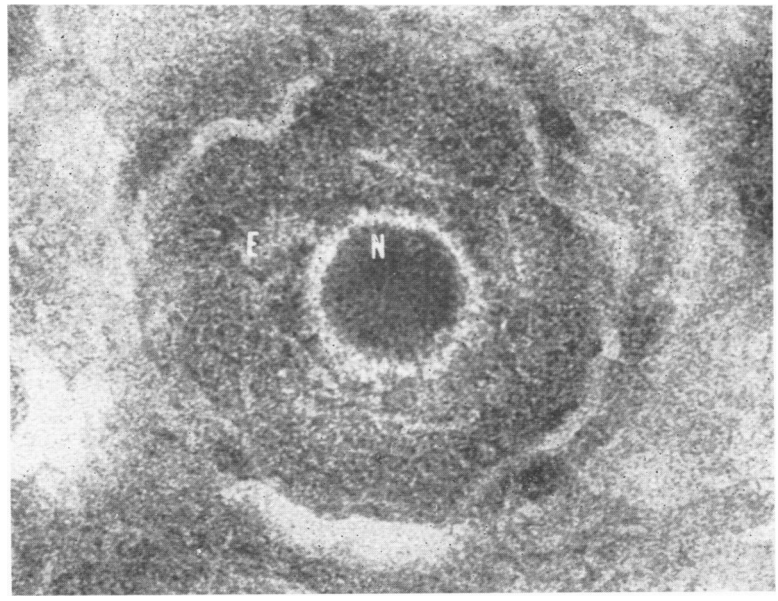

FIG. 1. *Electron micrograph of typical herpesvirus negatively stained with uranyl acetate. E, envelope; N, nucleocapsid.* × *111,000.*

rabbits by intramuscular injection with BPL (beta-propiolactone)-treated virus in Freund's incomplete adjuvant. Two weekly injections of BPL-treated virus in adjuvant were followed by one injection of untreated infected PRK tissue culture material. The animals were bled 2 weeks after the final injection.

Antisera prepared in rabbits against crude preparations of HSV strains US (type 1) and L-2 (type 2) were obtained from John Stewart, Center for Disease Control (CDC), Atlanta, Ga. Anti-varicella virus serum was prepared as described previously (5).

Human sera used in these studies were obtained from the CDC serum bank or from specimens submitted to CDC for serological testing. Herpes convalescent-phase sera were collected from humans with nongenital herpes infection.

Electron microscopy. Specimens were prepared by the pseudoreplica technique (14) and stained with 0.5% uranyl acetate. They were examined with a Philips 200 electron microscope.

Complement fixation. CF tests were performed by the microtiter LBCF test (2). Reference antigens and antisera were obtained from the Biological Reagents Section, CDC.

RESULTS

Electron microscopy shows that untreated preparations of VR #3-infected cell cultures contain approximately equal numbers of enveloped and nonenveloped particles, whereas only about 10% of the particles in similar preparations of MS are enveloped. In contrast, capsid CF antigens of these serotypes prepared by treatment with NP-40, filtration, and centrifugation contain only deenveloped forms of the viruses. A typical untreated enveloped herpesvirus is shown in Fig. 1. The virion is composed of an outer envelope (E) surrounding an icosahedral nucleocapsid (N). The virion may be compared with particles in Fig. 2 which were treated with NP-40 and partially purified. The outer envelope is completely dissolved, and the subviral particles are similar in morphology to other NP-40-treated herpesviruses (1). Electron microscopic examination of numerous detergent-treated preparations of herpes type 1 or type 2 indicated that all particles were free of envelope or envelope remnants. The preparations were highly reactive in CF but were not infectious in vitro. They also elicited CF but not neutralizing antibody when inoculated into laboratory animals.

The soluble and envelope CF antigens of VR #3 and MS are readily separable from particulate material by ultracentrifugation. These antigen preparations do not contain virions or deenveloped particles.

In Table 1, the CF activities of VR #3 and MS capsid, envelope, and soluble antigens and a reference egg antigen (VR #3) are compared by using experimentally produced herpesvirus anti-

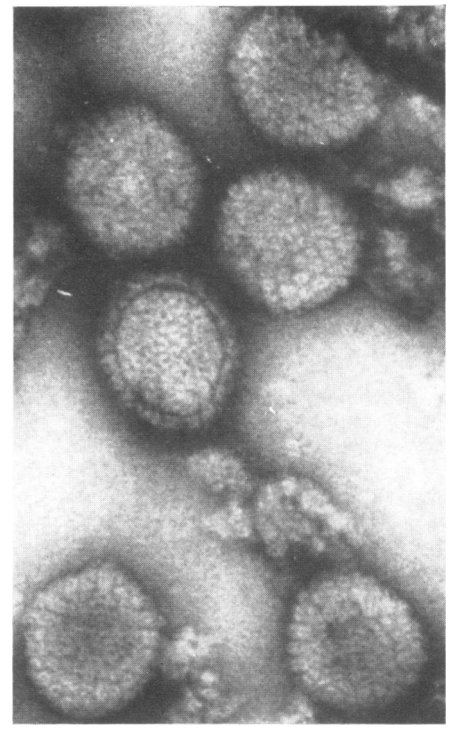

FIG. 2. *Electron micrograph of HSV particles treated with Nonidet P-40 and negatively stained with uranyl acetate.* × *140,000.*

serum. These data show that VR #3 and MS capsid antigens are highly cross-reactive in CF with various VR #3 immune sera. The antibody titer of animals immunized with preparations of partially purified enveloped or deenveloped VR #3 is not significantly different with antigens of either serotype. However, antiserum to deenveloped MS has a fourfold lower titer against the deenveloped VR #3 antigen than against its homologous antigen. The reference egg antigen (type 1) is also considerably more reactive with type 1 antiserum than with heterologous MS antisera. Envelope antigens are reactive with antisera to crude virus of both types. These antisera react at a higher titer when tested with homologous envelope antigen than when tested with the heterologous antigen. Antiserum to partially purified VR #3 contains a low level of antibody to VR #3 envelope antigen but no antibody to MS envelope antigen. No measurable levels of antibody could be detected when antiserum to partially purified MS was tested with envelope antigen of either type. Envelope antigens are not reactive when tested with antisera to deenveloped particles of both HSV VR #3 and MS. Soluble antigens of both HSV serotypes are cross-reactive

with experimental sera produced against crude virus preparations. Antibody levels of 1:32 to 1:64 are common with experimental antiserum. However, these antigens do not react in CF with antiserum to partially purified virus or deenveloped particles.

None of the HSV CF antigens are reactive with experimentally produced antiserum to VV. However, capsid, envelope, and soluble CF antigens of both serotypes are cross-reactive with antiserum to crude preparations of HVB. The level of antibody detected with capsid and soluble antigens of both serotypes (1:16 to 1:32) is similar to that obtained by testing these antigens against HSV type 1 antiserum.

Two groups of human sera were tested with capsid, envelope, and soluble antigens of strains VR #3 and MS and with reference antigen. One group consisted of 13 sera from individuals with recent clinical herpetic infections. The second group of sera was collected from 15 "normal" blood bank donors. Sera that were completely negative when tested with reference herpes antigen were not included in this group. CF titers

TABLE 1. *CF titer of serum with indicated antigen*[a]

Antiserum to	HSV reference egg antigen (VR #3)	VR #3 capsids	VR #3 soluble antigen	MS capsids	MS soluble antigen	Varicella virus	VR #3 envelope antigen	MS envelope antigen
Partially purified enveloped VR #3	128	128	<4	128	<4	<4	8	<4
VR #3 deenveloped particles	128	128	<4	128	<4	<4	<4	<4
Crude HSV strain L-2 (type 1)	128	64	64	32	32	<4	32	16
Partially purified enveloped MS	64	4	<4	32	<4	<4	<4	<4
MS deenveloped	16	16	<4	64	<4	<4	<4	<4
Crude HSV strain US (type 2)	128	16	32	32	64	<4	16	64
Varicella virus	<4	<4	<4	<4	<4	256	<4	<4
Herpesvirus B	32	32	32	32	16	<4	8	8

[a] Comparison of reactivity of reference, capsid, envelope, and soluble CF antigens of HSV type 1 and type 2 with experimental antiherpesvirus sera.

TABLE 2. *Distribution of CF titers of herpes convalescent sera and sera from normal blood bank donors*[a]

Serum	CF titer	HSV reference egg antigen (VR #3)	VR #3 capsids	MS capsids	VR #3 envelope antigen	MS envelope antigen	VR #3 soluble antigen	MS soluble antigen
Herpes convalescent sera (total = 13)	128	2	2	1	1	0	0	0
	64	2	0	0	1	0	0	0
	32	5	4	2	3	1	0	0
	16	3	2	4	6	4	1	0
	8	1	5	3	2	5	0	0
	<8	0	0	3	0	3	12	13
Normal sera (total = 15)	128	0	0	0	0	0	0	0
	64	0	2	1	0	0	0	0
	32	1	5	1	2	1	0	0
	16	4	4	8	6	5	0	0
	8	8	2	3	6	7	5	3
	<8	2	2	2	1	2	10	12

[a] Comparison of reactivity of reference, capsid, envelope, and soluble CF antigens of HSV type 1 and type 2 with sera from humans with a past exposure to HSV and from human beings convalescing from herpetic infection.

found in sera of these two groups are compared, by distribution, in Table 2.

When tested against capsid antigens of both serotypes, the two groups show approximately the same distribution pattern of antibody titers. Convalescent serum titers range from <8 to 128; titers of normal sera range from <8 to 64. The distribution of titers found among the "normal" group when measured against crude reference egg antigen shows 10 sera with antibody levels of ≤8. Of five sera with higher levels of antibody, four have titers of 1:16 and one has a titer of 1:32. In marked contrast, when convalescent sera were tested against reference egg antigen, most (12 of 13) of the serum titers were >1:8. The titers of 9 of these 12 are clustered from 1:32 to 1:128. When these sera were tested with envelope antigens of VR #3, the distribution of antibody titers of the two groups was similar to that obtained with crude reference antigen. Eleven of 13 convalescent sera have titers ≥1:16. One serum has a titer of 1:64 and one has a titer of 1:128. Eight of 15 "normal" sera have titers of ≥16 but none has a titer exceeding 1:32. Envelope antigens of MS react approximately the same with both groups of sera. Results of testing both groups of sera with soluble antigens indicate that measurable antibody to soluble antigen, when present, is very low. Only one serum, a convalescent specimen, shows a titer of ≥1:8.

Data presented in Table 3 show the complete range of CF titers found in a group of normal sera tested against HSV (VR #3) reference, capsid, envelope, and soluble antigens. The sera were chosen from control sera on file in the CDC serum bank and included sera from males and females, ages 7 to 71, from various geographical locations within the United States. Titers to both reference and capsid antigen are similarly distributed from <8 to 64 and 128. Titers to the envelope antigens are distributed from <8 to 32, but antibody levels to soluble antigen are low (≤16 or <4).

TABLE 3. *CF titer of 60 sera from normal blood bank donors tested with herpes simplex VR #3 antigens*[a]

Reciprocal of CF titer	HSV reference egg antigen (VR #3)	VR #3 capsids	VR #3 envelope antigen	VR #3 soluble antigen
128	0	1	0	0
64	1	4	0	0
32	5	18	6	0
16	22	12	18	4
8	14	7	16	17
<8	18	18	20	39
Total	60	60	60	60

[a] Comparison of reactivity of reference, capsid, envelope, and soluble CF antigens of HSV type 1 with sera from a randomized group of human beings with a past exposure to HSV.

DISCUSSION

NP-40 is a surface-active octyl phenol/ethylene oxide condensate which effectively dissolves cell membranes and dissociates the envelope(s) from the nucleocapsids of some herpesviruses (1, 10). The selective action of the detergent in dissociating the envelope of type 1 and type 2 herpes made it possible to obtain subviral CF antigen of these serotypes in sufficient quantities for serological studies. Electron microscopy revealed that detergent-treated herpes preparations contain only deenveloped forms of the virus. The particles were apparently not significantly altered either morphologically or antigenically; they retained the characteristic icosahedron configuration of herpes capsids, elicited CF antibody production in immunized animals, and actively fixed complement in reactions with human sera containing HSV antibody.

Sufficient quantities of antigenically active envelope material for serological studies were obtained by treatment of HSV with ether and high *p*H. Neither ether nor high *p*H, separately or combined, completely deenveloped the particles nor destroyed the structural integrity of the capsid. Envelope material was easily separated from capsids and capsids with envelope remnants by ultracentrifugation through sucrose. Envelope material derived from NP-40-treated HSV was not active in the CF test. The detergent apparently associates tenaciously with viral lipoprotein and, even in small quantities, inactivates complement.

Envelope antigens of HSV were not reactive in CF tests with anticapsid sera, indicating that the capsids used for immunization contained no antigenically active envelope components and that the envelope antigens contained no antigenically active capsid components. These findings confirmed results of electron microscopy which showed no capsids present in envelope antigen preparations. Envelope antigens were weakly reactive or nonreactive with antisera to purified virus. In this regard it is possible that CsCl altered the antigenic property of the HSV envelopes. Electron microscopy revealed that purified virus preparations used for immunization contained enveloped virus but that many particles had distorted envelopes.

Nonviral-associated CF antigens of both herpes types were readily obtained from cell-free culture fluids. They were physically separable from particulate material by ultracentrifugation and were

active in CF and indirect hemagglutination (13) tests but contained no virions or capsids. These CF antigens are probably the same "soluble" antigens as previously described by others in fluids of disrupted VR # 3-infected cell cultures (12). However, they do not appear to be virion precursors because they failed to react in CF with experimental antiserum to partially purified enveloped or deenveloped particles. Capsid, envelope, and soluble antigens were all markedly reactive with antisera to crude type 1 and type 2 HSV. These antisera contained significant amounts of antibody to all HSV antigens tested probably because they were prepared in a susceptible host with high-titered infectious virus, which had received no deleterious treatment such as CsCl centrifugation or ether treatment. It was noted that titers of these antisera were consistently higher when tested with homologous capsid, envelope, and soluble antigens than when tested with heterologous antigens of similar origin.

The finding that experimental antitype 1 capsid sera were more cross-reactive than similar preparations of type 2 herpes is somewhat at variance with neutralization tests which indicate that type 1 serum is better for typing isolates (8). We attribute these results to the use of partially purified antigens and their homologous antisera in the CF tests because no type differentiation could be detected when crude reference antigen was tested against antiserum to crude virus preparations. In the latter system, type specificity could easily be masked by high levels of antibody to soluble antigens (1:32 to 1:64).

Cross-reactions between HSV and VV could not be demonstrated with the antigens used in this study. From these findings it would appear unlikely that the heterotypic antibody response occurring in infections with these viruses (11) is due to a common envelope, internal, or soluble component. On the other hand, CF tests with hyperimmune rabbit anti-HVB serum indicate that HVB and type 1 and type 2 herpes contain common envelope, internal, and soluble antigens. It is, however, doubtful that antibody to HSV soluble antigen would be detected in sera from animals naturally infected with HVB because high levels of CF antibody to this antigen can only be detected in sera of animals immunized with crude material from virus propagated in vitro.

The data presented demonstrate that high levels of CF antibody to deenveloped HSV were present in the sera of individuals with a past history of exposure to the virus or from those convalescing from a recent herpetic infection. The range of antibody titers to these subviral components was of the same magnitude ($\sim$1:32) in both groups, but the latter group had a higher titer to reference antigen which contained virions, capsids, and soluble components. This finding suggests that CF antibody to the capsid component is longer lasting than that to viral envelope components or soluble antigens and is probably responsible for a large portion of the antibody detected in routine CF tests for herpes. This interpretation is supported by the findings of others which indicate that CF antigen appears in infected cells before virus (3) and that capsids combine with early 19*S* and late 7*S* but not with late 19*S* or early 7*S* antibody (7). Data of Nii et al. (9) which indicate that the capsid of HSV may be poorly antigenic were probably derived from experiments with sera which contained low levels of late 7*S* antibody. The preparations of capsids also fail to produce neutralizing antibody probably because envelope components are not present. Concomitantly, it is a priori not illogical to assume that antibody to a virus which is largely quiescent and requires an envelope for infectivity would be directed primarily against an internal viral component rather than an envelope or soluble component produced on active infection. Consonant with these observations, our data show that CF antibody to the HSV envelope is increased in convalescent sera relative to antibody levels in sera from healthy individuals with less recent exposure to herpes.

Testing of sera from healthy individuals with past exposure to herpes with reference, capsid, envelope, and soluble antigens showed that levels of antibody to reference and capsid antigens were approximately the same, whereas antibody to the envelope was at a lower level, and antibody to soluble antigen was 1:8 or less in all sera tested. The finding that reference antigen detected higher levels of antibody in herpes convalescent sera than did capsid antigen indicated that the envelope of HSV plays a major role in host antibody response to active herpes infection. This was confirmed by testing herpes convalescent sera against envelope antigen. These sera showed rises in antibody levels to the HSV envelope antigen.

These studies clearly show that type 1 and type 2 herpes share external, internal, and soluble antigens. Nevertheless, testing of experimental sera with different HSV antigens indicates that ascertaining antibody levels to separate viral components is pertinent in studies concerning HSV type-specific antibody response in human herpetic infection.

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