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Type Specificity of Complement-Fixing Antibody against Herpes Simplex Virus Type 2 AG-4 Early Antigen in Patients with Asymptomatic Infection

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We evaluated the type specificity of complement-fixing (CF) antibody against the AG-4 early antigen of herpes simplex virus (HSV) type 2 (HSV-2) by comparing a commercial AG-4 CF kit (Simplex-2; Gene Link Australia, Inc., Princeton, N.J.) with quantal microneutralization (MN) and absorption-Western blotting in testing sera from patients with and without a history of genital herpes. Sera characterized as HSV type 1 (HSV-1) or HSV-2 positive or negative by MN were selected and tested by CF, and those with discordant results were further analyzed for specific antibodies by absorption with HSV-1 or HSV-2 antigen and Western blotting with heterologous HSV proteins. A total of 34 of 42 (81%) sera HSV-2 positive by MN, 19 of 43 (44%) sera HSV-1 positive by MN, and 0 of 19 sera negative by MN were positive by CF. Absorption-Western blotting showed that 12 of 18 (67%) sera HSV-1 positive by MN but positive by CF had no HSV-2-specific antibody and that all 7 sera HSV-2 positive by MN but negative by CF had HSV-2-specific antibody. When MN and absorption-Western blotting data were combined to analyze patients with no history of genital herpes, 7 of 19 (37%) with no HSV-2-specific antibody were positive by CF, and 7 of 27 (26%) with HSV-2-specific antibody were negative by CF. The positive and negative predictive values for the CF test were 78 and 75%, respectively, in this group. The presence of antibody to the HSV AG-4 antigen does not discriminate sufficiently between HSV-1- and HSV-2-infected patients to be of value in predicting HSV-2 infection in the absence of symptomatic disease.

The need for a truly type-specific serologic test to distinguish between antibodies directed against herpes simplex virus (HSV) types 1 and 2 (HSV-1 and HSV-2, respectively) has been extensively documented (20). This need is becoming more compelling as the extent of genital HSV infections increases, accompanied by what appears to be a real increase in the incidence of neonatal infections (28) and heightened concern about the association between genital HSV-2 infections and cervical neoplasia (22).

The difficulty in distinguishing between antibodies directed against these two viruses lies in the fact that the viruses possess many immunogenic proteins in common (5, 11, 14). Prior infection with HSV-1 results in a boost in the production of type-common antibodies in response to infection with HSV-2 (17, 21, 24), frequently to the degree that the HSV-2-specific antibody response is blunted and masked by the development of type-common antibodies.

An early attempt to address this problem was the differential neutralization assay with quantal analysis (18, 23), and this system and others based on the same premise have remained the mainstays of HSV serology. More recently, analysis of individual proteins and polypeptides of HSVs has revealed that type-specific antigenic regions do exist (27), holding out promise of the development of assays for type-specific antibodies directed against such antigens. Recent examples are glycoprotein C_1 and glycoproteins G_2 and C_2 specific for HSV-1 and HSV-2, respectively (4, 7, 15, 31, 32). However, antibody assays using these glycoproteins are available only in research laboratories and are still in the process of development.

A complement fixation (CF) assay (Simplex-2; Gene Link Australia, Inc., Princeton, N.J.) utilizing an apparently HSV-2-specific antigen has recently been marketed in North America. The antigen, designated AG-4 early antigen, is a crude mixture of HSV proteins and polypeptides generated during the first 4 h of infection of HEp-2 cells by HSV-2 (12, 25). The CF assay was first used as a marker for cervical carcinoma (6), but in later reports, Arsenakis and coworkers reported that 88% of persons attending a sexually transmitted disease clinic with culture-positive HSV-2 infections had antibody to AG-4 (1–3). Advertisements for this product have suggested that physicians use the assay to identify women who are at risk of infecting their neonates with HSV-2 during parturition. However, the assay has not been investigated in patients with asymptomatic infections, and it is in this context that such a test would be most valuable, since in more than 70% of cases of neonatal HSV infection there is no maternal history of disease (28, 30).

In this report, we compare the AG-4 CF test with quantal microneutralization (MN) by using sera from individuals with and without a history of genital herpes. Sera with discordant results were further analyzed by a Western blot assay to identify type-specific antibodies.

MATERIALS AND METHODS

Sera. Sera were obtained from individuals attending the gynecology or herpes research clinics at the University of Washington Hospitals. They were selected from the serum bank on the basis of their anti-HSV-1 or anti-HSV-2 activity determined by MN. When possible, patient records were reviewed to determine whether the patient had ever had symptoms consistent with genital herpes simplex infection.

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In the cases of previously documented genital herpes infections, the viral isolates had been serotyped by direct immunofluorescence with monoclonal antibodies (Syva Co., Palo Alto, Calif.). All sera were less than 6 months old when tested and had been stored at −20°C.

CF assay. The CF assay was carried out in accordance with the instructions of the manufacturer. The antigen supplied in the kit is a crude lysate of HEp-2 cells infected with HSV-2 at a multiplicity of infection of 0.7 PFU per cell and lysed at 4 h after infection. The control antigen supplied is a lysate of uninfected HEp-2 cells. Rabbit complement was supplied by Flow Laboratories, Inc., McLean, Va., or M. A. Bioproducts, Walkersville, Md., and was used at a dilution of 1:45, as determined by the complement titration method detailed in the Simplex-2 package insert. The test was performed in 96-well, round-bottomed microtiter plates (Linbro, Div. Flow Laboratories, Inc., Hamden, Conn.). Sera were tested, as recommended, at a single dilution of 1:4, and results were expressed only as positive or negative. In addition to reading the CF results as recommended by the manufacturer (a positive result is less than 50% hemolysis estimated with cells in suspension), we centrifuged the microtiter plates at 600 × *g* for 3 min and scored the hemolysis on a scale of 0 to 5 (0 = total hemolysis, 5 = no hemolysis); a score of 3 or above was considered a positive result. The latter method gave clearer results when there was incomplete hemolysis.

MN assay. Sera were analyzed for the presence of neutralizing antibodies against HSV-1 and HSV-2 by an MN assay described previously (18, 23). Human fetal tonsil diploid fibroblasts were infected with HSV-1 strain E115 or HSV-2 strain 333. The presence of HSV-1 or HSV-2 antibody in the sera was determined by the 2:1 ratio (23) and the difference in potency of neutralization (18): sera with a 2:1 ratio of ≥100 and a difference in potency of neutralization of ≤0.05 were considered to have HSV-2-specific antibody; those with a 2:1 ratio of ≤85 and a difference in potency of neutralization of ≥0.5 were considered to have HSV-1-specific antibody. Sera that possessed indeterminate neutralizing antibodies (2:1 ratio, >85 but <100; difference in potency of neutralization, >0.05 but <0.5) were excluded from this study.

Absorption-Western blotting. Sera that gave discordant results in the MN and CF tests were further analyzed by absorption-Western blotting modified from the method reported by Bernstein et al. (8, 9). The modifications are described below.

(i) Absorption of sera. Human fetal tonsil diploid fibroblasts were infected with HSV-1 strain E115 or HSV-2 strain 333. When a 4+ cytopathic effect was observed, a lysate was prepared by sonication, treatment with 1% Nonidet P-40 in phosphate-buffered saline (pH 7.6) at 4°C overnight, and centrifugation at 10,000 × *g* for 20 min. The dissolved HSV proteins were then coupled to cyanogen bromide-activated Sepharose beads (Sigma Chemical Co., St. Louis, Mo.). Each serum was diluted 1:10 in 1% bovine serum albumin, and 2 ml of diluted serum was then incubated with 1 ml of HSV-1– or HSV-2–Sepharose at room temperature for 4 h and then twice at 4°C for 12 h, with a new Sepharose preparation being used for each incubation.

(ii) Western blotting. HSV-infected cell lysates were prepared as described above except that the solution used to dissolve the cell proteins contained 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, and 1% aprotinin in 10 mM phosphate buffer (pH 7.2). Proteins in the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by standard methods (10, 13). The proteins were then transferred electrophoretically to a 0.45-μm-pore-diameter nitrocellulose sheet at 30 V for 16 to 18 h in 25 mM Tris containing 192 mM glycine, 20% methanol, and 0.05% sodium dodecyl sulfate. The nitrocellulose was cut into strips, and 5 ml of 1:50 dilutions of absorbed and unabsorbed portions of the sera were incubated with HSV-1, HSV-2, and control (uninfected cell lysate) strips to give seven blots per serum.

Detection of antibody binding to the blots was accomplished with an avidin-biotin-horseradish peroxidase system (Vectastain ABC; Vector Laboratories, Burlington, Calif.). This system utilizes biotinylated goat anti-human immunoglobulin G with avidin and biotinylated horseradish peroxidase completing a sandwich configuration. The substrate is diaminobenzidine tetrahydrochloride.

Analysis of results. Sensitivity, specificity, and positive and negative predictive values were calculated by the following formulas (see Table 4): sensitivity = true-positives/(true-positives + false-negatives); specificity = true-negatives/(true-negatives + false-positives); positive predictive value = true-positives/(true-positives + false-positives); and negative predictive value = true-negatives/(true-negatives + false-negatives).

Using these parameters, we compared the CF test with MN as the reference test. The data were then reanalyzed so that the reference test included the MN results modified by the absorption-Western blotting results. In both cases, a true-positive was regarded as a positive CF result in a serum with HSV-2 specificity by MN or absorption-Western blotting, regardless of the presence of HSV-1 antibody on the Western blot; a false-positive was regarded as a positive CF result in a serum with HSV-1 specificity. True- and false-negatives were regarded as negative CF results in sera with HSV-1 and HSV-2 specificities, respectively. Since the purpose of the AG-4 CF test is to discriminate between HSV-1 and HSV-2 antibodies and not merely to detect the presence of HSV antibodies, the 19 sera without HSV antibodies were excluded from this analysis.

RESULTS

A total of 105 sera were tested by both MN and AG-4 CF assays. Of 42 sera HSV-2 positive by MN, 34 (81%) were positive by AG-4 CF, and of 43 sera HSV-1 positive by MN, 19 (44%) were positive by AG-4 CF (MN-1 CF+) (Table 1). Of 20 sera that were seronegative by MN, none were positive by AG-4 CF, and 1 exhibited persistent anticomplement activity even after prior incubation with complement and was therefore excluded from the results.

Patient records were retrievable for 67 (79%) of the seropositive patients. Of these patients, 49 (73%) had no history of symptomatic genital HSV infection, and 18 (27%) had a history consistent with symptomatic genital HSV infection; 8 of these 18 cases had been confirmed by culture. In the asymptomatic group of patients, of 27 sera HSV-2

TABLE 1. Frequency of AG-4 CF antibodies in sera tested by MN

CF result	No. (%) of sera with indicated antibody status determined by MN		
	HSV-1 positive	HSV-2 positive	Negative
Positive	19 (44)	34 (81)	0
Negative	24 (56)	8 (19)	19 (100)

TABLE 2. Frequency of AG-4 CF antibodies in sera according to history of genital HSV infection

CF result	No. (%) of sera determined to be positive for indicated antibodies by MN			
	Asymptomatic patients		Symptomatic patients	
	HSV-1	HSV-2	HSV-1	HSV-2
Positive	10 (45)	22 (81)	0	11 (92)
Negative	12 (55)	5 (19)	6 (100)	1 (8)

positive by MN, 22 (81%) were also positive by CF, and of 22 sera HSV-1 positive by MN, 10 (45%) were also positive by CF (Table 2). These percentages are almost identical to those in Table 1. However, in the symptomatic group of patients, only 1 discrepant result was observed (Table 2). Of the eight sera from patients with previous culture-proven HSV-2 infections, only five (62.5%) were positive by AG-4 CF.

In view of the fact that two of the sera characterized as HSV-1 positive by MN were from patients with previously documented HSV-2 disease and because of the literature demonstrating that prior HSV-1 infection may mask the HSV-2 antibody response when assayed by MN (17, 21, 24), one possible interpretation of the positive CF results with 19 sera that were HSV-1 positive by MN is that AG-4 CF was detecting HSV-2-specific antibody not detected by MN.

To resolve this question, we analyzed the 27 sera with discordant results (19 characterized as MN-1 CF+, 8 characterized by MN as HSV-2 positive but CF negative [MN-2 CF−]) by using the absorption-Western blot assay. We first tested this assay with previously characterized convalescent-phase sera from patients with culture-proven genital infections: seven patients with primary HSV-2 infections, three patients with primary HSV-1 infections, and five patients with nonprimary, first-episode genital HSV-2 infections; the Western blots showed 100% concordance with culture results. Figure 1 shows Western blots of three sera that were from asymptomatic study patients and that possessed different type-specific antibody configurations. Type specificity of the tested sera was assigned on the basis of the predominant absorption of homologous and cross-reacting antibodies (8). The type specificity of the predominant antibody was determined initially from the blots of unabsorbed sera; in serum 414 this was HSV-1, in serum 401 this was HSV-2, and in serum 1077 there were approximately equal amounts of HSV-1 and HSV-2 antibodies. The overall type specificity was interpreted by comparing the blots of absorbed sera. Serum 414 had only HSV-1 antibody: absorption with HSV-1 proteins removed nearly all antibodies (lanes A and B), but absorption with HSV-2 proteins left many HSV-1-reactive antibodies (lane C) and very little HSV-2-reactive antibodies (lane D). Serum 401 had only

TABLE 3. Antibodies detected by the absorption-Western blot assay in 25 sera with discordant MN and CF results

MN and CF result	No. of sera determined to be positive for indicated antibodies by absorption-Western blotting[a]		
	HSV-1	HSV-2	HSV-1 + HSV-2
MN-1 CF+	12 (7)	1 (0)	5 (3)
MN-2 CF−	0 (0)	3 (1)	4 (3)

[a] Data represent numbers of sera from symptomatic (asymptomatic) patients.

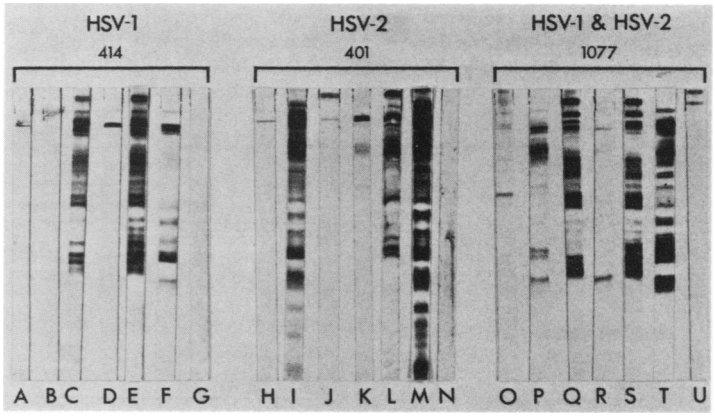

FIG. 1. Western blots of HSV proteins incubated with sera from patients with no history of genital herpes. Each lane is nitrocellulose paper containing electrophoretically separated HSV proteins. Aliquots of sera were absorbed with either HSV-1 or HSV-2 proteins and incubated with HSV-1 and HSV-2 strips. Unabsorbed aliquots of sera were incubated with HSV-1, HSV-2, and control (uninfected cell lysate) strips. Lanes are as follows: sera absorbed with HSV-1 proteins and blotted with HSV-1 proteins (lanes A, H, and O) and HSV-2 proteins (lanes B, I, and P); sera absorbed with HSV-2 proteins and blotted with HSV-1 proteins (lanes C, J, and Q) and HSV-2 proteins (lanes D, K, and R); unabsorbed sera blotted with HSV-1 proteins (lanes E, L, and S), HSV-2 proteins (lanes F, M, and T), and uninfected cell lysate (lanes G, N, and U).

HSV-2 antibody: absorption with HSV-2 proteins removed nearly all antibodies (lanes J and K), but absorption with HSV-1 proteins left many HSV-2-reactive antibodies (lane I) and very little HSV-1-reactive antibodies (lane H). Serum 1077 had both HSV-1 and HSV-2 antibodies: absorption with either protein left heterologous antibodies in considerable quantities (lanes P and Q).

One each of the 19 MN-1 CF+ sera and the 8 MN-2 CF− sera could not be tested further because insufficient serum was available. Of the remaining 18 MN-1 CF+ sera tested by absorption-Western blotting, 5 (31%) contained HSV-1 and HSV-2 antibodies, 1 contained only HSV-2 antibody, and 12 contained only HSV-1 antibody (Table 3). Of the 7 MN-2 CF− sera tested by this assay, 3 contained only HSV-2 antibody, and 4 contained both HSV-1 and HSV-2 antibodies. When these data were analyzed on the basis of the symptom status of the patients (Table 3), 7 of 10 (70%) MN-1 CF+ sera from asymptomatic patients contained no HSV-2-specific antibody, and 3 contained antibodies to both HSV-1 and HSV-2; all 4 MN-2 CF− sera from asymptomatic patients contained HSV-2-specific antibody.

On analysis of the 85 seropositive study sera, the sensitivity and specificity of the CF as compared with those of MN plus absorption-Western blotting were 85 and 65%, respectively; these figures were almost identical in asymptomatic patients (Table 4). The positive and negative predictive values in all patients were 75 and 77%, respectively, again very similar to those in asymptomatic patients. In symptomatic patients, these parameters were as follows: sensitivity, 92%; specificity, 100%; positive predictive value, 100%; and negative predictive value, 86%. However, the numbers were small (Table 2).

DISCUSSION

The results of this study demonstrate that the AG-4 antigen is capable of binding HSV-1 antibody in the CF

TABLE 4. Sensitivity, specificity, and positive and negative predictive values for the AG-4 CF test compared with MN and after reevaluation by absorption-Western blotting (WB) for all sera and for sera from asymptomatic patients

Patients	%			
	Sensitivity	Specificity	Predictive value	
			Positive	Negative
All				
CF vs MN	81	56	64	75
CF vs WB	85	65	75	77
Asymptomatic				
CF vs MN	81	54	69	71
CF vs WB	86	63	78	75

assay in 30% of all individuals and in 37% of asymptomatic individuals. This contrasts with the results of studies by Arsenakis et al., who reported positive CF rates of 10% (1 of 10) (1) and 6.25% (1 of 16) (3) in sera from patients with virologically confirmed clinical HSV-1 disease.

The rate of cross-reaction with HSV-1 antibody is the most significant issue when considering such a test. Because the prevalence of HSV-1 infections in young adults is high—40 to 90%, depending on the populations studied (19, 26, 29)—a cross-reaction rate of 30 to 37% with this test would give misleading results in precisely the patient groups for which such a test would be most valuable, in particular, pregnant patients with no history of genital herpes but also, for example, patients with genital ulcers but negative cultures. In addition, the use of this test to define the epidemiology of HSV-2 infections would significantly distort the results in almost all populations.

Earlier studies of the AG-4 antigen identified the antigen as a marker for the development of cervical intraepithelial neoplasia. Aurelian and co-workers showed that the antigen was an early product of infection of HEp-2 cells by HSV-2 and suggested that antibody to AG-4 was an identifier of women at risk of developing cervical intraepithelial neoplasia (6, 25). The relationship between AG-4 and HSV-1 infection was not explored.

Later studies by Arsenakis et al. showed that the presence of AG-4 antibody correlated better with acute clinical infection with HSV-2 than with the occurrence of cervical neoplasia (1–3). However, a recent report by this same group (12) in which the identity of the AG-4 proteins and polypeptides was investigated revealed that the in vitro production of AG-4 antigen by HSVs is dependent upon the multiplicity of infection: at a multiplicity of infection of 2.5 PFU per cell, AG-4 production by HSV-1 was detected, compared with a multiplicity of infection of 0.3 PFU per cell for HSV-2. A natural extrapolation from this in vitro work is that in vivo infection with HSV-1 will result in AG-4 antigen production and therefore a corresponding antibody response. The fact that 30% of our patients with antibody to HSV-1 alone also had antibody to AG-4 antigen suggests that there is a significant in vivo correlation with this in vitro observation.

In conclusion, although the AG-4 CF test may be useful in some cases of culture-negative, clinically evident genital HSV-2 disease, its lack of type 2 specificity as compared with that of MN and absorption-Western blotting renders it unsuitable as a routine test for identifying the presence of HSV-2-specific antibody in the general population. Use of the absorption-Western blot assay in our experiments confirmed that this assay affords excellent specificity in identifying HSV-1 and HSV-2 antibodies in cases of dual infection. However, while it is appropriate as a research tool, it is too cumbersome for routine diagnostic use. The need still exists for a sensitive and readily automated HSV-2-specific serologic test.

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