Comparison of Antibody-Dependent Cellular Cytotoxicity and Complement-Dependent Antibody Lysis of Herpes Simplex Virus-Infected Cells as Methods of Detecting Antiviral Antibodies in Human Sera

THANGAMANI SUBRAMANIAN AND WILLIAM E. RAWLS*

Department of Pathology, McMaster University, Hamilton, Ontario, Canada

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An antibody-dependent cellular cytotoxicity (ADCC) assay was used to detect antibodies to the herpes simplex viruses in human sera. The assay utilized the release of ^{51}Cr from BHK-21 cells infected with the viruses, hamster peritoneal exudate cells as effector cells, and antiviral antibodies in human sera. The technique was found to be far more sensitive than complement-dependent antibody lysis of infected cells and virus neutralization. The ADCC assay was useful in detecting antibodies in sera that had low titers or no antibodies detectable by other methods. In a sample of 100 sera from university students, 40 were positive by complement-dependent lysis whereas 73 were positive by ADCC. Of 400 sera from women with cervical cancer, 17 did not have detectable antibodies by microneutralization or complement-dependent lysis; however, all sera were positive by ADCC, suggesting that all of the women had been infected in the past with one or both types of herpes simplex virus.

The percentage of adults without detectable antibodies to herpes simplex viruses (HSV) has been found to vary in seroepidemiological studies carried out in different populations (4, 6, 10-13, 15-19, 21, 28, 31). Since minimal fluctuation in antibody titers has been observed in most individuals followed prospectively (3, 5, 7-9, 25), the absence of detectable antibodies implies that the individual had not been infected in the past. These seroepidemiological observations suggest population differences in past infections with the viruses; however, it is possible that antibodies do not persist at high levels in all infected individuals (3, 8, 30). Thus, the differences in detectable antibodies may reflect not only differences in past infection rates, but also differences in antibody persistence.

Recently, it was demonstrated that cell-mediated lysis of antibody-coated target cells is a sensitive method of detecting antibodies to HSV (20, 24, 27). In the present study, the antibody-dependent cellular cytotoxicity (ADCC) technique was utilized for quantitation of HSV antibodies in human sera. As reported by others (24, 27), we found the ADCC method to be much more sensitive than virus neutralization or complement-dependent antibody lysis of virus-infected cells. In addition, a significant proportion of sera from adults without detectable antibody by the latter methods were found

to contain antibodies detectable by the ADCC technique.

MATERIALS AND METHODS

Sera. Selected convalescent-phase sera from patients who experienced clinically apparent infections with HSV type 1 (HSV-1) or HSV type 2 (HSV-2) were used to examine the variables of the ADCC technique. Serum samples were collected from 100 students attending McMaster University. These samples were collected without regard to specific illness from students attending the health clinic and were felt to be representative of the study group. In addition, selected sera from previously described studies were utilized (12, 22, 29). These included 30 sera (12) and 34 sera (22) found to have low titers or no detectable antibodies to HSV-2 by microneutralization, and 60 sera (29) found to have low or no detectable antibodies by complement-dependent antibody lysis of virus-infected cells. The sera were stored at -35°C and heat inactivated at 56°C for 30 min prior to use in the assay procedures.

Target cells. The KOS strain of HSV-1 and the HV-219 strain of HSV-2 were used (26). Monolayers of BHK-21 cells in 75-cm² tissue culture flasks (Corning Glass Works, Corning, N.Y.) were infected at a multiplicity of 3 to 5 plaque-forming units per cell, and adsorption of the virus was for 1 h at 23°C . The monolayers were then overlaid with Eagle minimal essential medium supplemented with 5% fetal bovine serum and antibiotics (penicillin, 100 U/ml; streptomycin, 100 $\mu\text{g}/\text{ml}$). To the cultures was added 200 μCi of ^{51}Cr as sodium chromate (New England

Nuclear Corp., Boston, Mass.), and the cultures were incubated at 37°C. After 18 h of incubation, the cells were monodispersed by using 0.025% trypsin and 0.02% ethylenediaminetetraacetate in buffered saline without calcium and magnesium ions. The cells were then washed five times with tris(hydroxymethyl)aminomethane-buffered saline containing 2% fetal bovine serum. After the final wash, the cells were suspended in minimal essential medium with 10% fetal bovine serum at a concentration of 10^5 viable cells per ml. BHK-21 cells not infected with virus were labeled with ^{51}Cr in a similar fashion and served as controls.

Effector cells. Peritoneal exudate cells from golden Syrian hamsters (Lakeview/Charles River, Newfield, N.J.) were used as effector cells. Adult hamsters were injected intraperitoneally with 5 ml of sterile paraffin oil. The animals were sacrificed 3 days later, and the peritoneal exudate cells were harvested from the peritoneal cavity by lavage with Hanks buffered saline. The cells were washed twice with buffered saline and suspended at the required concentration in minimal essential medium containing 10% fetal bovine serum.

ADCC assay. The test serum was appropriately diluted in minimal essential medium containing 10% fetal bovine serum. To 0.2 ml of the diluted serum in glass tubes (12 by 75 mm) were added 0.4 ml of target cells and 0.4 ml of effector cells. The tubes were gently shaken and then incubated at 37°C in a humid atmosphere of 95% air and 5% CO_2 . At the end of the incubation period (usually 8 or 20 h), the tubes were thoroughly agitated and centrifuged at 1,500 rpm for 3 min (IEC PR-J centrifuge) to sediment unlysed cells and cell debris. A 0.5-ml sample was carefully removed from the top of the 1-ml reaction volume and transferred to a clean test tube. The radioactivity of each of the 0.5-ml fractions was counted in a Beckman automatic gamma counter. The percent specific release of ^{51}Cr was calculated by the formula:

$$\% \text{ specific release} = \frac{2 \times \text{cpm of top fraction}}{\text{cpm of top fraction} + \text{cpm of bottom fraction}} \times 100$$

where cpm is counts per minute. As controls, all assays included reaction mixtures to which diluent was added instead of effectors cells or instead of serum and in which uninfected cells were substituted for target cells. The assays were performed in triplicate, and the values from the three determinations were averaged. Analysis of variations of triplicate values indicated that specific release of greater than 5% above control values was unlikely to occur by chance ($P < 0.01$) and, therefore, were considered positive for antibody activity.

For quantitation, the data were normalized and expressed as percent cytotoxicity. This was accomplished by assigning the value of 0% to the release in control mixtures containing known antibody-negative serum and 100% to the release observed in mixtures containing excess antibody. The reciprocal of the serum dilution that produced 50% cytotoxicity was taken as the titer of antibody in the ADCC

assay. The method of expression of results is exemplified in the data shown in Table 1. The percent specific release for control mixtures was about 37, and that for the positive antibody control was 68. The values of 37 and 68% were taken as 0 and 100%, respectively, and the mean percent specific release for the unknown, 48, represents 35% on this scale.

Complement-dependent lysis of cells by antibody. Antibodies to the surface antigen of BHK-21 cells infected with HSV-1 and HSV-2 were also assayed by adding guinea pig complement to sensitized cells. Details of this procedure were previously described (14).

Neutralizing antibodies. The presence of neutralizing antibodies was detected by mixing virus and serum and then assaying for surviving plaque-forming virus. Details of the plaque reduction test are described elsewhere (23).

RESULTS

Variables of the ADCC technique. Since we wished to use the ADCC test for assaying antibody activity, several combinations of readily available target and effector cells were examined. These included BHK-21 and RK-13 cells infected with HSV-1 or HSV-2 as target cells and peritoneal exudate cells, peripheral blood lymphocytes, and spleens cells from rabbits and hamsters as effector cells. The most satisfactory results were obtained with virus-infected BHK-21 cells and hamster peritoneal exudate cells.

The influence of different concentrations of effector cells was examined by sensitizing ^{51}Cr -labeled BHK-21 cells that had or had not been infected with HSV-1 or HSV-2; sensitization was carried out by incubating the target cells

TABLE 1. Results of a typical ADCC assay using ^{51}Cr -labeled BHK-21 cells infected with HSV-1

Effector cells	Antibody to HSV-1	cpm of bottom fraction	cpm of top fraction	Specific release (%)	Cytotoxicity (%)
1:50 ^a	None	3,511 3,482 3,540	792 788 796	37 37 37	0
None	Positive control	3,122 2,936 3,029	676 686 681	36 38 37	0
1:50	Negative control	3,324 3,566 3,445	726 804 765	36 37 36	0
1:50	Positive control	2,940 2,842 2,891	1,520 1,484 1,502	68 69 68	100
1:50	Unknown serum	3,158 3,096 3,127	1,010 1,026 1,018	48 50 49	35

^a Fifty peritoneal exudate cells to one target cell.

with 1:50 dilutions of sera for 1 h. Different numbers of hamster peritoneal exudate cells were added, and 20 h later the lysis of target cells was assessed. The optimum target:effector cells ratio was found to be 1:50 for BHK-21 cells infected with HSV-1 (Fig. 1) and 1:200 for BHK-21 cells infected with HSV-2 (Fig. 2).

The rate of destruction of sensitized target cells was examined by sensitizing the cells with serum, adding peritoneal exudate cells at a ratio of 1:50 for HSV-1 and 1:200 for HSV-2, and then terminating the reaction after various periods of incubation. Specific destruction was evident after 4 h of incubation and increased rapidly thereafter (Fig. 3). Maximum specific lysis was attained by 8 h of incubation, after which increased lysis in the presence of antibody was associated with an increased nonspecific lysis. Somewhat similar results were obtained with cells infected by HSV-2; specific lysis was initially detected at 4 h and increased rapidly until 8 h. Nonspecific lysis at 20 h after infection was less for HSV-2-infected cells than for HSV-1-infected cells (data not shown). The differences between lysis in the presence of antibodies and in the absence of antibodies were

FIG. 1. Effect of varying numbers of effector cells on the lysis of BHK-21 cells infected with HSV-1. Symbols: ○, BHK-21 cells infected with HSV-1 and sensitized with serum containing neutralizing antibodies to HSV-1; ▲, BHK-21 cells infected with HSV-1 and sensitized with serum not containing detectable neutralizing antibodies to HSV-1; ●, BHK-21 cells infected with HSV-1 and not sensitized; △, uninfected BHK-21 cells sensitized with serum containing neutralizing antibodies to HSV-1.

FIG. 2. Effect of varying numbers of effector cells on the lysis of BHK-21 cells infected with HSV-2. Symbols: ○, BHK-21 cells infected with HSV-2 and sensitized with serum containing neutralizing antibodies to HSV-2; △, BHK-21 cells infected with HSV-2 and sensitized with serum not containing neutralizing antibodies; ●, uninfected BHK-21 cells sensitized with serum containing HSV-2 antibodies.

FIG. 3. Kinetics of lysis of BHK-21 cells infected with HSV-1 by hamster peritoneal exudate cells. Symbols: ○, BHK-21 cells infected with HSV-1 and sensitized with serum containing neutralizing antibodies to HSV-1; △, BHK-21 cells infected with HSV-1 and sensitized with serum lacking neutralizing antibodies to HSV-1; ●, uninfected BHK-21 cells sensitized with serum containing neutralizing antibodies to HSV-1.

maximal at 8 h for HSV-1-infected cells and at 20 h for HSV-2-infected cells.

The influence of decreasing concentrations of antibody is depicted in Fig. 4. With increasing serum dilution, there was a decrease in the percent cytotoxicity. The reciprocal of the serum dilution that produced a 50% cytotoxicity was taken as the antibody titer. In the ADCC assay, the slopes of the dose-response curves were relatively flat, and the titers of sera upon repeat assays were more variable than the titers obtained by complement-dependent lysis. Antibody titers for the type 1 serum used in the experiment shown in Fig. 4 were 1:20 by complement-dependent lysis and 1:290 by ADCC, whereas the titers for the type 2 serum were 1:125 by complement-dependent lysis and 1:5,500 by ADCC. Thus, the ADCC method was found to be more sensitive than the complement-dependent lysis of infected cells by antibody.

The rate of binding of antibody to the target cells was found to be very rapid (Fig. 5). The maximum rate of binding was observed in the first few minutes of the reaction, and binding

FIG. 4. Effects of serum dilution on cytotoxicity of BHK-21 cells infected with HSV-1 or HSV-2. Symbols: ○, BHK-21 cells infected with HSV-1, sensitized with varying dilutions of HSV-1 antiserum, and reacted with effector cells at a ratio of 1:50; ●, BHK-21 cells infected with HSV-1, sensitized with varying dilutions of HSV-1 antiserum, and lysed with complement; △, BHK-21 cells infected with HSV-2, sensitized with varying dilutions of HSV-2 antiserum, and reacted with effector cells at a ratio of 1:200; ▲, BHK-21 cells infected with HSV-2, sensitized with antiserum to HSV-2, and lysed with complement.

FIG. 5. Kinetics of binding of antibody to target cells. The kinetics of antibody binding to target cells was assayed by mixing 0.5 ml of a 1:5 dilution of serum with 10^7 target cells in 0.5 ml and incubating at 4°C. At intervals, 10 μ l of the reaction mixture was removed and diluted in 10 ml of cold diluent. The cells were washed three times and then mixed with effector cells. The percent cytotoxicity in these experiments is expressed in relation to the cytotoxicity obtained when excess antibody was not removed, which was taken as 100%. Symbols: ○, BHK-21 cells infected with HSV-2 and reacted with HSV-2 antiserum; ●, BHK-21 cells infected with HSV-1 and reacted with HSV-1 antiserum.

was essentially complete by 30 min. For the sera tested, 15 to 25% additional cytotoxicity was observed if the excess antibodies were not removed from the reaction mixture. The results of these experiments also demonstrate that induction of cell cytotoxicity is brought about by antibody bound to the target cells and not by a direct attachment of free antibody to effector cells.

Analysis of sera from college students. From the above analysis, a standardized test of ADCC was adopted in which HSV-1 target cells were reacted with effector cells at a ratio of 1:50 and incubated for 8 h, and HSV-2 target cells were reacted with effector cells at a ratio of 1:200 and incubated for 20 h. Sequential serum samples collected from two patients with acute ulcerative pharyngitis were analyzed for anti-

bodies by the ADCC method and by complement-dependent lysis. HSV-1 was isolated from throat swabs of both of these patients. The kinetics of the appearance of antibodies were similar in both patients by both techniques; however, the titers obtained by the ADCC method were much higher than those obtained by complement-dependent lysis (Fig. 6).

In preliminary analysis of sera from college students, we found a large percentage seronegative by complement-dependent lysis (data not shown). Therefore, sera were collected from 100 students and tested by complement-dependent lysis and by ADCC at a dilution of 1:10 (Table 2). All sera positive by complement-dependent lysis were also positive by ADCC; however, 33 sera positive by the ADCC method titrated less than 1:10 by complement-dependent lysis. These data suggest that a substantial proportion of the student population had been infected with HSV-1 in the past but did not possess antibodies of sufficient titer to be detected by complement-dependent lysis.

Analysis of sera from patients with cervical neoplasia. Antibodies to HSV-2 have been associated with squamous cell carcinoma of the

cervix, yet a small percentage of patients with cancer do not have detectable antibodies to HSV-1 or HSV-2. To evaluate whether the seronegativity of these women represented the absence of past infection or levels of antibody below detection by the usual assay methods, sera found negative by microneutralization or by complement-dependent lysis were analyzed by ADCC (Table 3). While 4.3% of women with invasive cancer lacked antibodies to HSV-2 by the conventional methods, all had antibodies detectable by the ADCC method. There was also a substantial decrease in the percentage of seronegative women with carcinoma in situ and of control women when their sera were analyzed by ADCC.

To confirm these findings, 50 sera from women with cervical neoplasia and 50 sera from control women were each divided into three samples and assayed for antibodies to HSV-1 and HSV-2 by three methods. These sera were selected on the basis of low or absent titers of antibody to HSV-2 as determined by microneutralization or by complement-dependent lysis.

TABLE 2. Seronegativity of college students as determined by ADCC and complement-dependent lysis

Method	No. tested	Antibodies to HSV-1 ^a	
		Positive	Negative
Complement-dependent lysis	100	40	60
ADCC	100	73	27

^a Sera tested at a dilution of 1:10.

TABLE 3. Seronegativity of cases of cervical neoplasia and control women as determined by ADCC

Disease state ^a	No. studied	Negative for antibodies to HSV-2 ^b			
		Conventional method ^c		ADCC	
		No.	%	No.	%
Invasive carcinoma	400	17	4.3	0	0
Carcinoma in situ	115	15	13	4	3.5
Controls	523	92	17.6	23	4.4

^a Study subjects were derived from three studies and are not comparable with respect to ages and socioeconomic status.

^b Negative at a serum dilution of 1:10.

^c Antibodies analyzed by complement-dependent lysis or by microneutralization.

FIG. 6. Antibody titers determined by ADCC and by complement-dependent lysis in sera of patients with acute herpetic pharyngitis. Symbols: ○, sera from patient 1 tested by ADCC; ●, sera from patient 1 tested by complement-dependent lysis; △, sera from patient 2 tested by ADCC; ▲, sera from patient 2 tested by complement-dependent lysis.

The results of these assays (Table 4) support the concept that some individuals without detectable antibody by the neutralization test or by complement-dependent lysis had antibodies detectable by ADCC. All of the sera that were negative by complement-dependent lysis but positive by ADCC were assayed by the ADCC technique, using uninfected BHK-21 cells as target cells; none contained antibodies to uninfected cells, suggesting that the lysis of infected BHK-21 cells in the ADCC represented virus-specific antibody. Furthermore, 21 of the sera were titrated for antibody by complement-dependent lysis and ADCC. A correlation was observed between titers obtained by the two methods (Fig. 7). Of the 10 sera with titers of 1:500 or less by ADCC, 7 had no detectable antibody by complement-dependent lysis. All sera with ADCC titers of greater than 1:500 had antibodies detectable by complement-dependent lysis.

DISCUSSION

Lysis of cells by antibody to surface antigens and effector cells from nonimmune animals is thought to represent the terminal sequence of events initiated by attachment of effector cells through Fc receptors to the antibody molecules (20, 27). Pertinent to this study is the exquisite sensitivity of this reaction for detection of antibody. Lysis of cells infected with HSV by antiviral antibodies and mouse peritoneal exudate cells (20) and human peripheral blood cells (24, 27) was previously described. We found that a continuous line of baby hamster kidney cells (BHK-21) infected with either HSV-1 or HSV-2 can be readily lysed with human antibody and hamster peritoneal exudate cells. As reported by others (24, 27), we found the ADCC assay capable of detecting antibodies to these herpesviruses at serum dilutions several hundred times greater than by the standard neutralization test or by complement-dependent antibody lysis of virus-infected cells. Shore et al. (27) were unable to differentiate past infections

with HSV-2 by the ADCC assay, and we also were unable to clearly make this distinction by the assay we developed (unpublished data). Although it provides no advantage over neutralization assays or assays in which complement is used with respect to ease of performance and cost, the ADCC test is clearly useful in detecting HSV antibodies at low concentrations.

Antibody titers to the HSV tend to remain relatively constant throughout adult life (5, 8, 9, 25, 31). Yet, when comparing populations living in different socioeconomic settings, differences are observed both in the percentage of the populations with detectable antibodies to the viruses and in the mean antibody titers of those who are seropositive (1, 4, 6, 10-13, 15, 17-19, 21, 28, 31). This suggests that environmental factors influence the antibody levels in humans. A decrease of antibodies to undetectable levels has been observed in a few children after primary herpetic gingivostomatitis (8, 30). Antibody titers appear to rise rapidly among these children, with the reappearance of virus in the oral secretions. These observations suggest that, after a primary infection, some individuals do not have a persistence of antiviral antibody detectable by standard methods. It is possible that establishment of latent infection may be required for the maintenance of antibodies at a given level. Individuals not establishing a latent infection during an initial exposure to the virus could become susceptible to subsequent reinfections during which they may become latently infected. Reinfections and/or recurrence may thus result in the development of increased levels of antibodies in certain patients (2, 3, 8, 25, 30). If this is true, the environment factors fostering increased exposure to the viruses would not only influence the probability of being infected initially, but also increase the probability of being reinfected. This could account for the observed differences in the percentage with antibodies and the differences in mean antibody titers among the seropositives in different social settings.

TABLE 4. Seronegativity of selected sera from women with cervical neoplasia and control women as determined by three techniques

Disease state	No. studied	Negative for HSV antibodies ^a					
		Neutralization		Complement-dependent lysis		ADCC	
		HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2
Cervical neo- plasia ^b	50	22	22	21	25	4	4
Controls	50	30	30	30	40	9	9

^a All assays were performed at a serum dilution of 1:10.

^b Cases of invasive carcinoma and carcinoma in situ included.

FIG. 7. Comparison of titers of antibodies to HSV-2 obtained by complement-dependent lysis and by ADCC.

By complement-dependent antibody lysis, we found antibodies to the HSV in 40% of the college students examined. This finding is in agreement with those of Glezen et al. (7) and Smith et al. (28), who found between 30 and 48% of college students with neutralizing antibodies to type 1 virus. However, by using the more sensitive ADCC assay, we were able to detect antibodies in 73% of our student population. Approximately 33% of the students appeared to have been infected in the past but did not maintain antibody titers sufficient to be detected by the less sensitive method.

Seroepidemiological studies have shown an association between antibodies activity to HSV-2 and carcinoma of the cervix (1, 4, 6, 10-12, 15-18, 21). According to the standard antibody assay techniques, not all women with cervical cancer show evidence of past infection with HSV-2 (21). This implies that infection by the virus may not be a necessary factor in the genesis of the cancer. However, we were unable to find a single serum without detectable antibodies to HSV-2 among 400 cancer cases. This observation suggests that essentially all women with cervical cancer have been infected with either HSV-1 or HSV-2. If, as postulated above, establishment of latency is required to maintain antibody titer levels detectable by standard assays, then our data suggest that some women may develop cervical neoplasia without becoming latently infected.

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