NOTES

Competitive Inhibition by Human Sera of Mouse Monoclonal Antibody Binding to Glycoproteins C and D of Herpes Simplex Virus Types 1 and 2

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A competitive enzyme-linked immunosorbent assay was used to test for human antibodies to antigenic sites on herpes simplex virus (HSV) glycoproteins C and D, which are recognized by mouse monoclonal antibodies. Antibodies capable of blocking the monoclonal antibodies were detected in the human sera, and the inhibition of binding correlated with the histories of herpetic infections. The binding of monoclonal antibody to glycoprotein C of HSV type 2 was inhibited primarily by sera from patients with recurrent herpes genitalis; however, the binding of the monoclonal antibodies to gC of HSV type 1 was inhibited by sera from patients previously infected with either HSV type 1 or HSV type 2. The observations suggest that the antigenic sites defined by the mouse monoclonal antibodies are recognized by the human host.

A number of antigenic sites have been delineated on glycoprotein D (gD) of herpes simplex virus type 1 (HSV-1) and HSV-2 (2) and on glycoprotein C (gC) of HSV-1 (6a). Each antigenic site appears to be composed of a single epitope or overlapping distinct epitopes (6a). Infection with HSV-1 or HSV-2 results in the production of type-specific as well as type-common antibodies to the viral glycoproteins (12). The type-specific antibodies might be directed against unique antigenic sites, or they might be directed against unique epitopes within an antigenic site also containing shared epitopes. With mouse monoclonal antibodies to epitopes on gC of HSV-1 and HSV-2 and on gD of HSV-2, we asked two questions in the present study. Are the antigenic sites of the HSV glycoproteins, as defined by studies in mice, also antigenic in natural infections of humans? If so, are the gC epitopes defined as type specific by mouse monoclonal antibodies associated with type-specific antibodies to this antigenic site in human sera? Based on our previous experience that monoclonal antibodies recognizing different epitopes within the same antigenic site were mutually competitive (6a), a competitive enzyme-linked immunosorbent assay (ELISA) was used to answer these questions. The underlying assumption was that human antibodies capable of inhibiting the binding of a mouse monoclonal antibody in this assay were directed against the same epitope or epitopes in close proximity.

Sera from patients selected on the basis of their experience with HSV-1 and HSV-2 were tested for their ability to block four monoclonal antibodies. These included sera from 14 children 1 year of age who lacked antibodies to either HSV-1 or HSV-2 (10), acute and convalescent sera from 10 patients with first-episode genital infections, paired sera from 10 patients who suffered from severe recurrent genital

herpes caused by HSV-2, single serum samples from 14 patients who suffered from periodic recurrent herpes labialis, and sera from 23 patients with cervical cancer and 27 control women matched for age and race (5). Two monoclonal antibodies, C7 and C16, were used to detect human antibodies to antigenic sites on gC of HSV-1 (6a), whereas monoclonal antibody 17αC1 was used to detect human antibodies to gC of HSV-2 (1). A monoclonal antibody, 17βA3, was used to assess antibodies to a type-specific epitope on gD of HSV-2 (8). Ascitic fluid preparations of the monoclonal antibodies were used, and their potency was determined by the ELISA.

An ELISA was used to quantitate mouse monoclonal antibodies to HSV-1 and HSV-2 glycoproteins, and antibodies in human sera were detected by blocking binding of these mouse monoclonal antibodies with the same assay. Antigens for the assay were prepared as previously described (11) with Vero cells infected with the KOS strain of HSV-1 and the 333 strain of HSV-2. To determine the concentration of the antigens, 50 μl of serially diluted antigen was placed in wells of Linbro microtiter plates (Flow Laboratories, Mississauga, Ont.) and incubated overnight at 37°C. The plates were then stored at -40°C until used. The antigen-containing plates were thawed, washed three times, and reacted with monoclonal antibodies. After incubation and removal of unbound antibody, goat anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Cappel Laboratories, Malvern, Pa.) was added and substrate was subsequently added. The antigen titer was taken as the reciprocal of the highest dilution yielding an optical absorbance of 0.05 or greater. Four times this concentration was used in the assays for antibodies.

The competitive ELISA was carried out by thawing the antigen-containing plates and washing them three times with a saline-Tween 20 solution (8.5 g of NaCl per liter plus

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0.05% Tween 20). Human sera were diluted 1:20 in phosphate-buffered saline-Tween 20-bovine serum albumin (0.05% Tween 20 and 0.1% bovine serum albumin), and 50 μ l was added to duplicate wells. After 30 min of incubation at 37°C, unbound antibody was removed by three washes with saline-Tween 20. A 50- μ l sample of monoclonal antibody diluted 1:100 in phosphate-buffered saline-Tween 20-bovine serum albumin was added to each well, and the plates were again incubated for 30 min at 37°C. After three washings with saline-Tween 20, 50 μ l of goat anti-mouse IgG conjugated with horseradish peroxidase diluted 1:200 in phosphate-buffered saline-Tween 20-bovine serum albumin was added to each well. The goat anti-mouse IgG (lot 17497; Cappel Laboratories) was adsorbed with 10% human serum lacking antibodies to HSV-1 and HSV-2 to remove antibodies cross-reacting with human IgG. The appropriate dilutions of these reagents were determined by prior titrations. The conjugated antibody was incubated in the plates for 30 min at 37°C, and then the plates were again washed three times with saline-Tween 20. Then 50 μ l of substrate was added, the plates were held at room temperature for 1 h, and the adsorbance was determined with an ELISA reader. The antibody activity in human sera was expressed as inhibition ratios, which were calculated by dividing the mean adsorbance obtained in wells receiving human sera by the mean adsorbance for wells receiving monoclonal antibodies only. The degree of inhibition of binding by monoclonal antibodies was inversely related to the dilution of the human serum when multiple dilutions were tested (data not shown). An estimate of intratest variation made by analyzing differences between duplicate determinations of all of the sera indicated that approximately 30% inhibition could result from test variables at a *P* value of 0.05. To reduce the effect of test-to-test variation, all sera were tested in two experiments for each monoclonal antibody.

If antibodies to the epitopes recognized by the mouse monoclonal antibodies were present in human sera, sera from patients with previous HSV-1 or HSV-2 infections should inhibit the binding of the monoclonal antibody to viral antigen. Thus, sera from persons who experienced recurrent herpes labialis or recurrent herpes genitalis as well as from children not infected with either virus were tested by competitive ELISA (Fig. 1). The inhibition ratios within each patient group varied considerably. However, the inhibition ratios for the monoclonal antibodies recognizing HSV-1 gC (C7 and C16) were generally lower for sera from patients who experienced recurrent herpes labialis than for sera from the other two patient groups. It is also apparent that sera from patients who experienced recurrent herpes genitalis inhibited the binding of the monoclonal antibody to HSV-2 gC (17 α C1) to a greater degree than did sera from the other patients. A similar pattern was not observed when the sera were assayed for inhibition of binding of the monoclonal antibody 17 β A3, which recognized the gD of HSV-2.

These data suggest that the responses of groups of patients differ according to previous experience with the viruses; however, the antibody responses of individual persons could not be identified with certainty because of the variability of the competitive ELISA. Therefore, the distributions of the inhibition ratios for the groups were tested for significant differences by using the *t*-test. In addition to the data shown in Fig. 1, the results of analyses of sera from two additional groups of patients were included. The neutralizing antibody titers of these latter patients were known, but their histories of herpetic infections were not. The mean inhibition ratios of the groups are presented in Table 1.

For monoclonal antibody C7, the mean inhibition ratio of sera from children without past herpes simplex infections was close to 1.00 and was significantly different from those of the other groups (*P* < 0.01). The amount of inhibition detected in sera from patients with recurrent herpes labialis was similar to that of women with cervical cancer and their controls. The inhibition ratios for patients with recurrent herpes genitalis was significantly less than for the antibody-negative children, but was significantly greater than the values obtained for the other three groups (*P* < 0.05). Similar relationships between inhibition ratios and patient groups were observed for monoclonal antibody C16. For the HSV-2 gC type-specific monoclonal antibody 17 α C1, inhibition ratios for patients with recurrent herpes genitalis were significantly less than inhibition ratios for the other groups (*P* < 0.01). The inhibition ratios of the other groups were not significantly different from those of the antibody-negative children, except for patients with recurrent herpes labialis (*P* < 0.05). The gD type 2-specific monoclonal antibody, 17 β A3, produced a different pattern. Mean inhibition ratios exceeded-

FIG. 1. Effect of patient history of HSV infections on the ability of sera to block binding of monoclonal antibodies. Sera from 1-year-old children who had no history of herpetic infection and who were seronegative for HSV neutralizing antibodies (HSV-), sera from young adults with histories of recurrent herpes labialis and without known genital herpes infections (HSV-1), and sera from patients with a history of recurrent genital herpes and without recurrent herpes labialis (HSV-2) were tested at a 1:20 dilution for their ability to block the monoclonal antibodies. The results are presented as inhibition ratios, which were derived by dividing the mean optical adsorbance value of duplicate wells receiving the human serum by the mean adsorbance value of wells receiving monoclonal antibody only.

TABLE 1. Inhibition of binding of monoclonal antibodies by sera from different patient groups

Patient group	No. of sera	Inhibition ratios (mean $\pm$ SD)			
		C7	C16	17 α C1	17 β A3
Children without HSV infection	14	0.97 $\pm$ 0.25	0.90 $\pm$ 0.25	0.73 $\pm$ 0.12	0.99 $\pm$ 0.26
Patients with recurrent herpes labialis	14	0.48 $\pm$ 0.18	0.32 $\pm$ 0.18	0.56 $\pm$ 0.15	0.86 $\pm$ 0.31
Patients with recurrent herpes genitalis	20	0.68 $\pm$ 0.26	0.51 $\pm$ 0.25	0.27 $\pm$ 0.10	1.14 $\pm$ 0.29
Patients with cervical cancer	23	0.47 $\pm$ 0.17	0.40 $\pm$ 0.22	0.64 $\pm$ 0.24	1.14 $\pm$ 0.31
Control women	27	0.47 $\pm$ 0.19	0.34 $\pm$ 0.21	0.67 $\pm$ 0.28	0.79 $\pm$ 0.27

ing unity were observed for patients with cancer and those with recurrent herpes genitalis. The values for these two groups were not significantly different from each other, but were significantly different from the mean inhibition ratios observed in sera from control women or from patients with recurrent herpes labialis ($P < 0.05$). These observations indicate that the inhibition of binding of the monoclonal antibodies correlates with the previous history of herpetic infections, except for monoclonal antibody 17 β A3.

Acute and convalescent sera from patients thought to have first-episode genital herpes were tested in the competitive ELISA to substantiate the correlation between infection and the development of antibodies recognizing the antigenic sites to which the monoclonal antibodies were directed (Table 2). The first serum samples were obtained during the initial clinic visit; neutralizing antibodies, which were detectable at a dilution of 1:10 or greater, were found in 3 of the 10 patients. A clear rise in neutralizing antibodies was not seen in the convalescent sera of these patients. For the remaining seven patients a rise of titers of less than 1:10 to greater than 1:10 was found between the acute and convalescent sera. HSV-1 strains were isolated from the lesions of four patients, and HSV-2 strains were isolated from the others (Table 2). In the three acute-phase sera with neutralizing antibodies, inhibition of binding of all four monoclonal antibodies was observed in one patient (no. 104), whereas in two patients (nos. 103 and 164) the binding of three of four monoclonal antibodies was inhibited. In the seven acute-phase sera lacking neutralizing antibodies, inhibition of binding of monoclonal antibody C16 was observed for patients 154, 106, and 162. Inhibition of binding of 17 α C1 was observed for patient 153 and inhibition of binding of 17 β A3 was observed for patients 165 and 166.

Among the patients with neutralizing antibodies in their acute-phase sera, only the inhibition ratios for 17 β A3 changed as the episode progressed. This included an increase in the inhibition ratio of patient 103 and a decrease in the ratio for patient 164. Among the patients without neutralizing antibody in acute-phase sera, decreases in the inhibition ratios were observed for all monoclonal antibodies for patient PF05 and for three of four monoclonal antibodies for patient 106. Antibodies inhibiting the binding of 17 β A3 and 17 α C1 were acquired in the convalescent period by patients 162 and 166, respectively. These data suggest that antibodies to the epitopes recognized by the monoclonal antibodies appear early in the course of infection. In addition, the responses do not appear to be totally type specific. Antibodies inhibiting the binding of the type 1 monoclonal antibody C7 were associated with three of four type 1 infections and with one of six type 2 infections. Those inhibiting monoclonal antibody C16 were associated with all four type 1 infections and four of six type 2 infections. Antibodies inhibiting the monoclonal antibody to gC of HSV-2, 17 α C1, were associ-

ated with three of four type 1 infections and with five of six type 2 infections. Antibodies blocking the binding of 17 β A3, a monoclonal antibody to gD of HSV-2, were associated with three of four type 1 infections and with five of six type 2 infections.

TABLE 2. Antibody responses associated with first-episode genital infections

Patient no.	Type HSV isolated ^a	Day of sample	Inhibition ratio ^b				Neutralization titer ^c	
			C7	C16	17 α C1	17 β A3	HSV-1	HSV-2
103	1	0	0.73	0.37	0.14	0.56	<1.00	1.35
		+23	0.80	0.53	0.32	0.81	<1.00	1.45
104	1	0	0.40	0.11	0.32	0.69	1.90	1.75
		+10	0.33	0.11	0.18	0.69	2.35	1.90
		+17	0.33	0.05	0.18	0.69	2.05	1.75
154	1	0	1.33	0.32	0.55	1.13	<1.00	<1.00
		+9	0.87	0.26	0.36	1.00	1.15	1.20
		+18	0.93	0.21	0.61	0.94	1.15	1.35
PF05	1	0	0.87	1.00	0.86	0.88	<1.00	<1.00
		+5	0.87	0.95	0.73	0.75	<1.00	<1.00
		+13	0.53	0.58	0.45	0.56	1.60	1.55
106	2	0	1.13	0.68	0.68	1.06	<1.00	<1.00
		+9	0.87	0.32	0.23	0.50	1.00	1.35
		+19	1.00	0.42	0.14	0.63	1.40	2.20
153	2	0	1.33	0.53	0.36	0.81	<1.00	<1.00
		+10	1.13	0.58	0.50	1.06	<1.00	1.35
		+18	1.27	0.37	0.41	0.94	<1.00	1.60
162	2	0	0.87	0.53	0.59	0.75	<1.00	<1.00
		+9	0.80	0.63	0.59	0.19	1.00	1.60
		+16	1.00	0.68	0.50	0.19	1.50	1.80
164	2	0	0.53	0.16	0.36	0.88	1.85	1.90
		+11	0.33	0.21	0.45	0.44	1.75	1.90
		+16	0.40	0.26	0.50	0.19	1.65	1.75
165	2	0	1.33	1.00	0.68	0.25	<1.00	<1.00
		+9	1.07	0.79	0.45	0.31	<1.00	1.40
		+16	0.80	0.74	0.64	0.25	1.00	1.75
166	2	0	1.13	1.11	0.91	0.31	<1.00	<1.00
		+9	1.00	1.00	0.59	0.19	<1.00	1.45
		+64	1.13	0.95	0.50	0.28	1.00	1.50

^a Viruses were isolated from the genital lesions and typed with monoclonal antibodies by a method detailed elsewhere (11).

^b Inhibition ratios of less than 0.70 (0.52 for 17 α C1) were taken as evidence for the presence of human antibodies that inhibit binding of monoclonal antibodies.

^c Neutralizing antibodies were quantitated by a previously described microneutralization test (10) and the titers are expressed to log₁₀.

The inhibition ratios obtained with the different monoclonal antibodies were assessed by the Pearson product-moment correlation coefficient for interrelatedness. A correlation coefficient of 0.646 was obtained when the inhibition ratios to monoclonal antibody C7 were compared with inhibition ratios obtained with monoclonal antibody C16. This correlation was highly significant ($P < 0.001$), and it was the only significant correlation found. Since monoclonal antibodies C7 and C16 recognize closely related epitopes on gC of HSV-1, whereas 17 α C1 and 17 β A3 recognize different epitopes, this finding provides further evidence that the competitive ELISA detected human antibodies to the antigenic sites recognized by the monoclonal antibody.

The interrelatedness of inhibition ratios obtained by the competitive ELISA and titers of neutralizing antibodies was also examined. Significant negative correlations ($P < 0.01$) were found between HSV-1 and HSV-2 neutralizing antibody titers and inhibition ratios for monoclonal antibodies C7, C16, and 17 α C1 when sera from patients with first-episode genital herpes were examined. This finding was expected, since acute-phase sera with little or no antibody activity were compared with convalescent-phase sera containing appreciable quantities of antibodies to multiple antigens, and a decrease in inhibition ratios would correlate with an increase in neutralizing antibody titers. Significant correlations were also found between HSV-1 neutralizing titers and inhibition ratios for C7, C16, and 17 α C1 in sera from patients with recurrent herpes genitalis (P values of 0.025, <0.001 , and 0.02, respectively). However, among these patients only inhibition ratios for C16 and 17 α C1 correlated significantly with neutralizing antibody titers to HSV-2 (P values of <0.001 and 0.009, respectively). In a larger sample of sera from women with cervical cancer and their controls, significant correlations were found only between HSV-1 neutralizing titers and inhibition ratios for C7 ($P = 0.04$) and C16 ($P < 0.001$) as well as HSV-2 neutralizing titers and inhibition ratios for C16 ($P = 0.009$). Thus, in all patient groups the most significant correlations were observed between neutralizing antibody titers to either virus type and inhibition ratios for monoclonal antibody C16. In contrast, the inhibition of binding of monoclonal antibody 17 β A3 was not correlated with neutralizing titers for any group.

There are limitations to an observational study such as the one we have presented, since the knowledge regarding all relevant variables is lacking. In our case, absolute knowledge of past infections with the HSVs was not known. The absence of a history of a herpetic infection does not exclude a subclinical infection. However, the patients with severe recurrent genital infections had neutralizing antibody patterns characteristic of patients infected with HSV-2 only (9), and the patients with recurrent herpes labialis had the demographic characteristics of persons at low risk of acquiring HSV-2 (13). Thus, we feel that improper classification of the patients to a degree sufficient to alter the conclusions is unlikely. A second unknown was the antigenic makeup of the viruses with which the patients were infected. With monoclonal antibodies, considerable variation in certain epitopes of HSV-1 and HSV-2 have been demonstrated (7), and the stability of the epitopes recognized by the monoclonal antibodies we used is not known. Despite these limitations, the data strongly suggest that antibodies to antigenic sites recognized by the mouse monoclonal antibodies are present in human sera. First, greater inhibition of binding of the monoclonal antibodies was observed in convalescent-phase sera than in acute-phase sera of patients with first-episode genital infections. This was especially

evident among paired sera lacking neutralizing antibodies in the initial sample (Table 2). Second, the degree of inhibition of binding by the monoclonal antibodies to gC epitopes correlated with the history of the type of herpetic infection experienced by the patient (Table 1). Finally, the specificity of the competitive ELISA was supported by a strong correlation of inhibition ratios only for monoclonal antibodies C7 and C16, which recognize epitopes clustered at the same antigenic site of gC of HSV-1 (6a).

Our observations also suggest that the epitopes recognized by monoclonal antibodies C7 and C16 are in close proximity to shared epitopes of gC of the two viruses, whereas the epitope recognized by monoclonal antibody 17 α C1 may function as a type-specific site on gC of HSV-2 in the natural infection. The concept that type-specific and type-common epitopes may reside in the same antigenic site of HSV glycoproteins has been substantiated by recently published experimental data. Within the first 23 N-terminal amino acids of gD of HSV-1 and HSV-2 there are two amino acid differences. With oligopeptides of various portions of the N-terminal region of the protein as antigens, three regions of antigenicity could be defined. A common region was defined by amino acids 11 to 19, and two HSV-2 gD-specific regions were defined by flanking amino acids. Thus, both type-specific and shared regions were found in the same antigenic domain (3). The DNA sequences of gC of HSV-1 and HSV-2 have been determined (4, 6); although a fair degree of heterogeneity exists between the two viruses, there are portions with a high degree of homology. Analysis of the nucleotide sequence of a mutant of HSV-1 resistant to neutralization by monoclonal antibody C16 suggested that the epitope recognized by this monoclonal antibody involved amino acid 129 (L. Holland, M. Levine, and J. Glorioso, unpublished data). This area of the molecule represents a major hydrophilic region where considerable homology is shared with gC of HSV-2. Zweig and co-workers (14) found antibodies raised against a synthetic oligopeptide of deduced amino acids 128 through 139 of HSV-1 gC to be cross-reacting. These observations support our finding by competitive ELISA of antibodies in human sera that cross-reacted to this antigenic site.

Monoclonal antibodies C7 and C16 sterically hinder the binding of each other; however, they can be assigned to separate epitopes on the basis of their ability to neutralize virus mutants selected by the monoclonal antibodies (6a). In human sera we found good correlations between inhibition ratios of these monoclonal antibodies and neutralizing antibody titers to HSV-1 and HSV-2. However, the inhibition against C16 was generally greater than that against C7, and for sera collected long after the initial infection only the inhibition ratios of C16 correlated with neutralizing activity. This finding implies that the molecular configuration recognized by monoclonal antibody C16 may be more relevant to neutralization in the natural setting than that recognized by C7.

The lack of inhibition of binding monoclonal antibody 17 β A3 by sera from patients with recurrent herpes genitalis was unexpected, since this monoclonal antibody recognized a type-specific epitope on gD of HSV-2 (8). Clear inhibition of binding of this monoclonal antibody occurred only when it was tested against convalescent sera of some patients with first-episode genital herpes (i.e., patients 162 to 166 in Table 2). The mean inhibition ratios for sera from patients with recurrent herpes genitalis and cervical cancer cases exceeded unity and were significantly different than those of patients with recurrent herpes labialis and those of control

women (Table 1). The reasons for this pattern of reactivity in the competitive ELISA are not readily apparent and will require further study. Further application of the competitive ELISA and monoclonal antibodies to specific epitopes on antigenic sites of the glycoproteins should permit resolution of this finding and possibly define the role of the antigenic sites.

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