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NOTES

Potent Neutralizing Activity Associated with Anti-Glycoprotein D Specificity Among Monoclonal Antibodies Selected for Binding to Herpes Simplex Virions

MICHAEL F. PARA,† MARY LYNN PARISH, A. GWENDOLYN NOBLE,‡ AND PATRICIA G. SPEAR*
Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

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Thirty-three monoclonal antibodies were selected for ability to bind to purified virions of herpes simplex virus and were shown by immunoprecipitation to react with one or another of the envelope glycoproteins. Only six of these antibodies exhibited potent neutralizing activity, and all six were specific for glycoprotein D. Two other anti-glycoprotein D antibodies and 25 antibodies specific for four other viral glycoproteins had much less potent, if any, neutralizing activity.

To obtain monoclonal antibodies useful for probing the activities of herpes simplex virus (HSV) envelope proteins, we isolated hybridomas selected for secretion of antibodies capable of binding to the surfaces of purified virions. Here we describe the isolation of these hybridomas and the characterization of the secreted antibodies with respect to isotype, antigen recognition, and neutralizing activity. Previously we showed that some of these antibodies can block HSV-induced cell fusion (25). Elsewhere (16) we document that some of the antibodies can block the adsorption of virions to cells and also show that potent neutralizing activity is not associated with blocking of adsorption.

Immunization of mice and isolation of hybridomas. Each of the BALB/c mice used as spleen donors for three independent fusions was injected at least once with purified UV-inactivated virions of HSV type 1 (HSV-1) strain HFEMsyn [HSV-1(HFEM)syn] (3) or HSV-2(G) (14). Some of the mice were also first injected with isolated glycoprotein E (gE) of HSV-1(HFEM)syn. Virions were purified as previously described (5, 39), and gE was isolated by affinity chromatography (3). For each injection the quantities of immunogen used were 1×10^9 to 2×10^9 PFU of virus (before UV inactivation) or the amount of gE purified from about 10^9 infected HEp-2 cells. Details of the immunization schedule for each fusion reaction are as follows. For fusion I, isolated gE was injected intraperitoneally (i.p.) in Freund complete adjuvant on day 0; gE was injected i.p. in Freund incomplete adjuvant on day 15; UV-inactivated HSV-1(HFEM)syn virions were injected intravenously on day 31; and the mouse was sacrificed on day 34. For fusion II, isolated gE was injected i.p. in Freund complete adjuvant about 5 months before beginning the course of immunization with purified UV-inactivated HSV-1(HFEM)syn virions; virions were injected i.p. with Freund incomplete adjuvant on days 0 and 6; virions were injected intravenously on day 14; and the mouse was sacrificed on day 17. For fusion III, purified UV-inactivated HSV-2(G) virions were injected i.p. with Freund complete adjuvant on day 0 and i.p. with incomplete adjuvant on day 6; virions were injected intravenously on day 41; and the mouse was sacrificed on day 44.

The methods used to produce and clone hybridomas and to prepare ascites fluids were those described by McKearn et al. (24) and in more detail by McKearn (22, 23). The myeloma cells used were Sp2/0-Ag14 (35). To detect antibodies in sera from the immunized mice and in media from the growing hybridoma cells, we performed a solid-phase radioimmunoassay. Purified virions were collected by centrifugation and suspended in 0.5 mM phosphate buffer (pH 7.4), and 50-μl samples containing 5×10^6 to 10×10^6 PFU were plated in 96-well polyvinyl trays. The trays were left overnight in the cold to permit adsorption of the virions to the polyvinyl, and then each well was treated with 100 μl of 5% bovine serum albumin in phosphate-buffered saline (10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$ [pH 7.4]) (PBS). After each well was washed 10 times with PBS, 25 μl of hybridoma medium or serum dilution was added for incubation at 37°C (1 h). After each well was again washed 10 times with PBS, 20 μl of ^{125}I-protein A (code IM.112, >30 mCi/mg, 100 μCi/ml; Amersham Corp., Arlington Heights, Ill.) diluted 1:100 in PBS containing 1% bovine serum albumin was added to each well for incubation at 37°C (1 h). After a final washing with PBS, the polyvinyl trays were exposed for about 8 h to X-ray film at −70°C with an intensifying screen.

Hybridomas that scored positive in this binding assay (115 in number) were then screened for neutralizing and immunoprecipitating activities of the secreted antibodies. The 39 hybridomas that were clearly positive in either of these assays were selected for the production of ascites fluids. Data on 33 of the 39 ascites fluids are presented here. The remaining six ascites fluids had little or no neutralizing activity.

* Corresponding author.
† Present address: Department of Medicine, Ohio State University, Columbus, OH 43210.
‡ Present address: University of Health Sciences, The Chicago Medical School, North Chicago, IL 60064.

TABLE 1. Properties of the anti-HSV monoclonal antibodies produced

Monoclonal antibodies	IgG subclass[a]	Precipitating activity[b]			Neutralizing activity[c]			
					HSV-1(HFEM)syn		HSV-2(G)	
		Glycoprotein precipitated	HSV-1(HFEM)syn	HSV-2(G)	No complement	Complement	No complement	Complement
I-1-7	3	gB	+	+	−	−		
I-59-6	3	gB	+	+	−	−		
I-84-5	2a	gB	+	+	−	−		
I-99-1	2a	gD	+	+	+	++	−[d]	−[d]
I-144-2	2a	gB	+	−	−	−		
I-188-5	2a	gD	+	+	+	+	+	++
I-206-7	2a	gD	+	−	−	−	−	−
I-252-4	2a	gB	+	−	−	+		
II-73-3	3	gC	+	−	−	−		
II-95-2	2a	gC	+	−	−	+	−	−
II-105-2	2a	gB	+	+	−	−		
II-125-4	2a	gB	+	+	−	−		
II-137-1	2a	gB	+	+	−	−		
II-387-1	2a	gB	+	+	−	−		
II-436-1	2a	gD	+	−	+	++	−[d]	−[d]
II-474-1	ND[e]	gC	+	ND	−	−		
II-475-1	2b	gC	+	−	−	−		
II-481B-2	2b	gE	+	−	−	−		
II-512-3	3	gC	+	−	−	−		
II-529-1	ND	gC	+	−	−	−		
II-603-2	3	gB	+	+	−	−		
II-659-3	1	gC	+	−	−	−		
II-694-6	2a	gB	+	+	−	−		
II-818-2	2a	gC	+	−	−	+	−	−
II-886-1	2a	gD	+	−	−	+	−	−
III-114-4	2b	gD	+	+	+++	+++	++	+++
III-174-1	2a	gD	+	+	+++	+++	+++	+++
III-188-4	2a	gC	−	+			−	−
III-195-2	3	gG	−	+			−	−
III-211-1	2a	gC	−	+	−	−	−	+
III-255-2	2a	gD	+	+	++	+++	++	+++
III-347-1	2a	gE	−	+			−	−
III-596-1	2a	gC	−	+			−	−

[a] All antibodies were of the IgG class. The subclass was determined by immunoprecipitation in agar with subclass-specific antiimmunoglobulin antibodies from Miles Laboratories, Inc. (Elkhart, Ind.) tested against fluids from the cultured hybridoma cells.

[b] Immunoprecipitations were performed as previously described (42) with extracts prepared from HEp-2 cells infected with HSV-1(HFEM)syn or HSV-2(G). The infected cells were incubated with [^{35}S]methionine or [^{14}C]glucosamine from 4 to 20 h after infection.

[c] Neutralizing activities were determined as described elsewhere (42) for representative batches of ascites fluids. Titers are expressed as the reciprocal of the dilution causing a 50% reduction in the number of PFUs. The actual titer of antibodies produced by a single cell line may vary somewhat from batch to batch of ascites fluid. The symbols given here denote ranges of titers as follows: −, <50; +, 50 to 9,000; ++, 10,000 to 99,000; +++, ≥100,000. Blank spaces indicate that the neutralization assays were not performed.

[d] −, Titer of <500 rather than <50 as elsewhere.

[e] ND, Not done.

Specificities of the monoclonal antibodies. The monoclonal antibodies resulting from fusions I and II (designated I-xx-x or II-xx-x, respectively [see Tables 1 and 2]) were induced in response to HSV-1(HFEM)syn, and those resulting from fusion III were induced in response to HSV-2(G). All 33 of the antibodies described here reacted with one or another of the virion envelope glycopolypeptides. Glycopolypeptides present in virions of HSV-1 and HSV-2 include gB, gC, gD, gE (37), and a species of 110,000 molecular weight (34) recently designated gH (4). The different forms of these glycoproteins produced by HSV-1 and HSV-2 share antigenic determinants but also differ to a greater or lesser extent in size and in structure of some antigenic determinants (37, 38). HSV-2 virions contain, in addition, a glycopolypeptide designated 92K or gG (20, 21, 33), the homolog of which has not yet been detected in HSV-1.

The antibodies described here, the immunoglobulin G (IgG) subclass of each, and the HSV glycopolypeptide recognized by each, according to the results of immunoprecipitation analyses done as previously described (42), are listed in Table 1. This table also indicates whether cross-reactivity with the antigen of the heterologous serotype could be detected by immunoprecipitation. Two criteria were applied for identification of the antigens that were precipitated. First, the electrophoretic mobilities of polypeptides precipitated from extracts of [^{35}S]methionine-labeled and [^{14}C]glucosamine-labeled infected HEp-2 cells were compared with those of polypeptides precipitated by previously characterized monoclonal antibodies or monospecific antisera. Second, sequential immunoprecipitations were done to ensure that the antibodies secreted by these hybridomas removed from antigen extracts the appropriate glycopolypeptide recognized by an antibody or antiserum of defined specificity. We previously presented the results of such experiments done with all the anti-gD antibodies and five of the anti-gB antibodies (25). Similar

TABLE 2. Titers of neutralizing ascites fluids

Ascites fluid	Neutralizing titer[a]							
	HSV-1				HSV-2			
	HFEM'syn		F		G		333	
	−C	+C	−C	+C	−C	+C	−C	+C
anti-gD								
III-114-4	100,000	>500,000	100,000	200,000	40,000	500,000	300,000	>500,000
III-174-1	200,000	200,000	500,000	>500,000	200,000	>500,000	100,000	400,000
III-255-2	60,000	200,000	100,000	300,000	40,000	100,000	40,000	100,000
I-188-5	1,000	5,000	20,000	100,000	7,000	50,000	3,000	50,000
II-436-1	4,000	10,000	20,000	60,000	<500	<500	<50	<50
I-99-1	50	10,000	<50	200,000	<500	<500	<50	<50
II-886-1	<50	200	<50	1,000	<50	<50	<50	<50
I-206-1	<50	<50	<50	<50	<50	<50	<50	<50
anti-gC								
II-95-2	<50	200			<50	<50		
II-818-2	<50	200			<50	<50		
III-211-1	<50	<50			<50	500		
anti-gB (I-252-4)	<50	100						

[a] Neutralizing activities were determined as described elsewhere (42). Titers are expressed as the reciprocal of the dilution of ascites fluid causing a 50% reduction in the number of PFUs for the virus strains indicated. Blank spaces indicate that the neutralization assays were not performed. −C, No complement; +C, complement was present.

experiments were also done with the other anti-gB antibodies and with all the antibodies that recognize HSV-1 gC (data not shown). Specificity of the antibodies reactive with HSV-2 gC and gE has previously been documented (27).

Neutralizing activities of ascites fluids. Neutralization assays were done exactly as described by Zezulak and Spear (42). Table 1 shows which of the antibodies exhibited neutralizing activity sufficient to cause a 50% reduction in PFU at ascites fluid dilutions of 1:50 or more. For all the anti-gD antibodies and any other antibodies that exhibited detectable activity, the neutralizing titers of ascites fluids were determined in assays against four HSV strains (Table 2). The large relative differences in the titers were due mostly to differences in intrinsic neutralizing potencies of the antibodies and not to differences in concentrations of monoclonal IgG in the ascites fluids. This was evident from electrophoretic analyses of the ascites fluids done to estimate the concentration of IgG and was also shown by neutralization assays done with purified IgG as described elsewhere (16).

All of the strong neutralizing antibodies were specific for gD, and all but one (7 of 8) of the anti-gD antibodies had some detectable neutralizing activity when tested at dilutions of 1:50 or higher. In contrast, only 4 of the 25 antibodies reactive with other glycoproteins exhibited neutralizing activity under these conditions and then only in the presence of complement.

It is likely that the eight anti-gD antibodies include at least seven distinct species, each of which may bind to different epitopes of gD. These antibodies were the products of hybridomas derived in three independent fusions, and they exhibited the different patterns of reactivity shown in Table 2. Moreover, when patterns of reactivity were found to be similar, the relevant antibodies belonged to different IgG subclasses (e.g., III-114-4 was 2b, whereas III-174-1 and III-255-2 were 2a). Eight gD epitopes were previously defined by use of other anti-gD monoclonal antibodies (11), and the locations of some of these epitopes on gD have been determined (6, 9, 12).

Neutralizing titers obtained in the presence of complement were in general two- to fivefold higher than in its absence. For I-99-1 in particular this enhancement of neutralizing activity by complement was very pronounced, similar to results reported elsewhere for some anti-gB and anti-gC monoclonal antibodies (17). It is also evident that neutralizing titers obtained in assays done with the virus strain used as immunogen were sometimes lower than those obtained with other virus strains (Table 2). We have no explanation now for this phenomenon.

DISCUSSION

It is well established that HSV gD can elicit and react with both cross-reactive and type-specific neutralizing antibodies (7, 8, 13, 18, 30, 36, 40, 41). The new information provided here is that gD probably elicits a greater number and variety of potent neutralizing antibodies than do other HSV virion antigens, at least among protein A-binding subclasses of IgG in mice.

Our results permit conclusions about the relative importance of the several HSV antigens in neutralization only if we did not inadvertently select against potent neutralizing antibodies of other specificities. We cannot now rule out the possibility that selection of protein A-binding antibodies (15, 19) introduced a bias with respect to the specificities of neutralizing antibodies obtained. We think this is unlikely, however, on the basis of a compilation of available data on other anti-HSV monoclonal antibodies selected by different criteria and without regard to protein A-binding capacity.

Table 3 gives data from several studies, including our own, in which relative neutralizing titers of monoclonal antibodies were presented. We note that, where anti-gD antibodies were included among those isolated, the antibodies which had the most potent neutralizing activity in the absence of complement were specific for gD. Holland et al. (17) described nine monoclonal antibodies selected for neutralizing activity in the presence or absence of complement. They found that only the single anti-gD antibody obtained had potent neutralizing activity in the absence or presence of complement, whereas seven anti-gC and one anti-gB antibodies neutralized only in the presence of complement. Showalter et al. (34) selected antibodies by immunoprecipi-

TABLE 3. Neutralizing activities of anti-HSV monoclonal antibodies characterized here and by others

Serotype of immunogen	Selection procedure	Glycoprotein recognized	No. of antibodies[a]			Range of titers[a]		Source or reference
			With neutralizing activity		No neutralizing activity detected	−C	+C	
			+ or − C	Only +C				
HSV-1	Immunoprecipitation	gB	1	3	5	800	25–3,200	34
		gC	2	6	0	25	800–1,600	
		gD	6	1	3	25–25,600	200–25,600	
		110K	4	0	0	25–800	25–800	
HSV-2	ELISA[b] and ^{125}I-protein A binding with infected cell lysates as antigen; indirect immunofluorescence on fixed cells	gB	0		1[c]		ND[c]	1, 2
		gC[d]	0		3[c]		ND	
		gD	1		1[c]	25	ND	
		gE	0		1[c]		ND	
		gG[e]	0		4[c]		ND	
HSV-1	ELISA with fixed infected cells	gB	0	1	1		40	32
		gC	1	0	0	640	>2,560	
		gE	0	0	1			
HSV-1	Neutralizing activity	gB	0	1	0		1,280	17
		gC	0	7	0		640–12,800	
		gD	1	0	0	12,800	102,400	
HSV-1 and HSV-2	^{125}I-protein A binding with purified virions as antigen	gB	0	1	10		100	This study
		gC	0	3	8		200–500	
		gD	6	1	1	50–200,000	200–>500,000	
		gE	0	0	2			
		gG	0	0	1			

[a] +C, Complement was present. −C, No complement. Range of titers reported for antibodies of the indicated specificity in neutralization tests against the virus used as immunogen. If no number appears, no neutralizing activity was detected.
[b] ELISA, Enzyme-linked immunosorbent assay.
[c] Neutralization tests done in the presence of complement were not reported. ND, Not done.
[d] Balachandran et al. (1, 2) designated this HSV-2 glycoprotein gF.
[e] Balachandran et al. (1, 2) designated this HSV-1 glycoprotein gC.

tation of solubilized proteins and showed that 23 of 41 antiglycoprotein antibodies had some level of neutralizing activity in the presence of complement (13 of these also neutralized in the absence of complement). The four antibodies with the highest titers in the absence of complement (≥1,600) were specific for gD. Although the study is not listed in Table 3 because quantitation of neutralizing activity was not presented, the first four anti-HSV monoclonal antibodies described (30) were selected for their neutralizing activity and shown to be specific for gD or gC.

Clearly, other HSV glycoproteins can also induce the production of neutralizing antibodies but are perhaps not so important in this regard. Purified gB, gC, and gE of HSV-1 (10, 26, 31, 40) and gC of HSV-2 (42) have each been shown to elicit neutralizing antibodies in rabbits, although most of the antisera had complement-dependent activity, and only weak activity was detected for anti-gE-1 and anti-gC-2. Comparative studies of immunogenicity in which neutralizing titers are assessed after injection under similar conditions of comparable amounts of the different HSV glycoproteins have not been reported to our knowledge. With regard to monoclonal antibodies, neutralizing activity has been detected with antibodies specific for gB, gC, gE, and gH (110K), as well as gD (Table 3; 28–30). Most of the anti-gB antibodies isolated thus far have no detectable neutralizing activity even in the presence of complement, and most of the anti-gC antibodies we isolated also had no detectable neutralizing activity. Among the 33 monoclonal antibodies described here, very few were specific for gE and gG and none for gH. Whether this reflects the relative inaccessibility of these antigens on virion surfaces or some other factor is at present uncertain.

The method of hybridoma selection used here required only that the secreted antibodies bind to the surfaces of purified virions and to protein A of *Staphylococcus aureus* (to facilitate identification of the recognized antigen). The antibodies that were selected, therefore, did not include any that failed to neutralize infectivity due to failure to bind to the virions (the trivial explanation), nor should there have been selection against nonneutralizing antibodies that can bind to virions. It is of interest that most of the antibodies characterized here did not have neutralizing activity. Characterization of the antibodies selected can ultimately permit discrimination between antigenic determinants that are or are not critical targets and elicitors of neutralizing antibodies. The results presented here, considered in conjunction with previously published data, permit some tentative conclusions to be drawn about the relative importance of the different HSV glycopolypeptides in eliciting and binding neutralizing antibodies.

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