Type-Common and Type-Specific Monoclonal Antibody to Herpes Simplex Virus Type 1

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Hybridoma cells produced by fusing mouse myeloma cells with spleen cells from mice primed with herpes simplex virus (HSV) type 1 yielded five clones producing neutralizing antibody against homologous virus. Two clones, HC1 and HC2, produced antibody capable of precipitating glycoprotein C and its precursor, whereas three clones, HD1, HD2, and HD3, produced antibody capable of precipitating glycoprotein D and its precursor. Antibody produced by the HC1 and HC2 clones neutralized HSV type 1 but not HSV type 2 or HSV type 1 strain MP, which is known to lack glycoprotein C. Antibody produced by the HD1 and HD2 clones neutralized both HSV type 1 and HSV type 2, whereas antibody produced by the HD3 clone neutralized HSV type 1 but not HSV type 2. The two clones which produced antibody to glycoprotein C and the two clones which produced type-common antibody to glycoprotein D were independently derived and not clonally related inasmuch as the antibody in each pair belonged to a different subclass of immunoglobulin.

Herpes simplex virus (HSV) types 1 (HSV-1) and 2 (HSV-2) are immunologically related, and hyperimmune sera produced against one virus will generally neutralize the heterologous serotype virus, although to a lower level (20, 21, 26). The antigenic specificity of the virus appears to be determined by the glycoproteins which are incorporated into the surface of the virion and, in the course of infection, into the various membranes of the infected cells (25). In both HSV-1 and HSV-2, five major virus-specific glycoproteins have been described and designated as gC, gB, gA, gE, and gD, in order of decreasing apparent molecular weight (1, 24). Previous studies have shown that antisera to HSV-1 glycoproteins D, C, A+B, and E are each able to neutralize virus but with different specificities (6, 7, 19, 23, 27, 29). Antibody to glycoproteins D, A+B, and E has been produced by immunization with purified antigens (6, 27). Antibody to glycoprotein C has been most commonly obtained by adsorption of antisera to wild-type strains with strain HSV-1 (MP), which lacks glycoprotein C (24). Anti-A+B and anti-D sera were able to neutralize both HSV-1 and HSV-2, whereas the anti-C serum neutralized the homologous wild-type virus but not HSV-2. The major problem with all of these antisera is that they are difficult to produce and are generally of low potency. Furthermore, such preparations frequently contain trace amounts of antibodies to

other glycoproteins and, therefore, the specificities of the antisera are difficult to prove unambiguously.

We report the production of monoclonal antibodies to glycoproteins C and D. These monoclonal antibodies show that glycoprotein C contains type-specific domains and that glycoprotein D contains both type-specific and type-common domains. Furthermore, the monoclonal antibodies can be used in immunofluorescence and neutralization tests and should prove to be useful reagents for the differentiation of HSV-1 and HSV-2.

MATERIALS AND METHODS

Cells and media. Human epidermoid carcinoma no. 2 cells (HEp-2) and African green monkey kidney (Vero) cells were grown in Dulbecco minimal essential medium supplemented with 10% fetal calf serum and maintained after infection in medium with 1% serum. The NS-1 cell line (14) was derived from MOPC-21, a BALB/c myeloma cell line. NS-1 and the hybrids derived by fusing NS-1 with mouse spleen cells were grown in RPMI 1640 supplemented with 15% horse serum.

Virus strains. The isolation and properties of a prototype of strains HSV-1(F), HSV-2(G), and HSV-1(MP), a spontaneously derived polycaryocyte-forming mutant obtained from the cyto-aggregating strain HSV-1(mP), have been described (8, 11, 12, 13). Strain 333 is an HSV-2 genital isolate. HTZ, HL4, and E377 are HSV-1 clinical isolates from the pharynx, lip, and eye, respectively.

Fusion of spleen cells with NS-1 cells and selection of antibody-producing hybrids. The pro-

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to col for producing the immunoglobulin-secreting hybrid cell lines used in this study was outlined by Oi and Herzenberg (17). Briefly, BALB/c mice were injected in the footpad with 10^4 plaque-forming units of HSV-1(HT2). After 4 weeks, the mice were primed (intraperitoneal injection) with soluble infected-cell extracts of HSV-1(F) (prepared as described below) in complete Freund adjuvant and with an aqueous antigen boost (intraperitoneal injection) administered 4 weeks later. After 3 days, the spleen of a hyperimmune mouse was removed, and the spleen cells were fused to an equal number of NS-1 cells with polyethylene glycol 1,000 (Sigma). The cultures were subjected to a 2-week selection regime with culture medium containing hypoxanthine, aminopterin, and thymidine (15). For screening the viable hybrids for cells producing the desired antibody, the culture fluids were tested in plaque reduction assays for neutralizing activity against HSV-1(F). For this test, 100 μ l of undiluted hybridoma cell culture fluid was mixed with 30 to 50 plaque-forming units of virus and incubated for 30 min at 37°C. The mixture was adsorbed to Vero cell monolayers, and the cells were grown for 3 days with a liquid overlay of Dulbecco minimal essential medium containing 0.1% pooled human gamma globulin. Hybridoma cultures which secreted a sufficient amount of antibody to neutralize 50% of the plaques were cloned by limiting dilution in the presence of BALB/c thymocytes. Clones which continued to produce neutralizing antibody were expanded. For producing ascites fluids from which concentrated immunoglobulin could be harvested, an intraperitoneal injection of 10^7 hybridoma cells was given to adult BALB/c mice.

Indirect immunofluorescence tests. HEp-2 cells were grown in monolayers on cover slips and infected with 30 to 50 plaque-forming units of HSV-1 or HSV-2. For producing foci of infection on an uninfected cell background, the cultures were incubated for 2 days with Dulbecco minimal essential medium containing 0.1% human gamma globulin. Antibody-containing culture media or ascites fluids were tested against unfixed infected cells to detect viral antigens on the cell surface. The infected cell monolayers were first washed with goat serum to eliminate nonspecific adsorption of immunoglobulin G (IgG) to Fc receptors (1, 28).

Preparation of radiolabeled antigen extracts. Monolayer cultures of HEp-2 cells were infected with virus at 20 plaque-forming units per cell and incubated from 5 to 24 h after infection with Dulbecco minimal essential medium containing 0.2 μ Ci of D-[1- 14 C]glucosamine hydrochloride per ml (45 to 60 mCi/mmol) (New England Nuclear). For some experiments, the cells were radiolabeled for 15 min at 5 h postinfection (pulse) and then washed with Dulbecco minimal essential medium and replenished with maintenance medium for the duration of the incubation period (chase). For radiolabeling all the viral proteins, a mixture of 14 C-amino acids (300 mCi/mmol) was added to a concentration of 2 μ Ci/ml to medium containing $\frac{1}{10}$ the amount of amino acids. After radiolabeling, the cells were harvested and sonicated in phosphate-buffered saline containing 1% sodium deoxycholate and Triton X-100 (Sigma) and centrifuged in an SW27.1 rotor at 25,000 rpm for 2 h at 4°C. Immune

precipitation reactions were done as previously described (24) with the following modification. The mixture of antigen and antibody was incubated for 2 h at 37°C, and then 5 mg of protein A-Sepharose CL-4B (Pharmacia) was added. In instances where the subclass of mouse IgG adsorbed poorly to protein A, an intermediate step was used in which the immune complex was precipitated by rabbit anti-mouse IgG (Miles). Protein A-Sepharose to which the immune complexes were bound was centrifuged at 4°C and repeatedly washed in buffer containing 0.1% sodium deoxycholate and Triton X-100. The immune precipitates were solubilized in sodium dodecyl sulfate for electrophoresis in 9.0% polyacrylamide gel slabs cross-linked with *N*-*N'*-diallyltartardiamide. After electrophoresis, the slabs were fixed in methanol and acetic acid and infused with an autoradiography enhancer (New England Nuclear), washed, dried on filter paper, and placed in contact with X-ray film at -70°C for fluorography (2).

RESULTS

Homogeneity of antibody secreted by cloned hybrids. Two tests for homogeneity were done. In the first test, the immunoglobulins produced by the various clones were tested in Ouchterlony double diffusion tests against rabbit anti-mouse immunoglobulin antisera. In these tests (not shown), the rabbit serum produced a single precipitin line with immunoglobulins from each of the cloned hybrids. Of five clones which were independently isolated, two produced antibody of the IgG2a subclass, and three produced antibody of the IgG1 subclass.

In the second test, the immunoglobulins were radiolabeled with 14 C-amino acids, and the radiolabeled IgG secreted into the extracellular fluid of cultured hybridoma cells was adsorbed to protein A, denatured in sodium dodecyl sulfate, and subjected to electrophoresis in 10% polyacrylamide gels. The electrophoretic profiles showed the limited heterogeneity in heavy and light chains (Fig. 1). The two clones, HC1 and HD1, which produced antibody of the IgG2a subclass, are shown. One heavy-chain band and one light-chain band were produced by each clone. The clones were independently isolated, and the electrophoretic mobility of their light chains differed. Antibody from clones HC2, HD2, and HD3 was of the IgG1 subclass, which bound less efficiently to protein A and yielded only a faint autoradiographic image. Analysis of the autoradiograms showed that each contained a single light chain and a heavy chain (data not shown).

Neutralizing activity of the immunoglobulin produced by hybridoma cells. Two types of neutralization tests were done. The first series of tests was designed to measure the neutralizing titer of the antibody produced by the

FIG. 1. Autoradiograms of ^{14}C -amino acid-radio-labeled IgG2a produced by hybridoma clones HC1 and HD1, denatured in sodium dodecyl sulfate, and electrophoretically separated in 10% polyacrylamide gels. L, Light chains.

different hybridoma clones against homologous virus. In this series of tests, the extracellular fluids or ascites fluids were diluted serially and mixed with 30 to 50 plaque-forming units of HSV-1(F), incubated at 37°C for 30 min, and then plated on Vero cells. The titer of the immunoglobulin was calculated from the amount required to reduce the number of virus plaques by 50%. The titer of the immunoglobulin released into the extracellular fluids ranged from 4 to 32 50% plaque-reduction units per 0.1 ml of immunoglobulin. The titer of antibody in ascites fluids was approximately 100-fold higher.

In the second type of neutralization test, immunoglobulins from the various hybridomas released into the extracellular fluids or ascites fluids of known titer against HSV-1(F) were tested against a number of HSV-1 and HSV-2 strains. Three significant observations, which are summarized in Table 1, emerged from these studies. Antibody produced by clones HC1 and HC2 neutralized the HSV-1(MP) strain but not the HSV-1(MP) or HSV-2(G) strains. Antibody titers of 128 to 256 and higher antibody concentrations in ascites fluids also failed to neutralize HSV-1(MP) and HSV-2(G). In view of the fact that HSV-1(MP) is a mutant of HSV-1(MP) lacking the ability to synthesize glycoprotein C, these results would suggest that HC1 and HC2 hybridomas synthesize antibody directed against glycoprotein C. The second observation of interest is that antibody produced by HD1 and HD2 hybridomas neutralized all of the strains in this study. The efficiency of neutrali-

zation of HSV-1(F), HSV-1(MP), and HSV-1(MP) was only slightly better than that of HSV-2 and, therefore, the antibody was directed against a type-common antigenic site. The third observation of interest is that antibody produced by clone HD3 neutralized all of the HSV-1 strains but not the HSV-2(G) strain and also failed to neutralize the unrelated HSV-2(333) strain. We conclude, therefore, that this antibody reacts with a type-specific antigenic site present in HSV-1 but not HSV-2 strains. Inasmuch as it neutralizes HSV-1(MP), it is probably not directed against glycoprotein C.

Detection of viral antigens on infected cells by indirect immunofluorescence tests with antibody from different clones. In this series of experiments, we tested the reactivity of two monoclonal antibodies with different specificities and a mouse hyperimmune antiserum to HSV-1 with unfixed HSV-1- and HSV-2-infected cells. The photomicrographs in Fig. 2 show that the hyperimmune antiserum reacted with HSV-1(F)- and HSV-2(G)-infected HEp-2 cells (panels a and b). As predicted by neutralization tests, however, the antibody produced by clone HC1 showed strong immunofluorescence with HSV-1(F)-infected cells but not with HSV-2(G)- and HSV-1(MP)-infected cells (panels c, d, and g), whereas clone HD1 reacted by immunofluorescence with cells infected with all of these strains (panels e, f, and h). In additional tests, antibody from clone HD3 reacted with HSV-1(F)-infected cells and weakly with HSV-2(G)-infected cells (data not shown). One interesting finding which emerged from this study concerned the pattern of immunofluorescence. The mouse hyperimmune sera produced a diffuse immunofluorescence on the surface of both HSV-1- and HSV-2-infected cells, whereas the monoclonal antibody produced discrete foci of immunofluorescence on the surfaces of the infected cells with which it reacted. The punctate nature of the immunofluorescence produced by the monoclonal antibody suggests either that

TABLE 1. Neutralizing activity of antibody from different clones in immune reactions with HSV-1 and HSV-2

Mono-clonal antibody	Neutralizing activity for:							
	HSV-1 strains						HSV-2 strains	
	F	mP	MP	HTZ	HL4	E377	G	333
HC1	+	+	0	+	+	+	0	0
HC2	+	+	0	+	+	+	0	0
HD1	+	+	+	+	+	+	+	+
HD2	+	+	+	+	+	+	+	+
HD3	+	+	+	+	+	+	0	0

FIG. 2. Photomicrographs of HSV-1- and HSV-2-infected HEp-2 cells in immunofluorescence reactions with hyperimmune antiserum and immunoglobulin produced by hybridoma clones. HSV-1(F) (a) and HSV-2(G) (b) with anti-HSV-1 mouse hyperimmune serum; HSV-1(F) (c) and HSV-2(G) (d) with immunoglobulin from clone HC1; HSV-1(F) (e) and HSV-2(G) (f) with immunoglobulin from clone HD1; HSV-1(MP) with immunoglobulins from hybridoma clones HC1 (g) and HD1 (h). The bar represents 25 μ m.

the individual glycoproteins aggregate on the plasma membrane or that they become aggregated after reaction with antibody.

Identification of the viral antigens which are targets of neutralizing antibody. The purpose of this series of experiments was to identify the antigens which reacted with the monoclonal antibody. The infected cells were labeled with either ^{14}C -amino acids or [^{14}C]glucosamine in the course of a short pulse (15 min) and then incubated in the absence of a radioactive precursor for 19 h (pulse-chase) or labeled continuously for 19 h. The immune precipitates obtained by the reaction with hyperimmune mouse antisera or monoclonal antibody (as described in Materials and Methods) were solubilized in sodium dodecyl sulfate and subjected to electrophoresis in polyacrylamide gels. As shown in Fig. 3, the hyperimmune mouse serum reacted predominantly with glycoproteins C and D of HSV-1 (slots 2 and 3) and glycoprotein D of HSV-2 (slot 1). Trace amounts of glycoproteins A and B were also precipitated from extracts of HSV-2-infected cells radiolabeled for 19 h (the autoradiographic images of these bands were very faint). The antiserum reacted with the product and precursor of glycoprotein D but predominantly with the fully glycosylated form of HSV-1 glycoprotein C (slot 3). In contrast,

antibody produced by clone HC1 (and HC2 with the same immunological specificity, not shown) precipitated both the precursor and the fully glycosylated form of glycoprotein C from HSV-1(F)-infected cells (Fig. 3). Antibody from these clones produced no detectable precipitates with extracts from HSV-1(MP)- or HSV-2(G)-infected cells (Fig. 4). Antibody produced by clones HD1 and HD2 (with the same immunological specificity) and also HD3 precipitated both the precursor and product forms of HSV-1 and HSV-2 D glycoproteins (Fig. 3 to 5). Because the immune precipitates shown in Fig. 5 were first precipitated by rabbit anti-mouse immunoglobulin and because the heavy chain of the immunoglobulin comigrated with glycoprotein D, the autoradiographic images produced by glycoprotein D in the immune precipitates were generally broader than those observed in the autoradiographic images of electrophoretically separated polypeptides present in non-precipitated extracts of infected cells. Evidence that the two bands precipitated by clones HC1 and HD1 are related as precursor and product is shown by pulse-chase experiments (Fig. 6). Only the slower-migrating species of glycoprotein C (gC) is precipitated by clone HC1, and the slower-migrating species of glycoprotein D (gD) is precipitated by clone HD1. In all of these

FIG. 3. Autoradiograms of electrophoretically separated [^{14}C]glucosamine-radiolabeled antigen (Ag) and immune precipitates of HSV-1(F)- and HSV-2(G)-infected HEP-2 cell extracts with mouse hyperimmune antiserum and monoclonal antibody. Immune precipitates of hyperimmune antiserum are shown with HSV-2(G) antigen radiolabeled from 5 to 24 h (slot 1), pulse-labeled and -chased HSV-1(F) antigen (slot 2), and long-labeled HSV-1(F) antigen (slot 3). Immune precipitates of 5- to 24-h-radiolabeled HSV-1(F) antigen with monoclonal antibody from hybridomas HD1 and HC1 are shown in the right panel. Apparent molecular weights of type 1 glycoproteins are as follows: gC, 133,000; gB, 125,000; gA, 119,000; and gD, 63,000. pgC, Precursor glycoprotein C; pgD, precursor glycoprotein D.

FIG. 4. Autoradiograms of [¹⁴C]glucosamine-radiolabeled antigen (Ag) (5 to 24 h) and immune precipitates of HSV-1(F), HSV-1(MP), and HSV-2(G) antigens with HC1 and HD1 hybridomal immunoglobulins. pgC, Precursor glycoprotein C; pgD, precursor glycoprotein D. ►, Immunoprecipitated glycoproteins.

FIG. 5. Autoradiograms of [¹⁴C]glucosamine-radiolabeled antigen (Ag) (5 to 24 h) and immune precipitates of HSV-1(MP) antigen with HD1, HD2, and HD3 hybridomal immunoglobulins. Immune precipitates and the control sample (Cont) were incubated with rabbit anti-mouse IgG, adsorbed to protein A-Sepharose, denatured, and electrophoretically separated.

experiments, the results obtained with [¹⁴C]glucosamine and ¹⁴C-amino acid antigens were essentially identical (Fig. 6).

DISCUSSION

In this paper we report the properties of five hybridoma clones produced by the fusion of

myeloma cells and spleen cells from mice immunized with HSV-1 (Table 2). Two of the clones, HC1 and HC2, produced antibody reactive with glycoprotein C of HSV-1, whereas the remaining three clones were directed against glycoprotein D. The two sets of clones were independently derived and are not clonally related. This is apparent from the observation that the two clones reactive with glycoprotein C, although identical in immunological specificity, differ in the immunoglobulin subclass produced. Thus, clone HC1 produced IgG2a, whereas clone HC2 produced immunoglobulin of the IgG1 subclass. In view of the molecular size of glycoprotein C (apparent molecular weight, 133,000), it is likely that HC1 and HC2 clonal antibody may be directed against different antigenic sites, although direct evidence for this point is not yet available. With respect to the clones which produce antibody against glycoprotein D, two of the clones produce antibody capable of neutralizing both HSV-1 and HSV-2, whereas one clone, HD3, neutralizes HSV-1 only. Furthermore, the two clones (HD1 and HD2) with apparently identical immunological specificity differ in the immunoglobulins they produce. Whereas clone HD1 produced immunoglobulin of the IgG2a subclass, clone HD2 produced immunoglobulin of the IgG1 subclass.

Results available to date have suggested that glycoprotein C of HSV-1 induces in hyperimmunized animals type-specific antibody (7, 19, 27, 29), and although we have produced only two clones which synthesize antibody against this glycoprotein, our results are consistent with this

FIG. 6. Autoradiograms of HSV-1(F) and HSV-2(G) radiolabeled antigen (Ag) and immune precipitates from reactions with HC1 and HD1 hybridomal antibody. Infected-cell extracts were radiolabeled with [^{14}C]-glucosamine (g) or ^{14}C -amino acids (aa) from 5 to 24 h postinfection or for a 15-min interval (pulse) and incubated in media without radioisotopes for 19 h (chase). pgC, Precursor glycoprotein C.

TABLE 2. Properties of the monoclonal antibody to HSV

Monoclonal anti-body	IgG sub-class	Result of neutralization tests for:			Glycoproteins immunoprecipitated from:			Result of immunofluorescence with:		
		HSV-1 strains		HSV-2 strain	HSV-1 strains		HSV-2 strain	HSV-1 strains		HSV-2 strain
		F	MP	G	F	MP	G	F	MP	G
HC1	2a	+	—	—	C	none	none	+	—	—
HC2	1	+	—	—	C	none	none	+	—	—
HD1	2a	+	+	+	D	D	D	+	+	+
HD2	1	+	+	+	D	D	D	+	+	+
HD3	1	+	+	—	D	D	D	+	+	+

hypothesis. We should note parenthetically that the function of glycoprotein C both with respect to the ability of the virus to infect cells and to the stability of the virus is uncertain (16). The glycoprotein appears not to be essential for virus replication in cell culture inasmuch as variants lacking the ability to produce glycoprotein C have been isolated (11).

Previous studies have reported that antisera against glycoprotein D neutralized both HSV-1 and HSV-2 strains, although by cross adsorption, antisera directed against the heterologous antigen could be adsorbed out, leaving type-specific antibody of relatively low potency (6, 20, 22). This report has shown unambiguously that glycoprotein D contains both type-common and type-specific domains. In light of the fact that of the three clones, one was type specific, it could be argued that the type-specific domain may be extensive and readily accessible to recognition by the immunological defense mechanism of

mice. Of particular interest is the observation that although antibody produced by clone HD3 did not neutralize HSV-2, it did precipitate glycoprotein D of HSV-2. Moreover, antibody from this clone reacted by immunofluorescence with HSV-1(F)-infected cells and (to a lesser degree) HSV-2(G)-infected cells. These results would argue that the specific HSV-1 antigenic site with which the antibody produced by clone HD3 reacts is present in HSV-2 glycoprotein D and is in part expressed on the surface of infected cells. However, in the HSV-2 virion, the site may not be exposed to the surface of the virion and therefore may not be accessible to the antibody. If this were true, the data would suggest that the conformation of HSV-1 and HSV-2 D glycoproteins may not be identical in the native state.

Rapid serotypic identification of HSV-2 strains remains an important diagnostic problem. The techniques generally used for strain identification are neutralization or immunofluo-

rescence by cross-absorbed antisera and analysis of the neutralization reaction in tests measuring the kinetics of the reaction. Neither technique is amenable for the rapid screening of isolates in diagnostic laboratories. The availability of monoclonal antibody offers valuable reagents for the rapid screening of isolates. It should be noted, however, that previous studies have shown that both HSV-1 and HSV-2 isolates can differ considerably from each other, both with respect to the electrophoretic mobility of their polypeptides (5, 10, 18) and to the nucleotide sequences in their deoxyribonucleic acids (9). In fact, recent reports suggest that no two epidemiologically unrelated HSV-1 and HSV-2 strains may be identical (3, 4). In light of these observations, it could be expected that HSV-1 and HSV-2 strains may differ with respect to the presence of individual type-specific antigenic sites. For these reasons, it is likely that the more valuable reagents for the rapid diagnosis of HSV-1 and HSV-2 strains would consist of mixtures of monoclonal antibodies each directed to a different type-specific antigenic determinant site. Studies on the variability of the antigenic sites defined by the clones described in this paper are now in progress.

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