Monoclonal Antibodies against Herpes Simplex Virus Type 1-infected Nuclei Defining and Localizing the ICP8 Protein, 65K DNA-binding Protein and Polypeptides of the ICP35 Family

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SUMMARY

The production and properties of monoclonal antibodies raised against herpes simplex virus type 1 (HSV-1)-infected cell nuclei are described. Biological and immunochemical assays revealed that these antibodies recognize four different proteins in HSV-1-infected cells. Four antibodies reacted with the major DNA-binding protein (ICP8) and six with the 65K DNA-binding protein. Two antibodies detected the ICP35 family of proteins and one antibody bound to a protein with an apparent mol. wt. of 60K. Immune electron microscopy showed that the major DNA-binding protein had a patchy distribution, whereas the 65K DNA-binding protein was evenly spread in the infected cell nuclei. The 60K protein as well as the polypeptides of the ICP35 family were preferentially found associated with the viral capsid.

INTRODUCTION

Monoclonal antibodies have been raised against different non-glycosylated and glycosylated proteins specified by herpes simplex virus (HSV). Some of these antibodies react type specifically, whereas others recognize epitopes common to both HSV type 1 (HSV-1) and HSV-2 (Zweig et al., 1979; Pereira et al., 1980; Balachandran et al., 1981; Showalter et al., 1981; McLean et al., 1982; Marlin et al., 1985). During the course of our studies on the immunological relationship between members of the Simplexviruses (Norrild et al., 1978; Ludwig, 1983), a subfamily of the Alphaherpesvirinae (Brown, 1986), we recently demonstrated that bovine herpes mammillitis virus (BHV-2)-specific monoclonal antibodies neutralize BHV-2, HSV-1 and HSV-2 by reacting with epitopes of the glycoprotein B. These antibodies detected epitopes located in the nuclei of infected cells (unpublished observations). These findings prompted us to initiate further studies with monoclonal antibodies. Here we report the isolation of monoclonal antibodies which interact specifically with the major DNA-binding protein ICP8 (Wilcox et al., 1980; Conley et al., 1981; Powell et al., 1981), the 65K DNA-binding protein (Bayliss et al., 1975; Marsden et al., 1987), the polypeptides of the ICP35 family (Preston et al., 1983; Braun et al., 1984) and a 60K protein. Immune electron microscopy was applied to localize the antigens in HSV-1-infected cells and to identify antigen-specific structures.

METHODS

Viruses and cells. HSV-1, strain F and HSV-2, strain G (Ejercito et al., 1968) as well as BHV-2, strain BMV (kindly provided by B. Roizman, University of Chicago, Ill., U.S.A.) were propagated in Vero cells, grown in Eagle's medium, Dulbecco's modification (Gibco), supplemented with 5% foetal calf serum. The BALB/c myeloma cell line P3-NS/1-Ag4-1 (NS-1) and hybridomas were grown in RPMI 1640 (Gibco Laboratories), containing 10 to 20% foetal calf serum, 2 mM-glutamine and 1 mM-pyruvate.

Monoclonal antibodies and proteins. Hybridoma cell lines were prepared as detailed by Köhler & Milstein (1975) and Köhler et al. (1976). Briefly, BALB/c mice were immunized with HSV-1-infected A31 cells, a permanent BALB/c 3T3 cell line (Lieber & Todaro, 1973). For each injection 10⁷ cells in 0-5 ml phosphate-buffered saline
(PBS) were mixed with an equal volume of Freund's adjuvant; the initial immunization was performed with Freund's complete adjuvant followed by booster injections containing Freund's incomplete adjuvant. Four to 5 days before fusion of the splenocytes with NS-1 cells the animals were inoculated with HSV-1 infected Vero cell nuclei. These nuclei were purified from approximately 5 × 10^7 infected cells by homogenizing the cells in a hypotonic buffer containing 0.5% NP40, followed by low speed centrifugation through a 20% sucrose cushion in PBS.

Monoclonal antibody 6898, directed against the 65K DNA-binding protein (unpublished observation) and the monoclonal antibody 1034, directed against ICP8, were kindly provided by Dr A. Cross, Institute of Virology, Glasgow, U.K. Purified 65K DNA-binding protein (Marsden et al., 1987) was provided by Dr H. S. Marsden, Institute of Virology, Glasgow, U.K. Monoclonal antibody LP1, directed against V\textsubscript{\textsuperscript{\textsubscript{86}}} was kindly provided by Dr A. Minson, University of Cambridge, Cambridge, U.K. A human serum with high neutralization titre against HSV-1 and a serum without reactivity to HSV-1 served as polyvalent control sera.

**ELISA.** Vero cells were infected with 2 × 10^3 p.f.u./cell on microtitre plates and fixed 22 h after infection with 3% formaldehyde in PBS or 0.1% glutaraldehyde in PBS. A plaque/focus immunoassay was performed as detailed elsewhere (Pauli et al., 1984).

**Neutralization assays.** Neutralization assays were performed with and without guinea-pig complement. Fifty μl of diluted antibodies was mixed with an equal volume of an HSV suspension containing 50 p.f.u. in a 24-well plate. After incubation for 30 min at 37 °C, complement was added in an appropriate dilution and incubated for a further 30 min at 37 °C. Inactivated complement served as a control. To each well 1 × 10^3 Vero cells in 100 μl of medium were added, and 200 μl of medium containing 1-6% carboxymethylcellulose was used as an overlay. The plates were evaluated 36 to 48 h later.

**Gel electrophoresis and immunoblotting.** Proteins were separated by SDS–PAGE using the buffer system of Laemmli (1970) and electrophoretically transferred to nitrocellulose sheets (BA83, Schleicher & Schüll) in a Bio-Rad transfer chamber as described by Burnette (1981). The nitrocellulose sheets were then incubated with PBS containing 3% bovine serum albumin, 0-1% Tween 20 and 0.01% NaN\textsubscript{3} for 1 h to block non-specific binding sites. The immunodetection of the antigen was carried out with a modification of the method described by Pauli et al. (1984). Briefly, the nitrocellulose sheets were incubated with monoclonal antibodies at 4 °C for 3 h. Bound antibodies were visualized by biotin-labelled anti-species IgG, streptavidin (Amersham), biotinylated alkaline phosphatase (Sigma) and a histochemical substrate [6 mg/ml fast red TR salt (Sigma), 0-8 mg/ml naphthol AS-MX phosphate (Sigma), 2 mM-MgCl\textsubscript{2} in 200 mM-Tris–HCl pH 8-0].

**Immunoprecipitation.** Infected cell lysates and antibodies were incubated overnight at 4 °C and the antibody-polypeptide complexes precipitated with goat anti-mouse IgG–agarose (Sigma) or Protein A–Sepharose (Sigma) for 3 h at 4 °C. Beads were washed four times with 0-6 M-LiCl containing 0-1 M-Tris–HCl pH 8-0 and 1% 2-mercaptoethanol. Bound proteins were eluted with 0-125 M-Tris–HCl pH 6-8, containing 2% SDS, 2% glycerol and 5% 2-mercaptoethanol.

**Extraction of infected cells.** Vero cells were infected with 1 to 2 p.f.u./cell and harvested 18 to 24 h later. Cell pellets were resuspended in lysis buffer (20 mM-Tris–HCl pH 7-4, 50 mM-NaCl, 0.5% NP40) and incubated for 30 min on ice. 0.5% sodium deoxycholate (DOC) was added shortly before the suspension was centrifuged (1 h, 100000g). Further details of this procedure have been reported (Pauli & Ludwig, 1977). Aliquots of the supernatant were stored at −70 °C until used. For SDS–PAGE analysis 2% SDS and 5% 2-mercaptoethanol were added.

**SDS lysates.** Cells were infected with 1 to 2 p.f.u./cell and harvested 18 to 24 h later. Equal volumes of pelleted cells and buffer containing 40% glycerol, 25% 2-mercaptoethanol, 10% SDS in 0.3 M-Tris–HCl pH 6.8 were mixed and ultrasonicated. The suspension was diluted 2.5-fold with H\textsubscript{2}O and clarified by centrifugation (10 min, 8000 g). The supernatant was used for SDS–PAGE.

**Purification of virus.** Cells were infected with 1 to 2 p.f.u./cell and harvested 24 h later. The supernatant was clarified by low speed centrifugation (10 min, 10000 g). The virus was centrifuged through 30% sucrose (5 ml) in TE buffer (10 mM-Tris–HCl pH 7-4, 3 mM-EDTA) in a SW27 rotor (Beckman) at 25000 r.p.m. at 4 °C for 1 h. The pellets were overlaid with one drop of TE buffer and incubated at 4 °C for 1 h. Virus pellets from six centrifugation tubes representing 200 ml of supernatant were resuspended in 1-0 ml of a double concentrated sample buffer containing 4% SDS, 4% glycerol and 10% 2-mercaptoethanol in 0.15 M-Tris pH 6.8.

**Immune electron microscopy (IEM).** Since the labelling of ultrathin cryostat sections appeared more sensitive than labelling of plastic-embedded sections (data not shown), we employed this procedure following described methods (Griffiths et al., 1983; Gelderblom et al., 1985). Briefly, HSV-1-infected Vero cells showing a pronounced cytopathic effect (20 h post-infection) were fixed in situ for 15 min with 0.1% glutaraldehyde in PBS containing 5% sucrose. The cells were scraped off the bottom of the culture vessel using a rubber policeman and sedimented. The resulting pellet was infiltrated stepwise with 0.3 M-, 1.2 M- and 2.3 M-sucrose in PBS and embedded in low melting point agarose. Small samples of such cryoprotected cells were mounted on specimen rods, fixed by rapid immersion into nitrogen slush (−210 °C) and sectioned with glass knives at a temperature of −80 °C in a Reichert
HSV-1-specific nuclear proteins

Table 1. Properties of monoclonal antibodies

<table>
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<th>Monoclonal antibody</th>
<th>Immunoglobulin subtype*</th>
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<th>Mol. wt.</th>
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<th>Virion§</th>
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* Subtype determined by Ouchterlony diffusion test using rabbit anti-mouse immunoglobulin subtype-specific sera (Miles Laboratories).
† ELISA was performed essentially as described (Pauli et al., 1984) on infected cells. Reaction pattern (n, nucleus; c, cytoplasm) and staining intensity (+/−, weakly stained; +, clear positive staining; ++, very intensely stained) were evaluated under a low power microscope.
‡ Antigens recognized in infected cell lysates after immunoblotting or immunoprecipitation.
§ Antigens detectable in purified virus after immunoblotting.

RESULTS AND DISCUSSION

Monoclonal antibodies

To define HSV-1-specific proteins in the infected nucleus BALB/c mice were immunized with HSV-1-infected A31 cells and boosted with infected nuclei before fusion of spleen cells with the mouse myeloma cell line NS-1. Thirteen monoclonal antibodies reacting with virus-specific antigens in the nucleus were selected by means of ELISA (Table 1). They all detected antigens in the nucleus and some of them showed a weak reaction with the cytoplasm. This result demonstrated that the polypeptides recognized were located mainly in the nuclei of infected cells. None of the antibodies was able to neutralize HSV-1, HSV-2 or BHV-2 in the presence or absence of complement. Two antibodies, Z3C2 and Z2B10, cross-reacted with BHV-2 in ELISA.

Western blotting of NP40/DOC lysates of HSV-1-infected cells enabled three different patterns of reactivity with the monoclonal antibodies to be recognized (Table 1). Seven monoclonal antibodies bound to a 60K polypeptide (Fig. 1, lanes 1 to 7), one antibody to a 130K polypeptide (Fig. 1, lane 8) and two antibodies detected a panel of several polypeptides between 35K and 48K together with two polypeptides of 67K and 70K and some faster migrating ones (Fig. 1, lanes 12 and 13). The monoclonal antibodies that did not recognize any polypeptides on Western blots (Fig. 1, lanes 9 to 11) immunoprecipitated a 130K polypeptide, supporting the hypothesis that they were directed towards epitopes present only on the native protein (Fig. 6). The three groups are represented in Table 1.
When purified virus was blotted, only three monoclonal antibodies bound to polypeptide bands (Fig. 2). Of the antibodies detecting the 60K polypeptide only Z2A9 recognized a similar polypeptide and an additional band of 39K (Fig. 2, lane 7). This antibody bound more strongly to its antigen when SDS lysates were used instead of NP40/DOC lysates (data not shown). These results suggested that two different proteins of 60K were detected by the monoclonal antibodies. The antibodies recognizing the 130K polypeptide in the infected cell lysate did not show any reaction with proteins of purified virus. The two antibodies that bound to several polypeptides in the range of 35K to 48K in the infected cell also bound to polypeptides in purified virus (Fig. 2, lanes 12 and 13). The two additional polypeptides of approximately 67K and 70K and the several faster migrating ones found in some preparations of infected cells were not detected in preparations of purified virions.

**Antibodies detecting two different 60K proteins**

The results described above suggest that two different 60K proteins were recognized by the monoclonal antibodies. One antigen was present only in lysates of infected cells and the other was present in both infected cells and virus. A protein with properties similar to the cell-associated 60K was described as the 65K DNA-binding protein (65KDBP) (Bayliss et al., 1975; Marsden et al., 1987). For further characterization we used a monoclonal antibody (6898;
unpublished observation), directed against the 65K_{DBP}. We verified that these six monoclonal antibodies did recognize 65K_{DBP} by two methods. First, purified 65K_{DBP} was recognized in Western blot analysis by six of the monoclonal antibodies as well as by the defined antibody 6898 (Fig. 3, lanes 1 to 7). Second, monoclonal antibody 6898 was used to immunoprecipitate 65K_{DBP} from lysates of infected cells. The immunoprecipitate was separated by SDS–PAGE and was found to be reactive in Western blotting with each of the six antibodies (data not shown). As expected from the described results, the monoclonal antibody Z2A9 was not reactive with 65K_{DBP} (Fig. 3, lane 8).

It is likely that antibody Z2A9 recognizes the virus-associated V_{MW}65 protein previously shown to be recognized by monoclonal antibody LP1 (McLean et al., 1982). However, Z2A9 recognized no common antigenic sites with LP1. This was shown by immunoprecipitation combined with Western blot analysis using LP1 (data not shown).

The monoclonal antibodies were characterized further by IEM of ultrathin cryosections of HSV-1-infected cells. Monoclonal antibody Z1F11, representative of the 65K_{DBP}-recognizing antibodies, reacted with epitopes distributed all over the nucleus in a diffuse manner (Fig. 4) whereas Z2A9 bound specifically to the nucleocapsid in the nucleus (Fig. 5).

The antibodies detecting the 65K_{DBP} are likely to be especially helpful because they detect antigenic determinants of both denatured (Fig. 3) and native protein (data not shown) which indicates that continuous epitopes are recognized (Eisenberg et al., 1985).

**Antibodies detecting ICP8**

Immunoprecipitation of antigens from NP40/DOC lysates of HSV-1-infected cells followed by immunoblotting with Z4B6 showed that three further monoclonal antibodies were directed
Fig. 3. Monoclonal antibodies specific for 65K$_{DBP}$. Purified 65K$_{DBP}$, electrophoresed in an 8.5\% SDS-polyacrylamide gel, was transferred and immunoblotted with monoclonal antibodies Z4D4 (lane 1), Z6F3 (lane 2), Z1A8 (lane 3), Z10C1 (lane 4), Z3H12 (lane 5), Z1F11 (lane 6), 6898 (lane 7, anti-65K$_{DBP}$) or Z2A9 (lane 8). Incubation of the detection system without monoclonal antibody is represented in lane 9.

against the 130K protein (Fig. 6, lanes 4, 5 and 6). The localization of the antigen in the nucleus and the molecular weight of the protein suggested that this protein might be identical to the major DNA-binding protein (ICP8) of HSV-1 (McLean et al., 1982; Littler et al., 1983). To investigate this possibility we compared the reactivity of a defined antibody directed against ICP8 with that of our antibodies. It could be demonstrated by use of anti-ICP8 monoclonal antibody 1034 that the 130K protein described above was identical to ICP8. However, all the monoclonal antibodies recognized the ICP8 migrating as a double band in immunoprecipitation (Fig. 6, lanes 2 to 6). Previous investigations (Knipe et al., 1982) have shown that the higher mol. wt. band (ICP8a) represents the precursor of the faster migrating protein (ICP8b). It was obvious that the antibodies precipitated more ICP8a than ICP8b although the latter protein seemed to be more abundant in Western blot analysis (compare Fig. 1 and 6). In immunoprecipitation the antibodies seem to have a higher affinity for the precursor (ICP8a) than for the mature protein (ICP8b).

IEM revealed that Z4B6 (representative of the 130K group) recognized antigen in a patchy distribution (Fig. 7), as shown by immunofluorescence by Quinlan et al. (1984). It is noteworthy that the two DNA-binding proteins seemed to be located in different compartments of the nucleus. The 65K$_{DBP}$ was found distributed throughout the whole nucleus (Fig. 4) whereas the appearance of ICP8 was restricted to electron-dense material (Fig. 7). These observations imply that the corresponding proteins might have different functions during virus replication.

**Antibodies detecting the ICP35 family**

Two monoclonal antibodies detected a number of antigens ranging in molecular weight from 35K to 48K (Fig. 1, 2, lanes 12 and 13). They seemed to be directed against p40/p80 or the ICP35 family of polypeptides. This follows from a comparison of the antigen profiles (Fig. 1 and 2) with
Fig. 4. Immune electron microscopy of an HSV-1-infected cell, showing part of both nucleus (n) and cytoplasm (c). Z1F11 specific for the 65K DNA-binding protein was incubated with a cryosection of infected cells and then treated with anti-mouse IgG-gold (5 nm). The detected antigen is present all over the euchromatin. Note that the condensed heterochromatin of the nucleus, the cytoplasm and the mature, extracellularly located viruses remained unlabelled. Bar marker represents 200 nm.
Fig. 5. Immune electron microscopy of part of the nucleus of an HSV-1-infected cell. Antigen labelled with antibody Z2A9 was found in direct contact with viral capsids, whereas other areas remained unlabelled. Bar marker represents 200 nm.
Mol. wt. (× 10^3)

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Fig. 6. Characterization of monoclonal antibodies reacting with the 130K protein. NP40/DOC lysates of HSV-1-infected Vero cells were incubated with monoclonal antibodies. Antibody–polypeptide complexes were precipitated with anti-mouse IgG–agarose beads and separated by SDS–PAGE, transferred and incubated with monoclonal antibody Z4B6. Antigens precipitated by antibody Z2B3 (lane 4), Z4G2 (lane 5), Z1H1 (lane 6) and anti-ICP8 monoclonal antibody 1034 (lane 2) were all recognized by antibody Z4B6 (internal control, lane 3), which was used for the immunodetection. Lane 1, incubation with Z1F11 (anti-65KDBp, negative antibody control); lane 7, incubation without the first antibody; lane 8, infected cell lysate sample. As expected, heavy (50K) and light (29K) chains of the first antibody used for precipitation were stained due to the detection system (see Methods).

Published data (Zweig et al., 1980; Braun et al., 1984). Some lower mol. wt. bands might result from degradation products. In the infected cell lysate these were detectable in varying amounts depending on the preparation investigated, but were not present in the mature virion (compare Fig. 1 and 2). The two slower migrating polypeptides (67K and 70K) only detectable in infected cells almost certainly correspond to p80 first described by Zweig et al. (1980) because p80 is a double as are the two slower migrating bands and because, like p80 (Zweig et al., 1980), the 67K and 70K proteins are not detectable in virions (Fig. 2).

IEM of ICP35 polypeptides was carried out with monoclonal antibody Z3C2. Label was found in direct association with capsids within the nucleus (Fig. 8). Preston et al. (1983) postulated that these polypeptides are essential for packaging and assembly processes of the viral DNA. These two antibodies cross-reacted with BHV-2-infected cells in Western blotting with polypeptides of comparable molecular weights (data not shown). These observations point to the conservation of these structures during the evolution of Simplexviruses.
Fig. 7. Immune electron microscopy of part of the nucleus of an HSV-1-infected Vero cell. Ultrathin cryosections of infected cells were incubated with monoclonal antibody Z4B6 specific for the 130K protein. The patchy distribution of the gold marker was observed all over the nuclei and correlated with electron-dense fibrillar masses of 0.8 to 1.6 μm diameter. Bar marker represents 200 nm.
Fig. 8. Immune electron microscopy of part of the nucleus of an HSV-1-infected Vero cell using monoclonal antibody Z3C2 specific for a 35K to 48K protein. The label was confined to small spots mainly in direct contact with viral capsids (arrows), but the cytoplasm was free of the gold marker. Bar marker represents 200 nm.
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REFERENCES


HSV-1-specific nuclear proteins


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