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Production and Characterization of Monoclonal Antibodies to Polyomavirus Major Capsid Protein VP1†

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Four hybridoma cell lines producing monoclonal antibodies against intact polyoma virions were produced and characterized. These antibodies were selected for their ability to react with polyoma virions in an enzyme-linked immunosorbent assay. The antibodies immunoprecipitated polyoma virions and specifically recognized the major capsid protein VP1 on an immunoblot. Distinct VP1 isoelectric species were immunoprecipitated from dissociated virion capsomere preparations. Two-dimensional gel electrophoresis demonstrated antibody reactivity with specific VP1 species. Monoclonal antibodies E7 and G9 recognized capsomeres containing VP1 species D, E, and F, while monoclonal antibodies C10 and D3 recognized capsomeres containing species B and C. Two of the monoclonal antibodies, E7 and G9, were capable of neutralizing viral infection and inhibiting hemagglutination. The biological activity of the monoclonal antibodies correlated well with the biological function of the species with which they reacted.

The polyomavirus genome codes for three structural proteins, VP1, VP2, and VP3, which form the icosahedral virion capsid. These proteins have structural as well as other biological functions in the virion particle. VP1 is the major capsid protein, forming nearly 80% of the total virion protein (11). The virion capsid is composed of 60 hexavalent and 12 pentavalent capsomere subunits (17). X-ray diffraction studies have suggested that both hexavalent and pentavalent capsomeres are structurally assembled as pentamers (31). The all-pentamer model requires that five subunits in the hexavalent capsomere interact nonequivalently with six neighboring pentavalent capsomeres, five of which are hexavalent and one pentavalent. It thus appears that the principle of quasiequivalence (14) is violated in the case of polyomavirus.

In addition to complex structural roles (11), VP1 is involved in several other important biological functions. These include association with the minichromosome (5), which very likely plays a role in regulation of transcription (9), and involvement as the virus attachment proteins (VAPs) for mouse kidney cells (MKC) and guinea pig erythrocytes (6). Previous work has demonstrated that VP1 is composed of multiple isoelectric species (22, 30). Our laboratory has shown that six species (designated A to F) are separable by isoelectric focusing (5), all of which have identical amino acid sequences as demonstrated by peptide mapping (2). Differences in the isoelectric points of the VP1 species are presumably based upon host-contributed posttranslational modifications which subsequently may allow variation in the protein conformation. These altered protein conformations are reflected in the wide range of biological activities associated with the VP1 protein (5).

Phosphorylation appears to account, at least in part, for the heterogeneity in VP1 pIs. Only species D, E, and F are phosphorylated (2, 5). These species are of particular interest because they function as the VAPs for MKC as well as guinea pig erythrocytes. Phosphorylation may alter the VP1 protein conformation thereby acting as a switching mechanism to change bonding specificity. This may allow the individual capsomere subunits to conform to the all-pentamer capsid model of Rayment et al. (31).

Polyclonal antisera have been useful in studying biosynthesis of viral polypeptides as well as biological roles of these polypeptides in complete virion particles (7, 29). The work reported here involves production and characterization of monoclonal antibodes (McAbs) against intact polyoma virions for use in further defining VP1 structural relationships and biological functions.

MATERIALS AND METHODS

Cell and virus propagation. Primary cultures of MKC were prepared as previously described (15, 33). Wild-type small-plaque polyomavirus was used to infect cells at a multiplicity of infection of 10. Infected cultures were maintained in serum-free Dulbecco modified Eagle medium (sf-DMEM) (28).

Virus purification. Virions were purified from the infected-cell lysate as described previously (28). CsCl gradients used to purify the virus were prepared as described by Brunck and Leick (13) and were described in greater detail previously (10). Polyoma capsids were purifed from infected-cell lysates as previously described (7). Purified virions and capsids were stored in the presence of the protease inhibitors aprotinin, leupeptin, phenylmethylsulfonyl fluoride, *N-p*-tosyl-L-lysine chloromethyl ketone, and L-1-tosylamide-2-phenylmethyl chloromethyl ketone (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 10 μg/ml each.

McAb production. (i) Immunization. Complete polyoma virions, isolated from a shallow CsCl gradient (28) and dialyzed overnight against 0.01 M Tris hydrochloride (pH 7.5), were used as the immunogen. BALB/c mice were immunized intraperitoneally with 10 μg of polyoma virions and boosted twice with the same quantity of antigen at monthly intervals. Mice received 25 μg of polyoma virions intraperitoneally on the final boost. Spleen cells were isolated from immunized mice 3 days after the final boost.

(ii) Fusion. The BALB/c mouse-derived myeloma cell line P3×63-Ag8.653 (Ag8) (24) was cultured in DMEM containing 10% fetal calf serum. Before the fusion, 2×10^7 myeloma cells and 1×10^8 immune spleen cells were washed three

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times in sf-DMEM and pelleted together in a 50-ml conical centrifuge tube at 500 × *g* for 10 min. The supernatant was removed, and the cells in the pellet were fused by the slow addition of 1 ml of 50% polyethylene glycol (Dutchland Hybrisure) in sf-DMEM over a 1-min period with gentle agitation. After the fusion, 1 ml of sf-DMEM was added dropwise over a 1-min period followed by the slow addition of 7 ml of sf-DMEM over a 2- to 3-min period. The resulting cell suspension was centrifuged for 10 min at 500 × *g*. The cell pellet was suspended in 100 ml of DMEM containing 10% fetal calf serum, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine. Primary mouse peritoneal macrophages (4×10^4 cells per well) were also included as a feeder layer. This cell suspension was distributed in Costar 96-well microtiter plates.

(iii) Selection and cloning. Wells showing cell growth were visually selected 10 to 14 days postfusion. Medium was removed from these wells to assay for the presence of immunoglobulin G (IgG) directed against polyomavirus, using an indirect enzyme-linked immunosorbent assay (ELISA). Once identified, colonies secreting antibodies against polyomavirus were cloned twice by limiting dilution. After cloning, colonies which retained polyomavirus-specific antibody production, as determined by ELISA reactivity, were expanded.

(iv) Ascites. BALB/c mice were injected intraperitoneally with 0.5 ml of Pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.) Seven days after Pristane priming, mice were injected with 5×10^6 hybridoma cells. Ascitic fluid was collected 10 to 14 days after injection of cells, and IgG was immediately purified.

Purification of monoclonal IgG. Cells and debris were removed from ascitic fluid by centrifugation at 500 × *g* for 10 min. Immunoglobulins were purified from the clarified ascitic fluid by sodium sulfate precipitation (25) followed by DEAE Affi-gel blue (Bio-Rad Laboratories, Richmond, Calif.) column chromatography modified as follows from the procedure of Bruck et al. (12). The sodium sulfate precipitate was applied to an Affi-gel blue column which had previously been equilibrated with 20 mM Tris hydrochloride–20 mM NaCl (pH 7.2) (column buffer). Proteins were eluted from the column by an NaCl step gradient in 20 mM Tris hydrochloride (pH 7.2). The columns were washed with column buffer followed by 25 mM NaCl buffer. Monoclonal IgG was eluted with 3 bed volumes of 50 mM NaCl buffer. Any protein remaining bound to the column was eluted with 100 mM NaCl buffer. Purity of the eluted fractions was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibody fractions eluted from the column during the 50 mM NaCl wash were pooled and used in all subsequent experiments.

ELISA. Optimal conditions for the ELISA were as follows. Polyoma virions were coated on microtiter plates at a concentration of 5 μg/ml in coating buffer (3.5 mM $NaHCO_3$, 1.5 mM Na_2CO_3, pH 9.6) for 1 h at 37°C and then overnight at 4°C. Unbound virus was removed by washing the plate three times in wash buffer (10 mM Tris hydrochloride, 0.05% Tween 20, pH 8.0). The supernatant medium from hybridoma cultures (50 μl), or a 1:50 dilution of serum, or a 1:250 dilution of purified IgG was added to individual microtiter wells and incubated at 37°C for 4 h. The plate was again washed, and 50 μl of alkaline phosphatase-labeled goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added to each well and incubated at 37°C for 1 h. The plate was washed three times with wash buffer and twice with deionized water. Substrate consisting of 50 μl of 2.5 mM *para*-nitrophenylphosphate in substrate buffer (10 mM 2-amino-2-methyl-1-propanol, 1 mM $MgCl_2$, pH 10.2) was added to each well, and color was allowed to develop at room temperature. All other dilutions were performed in dilution buffer (10 mM Tris hydrochloride (pH 8.5), 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 0.05% Tween 20). A_{405} was measured on a model EL307 Bio-Tek Enzyme Immunoassay Reader. All absorbances were standardized to preimmune serum which was designated zero absorbance.

Determination of McAb class and subclass. A modified indirect ELISA was used to determine that McAb class and subclass. Each antibody was coated on microtiter wells at a concentration of 5 μg/ml. Rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, and IgM (Bionetics Corp.) (10 μg each) were added to separate wells, and the degree of binding was determined by the addition of goat antirabbit-alkaline phosphatase conjugate and substrate.

Fab preparation. Monoclonal IgGs (10 mg) were incubated with 250 μg of papain immobilized on agarose beads (Pierce Chemical Co., Rockford, Ill.) in 1 ml of cleavage buffer (20 mM NaH_2PO_4, 20 mM cysteine hydrochloride, 10 mM EDTA-tetrasodium, pH 6.2). The suspensions were incubated for 12 h at 37°C with rocking. After cleavage, 3 ml of 10 mM Tris hydrochloride (pH 7.5) was added, and the suspensions were centrifuged to remove the immobilized papain. The supernatants were applied to 5-ml columns of protein A immobilized on agarose beads (Pierce Chemical Co.) previously equilibrated with 10 mM Tris hydrochloride, pH 7.5. The columns were washed with 15 ml of 10 mM Tris hydrochloride, pH 7.5. Monoclonal Fabs were collected in the eluate, and their purity was determined by SDS-PAGE.

Radioactive-labeling procedures. Purified polyoma virions, *Staphylococcus aureus* protein A, and monoclonal IgG were labeled in vitro with ^{125}I by the chloramine T method described by Frost and Bourgaux (19). The labeling reactions were quenched by the addition of unlabeled tyrosine to a final concentration of 0.1 mg/ml. Unbound ^{125}I was removed from all chloramine T-labeled substrates by centrifugation of the labeling mixture through a 2-ml Sephadex G-25 column at 2,000 × *g* for 10 min and overnight dialysis against 0.01 M Tris hydrochloride, pH 7.4.

Virions were labeled in vivo with [^{35}S]methionine (400 Ci/mmol; New England Nuclear Corp., Boston, Mass.) by removing the sf-DMEM 24 h postinfection, washing the cells twice with phosphate-buffered saline (PBS), and replacing the medium with sf, methionine-free Eagle medium containing 20 μCi of [^{35}S]methionine per ml. Cells were harvested 30 h postinfection by washing the monolayers twice in sf-Eagle and scraping the cells into 5 ml of sf-Eagle containing protease inhibitors as described above. The harvested cells were pelleted by low-speed centrifugation and resuspended in 1 ml sf-Eagle containing protease inhibitors. Nonidet P-40 was added to a final concentration of 0.1% (vol/vol). The resuspended cell pellet was vortexed for 30 s and incubated at 4°C for 15 min. Any remaining unbroken cells were pelleted by low-speed centrifugation. Nuclei were pelleted from the lysed cell suspension, washed twice in sf-Eagle, and pelleted again. The cytoplasmic supernatant was saved, and the nucleus pellet was suspended in 1 ml of RIPA buffer (20 mM MOPS hydrochloride [morpholinepropanesulfonic acid hydrochloride] [pH 7.0], 150 mM NaCl, 1mM EDTA, 0.1% [wt/vol] SDS) containing protease inhibitors. The resuspended nuclei were broken by homogenization (20 strokes) in a Dounce homogenizer. Unbroken nuclei were pelleted, and 100 μg each of DNase and RNase (Calbiochem-Behring, La Jolla, Calif.) were added to the supernatant.

Nucleases were also added to the cytoplasmic fraction collected previously.

Electrophoresis. SDS-PAGE was performed as described previously in 15% acrylamide gels with a 0.2% bisacrylamide cross-linker (10). Samples were prepared for two-dimensional electrophoresis as previously described (5).

Immunoblot analyses. Polyoma proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose paper as described by Bittner et al. (4). The residual binding potential of the nitrocellulose was blocked by incubation overnight with 0.05% Tween 20 in PBS (PBS-Tween) as described by Batteiger et al. (3), and the proteins were probed with the respective antibodies in PBS-Tween. After incubation for 12 to 18 h, the antibody was removed, and the nitrocellulose paper was washed five times, 10 min each time, with PBS-Tween. The nitrocellulose was then incubated for 4 h with 10^5 cpm/ml of either ^{125}I-labeled *S. aureus* protein A as described by Renart et al. (32) or ^{125}I-labeled goat anti-mouse IgG. Unbound protein A was removed by washing five times with PBS-Tween. Bound IgG was detected by autoradiography. In several experiments, McAbs were labeled directly with ^{125}I and used as the primary antibody probe. Incubation of the blots with *S. aureus* protein A or second antibody was omitted in these instances.

Dissociation of polyoma virions. Purified polyoma virions were dissociated by treament with 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)–3 mM dithiothreitol–0.15 M NaCl in 0.01 M Tris hydrochloride (pH 8.5) for 1 h as previously described (10).

Immunoprecipitation. Optimal conditions for immunoprecipitation are described as follows. Purified polyoma virions or dissociated virions were incubated with 10 μg of specific antibody overnight at 4°C after preadsorption with preimmune mouse serum (34). A 20% suspension of *S. aureus* (Calbiochem Pansorbin) (100 μl) was added and incubated for 30 min at 4°C. The precipitate was pelleted by centrifugation and washed four times in 50 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–0.05% Nonidet P-40–1% aprotinin (TNN buffer). The final pellet was suspended in 100 μl of 5 M urea in TNN buffer and incubated at room temperature for 15 min to elute the *S. aureus* cells from the antigen-antibody complexes. *S. aureus* cells were pelleted, and the supernatant was reprecipitated with fresh *S. aureus* cells after the addition of 900 μl of TNN. The precipitate was washed four times in TNN buffer, and proteins were eluted from the *S. aureus* cells by incubation in 50 μl of SDS-PAGE sample buffer for 20 min at room temperature. The immunoprecipitates were analyzed by SDS-PAGE.

Fluorescent-antibody neutralization assay. Polyoma virions were mixed with IgG dilutions ranging from 0.01 to 10^4 ng/ml and incubated for 1 h at 37°C. The virion-IgG mixture was used to infect MKC which were grown to confluency on glass cover slips. After adsorption for 1 h at 4°C, the infecting solution was removed, and the cells were washed. At 36 h postinfection, an immunofluorescence assay was performed (29) to determine the extent of infection. The assay was quantitated by counting multiple fields of cells and scoring for positive fluorescent nuclei.

Hemagglutination inhibition (HAI). Two fold dilutions of each McAb beginning with 10 μg/ml were mixed with 8 hemagglutinating units of polyomavirus and incubated at room temperature for 1 h. Guinea pig erythrocytes (0.75%) were added, and the hemagglutination pattern was allowed to develop at 4°C.

Competition of monoclonal IgG and Fab. Polyoma virions (50 ng) were coated on each well of a microtiter plate as described above. Coating was followed by the addition of 10 μg of unlabeled monoclonal IgG. After washing, 10^5 cpm of ^{125}I-labeled monoclonal IgG per ml was added and allowed to react for 4 h. Individual wells were washed, and bound ^{125}I-labeled IgG was quantitated in an LKB 1275 Minigamma counter. An identical experiment was also performed with monoclonal Fab fragments.

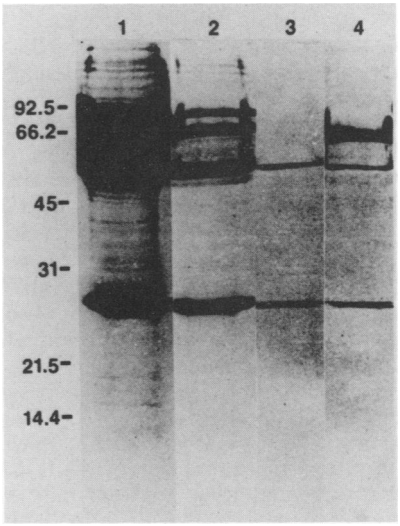

FIG. 1. SDS-PAGE analysis of McAb E7 purification. Sodium sulfate-precipitated antibodies were applied to an Affi-gel blue column and eluted with a 25 to 100 mM NaCl step gradient. Fractions collected during each wash were analyzed by SDS-PAGE and stained with Coomassie blue. Lane 1, Unpurified ascites; lanes 2 to 4, elution with 25 mM NaCl, 50 mM NaCl, and 100 mM NaCl, respectively. Numbers on left are molecular weight markers (×10^3).

RESULTS

Four hybridoma cell lines producing McAbs against intact polyomavirus resulted from a fusion of BALB/c mouse immune spleen cells and Ag8 myeloma cells. The four McAbs, designated C10, D3, E7, and G9, were purified from ascitic fluid by sodium sulfate precipitation followed by Affi-gel blue column chromatography. SDS-PAGE analysis demonstrated the purity of fractions eluted with each of the four McAbs. The electrophoretic profile obtained after purification of antibody E7 is shown in Fig. 1. All fractions collected during the 50 mM NaCl wash demonstrated only heavy- and light-chain IgG and lacked contaminating proteins. The 50 mM NaCl wash fractions of individual antibodies were pooled and used as the purified McAb preparation.

Considering the native conformation of the immunogen, our first goal was to demonstrate McAb reactivity to intact polyoma virions. Our initial screening of the hybridomas by ELISA showed that each McAb had equivalent binding capability to virions immobilized on plastic microtiter plates (data not shown). To show McAb recognition of virions in solution, ^{125}I-labeled polyoma virions were immunoprecipitated with each antibody (Fig. 2). Each antibody was able to immunoprecipitate intact virions, although variation in the total amount of virions precipitated was observed. No reactivity of normal BALB/c mouse IgG with virions was observed (Fig. 2, lane 6). The class and subclass of each McAb were determined by a modified ELISA (data not shown). McAbs C10 and D3 fell into the IgG1 class, while antibodies E7 and G9 were classified IgG2a. The reduced ability of McAbs C10 and D3 to immunoprecipitate polyoma virions

was attributed to the reduced binding capacity of *S. aureus* protein A for antibodies of the IgG1 class (26).

Several approaches were used to demonstrate antibody reactivity toward specific viral structural proteins. Immunoblot analyses of polyoma proteins (Fig. 3) showed exclusive reactivity of the McAbs with the major capsid protein VP1 as well as with 16,000- and 18,000-dalton VP1-derived fragments (16K and 18K fragments) which arise spontaneously upon SDS-PAGE (1, 7). Overexposure of the autoradiogram did not reveal any McAb reactivity with VP2 or VP3 (data not shown). Once again, no reactivity of normal BALB/c mouse IgG with viral proteins was observed (Fig. 3, lane 6). Initial attempts to detect antibody binding with ^{125}I-labeled protein A were unsuccessful (data not shown). The most sensitive and reproducible immunoblot assays resulted from the use of direct-labeled monoclonal IgG. The immunoblot results indicated that each McAb was capable of reacting with the denatured form of VP1. The difficulty encountered in demonstrating this reactivity suggests that McAb reactivity is greater with VP1 in its native conformation.

Another approach to demonstrate antibody reactivity to specific viral structural proteins involved determining the reactivity of each McAb to virion proteins in their native conformation. Virions were dissociated with EGTA and β-mercaptoethanol into capsomere subunits in which virion proteins appear to retain their native conformation. The capsomere preparations were immunoprecipitated with each McAb, and the precipitates were analyzed by SDS-PAGE (Fig. 4). These studies confirmed antibody reactivity toward VP1 but indicated that electrophoretic variants of the VP1 proteins were immunoprecipitated by each antibody. These variants migrated within the broad VP1 band recognized by polyclonal antisera (Fig. 4, lane 1). Antibody E7 immunoprecipitated VP1 proteins which migrated more slowly in SDS-PAGE than those immunoprecipitated by C10, D3, and G9. These studies suggested that the capsomere recognized by E7 contained VP1 proteins which were posttranslationally modified to a greater extent than those in capsomeres recognized by C10, D3, and G9. Several investigators (8, 18) including ourselves (7) have speculated that a serine protease may be involved in cleavage of the VP1 protein. However, the heterogeneity of McAb reactivity is probably not due to protease digestion of the VP1 protein since protease inhibitors were included during each step of the experiment. The McAbs appear to recognize specific VP1 conformations, since variation in reactivity is present when proteins in their native conformation are used as antigen, but the variation is absent when denatured proteins are used. This is exemplified by the homogeneous McAb reactivity observed after immunoblot analysis (Fig. 3), indicating that selective recognition of the VP1 species is conformation dependent. Overexposure of this autoradiogram did not reveal any VP2 or VP3 structural proteins in the immunoprecipitated capsomeres.

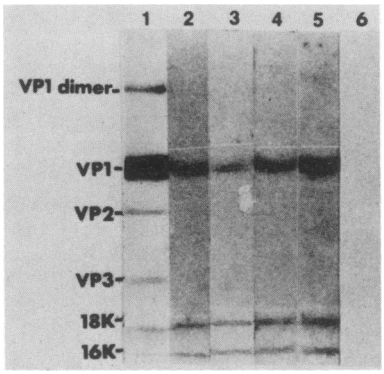

FIG. 3. Reactivity of McAbs to polyomavirus proteins on an immunoblot. Polyomavirus proteins were separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Individual lanes of the membrane were probed with different ^{125}-labeled IgGs and visualized by autoradiography. Lanes: 1, polyclonal rabbit IgG directed against dissociated polyomavirus; 2, McAb C10; 3, McAb D3; 4, McAb E7; 5, McAb G9; 6, normal BALB/c mouse IgG.

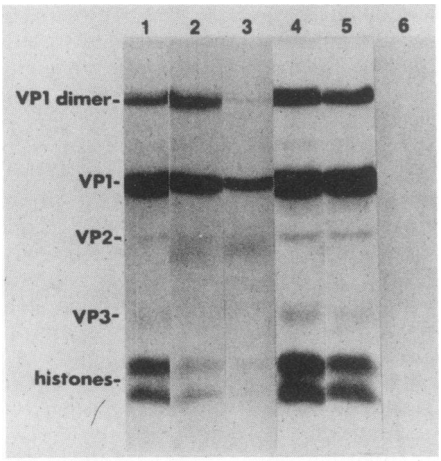

FIG. 2. Immunoprecipitation of intact polyoma virions. CsCl-purified virions were labeled with ^{125}I and immunoprecipitated with various McAbs. The precipitates were analyzed by SDS-PAGE. Proteins precipitated by each McAb are shown in the following lanes of an autoradiogram: 1, polyclonal rabbit antiserum directed against intact polyomavirus; 2, McAb C10; 3, McAb D3; 4, McAb E7; 5, McAb G9; 6, normal BALB/c mouse IgG.

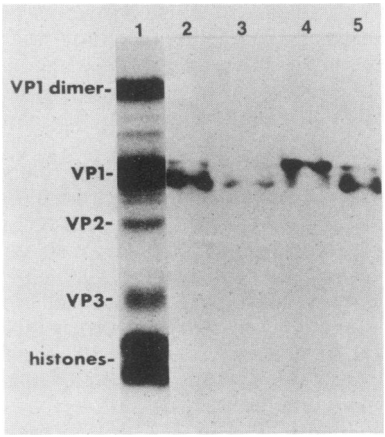

FIG. 4. Immunoprecipitation of dissociated polyoma virions. CsCl-purified polyoma virions were labeled with ^{125}I and dissociated as described in Materials and Methods. The dissociated capsomeres were immunoprecipitated with various McAbs, and the precipitates were analzyed by SDS-PAGE and autoradiography. Proteins precipitated by each IgG are shown in the following lanes: 1, polyomavirus; 2, McAb C10; 3, McAb D3; 4, McAb E7, 5, McAb G9.

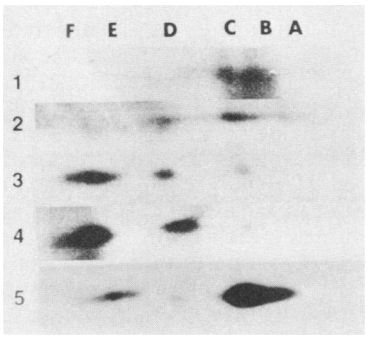

FIG. 5. Reactivity of McAbs to particular VP1 species. Polyoma virions were dissociated and immunoprecipitated as described in the legend to Fig. 4. Precipitates were analyzed by two-dimensional gel electrophoresis. Proteins precipitated by each McAb are shown in the following lanes: 1, McAb C10; 2, McAb D3; 3, McAb E7; 4, McAb G9; 5, ^{125}I-labeled polyoma virions. Positions of the VP1 species (A through F) are identified above the autoradiogram.

We next chose to investigate the possibility that the heterogeneous bands resulting from immunoprecipitation of dissociated virions arose from McAb reactivity with particular VP1 isoelectric species. ^{125}I-labeled polyoma virions were dissociated, immunoprecipitated, and analyzed by two-dimensional gel electrophoresis to visualize the VP1 species present in capsomeres recognized by each McAb (Fig. 5). The immunoprecipitated species were positioned by Coomassie blue staining of unlabeled marker virus which was run in each lane. Antibodies C10 and D3 recognized capsomeres which were enriched in VP1 species B and C, while antibodies E7 and G9 recognized capsomeres enriched in species D, E, and F. These studies indicate that VP1 species D, E, and F may be clustered in a unique set of capsomeres. In addition, this work supports our contention that the VP1 electrophoretic variants observed in Fig. 4 are not a result of protease digestion but rather represent particular VP1 species recognized by each McAb.

Each McAb was capable of selectively immunoprecipitating viral proteins from infected-cell lysates (Fig. 6). MKC labeled in vivo with [^{35}S]methionine were lysed 28 h postinfection. SDS-PAGE analysis of immunoprecipitates from infected nuclei demonstrated the presence of protein complexes, possibly assembly intermediates, present during the course of infection (35). These complexes contained all viral proteins and probably reflect the immature nature of the virions or assembly intermediates in the lysed MKC nuclei. The ratio of virion proteins appeared different from that observed in complete virions. However, Yuen and Consigli (35) have previously observed that early assembly intermediates have less VP1 than complete virions yet contain nearly a full complement of VP2 and VP3. SDS-PAGE analysis of immunoprecipitates from infected cytoplasm showed only VP1. This observation is consistent with the knowledge that polyomavirus structural proteins are translated in the cytoplasm and transported to the nucleus for assembly into viral particles (29). We found the use of nucleases necessary to release nascent VP1 from the cytoplasmic polysomes so that protein complexes were not precipitated. However, the use of RNase and DNase on nuclear preparations before immunoprecipitation had no effect on the complexes that were precipitated. Normal BALB/c mouse IgG did not precipitate any proteins from either infected cytoplasm or nuclei.

Since previous work had shown that VP1 species D, E, and F function as the VAPs and since two of the McAbs showed specificity for these species, the biological

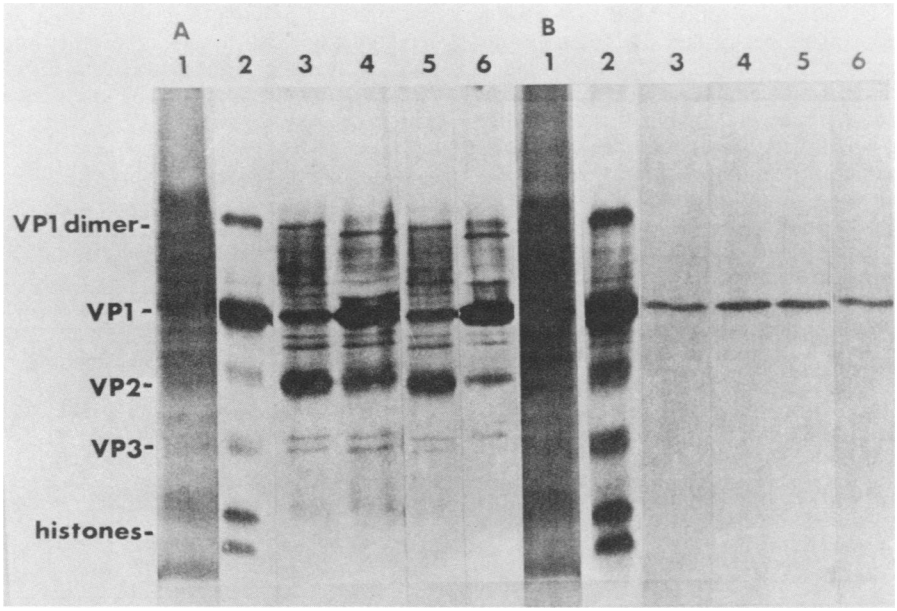

FIG. 6. Immunoprecipitation of virion proteins from infected-cell lysates. Infected MKC were labeled with [^{35}S]methionine and harvested 36 h postinfection. Isolation of nuclei and cytoplasm from infected-cell lysates is described in Materials and Methods. (A) Autoradiogram in which proteins were immunoprecipitated from infected nuclei by the following IgGs: lane 1, unprecipitated cell lysate; lane 2, polyclonal rabbit IgG directed against dissociated polyomavirus; lane 3, McAb C10; lane 4, McAb D3; lane 5, McAb E7; lane 6, McAb G9. (B) Autoradiogram of proteins immunoprecipitated from cytoplasm of infected cells. The lanes in panel B correspond to those described for panel A.

TABLE 1. Characterization of McAb biological activities

McAb	Neutralization activity (%)[a]	HAI activity[a]
C10	0	0
D3	18	0
E7	65	160,000
G9	43	2,500

[a] Neutralization and HAI experiments were performed as described in Materials and Methods. The data shown for neutralization resulted from treatment of virus with 10^2 ng of IgG before infection.

activity(s) of these antibodies was investigated. Fluorescent-antibody staining of MKC infected with virus that had previously been treated with McAb showed that two of the McAbs had neutralization activity. The results shown in Table 1 represent data obtained when 100 ng of McAb per ml was used to treat the virus before infection. McAbs E7 and G9 were capable of neutralizing 65 and 43% of the infection observed in control infections, respectively. These results indicate that McAbs E7 and G9 bind to the VP1 molecule at the attachment epitope or near enough that virus attachment is sterically inhibited. McAbs C10 and D3 showed greatly reduced abilities to neutralize polyomavirus infection and were not considered to be effective neutralizing antibodies. By increasing the concentration of McAbs E7 and G9 to 10^4 ng/ml, infection could be 100% inhibited. However, the highest concentrations of McAbs C10 and D3 used (10^4 ng/ml) were capable of neutralizing virus infection by less than 20%.

HAI studies (Table 1) showed that McAbs E7 and G9 both had significant HAI titers while McAbs C10 and D3 showed no HAI activity. The overlapping neutralization and HAI activities of McAbs E7 and G9 suggests that the attachment and hemagglutination epitopes may lie very close to one another on the VP1 molecule.

To determine whether any of the McAbs were reacting at the same site on the VP1 molecule, solid-phase competition radiommunoassay studies were performed. Polyoma virions were immobilized, and each unlabeled McAb was added separately, followed by ^{125}I-labeled McAb (Table 2). None of the McAbs could totally compete for binding of another McAb. Competitions performed with complete IgG molecules showed a large amount of competition between antibodies E7 and G9, yet this amount of competition was less than that observed for any of the McAbs with itself. In competitions performed with Fab fragments of each antibody, the degree of competition between McAbs E7 and G9 was decreased. We interpret these results to indicate that McAbs E7 and G9 recognize separate epitopes on the VP1 molecule which are located very near one another and that McAbs C10 and D3 have relatively lower affinity for their respective epitopes. It remains to be determined whether either of the neutralizing antibodies react specifically with an attachment epitope or if they simply bind near enough to an attachment epitope that they block the biological activity of that epitope.

DISCUSSION

McMillen ane Consigli (29) have shown that polyomavirus proteins migrating in the histone region of SDS-PAGE induced strong neutralizing and HAI antisera when injected into rabbits. These proteins were later identified as approximately 16,000- to 18,000-dalton spontaneous cleavage products of VP1 (7). Recently, Anders and Consigli (1) demonstrated that similar fragments can be generated chemically by formic acid cleavage of VP1. The antisera raised against spontaneously generated 16K and 18K VP1 fragments reacted specifically with 16K and 18K fragments generated by formic acid cleavage of the VP1 molecule. The 16K and 18K VP1 formic acid fragments are also strong immunogens for neutralizing and HAI polyclonal antisera. Our ability to demonstrate neutralizing and HAI biological activities associated with McAbs specific for the 16K and 18K VP1 fragments (Table 2) supports these previous observations and confirms the location of the hemagglutinin and virus attachment epitopes at least partially within the C-terminal 153 amino acids of VP1. In addition, preliminary studies in our laboratory demonstrated that in vitro surface labeling of polyoma virions failed to label the 29,000-dalton VP1 formic acid fragment (29K fragment), but labeled the 16K and 18K fragments very efficiently (D. G. Anders and R. A. Consigli, unpublished data). These studies indicated that the VP1 molecules which form the virion capsid structure are folded and assembled such that the C-terminal 16K and 18K fragments of VP1 are exposed while the N-terminal 29K region is buried or cryptic on the capsid surface. The ability of each McAb to recognize VP1 and 16K and 18K VP1 fragments with no reactivity in the 29,000-dalton region (Fig. 3) suggests that these four McAbs react with VP1 epitopes in the 16,000- to 18,000-dalton region of VP1. A Hopp and Woods hydrophilicity plot (23) calculated from the predicted amino acid sequence of VP1 (16) indicates that there are several very hydrophilic sequences within the 16,000- to 18,000-dalton region (data not shown). These hydrophilic sequences represent likely antigenic sites within this region (23). Reactivity with the 16K and 18K VP1 fragments is not surprising considering that the region appears to be highly accessible on the intact virion surface. Since these McAbs were produced to intact virions, one would not expect to see reactivity against the cryptic 29K fragment.

Electron microscopic studies have demonstated that attachment of polyoma capsids to MKC is followed quickly by endocytosis and transport of the internalized capsids to cellular lysosomes for degradation (27). This pathway is also followed by the majority of infecting virions. However, a

TABLE 2. Competition of McAb IgG and Fab[a]

McAb pretreatment	% ^{125}I-IgG bound with the following ^{125}I-IgG:				% ^{125}I-Fab bound with the following ^{125}I-Fab:			
	C10	D3	E7	G9	C10	D3	E7	G9
IgG								
C10	100	100	100	100				
D3	97	88	80	93				
E7	95	98	6	7				
G9	77	97	8	5				
Fab								
C10					ND	ND	100	100
D3					ND	ND	100	100
E7					ND	ND	12	25
G9					ND	ND	12	10

[a] Polyoma virions were immobilized and reacted with either unlabeled McAb IgG or unlabeled McAb Fab. Binding was followed by the addition of ^{125}I-labeled McAb IgG or McAb Fab as indicated. The percentages shown indicate the amount of ^{125}I-labeled antibody which binds after pretreatment with the appropriate unlabeled antibody. 100% binding was determined by binding ^{125}I-labeled antibody in the absence of competing unlabeled antibody. ND indicates that the particular antibody combination was not done.

small percentage of these particles are transported directly to the nucleus and initiate a productive infection. Subsequently, it has been shown that the different fates of virions and capsids within MKC resulted from the manner by which virions and capsids adsorbed to the MKC surface (6). Capsids could not compete efficiently with virions for binding to MKC due to the absence of the specific VAP, VP1 species E, from the capsid structure (5). The viral hemagglutinins VP1 species D and F are present in both capsids and virions and also function as the nonspecific VAPs for MKC (5). These studies indicated that at least two types of receptors for polyomavirus exist on the surface of MKC and that both specific and nonspecific VAPs exist on polyomavirus. Thus, it may be possible to neutralize polyomavirus infection without significant inhibition of virus binding or internalization.

The interaction of a virus particle with a host cell can be blocked at several steps with antibody to the virus. Inhibition of virus-cell interaction at one or more of these sites, including virus binding and internalization, may result in neutralization of virus infection. Since polyomavirus binds to both specific and nonspecific MKC receptors, it seems unreasonable to assume that an antibody which possesses neutralizing capability will necessarily be able to inhibit a significant amount of virus binding or internalization or both. Recent work in our laboratory has indicated that neutralizing McAbs E7 and G9 can block approximately 90% of virus binding (G. R. Griffith and R. A. Consigli, unpublished data). Nonneutralizing McAbs C10 and D3 block only 5% of virus binding. Internalization studies, performed as described by Griffith and Consigli (21), for the isolation of monopinocytotic vesicles containing virus showed that McAbs E7 and G9 could prevent over 99% of virus internalization, while McAbs C10 and D3 prevented less than 9% of virus internalization. In the case of polyomavirus, it appears that antibodies capable of neutralizing virus infection are also capable of blocking binding and inhibiting internalization. Therefore, these neutralizing McAbs appear to block infection at a very early step in the infection process probably involving virus interaction with the host-cell surface. Since two of the four McAbs characterized have neutralizing capability and since the VAP VP1 species E is a relatively minor component of the total VP1 protein, the VAP probably contains a highly immunogenic epitope.

The six isoelectric species of VP1 have identical amino acid sequences and appear to differ from one another only in the degree or type(s) of posttranslational modification(s). It has been suggested that the proteins have different conformations based upon the particular complement of posttranslational modifications which they possess (5). Recently, the reduced VP1 phosphorylation in polyomavirus *hr*-t mutants was found to be a significant factor in the ability of this mutant to assemble properly (20). These conformational alterations may be responsible for various biological activities associated with the identical VP1 amino acid sequences. Determination of the exact VP1 species specificity of each McAb has proven difficult because of our inability to separate the VP1 species while maintaining native protein conformation. We showed that the McAbs recognize conformational epitopes on the VP1 species (Fig. 4) and that these conformations must be maintained for the McAb to selectively recognize certain VP1 species (Fig. 3).

Brady et al. (11) have suggested that the 5S capsomeres obtained from EGTA and dithiothreitol dissociation of virions constitute the pentavalent subunits of the virion. This idea was confirmed by Bolen et al. (5), who also demonstrated that VP1 species D, E, and F are the primary components of the 5S capsomeres. Neutralizing McAbs E7 and G9 immunoprecipitate all three VAP species (D, E, and F) after virion dissociation into capsomere subunits. We suggest that these species are clustered in a subset of capsomeres which function in viral attachment and that these capsomeres are most likely located at the vertices of the virion in the pentavalent capsomeres described by Rayment (31). The absence of VP2 and VP3 in capsomeres immunoprecipitated by McAbs E7 and G9 suggests that these structural proteins are not present in capsomeres which make up the vertices of the virion structure.

The results of neutralization and hemagglutination studies (Table 1) suggest that the VAP and hemagglutinin lie very close to one another along the VP1 protein. This observation was confirmed by the competition studies performed with McAb IgG and Fab (Table 2). Although McAbs E7 and G9 appeared to compete with one another when IgG was used, the degree of competition was significantly reduced when monovalent Fab McAb fragments were used. We speculate that the conformation of species D and F results from a particular complement of posttranslational modifications and exposes the hemagglutinin epitope. Species E has a slightly altered conformation due to variation in posttranslational modifications, and thus the VAP epitope is more exposed. Phosphorylation is an important VP1 modification because the only phosphorylated species are the VAPs (species D, E, and F) (2, 5). This modification has been relegated entirely to the 29,000-dalton region of VP1, yet the biological activity of the VAPs appears to be partially if not entirely in the 16K and 18K VP1 fragments (1). It therefore appears that phosphorylation is not directly responsible for altering protein function but rather induces a conformational change in a distant region of the protein which allows different biological activities.

The data presented here demonstrate the structural and biological reactivity of four McAbs produced against intact polyoma virions. We showed that the VP1 epitope required for specific adsorption of polyomavirus to MKC lies very near the hemagglutination epitope. In addition, we showed that VP1 species D, E, and F are clustered in capsomeres on the virion surface. The four antibodies discussed here appear to have significantly higher reactivity against native-conformation proteins than against denatured proteins. The reactivity of these antibodies with specific VP1 species suggests that they recognize conformational epitopes which result from posttranslational modifications of the VP1 protein. We are currently expanding our panel of McAbs to include antibodies reactive against the denatured form(s) of VP1 and fragments of VP1 as well as the native and denatured forms of VP2 and VP3. The availability of a panel of McAbs with various specificities will be extremely useful for further study of polyomavirus protein structure and function.

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