NOTES

Isolation of a Monoclonal Antibody Against Viral Polypeptide VP2 of Simian Virus 40

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A stable hybridoma cell line, IIG8-203-2, that secretes a monoclonal antibody of the immunoglobulin subclass M was obtained by fusion of mouse myeloma cells with spleen cells of mice that had been immunized with the viral polypeptide VP2 of simian virus 40. The monoclonal antibody recognizes viral polypeptides that migrate with VP2 polypeptides in a sodium dodecyl sulfate-polyacrylamide gel. It also recognizes two intracellular polypeptides (29,000 and 37,000 daltons) in a detergent-insoluble fraction extracted 30 h after virus infection of TC7 cells.

Simian virus 40 (SV40) viral polypeptides VP1, VP2, and VP3 are structural polypeptides which are encoded by the late region of the SV40 genome. Virion proteins can be resolved into several bands, with molecular weights ranging from 10,000 to 50,000, by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (4). Seventy-five percent by mass of virion protein is polypeptide VP1, with a molecular weight of about 45,000. A second and third polypeptide (VP2 and VP3), with molecular weights of about 35,000 to 40,000 and 25,000, respectively, account for an additional 20%. Four other small polypeptides account for a total of about 10% of the virion protein; they have been shown to be host histones (13). The three structural polypeptides, VP1, VP2, and VP3, are encoded in the viral genome (16). It is believed that the amino-terminal sequence of VP2 is not present in VP3, but that otherwise the two amino acid sequences are identical (15, 16). These polypeptides are synthesized at a similar rate in the late phase of a productive infection, and they are metabolically stable (1, 6, 11, 14, 18). Very little is known about the biosynthesis of these polypeptides and their subsequent transport to the nucleus for virus assembly. To study the biosynthesis of these structural polypeptides, we have previously prepared monospecific antisera to VP1 and VP3 (8). However, the anti-VP3 antibody cross-reacts with VP2 as expected from the sequence data (15). Therefore, the goal of the present experiments was to prepare a monoclonal antibody which recognizes antigenic determinant sites specific to VP2.

For this purpose, 2-month-old female BALB/c mice were injected with about 10 to 15 μ g of VP2 polypeptides (7) purified on an SDS-polyacrylamide gel. Each mouse was injected intraperitoneally with about 0.3 ml of injection mixture, which consisted of about 60 μ g of VP2 polypeptide in 0.45 ml of phosphate-buffered saline (PBS), 0.45 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), and 0.3 ml of *Bordetella pertussis* vaccine fluid (Eli Lilly & Co., Indianapolis, Ind.). Subsequent injections were carried out at monthly intervals with about 0.3 ml of injection mixture, consisting of 60 μ g of VP2 polypeptide in 0.6 ml of PBS plus 0.6 ml of Freund incomplete adjuvant. One week after the third injection (week 10), 0.5 ml of blood was collected by tail bleeding. The antibody activity to the viral polypeptide in each serum was examined by indirect immunofluorescence on SV40-infected TC7 cells (a subline of African green monkey kidney cells) by the procedure described by Kasamatsu and Nehorayan (8). The infected cells showed immunostaining patterns (see Fig. 4d) identical to those observed with polyclonal anti-VP3 sera (see Fig. 4c). Two mice were sacrificed for hybridoma production on day 4 after the seventh and after the ninth injections of the antigen, respectively. Totals of 2×10^8 and 3×10^8 spleen cells were fused with 3×10^7 and 8×10^7 cells of the 8-azaguanine-resistant NS1/SP2 myeloma line, respectively, by the procedure described by Kohler and Milstein (12), using polyethylene glycol.

The cell suspension was distributed into several 96-well tissue culture plates at about 10^6

cells per well and cultured in 100 μ l of RPMI 1640 medium supplemented with 0.5 mM oxaloacetic acid–2 mM glutamine–1 mM pyruvate–100 U of penicillin per ml–100 μ g of streptomycin per ml–20% calf serum (complete RPMI 1640 medium). Twenty-four hours after plating, 100 μ l of HAT medium (complete RPMI 1640 medium supplemented with 1.0×10^{-4} M hypoxanthine, 4.0×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine) was added to start the selection of hybrid cells. The surviving hybrid cells were detected 8 to 16 days after the fusion, appearing as small colonies that grew upon the heavy lawn of dead cells. After 16 days, the surviving cultures were tested for the production of mouse immunoglobulins by a solid-phase ELISA assay (3) and for the production of specific anti-VP2 antibody by an indirect radioimmunoassay. The radioimmunoassay was performed as follows. Each well of a V-shaped 96-well microtiter plate (soft polyvinylchloride dish) was coated with 40 ng of purified VP2 polypeptide in 10 μ l of PBS containing 0.1% NaN_3 , 0.004% SDS, 4 mM Tris-hydrochloride (pH 8.3), and 7.6 mM glycine for 2 h at 37°C and for 5 to 6 h at room temperature. The room temperature incubation was performed on a rotator at about 60 rpm. Twenty-five microliters of 5% bovine serum albumin solution in PBS-azide (50 mM sodium phosphate [pH 7.3], 0.9% NaCl, and 0.1% NaN_3) was added to each well, and the incubation continued for 2 to 3 h at 37°C. The plate was used immediately or stored at 4°C until further assay. The wells were rinsed with PBS-azide and incubated for 3 h at 37°C with either 30 μ l of the supernatant of each culture or 30 μ l of a 1:100 dilution of anti-VP2 sera obtained from the mouse on which the splenectomy for the fusion was performed. The wells were then incubated with about 200 ng of affinity column-purified rabbit anti-mouse immunoglobulin in 30 μ l of PBS-azide-Tween 20 (PBS-azide containing 0.05% Tween 20) for 3 h at 37°C and, finally, with 10^5 cpm of ^{125}I -labeled protein A from *Staphylococcus aureus* in 25 μ l of PBS-azide-Tween containing 0.25% gelatin for 2 h at room temperature. Each well was washed three times with 0.1 ml of PBS-azide-Tween between the incubation procedures. The wells were excised and counted in a gamma counter. In such tests, the mouse anti-VP2 antiserum at a 1:100 dilution bound approximately 2,000 to 4,000 cpm of ^{125}I -labeled protein A, whereas undiluted culture fluids from positive hybrid cells bound in the range of 500 to 3,000 cpm above background. In two fusion experiments, 21 wells from the first fusion and 208 wells from the second fusion contained viable colonies. Sixty-five percent of the wells with viable colonies contained immunoglobulin-secreting colonies, and 2 wells

(first fusion) and 87 wells (second fusion) showed anti-VP2 activity by the radioimmunoassay. During successive passages, many colonies lost the ability to secrete antibody.

One hybridoma supernatant, from culture IIG8, was found to bind about sixfold more ^{125}I -labeled protein A than any other; culture IIG8 was therefore cloned twice at limiting dilutions. One stable subclone, IIG8-203-2, was grown in mass culture and characterized further. To increase the antibody titer, 2×10^6 cells from IIG8-203-2 were injected intraperitoneally into a (BALB/c $\times$ AKR) F_1 hybrid mouse that had been primed with pristane 14 days before the injection. The tumor was detectable 1 week later. The ascites fluid (total of 6 ml from two drainings) was drained on days 7 and 9 and stored at -20°C after the removal of debris by centrifugation at 1,000 rpm for 10 min and 10,000 rpm for 30 min in a Sorvall SS-34 rotor. The antibody titer of the ascites fluid was about 10^3 -fold higher than that of the tissue culture fluid from IIG8-203-2.

Immunoglobulin from the culture fluid of the cloned hybrid cell line was concentrated by ammonium sulfate precipitation (40% saturation) and examined by agar gel diffusion analysis

FIG. 1. A photograph of agar gel diffusion test for identification of the immunoglobulin class. The center well contained (A) 20 μ l of a 30-fold concentrated supernatant of culture IIG8-203-2 and (B) 20 μ l of 30-fold concentrated complete RPMI 1640 medium. The outer wells contained 20 μ l of: rabbit anti-total mouse IgG serum (mig), affinity column-purified rabbit anti-mouse IgG (1.2 mg/ml) (MIG), rabbit anti-mouse IgG1 (G1), rabbit anti-mouse IgG2a (G2a), rabbit anti-mouse IgG2b (G2b), or rabbit anti-mouse IgM (M). The immunodiffusion plates were incubated for 48 h at 22°C, washed extensively with PBS, stained with 0.1% Coomassie blue in 45% (vol/vol) methanol and 10% (vol/vol) acetic acid, and destained in 90% methanol and 2% acetic acid. An immunoprecipitin line was observed in the reaction to rabbit anti-total mouse IgG serum, affinity column-purified rabbit anti-mouse IgG, and to rabbit anti-mouse IgM. Immunodiffusion disks (pattern A) were purchased from Cappel Laboratories, Cochranville, Pa., and immunoglobulin class-specific reagents were purchased from Miles Laboratories, Inc., Elkhart, Ind.

with antisera prepared in rabbits against purified total mouse immunoglobulin G (IgG) and murine myeloma proteins. The IIG8-203-2 subclone was found to produce IgM (Fig. 1).

Identification of the polypeptide recognized by the monoclonal antibody secreted by culture IIG8-203-2 was achieved, using the procedure of Towbin et al. (17). VP2 polypeptides are visible as multiple bands in a region around the 40,000-dalton marker polypeptide (Fig. 2b, lane 1) on an SDS-polyacrylamide gel, flanked by VP1 and VP3 polypeptides of the disrupted virions. When these viral polypeptides were electrotransferred from a gel onto a nitrocellulose membrane and immunoreacted with the culture fluid, followed by rabbit anti-mouse immunoglobulin and 125 I-labeled protein A, one rather broad band in the 40,000-dalton region was detected by autoradiography (Fig. 2c, lanes 1, 2, and 3). A similar result was obtained when the ascites fluid was used for immunoreaction instead of the culture fluid (data not shown). On the other hand, the polyclonal anti-VP3 serum produced in rabbits reacts with VP2 and VP3 (Fig. 2a, lane 2). Thus,

the monoclonal antibody produced by culture IIG8-203-2 reacts with a polypeptide that migrated like VP2. This experiment does not distinguish between the two possibilities that (i) the monoclonal antibody recognizes an antigenic determinant site specific to VP2 but not present in VP3 or (ii) the antibody recognizes an antigenic determinant site of some different polypeptide, not VP2, that migrates at 40,000 daltons. The latter possibility is unlikely, since only SV40-infected TC7 cells show the immunoreactive intracellular polypeptide (Fig. 3) and cytoplasmic immunofluorescent staining (Fig. 4). Thus, the polypeptides recognized by the monoclonal antibody are contained in purified virions and are expressed in SV40-infected cells.

Intracellular polypeptides reactive to the monoclonal antibody and to polyclonal anti-VP3 antibody were examined by the procedure described above (Fig. 3). Several points can be made from this experiment. First, there are no obvious polypeptides which react with either monoclonal antibody or anti-VP3 in either the detergent-soluble or the insoluble fractions of

FIG. 2. Identification of the viral polypeptide reactive to the monoclonal antibody. About 10 μ g (lanes 1 of b and c), 5 μ g (lanes 2 of a, b, and c), and 1.5 μ g (lanes 3 of b and c) of purified SV40 virions (6) were disrupted, and viral polypeptides were separated on 15% SDS-polyacrylamide gels. A part of a gel was stained with Coomassie blue (b), and the other part of the gel (c) was used for the identification of the polypeptide immunoreactive to the monoclonal antibody as described previously (17). The viral polypeptides were transferred electrophoretically to nitrocellulose membranes from gels. The nitrocellulose membranes with the immobilized viral polypeptides were treated with 0.25% gelatin for 1 h at 37°C in buffer A (50 mM Tris-hydrochloride [pH 7.4], 0.15 M NaCl, 0.1% NaN₃) and reacted with a 1:150 dilution of rabbit anti-VP3 serum (a) (8), or with a 1:4 dilution of supernatant of culture IIG8-203-2 (c) in buffer A containing 0.25% gelatin for 8 h at 22°C. The nitrocellulose membranes were incubated with 125 I-labeled protein A (2×10^5 cpm/ml) in buffer A containing 0.25% gelatin for 1 h at 22°C., except that the nitrocellulose membrane in (c) was reacted with a 1:400 dilution of rabbit anti-total mouse IgG for 5 h at 22°C before the 125 I-labeled protein A incubation. The membranes were washed, with three changes of buffer A, for 1 h between each reaction. The dried nitrocellulose membranes were exposed to X-ray film (Cronex 4, Du Pont Co., Wilmington, Del.), using intensifying screens (Quanta II, Du Pont Co.). About 70, 6, and 10% of total viral polypeptides are VP1, VP2, and VP3, respectively. The viral polypeptides VP2 and VP3 often separate into closely migrating multiple bands on SDS-polyacrylamide gels (5). They are marked at the left side of (b) lane 1, as is VP1. The band position marked for VP1 for lane 2 (a) was determined by a reaction of the other half of the nitrocellulose membrane with anti-VP1. The 125 I-labeled molecular-weight-marker polypeptides, indicated with lines at the left side of lane 1 (a) and the right side of lane 4 in (b) and (c), are 68,000, 40,000, and 29,000 daltons.

FIG. 3. Autoradiographs of intracellular polypeptides reactive to the monoclonal antibody and to anti-VP3 antibody. TC7 cells, uninfected or infected with SV40 (10 PFU/cell, 30 h postinfection), were treated with cytoskeletal lysis buffer as described previously (10), and cellular proteins were separated into detergent-soluble and detergent-insoluble fractions. Intracellular polypeptides were separated on 15% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, and immunoreacted, as described in the legend to Fig. 2, with a 1:500 dilution of ascites fluid obtained from a mouse after pristane treatment and injection of clone IIg8-203-2 cells (lanes 1 through 9) or with a 1:150 dilution of anti-VP3 (lanes 10 through 19). Each sample contained either 10 μ g of polypeptides (lanes 2, 4, 6, 8, 11, 13, 15, 17) or 20 μ g of polypeptides (lanes 3, 5, 7, 9, 12, 14, 16, 18) of the detergent-soluble fraction from uninfected cells (lanes 2, 3, 11, 12) and from infected cells (lanes 6, 7, 15, 16) or the detergent-insoluble fraction from uninfected cells (lanes 4, 5, 13, 14) and from infected cells (lanes 8, 9, 17, 18). Lane 19 contained 5 μ g of viral polypeptides. Bands corresponding to viral polypeptides VP2 and VP3 which are immunoreactive to anti-VP3 are marked at the right side of lane 19. Lanes 1 and 10 show 125 I-labeled marker polypeptides (68,000, 40,000, 29,000, a doublet of 18,400 and 17,500, and 14,300 daltons from the top). The nature of the three to four very weak bands which appear at about 15,000 daltons in the insoluble fractions of both uninfected and infected cells (lanes 4, 5, 8, 9, 13, 14, 17, and 18) is not known at the present time. These bands are detected by rabbit control serum (data not shown), rabbit anti-VP3 serum, and by the ascites fluid used in this study. At this stage of infection 75% of total cellular VP3 polypeptides are present in the detergent-insoluble fraction of infected cells, and the rest are present in the detergent-soluble fraction (unpublished results). The 10 μ g of protein applied to each lane of the gel corresponds to the amount of protein isolated from the soluble fractions of 6×10^4 cells and the insoluble fraction of 3×10^5 cells. The failure to detect VP2 and VP3 polypeptides in the gel lanes of the soluble fractions of infected cells (lanes 15 and 16) by anti-VP3 is due in part to the low concentration of these polypeptides in that fraction and to the difference in the total number of cells from which the proteins are isolated.

uninfected TC7 cells, except for a few polypeptides, at about 15,000 daltons, which are detected in the insoluble fractions of both uninfected and infected cells (Fig. 3, lanes 4, 5, 8, 9, 13, 14, 17, and 18). The nature of these polypeptides is unknown at the present time. Second, in the experiment shown in Fig. 3 (lanes 8, 9, 17, and 18) in which we examined equal weights (10 μ g) of protein in the detergent-insoluble or detergent-soluble fraction of infected cells, we observed a much greater amount of polypeptides which react with anti-VP3 in the detergent-insoluble fraction. In agreement with this, other experiments (data not presented) have shown that about one-fifth of the total cell protein is in the detergent-insoluble fraction, whereas about 75% of VP3 polypeptides are present in the insoluble fraction. Thus, the ratio of VP3 polypeptides in insoluble and soluble fractions is about 15:1. These results therefore suggest that the major portion of the immunoreactive polypeptides is localized in the nucleus or in cytoplasm bound to the cytoskeleton (2) or both. In

this fraction, at least two polypeptides are immunoreactive to the monoclonal antibody (about 37,000 and 29,000 daltons), and two are immunoreactive to anti-VP3 (about 37,000 and 25,000 daltons). The 37,000-dalton polypeptide(s) detected in both immunoreactions is slightly smaller than the polypeptide identified by the monoclonal antibody in disrupted virions (40,000 daltons). Faint 40,000-dalton polypeptides are visible in both autoradiographs after a longer exposure time. Thus, the 37,000-dalton polypeptide may be an unmodified form of the 40,000-dalton polypeptide. The 29,000-dalton polypeptide detected with the monoclonal antibody seems to be different from the 25,000-dalton polypeptide detected with anti-VP3, in that the difference in the mobilities of these polypeptides is reproducible. This implies that the 29,000-dalton polypeptide does not contain any of the antigenic determinant sites recognized by anti-VP3. At present we do not know the origin of this 29,000-dalton intracellular polypeptide.

The staining patterns given by the monoclonal

FIG. 4. Fluorescence micrographs of stained cells. Cells were grown on cover glasses, infected with SV40, and fixed and prepared for immunofluorescence analysis as described previously (8). The first antibody reaction was as follows: uninfected cells reacted with a 1:100 dilution of ascites fluid (a). Infected cells (29 h postinfection) reacted with a 1:100 dilution of ascites fluid (b), a 1:50 dilution of rabbit anti-VP3 serum (c), or a 1:25 dilution of mouse anti-VP2 serum (d). The second reaction was either with fluorescein-labeled goat anti-mouse IgG (a, b, and d) or with fluorescein-labeled goat anti-rabbit IgG (c). Bar = 40 μ m.

anti-VP2 and the polyclonal anti-VP3 are discrete and nonoverlapping. As shown in Fig. 4b, the staining pattern with the monoclonal antibody lies only in the cytoplasm of infected cells and is quite distinct from the nuclear staining pattern observed in most of the cells with anti-VP3 serum (Fig. 4c) and anti-VP2 serum (Fig. 4d). Uninfected cells showed very little staining with monoclonal antibody (Fig. 4a). The experiments show that VP2 is present in infected cells in both the cytoplasm and the nucleus. However, the monoclonal antibody does not recognize the antigenic determinants exposed in the nuclear form of VP2. The polyclonal anti-VP3 may not recognize the determinants expressed in the cytoplasm; alternatively, the concentration of VP2 antigen in the cytoplasm may be too low to be detected by this antiserum. Furthermore, the monoclonal antibody does not precipitate [4,5- 3 H(N)]lysine-labeled SV40 virions in indirect immunoprecipitation (unpublished data), although we have observed previously that the

polyclonal anti-VP3 antibody does immunoprecipitate virions (8). This suggests that the antigenic determinant site in the nucleus for the monoclonal antibody is hidden inside the virus particle or capsomeres and is unavailable to the antibody.

Our previous study showed that several mutations within the coding sequence of VP1 alter the subcellular localization of VP2 or VP3 (or both) in infected cells (9). From this study we have postulated the involvement of VP1 polypeptide in the transport of VP2 or VP3 (or both) from cytoplasm to nucleus for virus assembly. However, it was not possible to distinguish intracellular forms of VP2 polypeptides from VP3 polypeptides with the polyclonal anti-VP3 antibody. The monoclonal antibody to VP2 will be useful for the study of the biosynthesis of VP2.

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