

An Intermediate Filament-associated Protein, p50, Recognized by Monoclonal Antibodies

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ABSTRACT Monoclonal antibodies (mAB) were raised to be used as probes to identify cytoplasmic components associated with intermediate filaments (IF). Four hybridomas (B27, B76, B78, and B100) secreting mAB were generated by fusing mouse myeloma cells with the spleen cells of mice immunized intraperitoneally with Triton-high salt insoluble materials from BHK-21 cells. This insoluble material consists mostly of IF, a small number of microfilaments, and some polyribosomes. Biochemical studies show that the Triton-insoluble materials contain many proteins, including vimentin (decamin) and desmin. Immunofluorescence microscopy of BHK-21 cells stained with the four mAB showed that these mAB decorate the IF in a dotted pattern. Double staining with polyclonal antibody to vimentin confirmed the reactivity of the mAB with the IF. These mAB also stained the vimentin-containing filament system in a variety of other cells including epithelial cells (PTK1 and HeLa) and cells of astroglial origin. Histological studies showed that mAB-B100 stained many types of tissue including epidermis, smooth muscle, and subdermis pericytes, but not the white matter nor the gray matter of the cerebellum and spinal cord. Immunoelectron microscopy with colloidal gold has shown that the mAB-B100 decorated the IF in clusters or aggregates around proteinaceous materials associated with the filaments. Results of immunoprecipitation indicate that mAB-B100 reacted with a protein of 50,000 daltons. These findings suggest that the mAB-B100 we have developed recognizes one of the many components of what appears to be an integrated cytoskeletal structure connected with intermediate filaments.

Intermediate filaments, with a diameter of ~ 10 nm, comprise a major component of the cytoskeleton found in most eucaryotic cells. According to their biochemical and immunological characteristics (for review see reference 8), these filaments can be separated into five subclasses: (a) Vimentin (decamin), the single polypeptide constituent of the fibroblast intermediate filament, has a molecular weight of 57,000. (b) Desmin is a 53-kdalton protein constituent of the intermediate filament located in smooth muscle cells. (c) Keratin, multiple polypeptides with molecular weights ranging from 40,000–70,000, make up the intermediate filaments in epithelial cells. (d) Glial filament protein, a protein of 51 kdaltons, is the protein constituent of the glial filaments. (e) Neurofilaments, the intermediate filaments in neurons, are composed of three polypeptides with molecular weights of 200,000, 150,000, and 70,000.

These filaments form a complex intertwining network among the various cellular organelles such as mitochondria, pinocytotic vesicles, lysosomes, and nuclei. While much is known about the biochemical nature of the basic subunits of these filaments and the ultrastructural integrity of these structures in cells, their functions are not yet understood in molecular terms owing to the lack of specific methods for disrupting them and thereby investigating their function. We have previously reported on the positive correlation between alterations in filament organization and changes in certain intracellular motile functions¹ (12, 19, 20). For example, (a) there is a close spatial relationship between the pattern of saltatory movements and the distribution of intermediate filaments in

¹ Wang, E., J. Michl, L. M. Pfeffer, S. C. Silverstein, and I. Tamm, submitted manuscript.

the cells (19); (b) nuclear positioning and movement in SV-5 virus-infected baby hamster kidney (BHK)² syncytia is dependent upon the organization of intermediate filaments (20); and (c) the rate of pinocytosis fluctuates depending upon the organization of filament distribution in the cytoplasm of macrophages¹ (12).

Structural entities such as side arm protrusions have been observed in association with intermediate filaments in cultured fibroblasts (5, 21), as well as in nerve axons (13, 16, 25). The discovery of the cytoplasmic microtrabecular lattice system (23) leads to the hypothesis that cytoskeletal elements are interconnected within a complex network of many different kinds of proteinaceous materials. The finding of such a lattice suggests the hypotheses that the three systems of filaments are connected with each other through many structural linkages to form a matrix within which intracellular organelles are positioned and moved about within the cell.

In the present paper we will report that a protein of 50 kdaltons, recognized by monoclonal antibodies, represents a new component that is associated with intermediate filaments in many, but not all tissues.

MATERIALS AND METHODS

Cells: The cells used in this study included two human malignant astrocytoma lines, U-251-5 (positive for glial filament expression), and U-251-6 (negative for glial filament expression), BHK-21 and PTK1 cells (obtained from the American Type Culture Collection, Rockville, MD), and human skin fibroblasts (obtained from The Human Genetic Mutant Cell Repository, Camden, NJ). BHK-21 cells were grown in reinforced Eagle's medium containing 10% calf serum and 10% tryptose phosphate broth as described previously (19). The other cell lines were grown in the same medium containing 10% fetal calf serum and 1% nonessential amino acids. All cultured cells were maintained in 5% CO₂ in a humidified incubator at 37°C.

Preparation of Monoclonal Antibodies: Mouse monoclonal antibodies were produced as described by Kohler and Milstein (7) as modified by Dippold et al. (2). Crude cytoskeletal preparations (CSK), composed mainly of intermediate filaments and some microfilaments and polyribosomes, were prepared according to the method described by Burr et al. (1). SDS gel electrophoresis of this CSK shows the presence of many proteins. These cytoskeletal preparations are commonly termed high-salt Triton-insoluble materials. Such materials isolated from 1×10^7 BHK-21 cells were used for each injection. Two female BALB/c mice were immunized. For the first immunization, CSK from 1×10^7 cells was mixed with an equal volume of complete Freund's adjuvant and inoculated subcutaneously. In four subsequent immunizations, CSK isolated from the same number of cells was injected in the absence of adjuvant into the peritoneal cavity at 2-wk intervals. 3 d after the final immunization, the mice were sacrificed and their spleen cells fused with the mouse myeloma NS-1 cell line at a ratio of 5:1 (spleen cells/myeloma cells) using 0.2 ml of 40% polyethylene glycol in 15% DMSO at 37°C for 2 min. After diluting the polyethylene glycol with 10 ml RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum, 2×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 3.2×10^{-5} M thymidine (HAT medium), $\sim 2 \times 10^5$ cells in 1 ml HAT medium were added to each well of the 24-well Costar plates (Costar Co., Cambridge, MA) with preplated macrophage culture as feeder layer. The feeder monolayer cultures were prepared from peritoneal macrophages isolated from the same type of BALB/c mice according to the procedure described by Michl et al. (10). After the cultures were grown for 10 d in HAT medium, cultures were fed with RPMI 1640 medium containing 10% fetal calf serum, 2×10^{-4} M hypoxanthine, and 3.2×10^{-5} M thymidine (HT medium).

Samples of supernatant were collected from wells containing one or more readily visible subconfluent colonies, and screened for antibodies to cytoskeletal components of BHK-21 cells by immunofluorescence microscopy. Antibody-producing hybrids were cloned a minimum of three times by the limiting-dilution method in 96-well Costar plates with attached feeder layer cells. After cloning, cultures were expanded in 24-well plates and then inoculated subcutaneously into Swiss nude mice and (BALB/c \times C57 BL/6) F1 hybrids. Supernatants of the growing cultures, nude mouse sera, and ascites were collected and stored at -70°C to be used as antibody sources for microscopic

and biochemical studies. The antibody subclass was determined by double diffusion in Agar with anti-Ig heavy-chain-specific reagents (Rainin Co., Ridgefield, NJ) (14).

Screening for Hybridoma Production: Hybridoma-secreted antibodies to the components of cytoskeletal systems were screened directly on fixed cells by immunofluorescence microscopy. BHK-21 fibroblasts were grown on Shandon multispot slides (Cappel Laboratories, Cochranville, PA) for 48 h. The slides were fixed in 3.7% formaldehyde in PBS for 30 min at room temperature. After rinsing with PBS, the cells were extracted with acetone at -20°C for 2 min. The slides were incubated at room temperature with normal goat serum (0.05 mg/ml) (Antibodies Inc., Davis, CA) for 10 min, followed by PBS rinsing and overnight incubation at room temperature with the hybridoma supernatant (50 μ l per well). After three washes with PBS, the slides were incubated for 30 min at room temperature with a mixture of rabbit anti-mouse IgG (0.04 mg/ml) and rabbit anti-mouse IgM (0.04 mg/ml). Following PBS rinsing, these slides were then incubated with fluorescein-conjugated goat anti-rabbit IgG (0.05 mg/ml) (Antibodies Inc.) for another 30 min before the final rinse with PBS and mounting. The slides were then examined with a Zeiss photomicroscope III equipped with epifluorescence illumination and a $\times 63$ lens.

Indirect Immunofluorescence Microscopy: Cells were grown on no. 1 glass coverslips for 48 to 72 h. These coverslips were then processed for staining with monoclonal antibodies following the same procedure as described for screening of the hybridomas. In instances where double-label indirect immunofluorescence studies were performed, the coverslips were initially incubated with the mouse monoclonal antibodies overnight, followed by incubation with fluorescein-conjugated goat anti-mouse IgG for 30 min, and then incubated with polyclonal rabbit anti-vimentin IgG for 30 min (20), followed by rhodamine-conjugated sheep anti-rabbit IgG (Antibodies Inc.) for 30 min. All incubations were at 37°C and they were followed by three 20-min washes in PBS. The coverslips were mounted as described above, and examined under the photomicroscope with appropriate filters for fluorescein and rhodamine molecules.

Indirect Immunoelectron Microscopy: Colloidal gold particles, ~ 5 nm-diam, were prepared according to the procedure described by Faulk and Taylor (4). These particles were then conjugated with protein A (Sigma Chemical Co., St. Louis, MO) following the procedure for gold-protein conjugation described by Horrisberger et al. (6).

Localization of monoclonal antibodies with this gold-protein A complex was performed by a procedure similar to that for immunofluorescence microscopy. Initially, cells were grown on Thermanox plastic coverslips (Flow Laboratories, Inc., McLean, VA) for 48 to 72 h. The cells were then fixed with 3% paraformaldehyde in PBS at pH 7.2 for 30 min at room temperature. After rinsing in PBS three times, the fixed samples were extracted with 0.2% Triton in PBS at room temperature for 10 min. After rinsing with PBS containing BSA (5 mg/ml), the samples were incubated with supernatants of the hybridoma cultures overnight at room temperature. These samples were rinsed again with PBS containing BSA and incubated with a mixture of rabbit anti-mouse IgG and rabbit anti-mouse IgM for 2 h at room temperature. Afterwards, the samples were rinsed with PBS containing BSA and then incubated with 5 nm gold-protein A conjugates for another 2 h before the final rinsing with PBS containing BSA. The samples were then processed for electron microscopy as described previously (20).

Immunoabsorption: Four positive hybridomas (B27, B76, B78, B100), which secreted antibodies to components of intermediate filaments, were selected. These antibodies were tested for possible cross-reactivity with vimentin by immunoabsorption. Vimentin was isolated from BHK-21 cells as described by Starger and Goldman (17). B100 was adsorbed with purified (1 mg/ml) vimentin as described elsewhere (9). Nude mouse serum diluted 1:100 was used as the source of monoclonal antibodies (mAB)-B100 antibody. mAB-B100 was incubated at 4°C overnight with the suspension of purified filament protein (0.2 mg/ml final concentration), which contained only vimentin. The filaments were then removed by centrifugation at 33,000 rpm for 30 min at 4°C. The supernatants were tested for residual reactivity by indirect immunofluorescence microscopy.

Immunoblotting: Immunoblotting was performed as described by Towbin et al. (18), with minor modifications. The CSK used as the immunogens were run on a 7.5% polyacrylamide gel. The protein bands were transferred onto nitrocellulose paper using a Bio-Rad transblotting unit (Bio-Rad Laboratories, Richmond, CA). The nitrocellulose paper was incubated overnight with 5% BSA in PBS at room temperature. The paper was then incubated with the monoclonal antibody for 1 h at room temperature followed by five 5-min washes in PBS. The paper was then incubated with biotinylated horse anti-mouse Ig (Cappel Laboratories) for 1 h at room temperature. After five 5-min washes in PBS, the paper was incubated with avidin and biotinylated peroxidase. The paper was washed 4 min with PBS and developed with 0.03% diaminobenzidine in combination with H₂O₂ in 0.05 M Tris buffer pH 7.9 for

² Abbreviations used in this paper: BHK, baby hamster kidney; CSK, crude cytoskeletal preparation; mAB, monoclonal antibodies.

5 min. After one wash in PBS for 30 min, the nitrocellulose paper was dried.

Immunoprecipitation: Protein A-Sepharose beads were incubated for 1 h at 4°C with a mixture of rabbit anti-mouse IgM (0.4 mg/ml) and rabbit anti-mouse IgG (0.4 mg/ml). Beads were pelleted, washed three times with PBS, and incubated for 3 h at 4°C with mAB (supernatants or ascites fluid). They were then washed three times with PBS and stored overnight in PBS at 4°C. U251-5 cells were labeled for 16 h with 0.125 mCi/ml [³H]leucine in Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum and 10% of the amount of leucine used normally. Cell monolayers were washed three times with cold PBS and lysed in cold RIPA buffer which contains 1 ml 10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Trasylyol, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride. The lysate was scraped from the plate, incubated on ice for 10 min, and then clarified by centrifugation for 15 min at 15,000 g. Aliquots of the lysate containing equal amounts of cell protein were incubated with the prepared protein A-Sepharose-antibody complexes for 6 h at 4°C (all incubations of protein A-Sepharose beads with antibody or antigen were performed on a wrist action shaker). Immune complexes were pelleted, washed five times with RIPA buffer, resuspended in SDS sample buffer, and boiled. Samples were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was prepared for fluorography by a -30 min incubation in 1 M sodium salicylate, and then dried.

Histological Studies: Tissue localization of antigens that react with mAB-B100 was primarily done on frozen tissue sections. 13-d-old chick embryos and adult rat cerebellum were frozen and cut with a cryostat microtome. Sections of the frozen tissue were stored at -160°C. When used, these sections were fixed with 3% paraformaldehyde in PBS for 10 min and rinsed with PBS afterwards. Supernatants of the hybridoma cultures were incubated with these tissue sections overnight at room temperature. After rinsing thoroughly with PBS, the samples were incubated with fluorescein-conjugated goat antimouse IgG (Cappel Laboratories Inc., Cochranville, PA) for 30 minutes before rinsing again with PBS, and mounted for examination with the fluorescence microscope.

RESULTS

Production and Isolation of Monoclonal Antibody

The spleen cells from two immunized BALB/c mice were fused with the mouse myeloma cell NS-1 in two separate fusions. From two fusions, 480 wells were screened for antibodies to cytoskeletal components by indirect immunofluorescence microscopy, as described in Materials and Methods. BHK-21 cells containing vimentin and desmin, and normal human skin fibroblasts containing only vimentin, were used as target cells to screen for reactivity against the intermediate filaments. Four of the 480 supernatants (B27, B76, B78, and B100) contained antibodies that recognized intermediate filaments in both BHK-21 and human skin fibroblasts. After four successive clonings in culture the hybridoma line B100 was selected for growth as a subcutaneous tumor in nude

mice and as an ascitic tumor in FI hybrids. The antibody designated mAB-B100 was found by Ouchterlony double diffusion (14), to be an IgG2b antibody.

Microscopic Localization

Indirect immunofluorescence staining of BHK-21 cells and human skin fibroblasts by mAB-B100 revealed a fibrous pattern extending from the perinuclear region to the periphery of the cell (Fig. 1, *a* and *b*). However, unlike the pattern observed in staining with polyclonal antibody to vimentin, the staining of filamentous structures was discontinuous in the form of dots. The pattern of dotted filaments is most obvious in areas where large numbers of filaments were arranged in parallel and formed large bundles. Unlike the periodicity seen along microfilament bundles and the muscle sarcomere, the physical positioning of these dots along the filaments does not display discernible regularity, nor are the dots of different filaments positioned in register. In addition, mAB-B100 gives a unique patchy staining pattern in the nuclear matrix (Fig. 2). To ascertain whether the dotted staining pattern of the filaments in fact represented the vimentin-containing filament system in the cell, we used the rabbit polyclonal antibody to vimentin in combination with staining with mAB-B100. Fluoresceinated goat antimouse IgG was used to locate the mouse monoclonal antibody in the cell, and rhodamine isothiocyanate conjugated sheep anti-rabbit IgG was used to locate the rabbit polyclonal antibody. Fig. 3 demonstrates the filamentous pattern observed with mAB-B100 as well as with the rabbit polyclonal antibody to vimentin in senescent human skin fibroblasts. Due to the limitation of the exciting filters, it is difficult to detect the dotted pattern of staining with mAB-B100 when another polyclonal antibody was used in the combined staining reaction. Epithelial cells (PTK1) were also used for localization of the filament system positive for staining with mAB-B100. Fig. 4 illustrates the double immunofluorescence patterns after staining with both mAB-B100 and polyclonal antibody to vimentin. Perinuclear filaments, representing the vimentin-containing system, are stained with mAB-B100. Lack of cross-reactivity of mAB-B100 with keratin filaments is recognized by the absence of any staining of filaments in the periphery of cells, where the cytoplasm is filled with a network of tonofilaments.

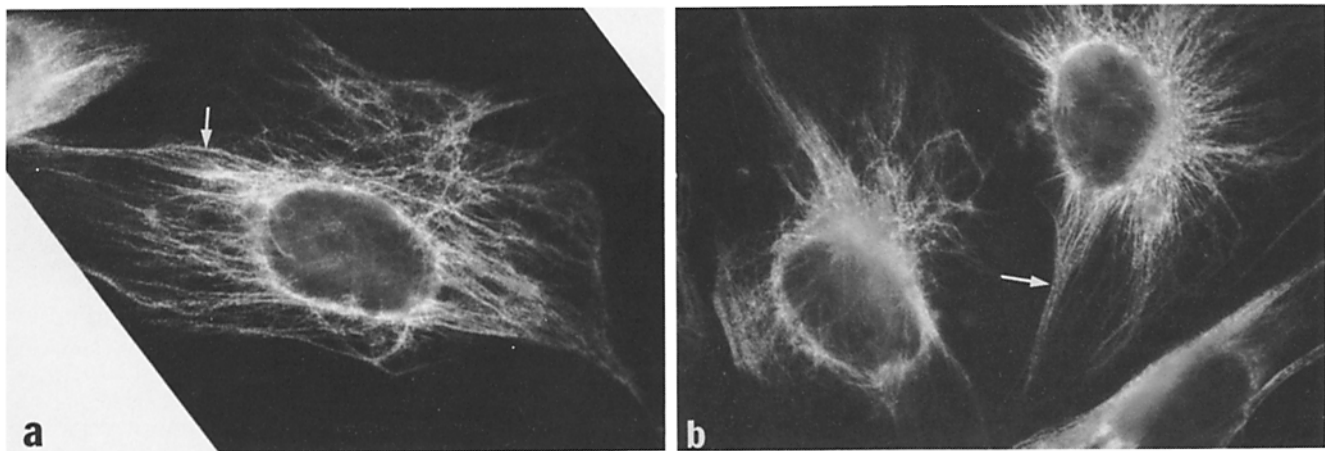


FIGURE 1 Immunofluorescence microscopy of the staining pattern of mAB-B100 in cultured baby hamster kidney (BHK)-21 cells (*a*) and in human skin fibroblasts (*b*). The arrows indicate the dotted composition of individual filaments, which is distinctly different from the staining pattern seen with polyclonal antibodies to vimentin. (*a*) $\times 700$; (*b*) $\times 600$.

The association of mAB-B100 with vimentin-containing filaments was further substantiated by the reaction of the antibody with tissue sections of 13-d-old chick embryo and adult rat cerebellum. Our results showed that in the chick embryo mAB-B100 reacted with most of the vimentin-associated tissue area such as the epidermis in the skin layer, the smooth muscle of the heart, and subdermis pericytes, but not the spinal cord. The staining reaction of mAB-B100 in rat cerebellum was absent in both white and grey matter, but positive in the area of blood vessels. Fig. 5 demonstrates the filamentous system decorated by mAB-B100 in vascular endothelial cells. The lumen of a blood vessel wall was sectioned obliquely to show the interior surface lining of the vessel composed mainly of endothelial cells (Fig. 5*a*). With fluorescence microscopy, the cells are seen to contain vimentin filaments (Fig. 5*b*), which can be simultaneously decorated with mAB-B100 (Fig. 5*c*).

Results of indirect immunoelectron microscopy with 5-nm

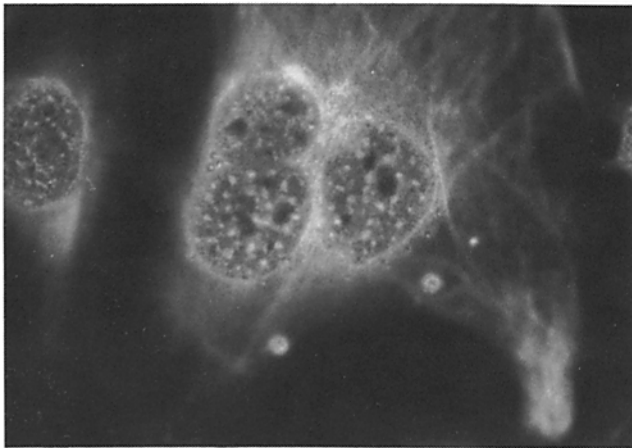


FIGURE 2 The staining pattern of mAB-B100 in the nuclear matrix in baby hamster kidney cells. $\times 750$.

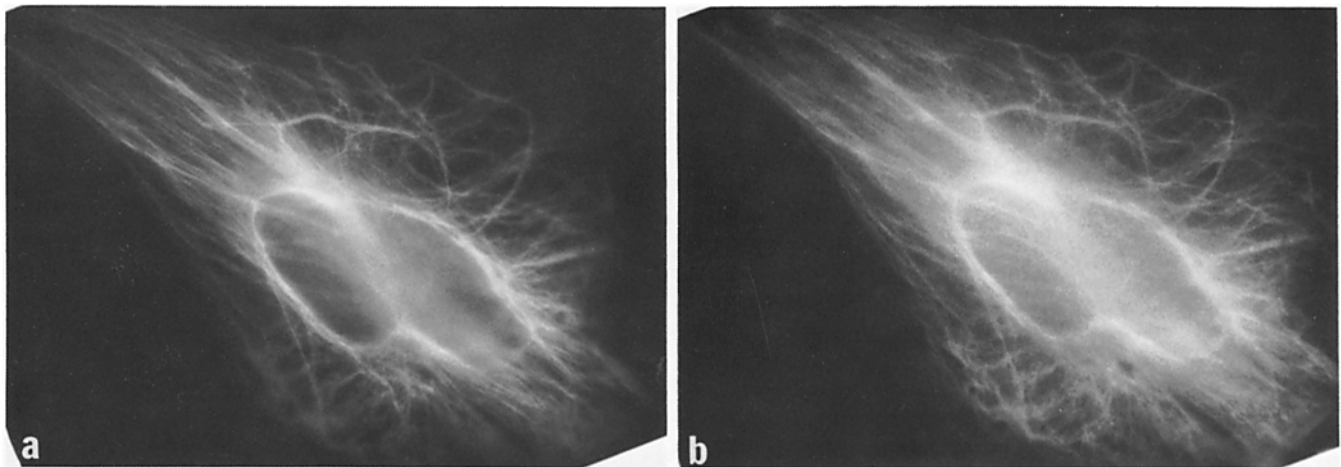


FIGURE 3 Simultaneous staining with mAB-B100 and rabbit polyclonal antibody to vimentin. For the mouse monoclonal antibody, fluorescent goat anti-mouse IgG was used, and rhodamine-conjugated sheep anti-rabbit IgG was used for labeling the polyclonal antibody. The visualization of the staining patterns of the two antibodies was done with phase contrast optics as well as epifluorescence illumination in combination of excitation filters at 490 nm for the rhodamine fluorochrome and 546 nm for the fluorescence fluorochrome. An additional filter (narrow band green filter with band path between 520–560 nm) was used to insure the complete separation of the two staining patterns. Senescent human fibroblasts were fixed, extracted, and processed for staining as described in Materials and Methods. The photographic recording was done with Kodakectochrome ASA 400 film and printing on black and white Ilford paper. (a) mAB-B100 staining pattern; (b) the same network stained by rabbit polyclonal antibody to vimentin. $\times 840$.

colloidal gold particles established that mAB recognizes amorphous proteinaceous materials associated with the intermediate filaments. In general, the gold particles are found in clusters outlining electron-dense areas superimposed on the filaments in BHK-21 cells (Fig. 6, *a* and *b*). The areas demarcated by the gold particles do not present a specific shape or size. Some gold particles, however, were not seen in clusters, but rather decorated the filaments themselves. Nevertheless, cluster formation in colloidal gold labeling of filaments represents the predominant pattern. In contrast, the immunogold labeling pattern with polyclonal antibody to vimentin takes the form of linear arrays of dots, with most of the gold particles decorating the filaments themselves (Fig. 7). The validity of the observations concerning ultrastructural localization of mAB-B100 was verified when in the initial incubation, mAB-B100 was replaced with the supernatant of another mAB-secreting hybridoma. Fig. 8 demonstrates the lack of labeling of filaments in BHK-21 cells when a monoclonal antibody to the 200-kdalton subunit of neurofilaments was used. This result is consistent with the fact that BHK-21 fibroblasts do not contain neurofilaments. The clustered pattern of mAB-B100 staining thus accurately represents the location of a protein associated with the vimentin-containing filament system in cultured cells.

Biochemical Characterization

To further characterize the specificity of mAB-B100, the antibody was adsorbed with purified vimentin. The adsorbed antiserum was then used to stain BHK-21 cells and human skin fibroblasts. As shown in Fig. 9, adsorption with the purified protein did not affect the ability of mAB-B100 to stain intermediate filaments in both BHK-21 cells and human skin fibroblasts. These results suggest that the antigenic determinant recognized by mAB-B100 is not present in the basic subunit, vimentin, of the intermediate filaments.

The specific reaction of mAB-B100 with a protein of 50 kdaltons was demonstrated by immunoprecipitation (Fig. 10),

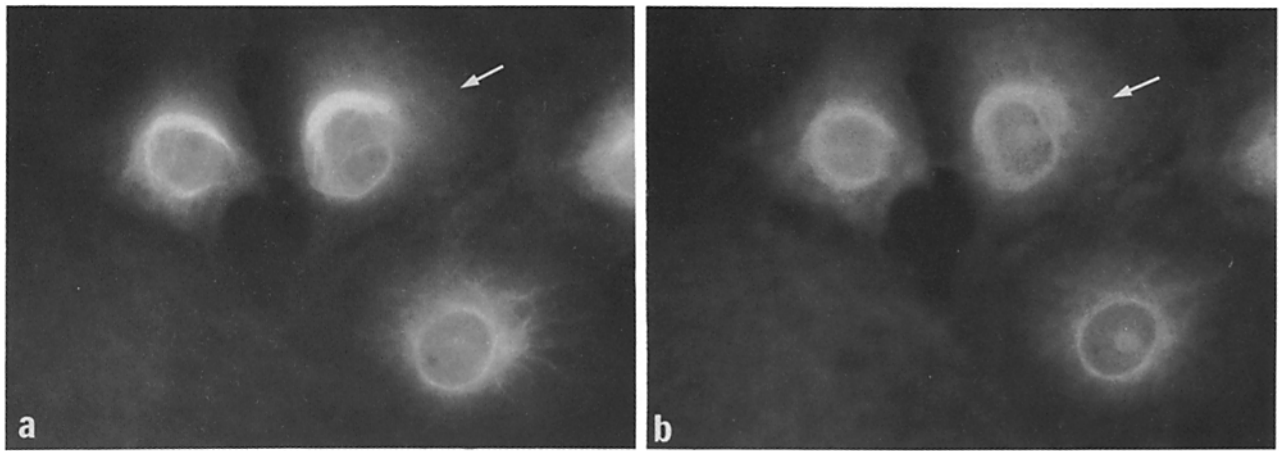


FIGURE 4 Simultaneous staining with mAB-B100 and rabbit polyclonal antibody to vimentin in cultured epithelial (PTK1) cells. The arrow indicates the absence of staining with either antibody in the cell periphery. The coincident staining reaction between mAB-B100 and polyclonal antibody to vimentin indicates the close association of p50 to intermediate filaments. The cells were processed, examined, and photographed in the same way as described for Fig. 3. (a) mAB-B100 staining pattern; (b) the same perinuclear region stained by the rabbit polyclonal antibody to vimentin. $\times 780$.

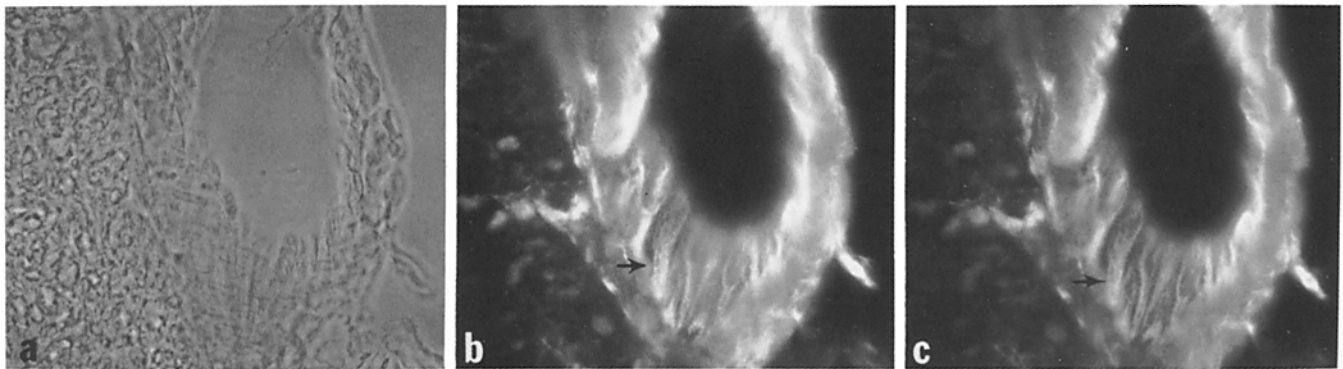


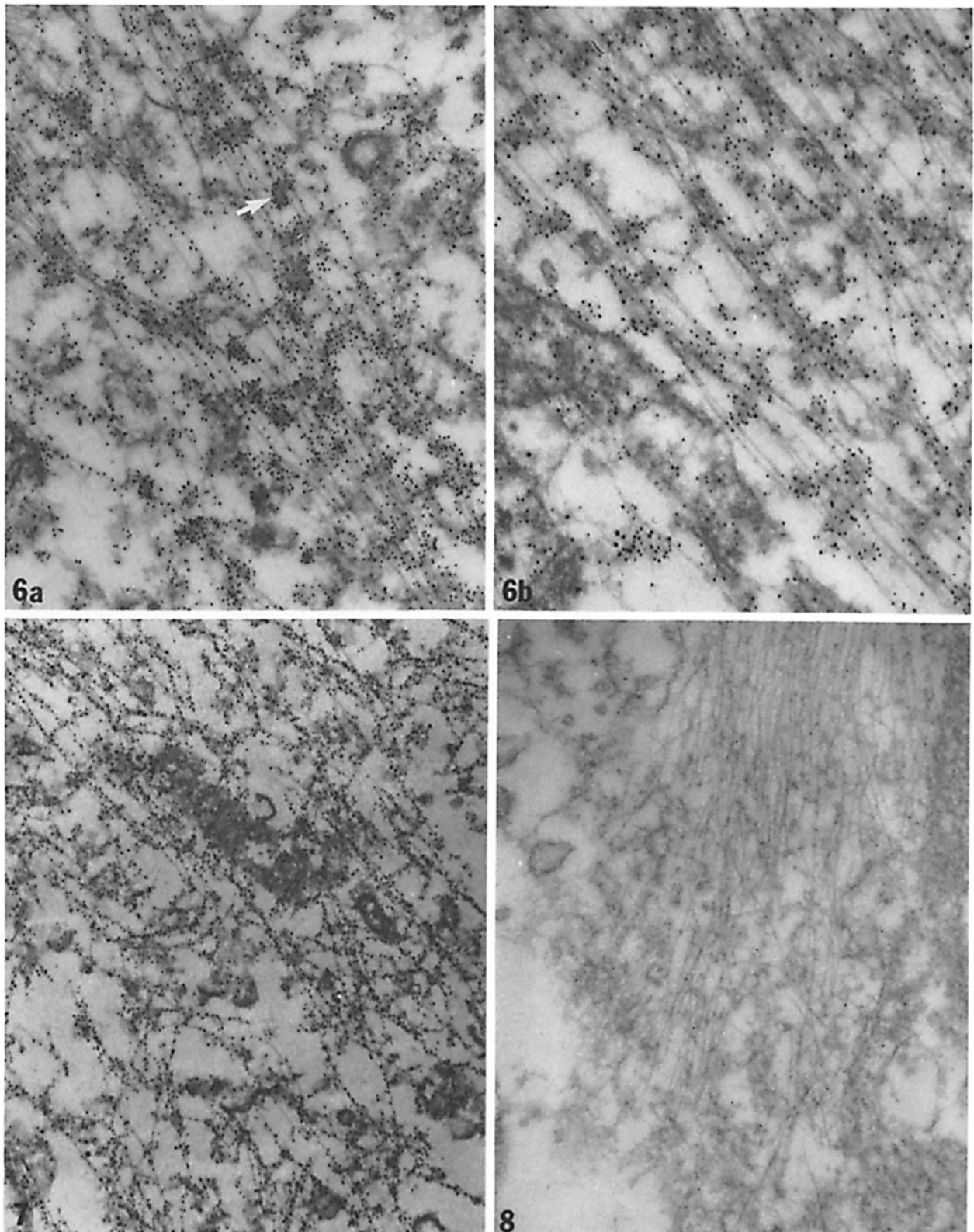
FIGURE 5 The staining reaction with mAB-B100 in vascular endothelial cells in the rat cerebellum. The frozen samples of rat cerebellum were sectioned and processed for double fluorescence microscopy as described in Materials and Methods. The examination and photography of the stained specimens were performed in the same way as described for Fig. 3. The arrows indicate the presence of filaments identified in the phase-contrast micrograph (a) by staining with both mAB-B100 (b) and polyclonal antibody to vimentin (c). $\times 500$.

using a cell extract from human glial astrocytoma cell line (U-251-5). Lane 1 represents the molecular weight protein standards ranging from 30,000 to 92,000. All the precipitated protein bands shown in lane 4 are the nonspecific reaction products of the nonselective reactions between rabbit anti-mouse serum and the cytoplasmic extracts. To enhance the efficiency of binding between antigen and antibody, the nude mouse ascites fluid of mAB-B100 was developed. When 25 μ l of the ascites fluid of mAB-B100 was used, a specific band at 50.5 kdaltons was found among the nonspecific bands (lane 3). When half the amount of the ascites fluid (12.5 μ l) was used, only a very faint band was observed at the 50.5-kdalton position (lane 2). When the monoclonal antibody to the 200-kdalton subunit of neurofilaments was used as the initial antibody for the incubation, the specific band was not seen (lane 5). The reactivity between p50 protein of the cytoskeletal extract and monoclonal antibody (mAB)-B100 was verified by the immunoblotting technique (Fig. 11). Fig. 11 demonstrates the reactivity of mAB-B100 with a protein of 50 kdaltons at the position below the glial filament protein (lane 1). The monoclonal antibody also recognized several minor bands below the major band of 50 kdaltons. These minor

bands are probably the proteolytic breakdown products of p50. The lack of reactivity for mAB-B100 with either vimentin or glial filament, or other high molecular weight proteins was evidenced by the absence of protein bands in area above the p50 band (lane 1). The specificity of mAB-B100 to p50 protein was further proven by the absence of reactivity when the supernatant of mAB to the 200-kdalton subunit of neurofilament was used to substitute mAB-B100 during the initial incubation (lane 2). The protein profile used for the immunoblotting is shown to contain two major protein bands identified as vimentin and glial filament (lane 3).

DISCUSSION

Recently, proteins associated with intermediate filaments have been recognized as important components in the cytoskeletal system. Such elements may not only stabilize or maintain the geometric organization of the network formed by filaments, but also serve as structural links integrating intracellular organelles into the scaffold of the cells for positioning and movement in the interior of cells. We have described in the present report a mouse monoclonal antibody



FIGURES 6-8 Fig. 6: Clustered pattern of colloidal gold decoration of intermediate filaments revealed by staining BHK-21 cells with mAB-B100. Immunoelectron microscopy of formaldehyde-fixed and Triton-extracted samples of BHK-21 cells. The detailed procedure for labeling with 5 nm colloidal gold-conjugated protein A is described in Materials and Methods. Note that the majority of gold particles are positioned in clusters around proteinaceous regions (arrow) along the intermediate filaments. (a) $\times 79,000$; (b) $\times 89,000$. Fig. 7: The linear pattern of colloidal gold decoration of intermediate filaments revealed by staining BHK-21 cells with rabbit polyclonal antibody to vimentin. This linear pattern shows that the core of these filaments is indeed composed of vimentin. $\times 60,500$. Fig. 8: Absence of colloidal-gold decoration on intermediate filaments when the fixed and extracted samples of BHK-21 cells were incubated with the monoclonal antibody mAB-R2, which is an antibody to the 200-kdalton subunit of neurofilaments. The samples were processed in the identical manner as described for Fig. 6. $\times 60,000$.

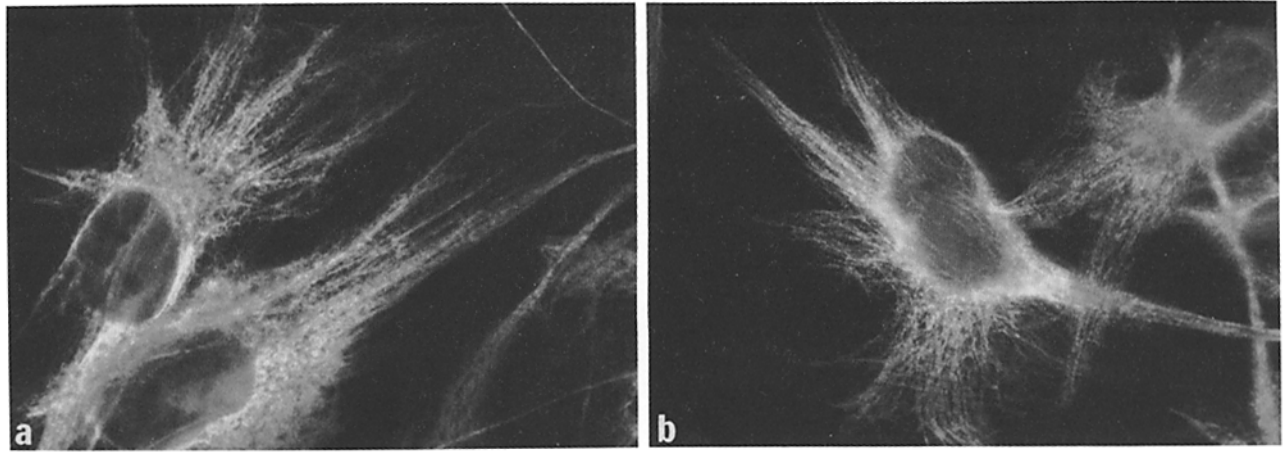


FIGURE 9 The dotted staining pattern of the filamentous network is not altered by prior incubation of mAB-B100 with purified vimentin isolated from baby hamster kidney cells. (a) Human skin fibroblasts; (b) baby hamster kidney fibroblasts. $\times 700$.

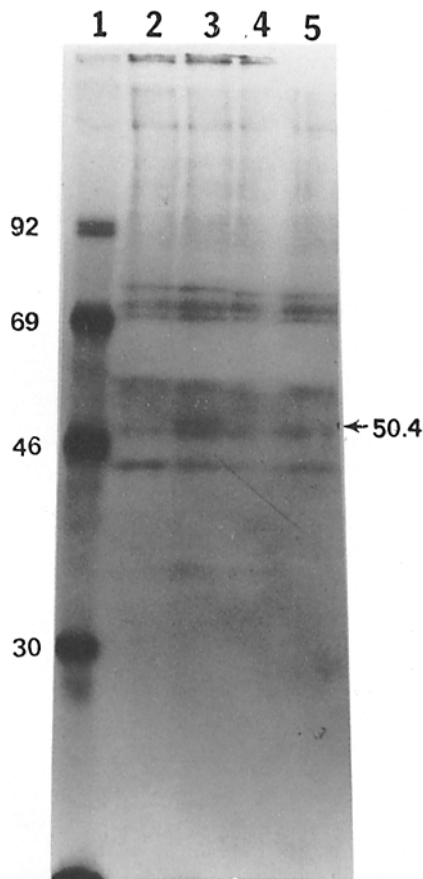


FIGURE 10 Immunoprecipitation of CSK preparations isolated from glial astrocytoma cells (U-251-5) with mAB-B100 and mAB-R2, an antibody to the 200-kdalton protein of neurofilaments. Lane 1, protein standards of 30–92 kdaltons; lane 2, a weaker immunoprecipitation reaction with 12.5 μ l of ascites fluid containing mAB-B100; lane 3, note the specific protein band at ~ 50.4 kdalton in addition to the bands seen in lane 2, precipitated with 25 μ l of ascites fluid containing mAB-B100 (arrow); lane 4, reaction products when the growing of medium of mAB-B100 hybridoma was used; these proteins were immunoprecipitated nonspecifically due to the nonselective reaction of the rabbit antimouse IgG and protein A. Similar nonspecific protein bands were seen when the initial incubation with the growing medium was eliminated; lane 5, the absence of protein band at 50.4 kdalton from the precipitates acquired by incubating the CSK extracts with mAB-R2, a monoclonal antibody to 200,000 subunits of the neurofilaments, which are not found in the glial astrocytes.

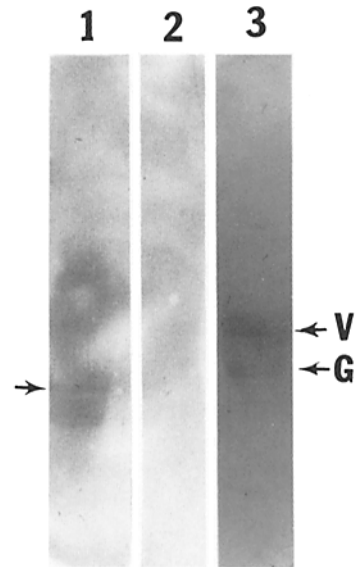


FIGURE 11 Immunotransblotting of CSK isolated from glial astrocytoma cells (U-251-5) with mAB-B100 and mAB-R2, an antibody to the 200 kdalton protein of neurofilaments. Lane 1, reaction product of the incubation between CSK protein profile on the nitrocellulose paper and mAB-B100. Note the presence of a major band at 50 kdaltons (arrow). The minor bands present at positions below the major band (p50) probably result from the action of protease during the extraction procedure. No protein band with electrophoretic mobility above the 50-kdalton region was noticed; lane 2, the absence of protein band at 50.4 kdalton from the precipitates acquired by incubating the CSK extracts with mAB-R2, a monoclonal antibody to 200,000 subunits of the neurofilaments, which are not found in the glial astrocytes; lane 3, SDS PAGE showing the migration of cytoskeleton extract used for the immunotransblotting assay. Vimentin (V) and glial filament (G) are shown as the two major proteins contained in the extract.

(B100) that recognized a protein (p50) of molecular weight 50,000 that is a candidate for being such an element. The association of this protein with intermediate filaments was demonstrated by staining intermediate filaments with mAB-B100 as well as polyclonal antivimentin antibody. Localization by immunoelectron microscopy, using 5 nm colloidal gold particles conjugated to protein A, revealed clusters of this 50-kdalton protein along the intermediate filaments of 10-nm-diam.

The distinctive feature of the p50 protein as a filament-associated protein, rather than a core unit of the filaments, is supported by the following additional evidence: (a) by immunoblotting and immunoprecipitation, the monoclonal antibody did not react with proteins positioned at regions higher than 50 kdaltons, where purified proteins such as vimentin, desmin, or glial filament protein were located; (b) incubation with purified vimentin isolated from BHK-21 cells did not remove the capability of mAB-B100 to stain the filaments (additional experiments on absorbing mAB-B100 with purified glial filament protein or desmin have given staining patterns similar to those obtained with unabsorbed serum). These results are entirely consistent with the finding that, at the ultrastructural level, p50 protein and vimentin displayed different patterns of protein arrangement. A similar argument supports the nonrelatedness between p50 and glial filament protein in that rabbit glial filament protein polyclonal antibody stains intermediate filaments in the same linear arrays as antivimentin IgG in glial astrocytes and not in the clustered pattern seen with mAB-B100. The absence of staining by mAB-B100 in the cell periphery of PTK1 cells suggests that p50 is not associated with the keratin-containing filament system.

Side-arm structures extending laterally from the intermediate filaments have been repeatedly observed in many systems (5, 13, 16, 19–21, 24). In particular, the uniform side-arm extensions of neurofilaments are readily recognized in nerve axons (13, 16, 24). It has been suggested that these structures are the morphological representations of a protein complex associated with the filaments. Recently, several reports (9, 22) identified these side arms as being composed of the 200-kdalton protein subunit of the neurofilament proteins, with the 68-kdalton protein forming the central core. By physicochemical methods, Runge and Williams (15) have produced an ATP-dependent microtubule-neurofilament complex in vitro. We have suggested that a complex relationship that governs various physiological functions, may exist between microtubules and vimentin-containing filaments, or among the filaments themselves, in cultured fibroblasts (19). A model of the intermediate filament complex, with or without microtubules, formed by cross-bridges has since been postulated from morphological observations (5, 13, 16, 19–21, 24) and from in vitro biochemical analysis (14). Therefore, it appears that protein p50 may be a member of the side-arm complex associated with the vimentin-containing filaments.

The dotted filament pattern of mAB-B100 staining is very similar to that reported by Zumbe et al. (25) with a monoclonal antibody against eucaryotic mRNA 5'-cap binding protein in BHK-21 cells. They find that this monoclonal antibody reacts specifically with the 50-kdalton component of the 5'-cap binding protein complex. It is possible that the 50-kdalton protein that we have detected is related to the protein in the 5'-cap binding protein complex. However, we do not rule out that the similarity between the staining reactions of Zumbe et al. and those of B100 antibody may be fortuitous due to common antigenic sites existing between the intermediate filament proteins and other cellular proteins, which occurs in high frequency due to the specificity of monoclonal antibodies. For example, the mAB to thymidine kinase can decorate the vimentin-containing system in fibro-

blasts (3).

The identification of the p50 as a protein associated with intermediate filaments should lead to an investigation of the complex molecular interaction of filaments with their environment. The proof of the possible involvement of p50 in the functioning of intermediate filaments awaits further experiments, such as analysis of intracellular motility in cells injected with mAB-B100.

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