Monoclonal antibody analysis of keratin expression in the central nervous system

(Purkinje cell/intermediate filaments/hybridoma)

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ABSTRACT   A monoclonal antibody directed against a 65-kDa brain protein demonstrates an epitope found in keratin from human epidermis. By indirect immunofluorescence, the antibody decorates intracytoplasmic filaments in a subclass of astrocytes and Purkinje cells of adult hamster brain. Double-label immunofluorescence study using antibody to glial fibrillary acidic protein and this antibody reveals the 65-kDa protein to be closely associated with glial filaments in astrocytes of fetal mouse brain cultures. Immunoblot analysis of purified human epidermal keratin and hamster brain homogenate confirms the reactivity of this antibody to epidermal keratin polypeptides. All the major epidermal keratins were recognized by this antibody. It did not bind to the remaining major intermediate filament proteins. These findings suggest that monoclonal antibody 34C9 recognizes a cytoskeletal structure connected with intermediate filaments. In addition, the monoclonal antibody demonstrates that epidermal keratins share an epitope not only among themselves but also with a "neural keratin."

We have investigated the expression and structural arrangement of a cytokeratin epitope in normal hamster and mouse central nervous tissue by using a monoclonal antibody as an immunological probe. We report the detection of this 65-kDa protein, associated with intermediate filaments, in the cytoskeletal structures of Purkinje cells and a subclass of astrocytes that has an epitope characteristic of simple epithelia.

Cytokeratin filaments of epithelial cells are a subclass of about 20 different polypeptides (1). Their phenotypic expression is developmentally regulated, is restricted in distribution among cell types and tissues, and is linked to the differentiated state of the cell (2, 3). Thus, the compositional profile of cytokeratin in a given tissue reflects the presence of a cell type-specific subset of several cytokeratin polypeptides.

These filaments form a complex cytoplasmic network among cellular organelles, and they often span the cell interior from nuclear membrane to the cell periphery. These filaments interconnect with microfilaments and microtubules. Within this cytoplasmic lattice system are intrafilamentous matrix proteins (4, 5), and several of these proteins have been reported to be specifically associated with intermediate filaments (6, 7). Evidence suggests that these intermediate filament-associated proteins (IFAPs) may have the ability to aggregate intermediate filaments and act as linkers for defining the space between intermediate filaments (8).

MATERIALS AND METHODS

Immunization of Mice. Two-month-old female BALB/c mice were immunized by footpad inoculation with 0.1 ml of a 10% suspension of Creutzfeldt–Jakob disease virus-infected mouse brain in complete Freund's adjuvant. The homogenate of brain infected with Creutzfeldt–Jakob disease virus was prepared from NIH Swiss mice inoculated with the mouse-adapted Fujisaki strain of the virus (9). One month later the mice were inoculated intravenously with 0.1 ml of the brain preparation without adjuvant. Spleens were removed 3 days later for fusion.

Production of Hybridomas. The mouse myeloma line NS-1 used in this study is a variant of BALB/c MOPC/21 cell line (10). The hybridization technique used was the procedure of Köhler and Milstein (11) as modified by Kennett et al. (12). The modifications have been described in detail (13).

Tissue Preparation. Golden Syrian hamsters were killed by decapitation, and their brains were removed and immersed in isopentane at $-30°C$ to preserve the normal cytoarchitecture of cells. After fixation, brains were stored at $-70°C$. Cryostat sections 8 μm thick through a coronal plane were prepared and used for indirect immunofluorescent staining as previously described (14).

Cell Cultures. Fetal mouse brain cultures were established and maintained in our laboratory as described by Sotelo et al. (15). For indirect immunofluorescence, cells grown on glass coverslips were washed three times with phosphate-buffered saline (PBS; Quality Biological, Gaithersburg, MD) and then permeabilized with methanol ($-20°C$) for 10 min followed by acetone for 10 sec. After washing three times, cells were incubated for 30 min with undiluted monoclonal antibody 34C9 culture supernatant, after which they were washed three times in PBS. Fluorescein-conjugated rabbit anti-mouse IgG (Miles) at a dilution of 1:16 in PBS was then added, and the cells were incubated for 30 min. All washes and incubations were carried out at room temperature.

Electrophoretic Blotting Procedure. Hamster brain homogenate and human epidermal keratins (Sigma) were used for immunoblots. Proteins were separated on a NaDodSO$_4$/10% polyacrylamide gel by use of the discontinuous buffer system of Laemmli (16) and transferred electrophoretically to nitrocellulose sheets according to the method of Towbin et al. (17). After transfer, the electrophoretic blots were incubated with 5% goat serum in PBS for 2 hr at 37°C. The blots were incubated for 6 hr at room temperature with the monoclonal antibody. The blots were then allowed to react with the avidin-biotin-peroxidase complex (Vectastain kit; Vector Laboratories, Burlingame, CA) and visualized with 4-chloro-1-naphthol (Sigma).

RESULTS

A hybridoma clone producing an anti-keratin antibody, designated 34C9, was derived from a fusion of NS-1 myeloma cells with spleen cells from BALB/c mice immunized with mouse brain infected with Creutzfeldt–Jakob disease virus. The antibody was detected by indirect immunofluorescence

Abbreviations: IFAP, intermediate filament-associated protein; GFAP, glial fibrillary acidic protein.

on air-dried frozen sections of adult hamster cerebellum. Astrocytes in the granular layer showed a diffuse fluorescence within the cytoplasm and a fibrillar pattern in their processes (Fig. 1). In contrast, astrocytes in the white matter and Bergmann glial fibers of Golgi epithelial cells in the molecular layer were not stained. Dense staining of intracytoplasmic loops was detectable in Purkinje cells (Fig. 1 *Inset*). The loops were compact, wider, and clearly differentiable from glial fibrils. Other neurons in the cerebellar cortex did not stain. 34C9 antigen expression was detectable in ependymal cells, where the staining was situated in the apical region of the cell (Fig. 2).

The intracytoplasmic distribution of 34C9 antigen *in vitro* was examined in fetal mouse brain cultures by immunofluorescence. As shown in Fig. 3, 34C9 antigen-specific fluorescence is arranged in fibrils that extend throughout the cell body, often showing a higher density in the nuclear vicinity in cells morphologically identified as astrocytes. To confirm the astrocytic nature of the 34C9 antigen-positive cell and to compare the organization of keratin and glial fibrillary acidic protein (GFAP) fibers, double labeling with anti-GFAP antibody as a marker for astroglia was used. A comparison of keratin and GFAP-specific fluorescence reveals that the distribution of keratin coincides with that of GFAP (Fig. 4). The results shown in Fig. 4 indicate that GFAP and keratin are coordinately expressed in astrocytes.

To determine the molecular specificity of the 34C9 antigenic determinant, we prepared adult hamster brain homogenate and keratin from human epidermis and analyzed them by one-dimensional NaDodSO$_4$ gel electrophoresis and immunoblotting. Immunoblot analysis (Fig. 5) showed that 34C9 antibody reacted with a 65-kDa protein of hamster brain preparation (lane a) and with the multiple keratins from human epidermis cells (lane b).

DISCUSSION

The central nervous system is a perfect model of specific distribution of intermediate-sized filaments among different classes of cells: neurofilaments of neurons; desmin, vimen-

FIG. 2. Immunofluorescent staining pattern by monoclonal antibody 34C9 in ependymal cells of adult hamster brain. The fluorescence is seen in the apical region of the cell in a cap distribution. (×500.)

tin, and glial filaments of astrocytes; and cytokeratins in ependymal cells. To our knowledge, until our demonstration of cytokeratin in ependymal cells, cytokeratin had not been observed in neural cells (18). In the present study we have demonstrated by immunocytochemical analysis the presence of a cytokeratin epitope within cytoplasmic loops in Purkinje cells, ependymal cells, and a subclass of astrocytes in the
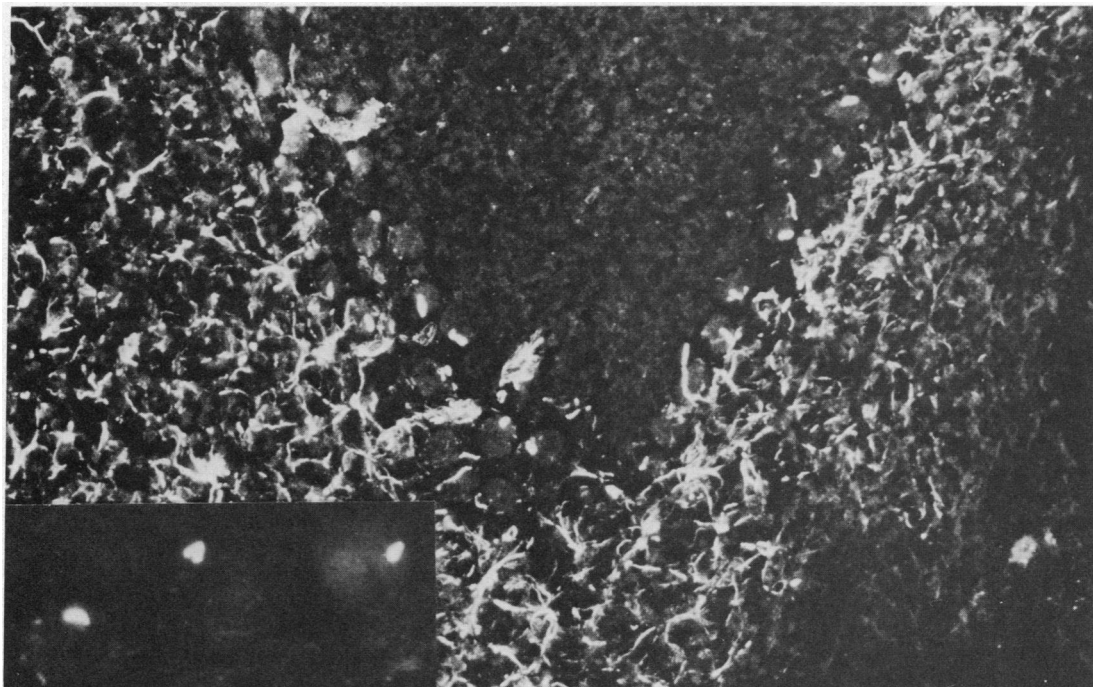

FIG. 1. Immunofluorescent staining pattern of monoclonal antibody 34C9 in the adult hamster cerebellum. In the granule cell layer, a diffuse fluorescence of the cytoplasm of astrocytes is seen. (×250.) (*Inset*) Higher magnification (×690) shows the labeling of intracytoplasmic loops within Purkinje cells.
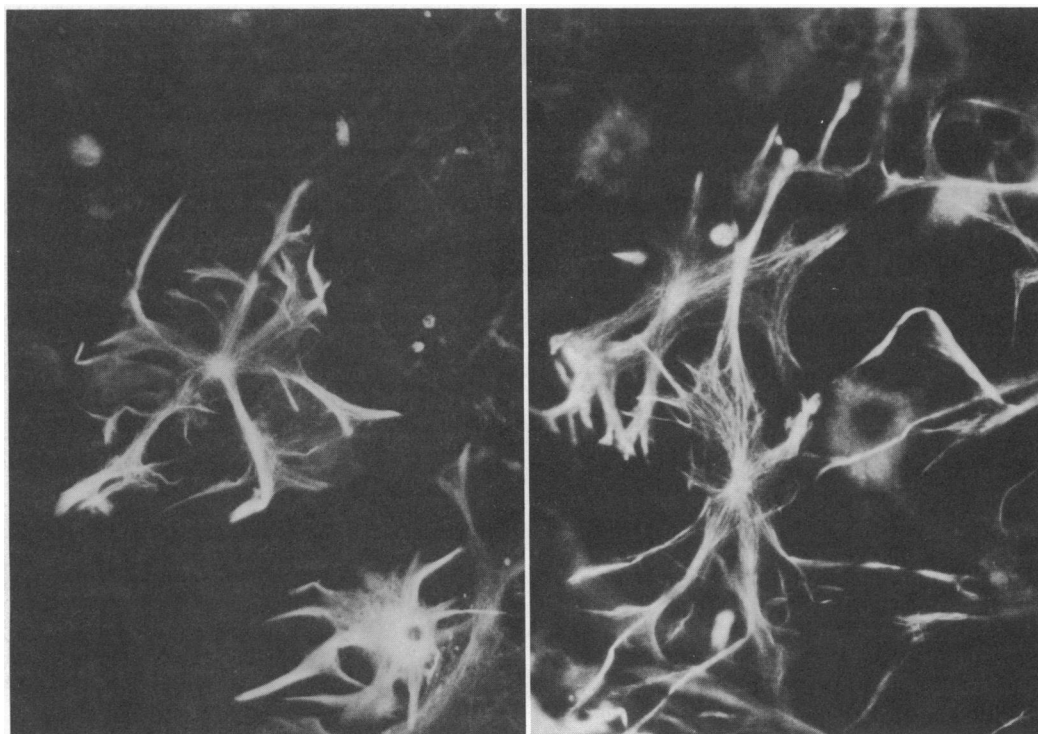
FIG. 3.   A composite of photomicrographs of monoclonal antibody 34C9 reaction with astrocytes in a fetal mouse brain culture. Cultures were maintained *in vitro* for 9 days and fixed in methanol/acetone before staining. Astrocytes exhibit intrafilamentous fluorescent staining extending into the periphery of the cell processes. (×210.)

granular layer of the cerebellar cortex. The 34C9 cytokeratin antigen was not detectable in other neural cells.

Immunoblot analysis of the molecular specificity of 34C9 antigen revealed an interesting relationship with the cytokeratins from human epidermis and with a 65-kDa brain protein. Tseng *et al.* (19) reported a monoclonal antibody (AE3) specific for 58- and 65- to 67-kDa keratins that reacted with

a 66-kDa protein in some nonepithelial cells, including brain, and suggested that this protein may be related to the 66-kDa protein (IFA) described by Pruss *et al.* (20).

The results reported here differ from the above in several aspects. Unlike AE3 antibody of Tseng *et al.* (19), 34C9 monoclonal antibody detected an antigenic determinant present on all keratins from human epidermis. Second, as
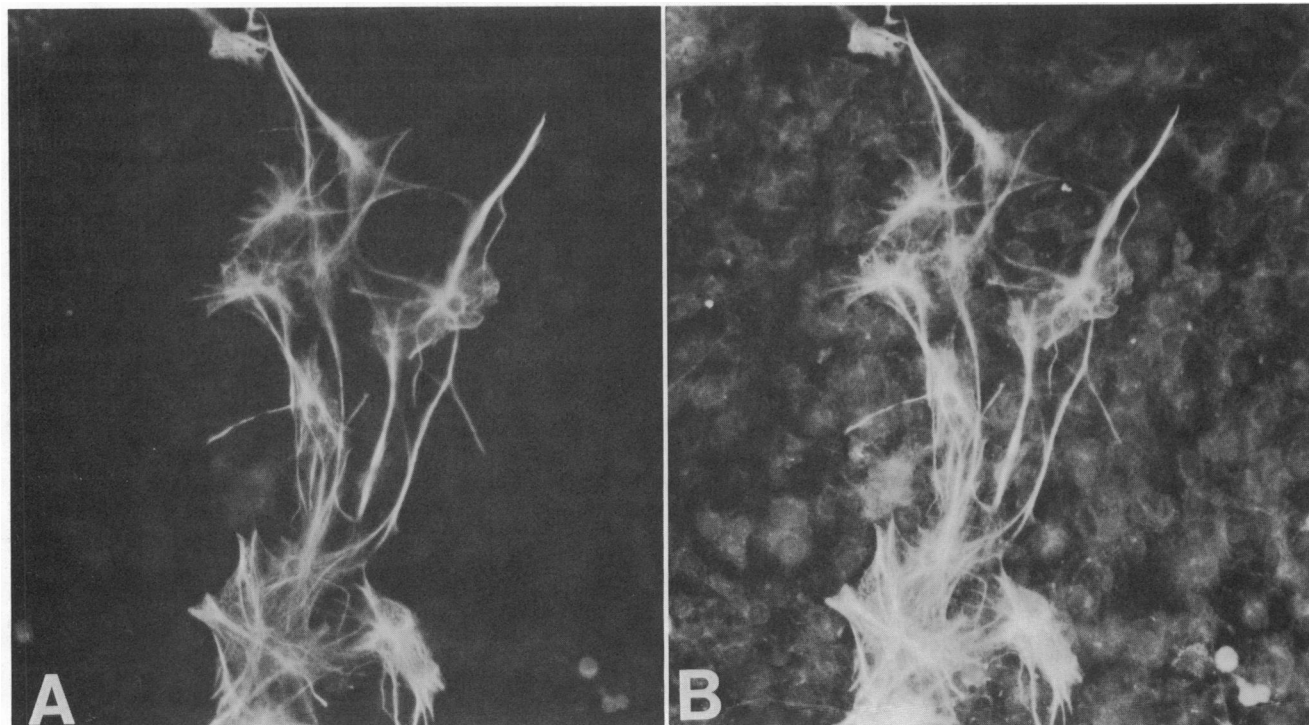

FIG. 4.   Double immunofluorescence labeling of cultures of fetal mouse brain cells with anti-GFAP (*A*) and anti-34C9 (*B*) antibodies. The codistribution of staining patterns of the two antibodies is seen in filaments of astrocytes. (×210.)

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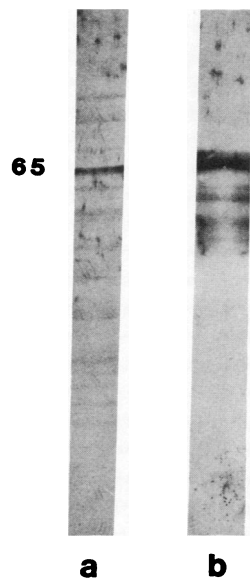*Proc. Natl. Acad. Sci. USA 84 (1987)* 3485

65

a   b

FIG. 5. Immunoblot analysis of adult hamster brain homogenate and a preparation of human epidermal keratins. The proteins were separated on NaDodSO$_4$/polyacrylamide gels and electrophoretically transferred onto a nitrocellulose sheet and incubated with monoclonal antibody 34C9. Lane a, adult hamster brain. Lane b, human epidermal keratins. Molecular mass in kDa is indicated on the left.

opposed to the IFA protein, which is associated with all five classes of intermediate filaments, 34C9 determinant was preferentially located in Purkinje cells and in a subclass of astrocytes. However, it is possible that 34C9 antigenic determinant is located on an unrelated restricted subunit protein that is not associated with all classes of intermediate filaments.

It seems likely that monoclonal antibody 34C9 recognizes a highly conserved domain of epithelial keratin that does not appear to have an exclusive tissue distribution. Franke *et al.* (3) showed that the pattern of keratin polypeptides is characteristic for a specific tissue or cell type and that this specificity may reflect the different levels of differentiation of cells. There is a common ectodermal origin of epidermis and brain, and this could explain the conserved epitope in brain. The reason for the preferential concentration of 34C9 keratin epitope in Purkinje cells and granular layer astrocytes for the construction of filaments of the same morphology in other neural cells is not clear.

The cellular variation of 34C9 expression may indicate a subtle tailoring of the resulting filament to fit the individual needs of the cell. Furthermore, the observed differences may be related to regulation of cell differentiation rather than to functional differences of the filaments.

Astroglia cells of fetal mouse brains, when cultured *in vitro*, retain their differentiated properties; they express GFAP and the keratin epitope. This suggests that anatomical-site effect can be excluded as an explanation for the keratin epitope expression in the cerebellar cortex and for the previously unreported expression in neural cells. At present, the spatial relationship between the 34C9 antigen and GFAP has not been determined. The major nonepithelial intermediate filament proteins—i.e., desmin, GFAP, and vimentin—form homopolymers and can form copolymers *in vitro*. If the 65-kDa protein is a restricted intermediate filament subunit protein, copolymerization with GFAP may be the interpre-

tation. An alternative explanation may be that the 65-kDa protein is an IFAP. The absence of binding of monoclonal antibody 34C9 to GFAP, vimentin, or the neurofilament subunit proteins when applied to nitrocellulose transfers of NaDodSO$_4$/polyacrylamide gels containing the purified preparations of subunit proteins lends support to an IFAP role (data not shown). Recently several IFAPs have been demonstrated in central nervous system tissue (6) and in diverse mammalian cell types (7, 21). In particular, a 48-kDa protein termed fillagnin is found associated with keratin filaments (22).

In addition, the intracytoplasmic loop structures observed in the Purkinje cells are very similar to the loops we previously described in mouse brain neurons (18). This observation led to the suggestion that these loop structures may indicate the presence of an intermediate filament organizing center (IFOC) (23). Therefore, a correlation between the IFOC and assembled intermediate filaments is indicated by the presence of the 65-kDa protein with both structures. The exact physiological role of the 65-kDa protein and its influence on the cytoskeleton of the cells of the central nervous system in which it can be detected remain to be determined.

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