Generation of Monoclonal Antibodies Recognizing Neuronal Elements in Formalin-fixed Paraffin-embedded Human Tissue

JACK P. ANTEL, M.D., JAYA KUCHIBHOTLA, M.Sc., AND KARI STEFANSSON, M.D.

Abstract. We used formalin-fixed human spinal cord and dorsal root ganglia as immunogens to generate monoclonal antibodies (mAb) which immunohistochemically react with neurons in formalin-fixed human tissue sections. Three of the mAb recognized all neuronal populations studied, including those in spinal cord, dorsal root ganglia, cerebellum, and cerebrum. A fourth mAb recognized neurons within spinal cord, dorsal root ganglia and dentate nucleus of cerebellum but not those in cerebrum or cerebellar hemispheres. This mAb, unlike the other three, did not recognize murine neurons. These data indicate the feasibility of generating mAb suitable for analysis of human pathological material in its most readily available form, formalin-fixed paraffin-embedded tissue.

Key Words: Antibodies, monoclonal; Central nervous system; Neurons; Peripheral nervous system.

INTRODUCTION

Monoclonal antibodies (mAb) are powerful agents with which to identify and subsequently characterize specific cell populations within the nervous system. Monoclonal antibodies are described which recognize surface membrane and intracytoplasmic determinants of various subclasses of neurons in an array of invertebrate and nonhuman vertebrate species (1-16). Some of these mAb do cross-react with human tissues. Miller and Benzer (2) found that some mAb raised against Drosophila melanogaster head, brain or retina recognize neuronal determinants on unfixed cryostat sections of human central nervous system (CNS); at least two of these mAb recognize only spinal cord determinants. None of these mAb, however, recognize formalin-fixed human tissues.

For studies of human pathological material, the most readily available tissues are those which are formalin-fixed and paraffin-embedded. Some mAb which recognize neurons in fixed animal tissues are reported. McKay and Hochfield (12) used paraformaldehyde- and glutaraldehyde-fixed spinal cord to raise mAb which immunohistochemically recognized CNS neuronal elements in fixed tissue sections of cats. Eisenbarth et al (7), using glutaraldehyde-fixed cells as immunogens, obtained mAb which recognize the plasma membrane of cell bodies of rat neurons in the CNS and peripheral nervous system (PNS) (7). In this report, we describe mAb which recognize neuronal elements in formalin-fixed paraffin-embedded human tissue.

From the Department of Neurology, and Brain Research Institute, University of Chicago, Chicago, Illinois.

Correspondence to: Dr. Jack Antel, Dept. of Neurology, University of Chicago, 5841 Maryland Ave., Chicago, IL 60637.

Supported by grants from the Amyotrophic Lateral Sclerosis Society (ALSSOA), Muscular Dystrophy Association, and NIH grant #PO 21442-01.
MATERIALS AND METHODS

Two approaches were followed in generating the mAb described in this report: 1) unfixed tissues were used as immunogens and for the initial immunohistochemical studies, 2) formalin-fixed tissues were used for both immunization and immunohistochemical studies.

Immunogens: 1) For studies of unfixed tissues, the immunogens used were a) motor neurons isolated from fresh bovine spinal cords using the procedure of Capps-Covey and McIlwain (17) and b) grey matter from unfixed human spinal cord obtained at autopsy performed 12 hours (h) postmortem on a 55-year-old man who died from a myocardial infarction; 2) for studies with fixed tissue, the immunogens used included a) grey matter from adult spinal cord, b) dorsal root ganglia (both 2a and 2b were from the same autopsy as 1b but were used after 7 days fixation in 40% buffered formaldehyde), c) spinal cord from a 30-week-old human fetus obtained at autopsy performed 24 h after death and fixed for seven days in 40% buffered formaldehyde. The immunogens used are outlined in Table 1.

Generation of mAb: The immunization procedure involved injecting the footpads of Lewis rats with immunogen emulsified in complete Freund's adjuvant. Each animal received two or three such injections at two- to three-week intervals; three days before fusion, animals were boosted intraperitoneally with antigen without adjuvant. Spleen cells were fused with the mouse myeloma line SP2/0 Ag 14. Hybridomas were screened by immunohistochemically staining tissue sections. Those hybridomas selected for further analysis were cloned by limiting dilution.

- Tissue Sections: Sections of unfixed tissue were prepared from autopsy tissue from young individuals, both males and females, who died without a neurological disease. The tissue was obtained a few hours after death and frozen in isopentane precooled in liquid nitrogen. Sections of fixed tissue were prepared from blocks of paraffin-embedded formalin-fixed tissue also from young individuals without neurological disease. The paraffin sections were deparaffinized in xylol and hydrated through decreasing concentrations of ethanol. Tissue sections examined included spinal cord (the tissue used to screen hybridomas), brain stem, cerebellum, cerebrum including neocortex and hippocampus, dorsal root ganglia, adjacent peripheral nerve, liver, kidney, and skeletal muscle. No attempt was made to examine specific levels of spinal cord with each mAb.

Tissue sections were also prepared from formalin-fixed and unfixed spinal cord and brain of adult SJL mice.

Immunohistochemical Studies: Both in the initial screen of hybridoma supernatants and in subsequent studies using supernatants from cloned cell lines, a peroxidase-antiperoxidase (PAP) technique described below was used (18, 19). For mAb raised using non-fixed immunogens, initial immunohistochemical studies were conducted on unfixed tissue sections and then subsequently evaluated on fixed tissue sections. For mAb raised using fixed immunogens the reverse order was followed. Staining of mouse tissue was performed as the last step in the analysis of all of the mAb.

The first step of the staining procedure of formalin-fixed tissues was to incubate the slides for 30 minutes (min) in 3% v/v hydrogen peroxide (H₂O₂) in 0.9% NaCl-0.5 M Tris HCl buffer at pH 7.6. This buffer was used throughout the procedure. The slides were then washed in buffer. All washes were done with four changes of buffer for seven min each. The sections were then overlaid for 15 min with normal serum, from the species (goat) donating the linking antibody, diluted 1:10 in buffer, and then blotted but not washed. The 1:10 goat serum in Tris-HCl buffer was used for all subsequent dilutions. The next 30-min overlay was with the mAb or animal sera to be tested, followed by a wash. The sections were then overlaid for 30 min with a 1:20 dilution of goat anti-rat IgG, followed by a wash. The last overlay was for 30 min with a solution of horseradish peroxidase–rat anti-peroxidase immune complex (Sternberger-Meyer Immunocytochemicals, Inc.) diluted 1:40, also followed by a wash. Thereafter, the slides were put into solution containing 0.25 mg/ml of 3',3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and 0.005% H₂O₂ v/v in saline-Tris buffer for approximately four min and washed. The sections were either counterstained with hematoxylin or had no counterstain.

Fig. 1. A frozen section of a human spinal cord stained immunohistochemically with mAb 224. The figure shows an intensely stained large anterior horn neuron. The staining is cytoplasmic and nuclear. × 500.

The staining procedure for unfixed tissues was as above except that tissues were exposed initially to serial ten-min incubations with 0.1% v/v, 0.2% v/v, and 0.3% v/v solutions of \( \text{H}_2\text{O}_2 \).

*Isotyping of mAb:* The isotyping was done with an immunodot method (20) using the Bio-Dot Microfiltration Apparatus from BioRad Laboratories (Richmond, CA). Antibodies against rat Ig subclasses were obtained commercially (Miles).

Attempts to Identify the Molecules Carrying the Antigenic Determinants Recognized by the mAb

*Immunoblot* (21): As sources of antigens we used, we used pieces of whole human or bovine spinal cord, grey matter from bovine or human spinal cord, large anterior horn cells (AHC) isolated from bovine spinal cord (17), and Purkinje cells isolated from bovine cerebellum according to the method of Sellinger et al (22). Both the AHC preparation and the Purkinje cell preparation were contaminated with a small amount of capillaries and smaller cells. We also used neurofilaments isolated from bovine spinal cord (23) provided by Dr. W. W. Schlaepfer, Department of Pathology, University of Pennsylvania. The tissues and the isolated cells were homogenized directly in a mixture containing 1% v/w sodium dodecyl sulfate, 1% v/v 2-mercaptoethanol and 5.7 M urea. The isolated neurofilaments were solubilized in the same mixture. The antigens were heated to 100°C for two min before electrophoresis. Proteins (peptides) in the antigen preparations were separated by polyacrylamide slab gel electrophoresis using both the system of Laemmli et al (24) and the system of Fairbanks (25). Approximately 500 \( \mu\)g of protein was applied to each 0.75-mm × 13.4-cm gel. Molecular weight markers from BioRad (Richmond, CA) were used.

The unstained gels containing the separated proteins from the antigen mixtures were overlaid with nitrocellulose paper and the bands transferred electrically onto the paper by use of the BioRad Trans-Blot (BioRad Laboratories, Richmond, CA) at 60 V for three h in 20 mM Tris-
Fig. 2. A frozen section of a human cerebellum stained immunohistochemically with mAb 211. The figure shows darkly staining Purkinje cells (arrow) as well as cells in the granular cell layer (upper left). There is negligible staining in the molecular layer (lower right). × 300.

HCl buffer at pH 7.5 with 20% methanol. The nitrocellulose sheets were cut into strips and the strips were placed individually into capped test tubes. The protein binding capacity of the nitrocellulose strips was saturated by incubating them overnight in 5% bovine serum albumin (BSA) in 0.05 M Tris-HCl saline buffer pH 7.6 at room temperature. The next step was 20 min equilibration with 10% normal goat serum in 0.05 M Tris-HCl saline buffer at pH 7.6 containing 0.05% Nonidet P-40 (Particle Data Laboratories, IL). This same medium was used for all subsequent washes and dilution. Equilibrated strips were next incubated for one h with the mAb to be tested. Subsequently the strips were washed with three changes of medium, each lasting 15 min. Next came one-h incubation with peroxidase-conjugated rabbit antibodies against rat immunoglobulins (Dako Corp., CA), diluted 1/100, followed by another three washes for 15 min each. The peroxidase activity was revealed by incubation in Tris-HCl saline at pH 7.6 containing 0.5% H₂O₂ and 25 mg/ml of 3',3'-diaminobenzidine tetrahydrochloride.

Detection of Binding of mAb to Lipids on Thin-layer Chromatography (TLC) Plates

Polar lipids and neutral lipids were extracted from human spinal cords using the method of Folch-Pi et al (26). Extracted gangliosides or neutral lipids were separated by TLC, using aluminum backed TLC plates (Silicapel 60; Merck, Dormstadt, Federal Republic of Germany). The plates were developed as follows: a) for the gangliosides—chloroform : methanol 0.2% CaCl₂ in H₂O (55–45–10 by volume), dried and redeveloped in chloroform : methanol 0.2% KCl in 2.5 M NH₄OH (50–40–10 by volume); b) for the neutral lipids—chloroform : methanol H₂O (100–42–6 by volume). After the development the plates were dried and cut into 1.0-cm-wide strips that were placed individually into test tubes. The binding of the mAb to lipids
TABLE 1
Immunohistochemical Staining Pattern of Neuron-directed Monoclonal Antibodies (mAb)

<table>
<thead>
<tr>
<th>Tissue used</th>
<th>mAb 211</th>
<th>224</th>
<th>648</th>
<th>182</th>
<th>299</th>
<th>395</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human unfixed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinal cord</td>
<td>+*</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+</td>
<td>+†</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Human fixed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinal cord</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Purkinje cells</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Granular cells</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dentate nucleus</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mouse unfixed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinal cord</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mouse fixed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinal cord</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ig isotype</td>
<td>(\text{IgG}_1)</td>
<td>(\text{IgG}_1)</td>
<td>(\text{IgM})</td>
<td>(\text{IgM})</td>
<td>(\text{IgG}_{	ext{H}})</td>
<td>(\text{IgM})</td>
</tr>
<tr>
<td>Immunogen used</td>
<td>Bovine AHC‡ + human spinal cord grey matter</td>
<td>Human fetal spinal cord</td>
<td>Human dorsal root ganglia</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Staining (+) or nonstaining (–) of the neuronal population by each of the mAb is indicated for each tissue studied.
† Staining of Purkinje cells and granular cell layer.
‡ AHC: Anterior horn cells.

on the TLC plates was demonstrated with the same indirect peroxidase method used to demonstrate binding of mAb to proteins on the nitrocellulose in the immunoblot method.

RESULTS

Immunochemical Studies: All mAb described in this report stained intracytoplasmic structures.

a. Monoclonal antibodies derived from immunization and initial screening with non-fixed tissue—Two mAb (224 and 211) were obtained which stained predominantly neuronal cell somas in non-fixed tissue (Figs. 1, 2). Some regional specificity was observed in that mAb 224 did not stain neurons within cerebral cortex (Table 1). Neither of these mAb stained formalin-fixed tissues nor did they stain unfixed mouse tissue.

An additional mAb 107 obtained from the same immunization stained central and peripheral nervous system axons in unfixed and fixed human and mouse tissues (Fig. 3). This mAb as mentioned below, by immunoblot procedure, recognized the 200,000-dalton component of the neurofilament triplet.

b. mAb derived from immunization and initial screening with formalin-fixed
tissue—Four mAb were generated which predominantly stained neuronal elements of formalin-fixed human spinal cord. All of these mAb stained the cell soma darkly (Table 1, Figs. 4–7); some staining of nerve cell processes was also noted. Neuronal staining was observed in anterior and posterior horns of the spinal cord as well as in the intermediolateral cell column with all these mAb. Two of the mAb, 299 and 182, stained ependymal cells. Monoclonal antibodies 182, 648, and 395 were subsequently found to stain neurons throughout the nervous system, as summarized in Table 1. These mAb also stained neurons in formalin-fixed mouse tissue sections.

Monoclonal antibody 299 displayed specificity with regard to staining intensity of neurons in different regions of the human nervous system. Non-spinal cord neurons stained were those in dorsal root ganglia, albeit rather weakly, and dentate nucleus of the cerebellum. Neurons in cerebral cortex, cerebellar hemispheres, and brain stem nuclei were not stained. In contrast to the other mAb, mAb 299 did not stain mouse tissue. On some human spinal cord sections, mAb 299 stained both leptomeninges and blood vessels. This staining was, however, variable from section-to-section and from one day to another. We have no explanation for this variability.

A number of mAb were also derived during these studies which immunohistochemically recognized neurons as well as other neural components with apparently equal affinity. Monoclonal antibody 554 (Fig. 7A) stained both neurons and myelin; its specific immunohistochemical characteristics are presented later.

Control Studies: Staining with the bridging antibody alone (goat antibodies against rat immunoglobulin) did at times result in some staining of reactive fibrillar astro-
Fig. 4. A section of a formalin-fixed paraffin-embedded human spinal cord stained with mAb 182. The figure shows dark staining of ventral horn neurons and some cell processes in the ventral horn. Nomarski optics, ×250.

cytes in the subpial area of human spinal cord but no other staining. Anti-acetylcholine receptor mAb (provided by Dr. David Richman, University of Chicago) of IgG and IgM subclasses produced the same staining of human spinal cord as did the bridging antibody control.

**Molecules Carrying the Determinants with Which the mAb React**

None of the mAb listed in the table could be demonstrated to react with determinants preserved on separated proteins or lipids. However, other mAb derived from immunizations with either fixed or non-fixed tissues were found to recognize such determinants.

Monoclonal antibody 107 (Fig. 3) bound to a protein with molecular weight of approximately 200,000 on immunoblots of human and bovine neural tissues. When applied to nitrocellulose containing electrophoresed neurofilament proteins it bound to the 200,000-dalton protein of neurofilaments.

Monoclonal antibody 554 (Fig. 7A) bound to a very high molecular weight protein (>300,000 daltons) on immunoblots of proteins from isolated bovine AHC (Fig. 7B). The protein was not detected on immunoblots of proteins from isolated myelin, unfractionated spinal cord, or isolated Purkinje cells. This may indicate that this protein is a minor component of myelin but a major component of the spinal cord AHC. Monoclonal antibody 554 also binds to a fast moving polar glycolipid from human spinal cord (Fig. 7C). It is unlikely that the polar glycolipid accounts for any of the immunohistochemical staining of the formalin-fixed paraffin-embedded tissue.
Fig. 5. A) A section of a formalin-fixed paraffin-embedded human spinal cord stained with mAb 395. The figure shows staining mainly of small neurons. Nomarski optics, ×100. The inset shows a higher power view of a small ventral horn neuron. B) A section of formalin-fixed paraffin-embedded mouse cerebrum stained with mAb 395. The figure shows intense staining of neuronal perikarya in the hippocampus.
since the tissue has been repeatedly soaked in 100% ethanol and xylene, a procedure which dissolves away most of the lipids. Since the 554-epitope is present both on a glycolipid and a protein, it very likely consists of a carbohydrate moiety; however, that remains to be proven.

DISCUSSION

Human neurons either in health or in disease have not been subjected to rigorous study because of difficulties in obtaining suitable material. In this paper, we report on our experience with developing mAb recognizing human neurons and describe some of the properties of the mAb, based predominantly on immunohistochemical criteria. Those mAb obtained by immunizing and screening with formalin-fixed immunogens recognize only formalin-fixed tissue; those obtained by immunizing and screening with unfixed tissue do not recognize formalin-fixed tissue. The latter finding is not invariable, however, in either our experience as evidenced by our anti-neurofilament mAb which recognizes both fixed and unfixed tissue and the experience of others (11). Our data do emphasize the need for specifically designing immunization and screening strategies to generate mAb applicable for use on formalin-fixed human tissue. Such reagents may prove useful for study of human pathological material including both neuronal degenerative diseases and neuronal derived neoplasms.

All of the mAb described in this report recognize intracytoplasmic determinants of neurons. To establish immunohistochemically whether concurrent plasma membrane staining is present would require use of intact cells. In this regard Dodd et al (16) demonstrated that mAb recognizing intracellular constituents in subpopulations of dorsal root ganglia neurons on tissue sections can also recognize surface membrane determinants when whole cells are stained.
With regard to species specificity, the mAb described here vary in their recognition of mouse neuronal cells. The mAb staining unfixed tissue and one of the mAb (299) staining formalin-fixed tissue do not stain mouse tissue, whereas the remaining mAb do cross the species barrier. This experience is in keeping with the variability seen in previous studies with mAb. Our data again emphasize the need for initial selection of mAb on human tissue if study of human material is the goal.

The immunohistochemical staining pattern observed with mAb 299 illustrates that our approach can be used to delineate properties distinctive of subgroups of human neurons. Monoclonal antibody 299 recognizes neurons throughout the spinal cord, in dorsal root ganglia, and in dentate nucleus of the cerebellum; mAb 299 does not stain cortical or subcortical neurons. Development of mAb selective for specific neuronal populations would provide reagents valuable for determining what constituents unique to a restricted neuronal group may underlie or contribute to selective vulnerability to inherited or acquired disease.

Monoclonal antibody 554, which immunohistochemically reacts with both neurons and myelin, recognizes both a protein and glycolipid on immunoblots indicating it may be binding to a carbohydrate moiety. Monoclonal antibodies directed against carbohydrate moieties may be of specific interest with regard to motor neuron disease since Dawson and Stefansson (27), in an analysis of spinal cord tissue from patients with amyotrophic lateral sclerosis (ALS), and Kundu et al using ALS skeletal muscle (28), have described differences in ganglioside composition between ALS and control tissues.

Identification of the molecules carrying the antigenic determinants recognized by the mAb, other than 107 and 554, remains a problem. Our denaturing gel system may fail to retain antigenic determinants on proteins for several reasons. In this system, antibodies directed against discontinuous antigenic determinants are not likely to react with anything on the nitrocellulose even though the gel may have contained proteins which in their native states carried the complimentary antigenic determinant. The polyacrylamide gel systems we used excludes proteins (peptides) that are smaller than ca. 10,000 daltons and larger than ca. 350,000. Despite the difficulties with identification of the molecules carrying the antigenic determinant recognized by the mAb, our data do indicate the feasibility of developing reagents suitable for the analysis of neuronal elements in preserved human tissue. This approach has an obvious application to the study of human pathological material.

Fig. 7.  A) A section of a formalin-fixed paraffin-embedded human spinal cord immunohistochemically stained with mAb 554. There is staining both of neurons in the anterior horns and myelin in the white matter. Nomarski optics. ×75. The inset contains a higher power view showing staining of myelin in the anterior commissure and an anterior corticospinal tract. B) The figure shows two nitrocellulose strips containing electrophoresed proteins from isolated bovine AHIC. Strip 1 was stained with a control rat IgM mAb. Strip 2 was stained with mAb 554. The control mAb is negative but mAb 554 binds to a protein doublet with approximate molecular weight of 300,000. C) The figure shows five strips of aluminum-backed TLC plate. Strips 1–3 contain separated polar lipids from human spinal cord and 4 and 5 contain separated neutral lipids from human spinal cord. Strips 1 and 4 were stained with mAb 554; Strips 2 and 5 were stained with a control rat IgM mAb and Strip 3 was incubated directly with peroxidase-labeled rabbit antibodies to rat immunoglobulins; mAb 554 binds to a rapidly moving polar lipid. The controls are negative.

*J Neuropathol Exp Neurol, Vol 44, November, 1985*
REFERENCES

1. Reichardt LF. Immunological approaches to the nervous system. Science 1984;225:1294-9
7. Eisenbarth GS, Walsh FS, Nirenberg M. Monoclonal antibody to a plasma membrane antigen of neurons. Proc Natl Acad Sci USA 1979;76:4913-7
22. Sellinger OZ, Legrand J, Clos J, Obilsson M. Unequal patterns of development of succinate-dehydrogenase and acetylcholinesterase in Purkinje cell bodies and granule cells isolated in bulk from the cerebellar cortex of the immature rat. J Neurochem 1974;23:4137-44


(Received 17 April 1985/Accepted 17 June 1985)

MS85-20