Monoclonal antibodies distinguish antigenically discrete neuronal types in the vertebrate central nervous system

(neuronal diversity/hybridoma/spinal cord/trigeminal nucleus/cerebellum)

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ABSTRACT   Eight hundred hybridoma lines were generated from mice immunized with the fixed gray matter of cat spinal cord. Of these lines, 47 were positive when screened immunohistochemically against sections of the cat spinal cord. Twenty-nine lines secreted antibodies that bound to neuronal antigens. Of these, 16 bound to axons only, 8 bound to axons and cell bodies, and 5 bound to cell bodies only. Eighteen lines secreted antibodies that bound to glial cells. Five lines that secreted antibodies that intensely stained spinal cord sections were cloned and screened against other parts of the central nervous system. Each of these five antibodies bound to specific subsets of neurons. For example, in the spinal cord, one antibody (Cat-301) recognized a surface determinant on the dendrites and cell bodies of neurons that, in morphology and location, resemble long-distance projection neurons. A second antibody (Cat-201) recognized an antigen in axons and in the cytoplasm of neuronal cell bodies that may be a subset of those recognized by Cat-301. A third antibody (Cat-101) recognized only axons. The subcellular localization of the antigen recognized by each antibody is the same in all areas of the central nervous system we have examined. The fact that each of the antibodies described here has a restricted distribution in the central nervous system shows that there is a high degree of molecular diversity among vertebrate neurons and that hybridoma technology can be used to explore this diversity. This class of reagents should be a useful addition to the many established techniques for studying the organization of the vertebrate central nervous system.

Neurons have many features that distinguish them from other cell types. Molecular differences between neurons have been postulated to account for the specificity of neuronal interactions and for the many physiological and anatomical properties that differentiate neurons from one another. Hybridoma technology has been used to show that there are many antigenic differences among neurons in the leech (1). This paper reports that monoclonal antibodies can also be used to show molecular differences among neurons in the vertebrate nervous system.

In this paper we report a group of antibodies that were raised against the gray matter of the cat spinal cord. The antibodies distinguish different neuronal types both in the spinal cord and in other areas of the cat nervous system. These results and other recent reports (2–8) suggest that the number of antigenically distinct subtypes of vertebrate neuron is large. Furthermore, markers that distinguish neurons in the adult nervous system may give us insight into features of the organization of the central nervous system which are not amenable to study by present techniques. One example is the application of these antibodies to studies of neuronal development.

METHODS

Spinal cord from adult cats perfused intravascularly with fixative was used both for the initial antigen and for the immunohistochemical screen. The immunization schedule was chosen because of our previous success using a similar protocol in raising antibodies that bind to specific neurons in the leech (1). It is hard to control the previous immunological experience of any one mouse but in this paper we present results from three mice which differed in the range of hybridomas we obtained. Mice were immunized by intraperitoneal injection on three occasions (days 1, 14, and 44) with the fixed, dissected, gray matter of the cervical spinal cord that had been homogenized in phosphate-buffered saline and suspended in an equal volume of Freund complete adjuvant. Two of the animals were immunized with spinal cord layers I–VI fixed in 4% paraformaldehyde; the third received tissue fixed in 1% paraformaldehyde/1% glutaraldehyde. Each animal received 50 mg (wet weight) of tissue at each immunization. The final injection in all three animals was intravenous and consisted of 20 mg (wet weight) of unfixed gray matter of cervical spinal cord that had been homogenized and boiled in 100 μl of Laemmli loading buffer (9) and diluted 1:5 in phosphate-buffered saline before injection.

Three days after the last injection, spleen cells from each mouse were dissociated, fused with cells of the NS-1 myeloma line, and plated at low dilution according to the method of Galfre et al. (10). The supernatant from hybrid cell colonies was screened immunohistochemically as described below. Positive lines were cloned twice in soft agar and stored frozen at −70°C and in liquid nitrogen. Ascites fluids from all five of these lines have a very high titer by immunohistochemical measurement and the distribution of antigen in the central nervous system does not vary over a 1:1,000 dilution. The immunoglobulin class secreted by these hybridoma lines was determined by the standard Ouchterlony double-diffusion assay using class-specific anti-mouse immunoglobulins purchased from Litton Bionetics (Bethesda, MD).

For immunohistochemical studies, 50-μm-thick Vibratome sections of 4% paraformaldehyde-fixed spinal cord (or other areas of the central nervous system) were dehydrated into xylene (to extract lipids and increase permeability), rehydrated, and incubated with the supernatant (full strength) from hybrid cell lines with 2% (vol/vol) Triton X-100 overnight. Sections were washed and incubated with horseradish peroxidase-conjugated antibody [anti-IgG heavy and light chains, sheep, rabbit, or goat anti-mouse (Cappel)] for 2 hr, washed, processed for visualization of the enzyme with 3, 3′-diaminobenzidine, and mounted on slides. Each series of sections processed included three controls: (i) buffer control; (ii) negative control (a mouse monoclonal IgG1 antibody that binds to a neuronal antigen in the leech); (iii) positive control [a mouse monoclonal IgG1 antibody that binds to α-tubulin (a gift of S. Blose)].

RESULTS

Of 800 hybridoma cell lines, 47 were positive when screened immunohistochemically against the cat spinal cord. The positive antibodies fell into four classes on the basis of the neural elements they recognized: axons only (Fig. 1B; 16 hybridoma lines); axons and cell bodies (Fig. 1C; 8 lines); neuron cell bodies and dendrites only (Fig. 1D; 5 lines); and glial cells (18 lines). Many of the antibodies in the first three classes appeared to recognize subsets of neurons in the spinal cord. We cloned five

hybridoma lines which secreted antibodies that intensely stained spinal cord sections and studied the cellular distribution of the antigen recognized by these antibodies in other areas of the central nervous system. Although the antibodies were raised against the cervical spinal cord, they recognized subsets of neural elements in other areas of the central nervous system as well (Figs. 2 and 3). The majority of antibodies recognized either axons (24 lines) or glia (18 lines), suggesting that antigens in these components of the nervous system are immunodomi-
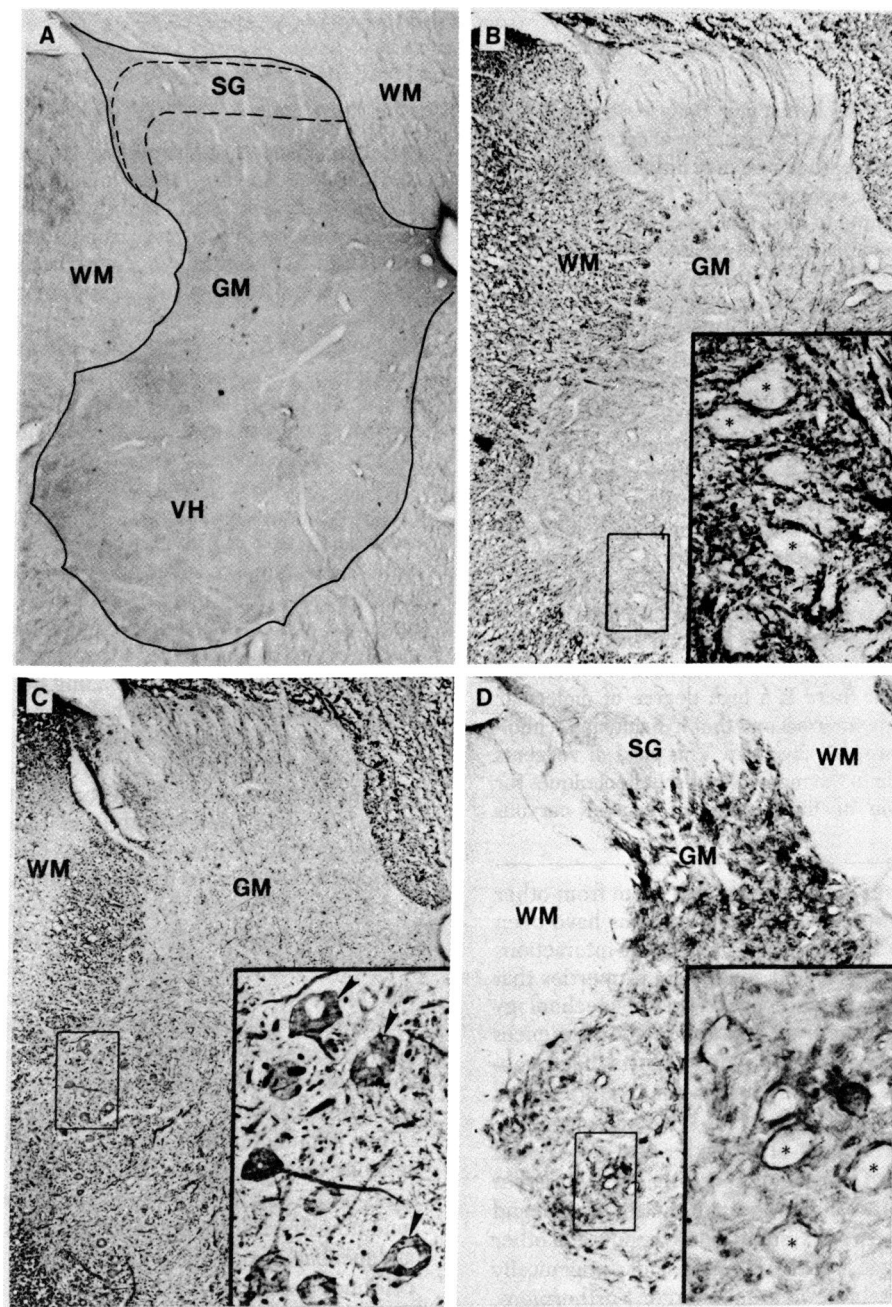

FIG. 1.   Transverse sections (50 μm thick) of the cervical spinal cord illustrate the staining patterns achieved with four different monoclonal antibodies (A). An antibody that did not bind to spinal cord sections left both the gray (GM) and white matter (WM) unstained. SG, substantia gelatinosa; VH, ventral horn. (B) Cat-101 bound to axons in the white matter (WM) and gray matter (GM). In the ventral horn, antibody-labeled axons outline the unstained, negative images of cell bodies. The small rectangular area is shown at higher magnification (Inset) with unstained cell bodies (asterisks) outlined by stained processes. (C) Cat-201 bound to axons in the white (WM) and gray (GM) matter and to cell bodies. Prominently stained cell bodies are found in the ventral horn motoneuron pools. The Inset shows antibody-staining localized to the cytoplasm of labeled cell bodies (arrowheads). (D) Cat-301 bound to neuron cell bodies and proximal dendrites in many areas of the spinal cord gray matter (GM) but not to axons. The substantia gelatinosa (SG) contains no antibody-labeled cell bodies. The large ventral horn neurons (asterisks) shown enlarged in the Inset demonstrated that Cat-301 binds to the surface of neurons. (×7.5; Insets, ×32).
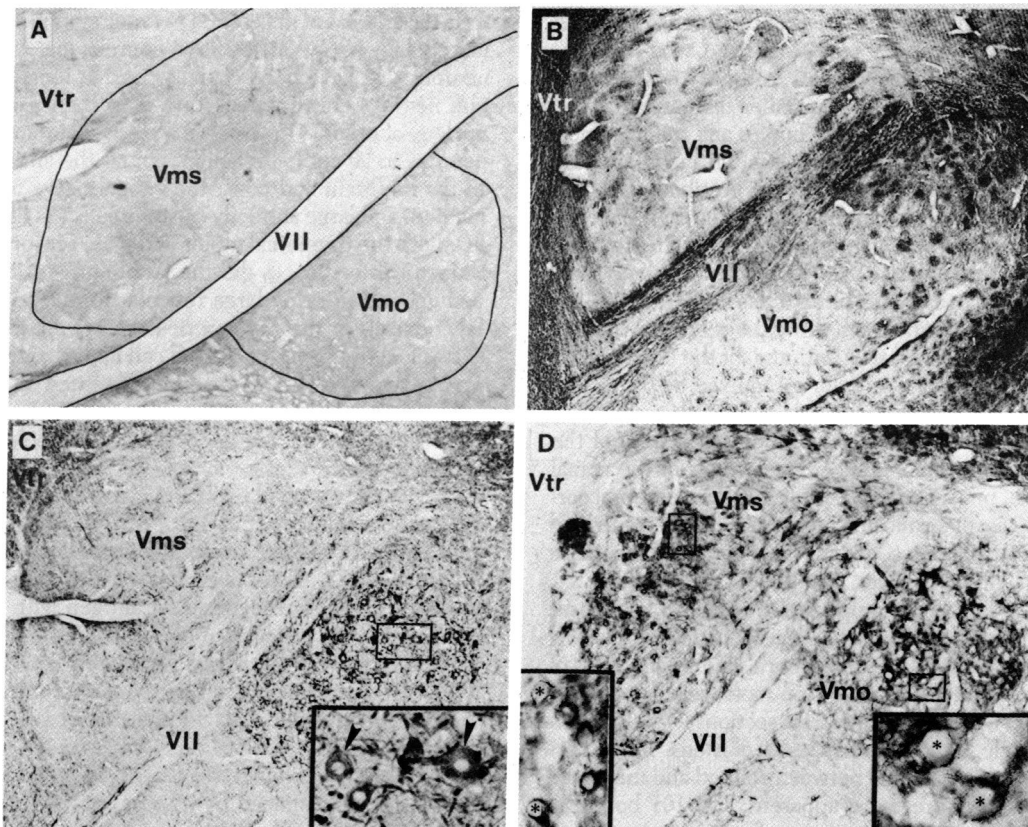
FIG. 2. The pons at the level of the trigeminal main sensory (Vms) and motor (Vmo) nuclei. (A) An antibody that did not bind to sections of the pons leaves axons in the spinal V tract (Vtr) and the facial nerve (VII) and cell bodies in the trigeminal motor (Vmo) and main sensory (Vms) nuclei unstained. (B) Cat-101 bound to axons in the facial nerve (VII) and the tract of the trigeminal nerve (Vtr) but not to cell bodies. (C) Cat-201 bound to cell bodies in Vmo but not in Vms. The rectangular area is enlarged (*Inset*) to show Vmo cell bodies (arrowheads) with cytoplasmic antibody staining. (D) In contrast, Cat-301 bound to cell bodies in both Vmo and Vms. *Left Inset* shows the rectangle in Vms enlarged; and *Right Inset* shows the rectangle in Vmo enlarged. In both cases, antibody bound to the surface of labeled cell bodies (asterisks). (×6; *Insets*, ×22.)

nant. The distribution of different antibodies also varied from one mouse to another. For example, 13 of the 18 anti-glial antibodies but only 2 of the 24 anti-axon antibodies were generated from the mouse immunized with spinal cord fixed in 1% paraformaldehyde/1% glutaraldehyde.

The differences in staining patterns among three of these

antibodies were apparent in sections of the cervical enlargement of the cat spinal cord. Antibody Cat-101 bound to axons in both the gray and white matter of the spinal cord (Fig. 1B). In the ventral horn of a spinal cord section stained with Cat-101, stained axons outlined the negative images of unstained motoneurons (Fig. 1B Inset). The determination that an antibody
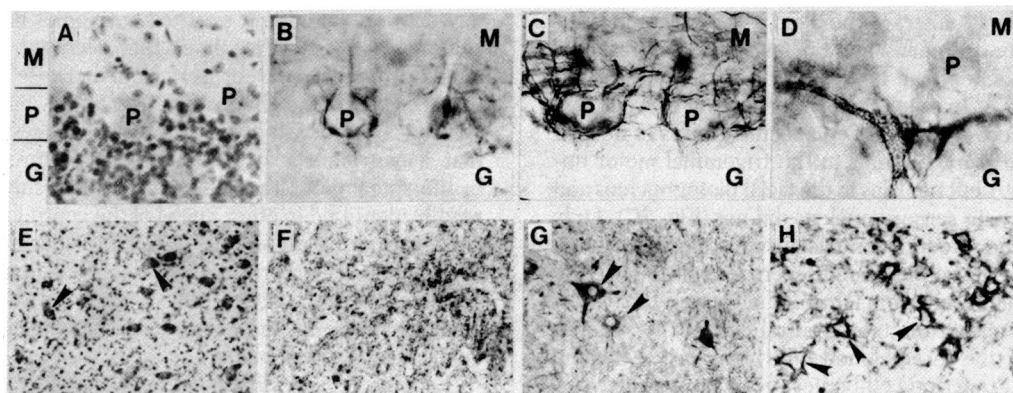

FIG. 3. Cerebellum labeled with monoclonal antibodies. (*A–D*) Cerebellar folia. (*E–H*) The deep cerebellar nuclei. (A) Nissl-stained section demonstrating cell bodies in the molecular layer (M), Purkinje cell layer (P), and granule cell layer (G). (B) Cat-101 bound to axons in the Purkinje cell layer and outlined the unstained Purkinje cell bodies (P). (C) Cat-201 also bound to axons surrounding Purkinje cell bodies (P) and to a number of axons in the deep part of the molecular layer (M). No cell bodies were labeled by Cat-201 in the cerebellar cortex. (D) Cat-301 bound to only a few cell bodies in the cerebellar cortex. The relative number of these cells and their location and morphology conform to features described for Lugaro cells. The two shown here illustrate the characteristic surface binding sites of Cat-301. (E) Nissl-stained section illustrates the density and sizes of neuron cell bodies (arrowheads). (F) Cat-101 bound to axons but not to cell bodies. (G) Cat-201 bound to axons and to a small number of cell bodies (arrowheads). (H) Cat-301 bound to the surface of a large number of cell bodies (arrowheads) but not to axons. (×15.)

recognizes axons was made on the basis of the intense staining of white matter relative to gray matter and the axon-like trajectories of antibody-identified elements. Although it is possible that such antibodies recognize myelin rather than axons themselves, the lack of staining of glial cell bodies and the presence of staining on some unmyelinated axons (Fig. 3 *B* and *C*) suggest that this class of antibody recognizes axons. Antibody Cat-201 bound to both axons and neuronal cell bodies (Fig. 1*C*). Labeled cell bodies were found in the ventral horn (Fig. 1*C Inset*) and occasionally in the deep layers of the dorsal horn. Antibody Cat-301 bound to neuron cell bodies but not axons (Fig. 1*D*). Cat-301-positive cell bodies were found throughout the gray matter except in layer II (the substantia gelatinosa) of the dorsal horn. Fig. 1*D Inset* shows that the antigen recognized by Cat-301 is distributed on the surface of cell bodies and dendrites of large ventral horn neurons. Antibody staining of other areas of the central nervous system (including several levels of the spinal cord, the medulla, pons, midbrain, diencephalon, cerebral cortex, cerebellum, corpus callosum, and optic tract) confirmed that the neuronal antibodies (Cat-101, -201, and -301) recognize subsets of neural elements (Figs. 2 and 3).

Cat-101. Cat-101, an IgG2a, appeared to bind only to a subset of axons in the spinal cord. The white matter, fascicles of axons coursing vertically through the dorsal horn, and the fascicles of motoneuron axons that form the ventral roots were not stained homogeneously (Fig. 1*B*). In spinal cord sections it was difficult to distinguish single homogeneous classes of axons. In the cerebellum, however, distinctions between stained and unstained populations of axons were more apparent. Cat-101 bound to axons around the Purkinje cell bodies, conforming to the pattern described for basket cell axons, and to only a few axons in the deep part of the molecular layer (Fig. 3*B*). The white matter of the cerebellar cortex also bound the antibody, as did a large number of axons in the pons (Fig. 2*B*) including the facial nerve and the spinal tract of the trigeminal nerve. Many areas that are rich in axons did not react uniformly with this antibody, as seen in the molecular layer of the cerebellar cortex. Consistent with the spinal cord observations, no stained cell bodies have been found in any area of the central nervous system we have examined with this antibody.

Cat-201. This IgA antibody stained cell bodies and axons in the spinal cord (Fig. 1*C*). In the cerebellar cortex, it bound to axons both in the Purkinje cell layer and in the molecular layer (Fig. 3*C*). In the molecular layer, the antigen recognized by Cat-201 was associated with fibers in the deep part of the molecular layer. Cat-201 bound to many cell bodies in the ventral horn and to small numbers of cell bodies in the dorsal horn of the spinal cord (Fig. 1*C*). In other areas of the central nervous system, Cat-201 selectively recognized specific groups of neuron cell bodies. In the pons, neurons in the trigeminal motor nucleus bound Cat-201 but neurons in the facial motor nucleus and in the trigeminal main sensory nucleus did not (Fig. 2*C*). Only a few cells in the deep cerebellar nuclei were recognized by Cat-201 (Fig. 3). Large neuron cell bodies were recognized by Cat-201 in the red nucleus, in layers III and V of the cerebral cortex (pyramidal cells), and in the most ventral part of the superior colliculus. Two of the three cloned lines secreting antibodies that stained both axons and cell bodies recognized similar specific groups of neuron cell bodies. Both of these lines secreted IgA antibodies and were derived from the same mouse, suggesting that they may be the same idiotype.

Cat-301. Antibody Cat-301, an IgG1, stained the cell bodies and proximal dendrites of many medium and large neurons in the spinal cord (Fig. 1*D*). Nissl-stained sections demonstrated the presence of small and medium-sized antibody-negative neurons in laminae that also contained antibody-positive neurons.

As in other areas of the central nervous system, the location and morphology of Cat-301-positive neurons matched that of some neurons that have been characterized as long-distance projection neurons (12) (neurons that send their axons beyond the nucleus of origin). In the spinal cord, many motoneurons and neurons in layers I, IV, and V bound the antibody (Fig. 1*D*). In the midbrain and pons, the cranial nerve nuclei contained medium and large antibody-positive cells. As illustrated in Fig. 2*D*, both the trigeminal motor and main sensory nuclei contain stained cell bodies, as did the facial motor nucleus. This antibody bound to many large reticular formation neurons but to few neurons in the periventricular gray matter. The cerebral cortex had two bands of labeled cell bodies, one in layer III and one in layer V. The cerebellum presented an interesting distribution of antibody-positive cells. Cat-301-positive neurons were numerous in the deep cerebellar nuclei (Fig. 3*H*) but sparse in the cerebellar cortex (Fig. 3*D*). Purkinje cells were negative. Only a small population of cells in the cerebellar cortex was recognized by Cat-301 (Fig. 3*D*). The location and morphology of these large, rare cells match features described for Lugaro cells (11). Light microscopy of the spinal cord shows that antibody Cat-301 recognized an antigen with a discontinuous distribution on the surface of cell bodies and dendrites. This same distribution was found for all cell bodies recognized by Cat-301 throughout the nervous system. Similarly, at the light microscope level the cytological localization of the other monoclonal antibodies, Cat-101 and -201, was the same in all areas of the central nervous system. This suggests that in each case the antigen recognized by a given antibody is similar (or the same) throughout the brain, but confirmation will require ultrastructural and biochemical data.

DISCUSSION

In this paper we report the distribution of antigens recognized by monoclonal antibodies raised against the gray matter of cat spinal cord. The assay for antibodies of interest was their ability to bind to neuronal antigens found on fixed Vibratome sections of the cat thoracic and cervical spinal cord. We used two regions of the cord in this initial screening to control for molecular diversity within the cord itself. The central feature of this assay was use of the precise anatomical organization of neurons in the central nervous system to detect molecules present in subsets of neurons. In screening 800 primary hybridoma lines in this way, 29 were found that secreted antibodies that bound to cat neuronal antigens. Five lines that secreted antibodies that bound strongly to neurons were cloned. These antibodies were then used to probe the distribution of the antigens they recognized in other parts of the central nervous system. We argued that, if many molecularly distinct types of neurons were present in the spinal cord, then related neuronal subtypes might be readily identified in other nuclei of the central nervous system. In this paper we show that these antibodies define subclasses of neurons. Whether antibody-identified neurons also share additional characteristics that distinguish them from other groups of neurons (for example, a common neurotransmitter) remains to be determined.

Each of the five cloned cell lines secretes an antibody that binds to subsets of neuronal elements in the central nervous system of the cat. The specificity of antibody binding is particularly apparent when we compare the binding of different antibodies in any one area of the cat brain. In this paper we show the binding patterns of three monoclonal antibodies in the spinal cord (Fig. 1), the trigeminal nuclei of the pons (Fig. 2), and the cerebellum (Fig. 3). In each case, different subsets of neurons are recognized by different monoclonal antibodies. For

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example, antibody Cat-201 binds to neuronal cell bodies only in the trigeminal motor nucleus (Fig. 2C). In contrast antibody Cat-301 binds to the surface of neuronal cell bodies in both the trigeminal motor and main sensory nuclei. These two antibodies also bind to distinct sets of neurons in the cerebellum (Fig. 3). The discussion below focuses on one of the questions posed by these results: How many antigenic classes of neurons exist in the mammalian brain?

Our results on the vertebrate central nervous system show two features which clearly distinguish them from our results with antibodies raised against the leech nerve cord (1). (i) The staining patterns on spinal cord sections of the 29 neuronal antibodies indicate that they all bind to subsets of neuronal elements. This has been confirmed in other areas of the central nervous system with five cloned hybridoma lines. (ii) Only a few of the hybridoma lines tested secreted antibodies against cat neuronal antigens. In contrast, of 500 hybridoma lines raised and screened against the leech nerve cord, 300 secreted antibodies that bound to neuronal antigens; about 80% of these bound uniformly to the leech central nervous system; 67 of these antibodies recognized subsets of neurons (1). In this study we screened 800 cell lines against the cat spinal cord; only 29 of these bound to cat neuronal antigens and all 29 appeared to recognize subsets of neurons. The low number of hybridomas secreting antibodies against cat neuronal antigens and the specificity of those antibodies that are generated (in contrast to the results in the leech) may be explained by a nonrandom immune response. The vertebrate immune system may favor antibodies against rare antigens which distinguish subtypes of neurons because mice may be tolerant to more prevalent neuronal antigens.

Many immunohistochemical studies have shown that neurons are biochemically diverse. Antisera raised against purified antigens such as neuropeptides and neuronal enzymes show these antigens to be present in subsets of neurons (for example, see refs. 13 and 14). Even antisera against neuron-specific enolase, often regarded as a general marker for neurons, fails to recognize all neurons in the adult central nervous system (15). Monoclonal antibodies against vertebrate neuronal antigens have been raised by other workers (2–8). Antibody CE-5, generated by immunizing mice with membranes from 7-day postnatal rat dorsal root ganglia (3), selectively recognizes long-distance projection neurons in several areas of the mammalian central nervous system, including the cerebellar Purkinje cells. Hawkes et al. (7) raised monoclonal antibodies against the partially purified synaptosomal proteins of rat cerebellum. They obtained many different distributions of antigen within the cerebellum, reflecting the highly specialized morphology of cerebellar axons and synaptic terminals. They were confident that in one case the antigen was expressed by a single type of neuron. Monoclonal antibodies that distinguish neuronal types in the cerebellum and elsewhere in the rat central nervous system were obtained in another recent study in which unfixed rat hypothalamic homogenate was used as the antigen (8). In this study we chose not to purify a particular class of immunizing antigen (we only excluded some myelinated axons by dissection because myelin is known to be highly immunogenic) and to focus on antigens that distinguish neuronal cell bodies. In this respect and in other respects, the strategy used in this study differs from that chosen by others. However, even though we have chosen different strategies, all of these studies indicate that

many molecular features distinguish vertebrate neurons from one another.

In this paper we describe two antibodies (Cat-201 and -301) that bind to neurons that resemble long-distance projection neurons in location and morphology. Each antibody apparently recognizes a distinct antigen because Cat-201 binds to a cytoplasmic antigen (Figs. 1C and 2C Insets) whereas Cat-301 recognizes a surface antigen (Fig. 1D and 2D Insets). Cat-301 binds to neurons in many nuclei of the central nervous system, but Cat-201 binds to neurons in many fewer locations. For example, in the pons, Cat-301 recognizes neurons in both the trigeminal main sensory and motor nuclei but Cat-201 only recognizes neurons in the trigeminal motor nucleus (Fig. 2). In the pons and spinal cord the laminar locations of Cat-201-positive neurons is a subset of the laminar locations of Cat-301-positive neurons. The morphology of positive cells in a given lamina is the same with both antibodies. It appears therefore that, in some areas of the central nervous system, Cat-201-positive cells may be a nested subset of Cat-301-positive cells. In contrast, antibody CE-5, described by Wood et al. (3), binds to a subset of long-distance projection neurons which overlaps only in part with the sets of Cat-201- and 301-positive neurons. The population of neurons recognized by CE-5 matches that described here for Cat-301 in many areas of the central nervous system. However, in the cerebellar cortex, neither Cat-201 nor Cat-301 recognizes Purkinje cells but antibody CE-5 does. These results indicate that there are at least three different subclasses within the population of long-distance projection neurons. This possibility illustrates the potential value of the association of specific antigens with particular neuronal subclasses. Future studies may indicate how these molecular features correlate with known anatomical, physiological, or developmental features of long-distance projection neurons.

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