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# A surface antigen expressed by a subset of neurons in the vertebrate central nervous system

(monoclonal antibodies/spinal cord/synaptic structure/neuroimmunology)

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**ABSTRACT** **Many hypotheses for the specificity of connections in the nervous system postulate the presence of surface chemical differences between neurons. Hybridoma technology offers a potential route to identify such surface antigenic differences between neurons. Monoclonal antibody Cat-301 was one of a panel of antibodies generated by immunizing mice with homogenized adult cat spinal cord. At the light microscopic level, Cat-301 recognizes a subset of neurons in many areas of the vertebrate central nervous system. This report shows at the ultrastructural level that Cat-301 binds to a surface antigen on neurons in the intact vertebrate central nervous system. Cat-301-positive neurons carry the antigen on cell bodies and proximal dendrites but not on axons. Using secondary antibody labeled with horseradish peroxidase, we show that antibody binding sites are present along the surfaces of neurons and extend around presynaptic profiles but are excluded from the synaptic cleft. The distribution of the Cat-301 antigen at central synapses is similar to that described for some components of the extracellular matrix of the neuromuscular junction. This study demonstrates that a specific surface antigen is found on a subset of neurons and suggests that other surface markers may be present on other subsets of mammalian central nervous system neurons. Antibodies against this antigen and other surface antigens may allow insight into the mechanisms involved in the formation and maintenance of synaptic connections in the central nervous system.**

Monoclonal antibodies have been generated against both simple and complex neural antigens. The application of hybridoma technology to simple identified antigens, such as the sodium channel (1) and the acetylcholine receptor (2), which have been characterized biochemically and physiologically, provides a means of identifying the components of a particular molecule and of obtaining probes that are specific for different antigenic determinants on these molecules. Importantly, hybridoma technology also allows specific antibodies to be generated against previously uncharacterized components of complex antigens such as the neuromuscular junction (3), synaptosomal preparations (4), or large parts of vertebrate (5–7) and invertebrate (8) nervous systems.

In this paper, we report on a monoclonal antibody to an antigenic determinant that is associated with the surface of a subset of central neurons in the intact vertebrate nervous system. The identification of antigens on the surface of subsets of neurons may have implications for the developmental problem of how specific connections are established and maintained in the nervous system.

## METHODS

Monoclonal antibodies were generated by immunizing mice with the fixed grey matter of the cat cervical spinal cord. Resulting hybrid cell lines were screened immunohistochemically on 50-$\mu$m-thick Vibratome sections of cervical and thoracic cord. The details of the immunization and screening procedures have been reported previously (9). The hybridoma line that secreted antibody Cat-301 was stabilized by cloning three times in soft agar. Ascitic tumor fluid containing high-titer antibody has been obtained from pristane (2,6,10,14-tetramethylpentadecane)-primed mice injected with Cat-301 hybridoma cells.

Adult cats were perfused intravascularly with a fixative containing either 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer or 1% paraformaldehyde/1% glutaraldehyde in 0.1 M sodium phosphate buffer. Fixed tissue was stored at 4°C in buffer with sodium azide (to prevent bacterial growth) for periods of 1 day to several months. The storage time did not affect the distribution or intensity of antibody staining. For light microscopic immunohistochemistry, 50-$\mu$m-thick Vibratome sections of the spinal cord (or other areas of the central nervous system) were incubated sequentially in monoclonal antibody Cat-301 for 12–20 hr (full-strength supernatant or ascites fluid at established dilutions), horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 2 hr (at a dilution of 1:100), and 3,3′-diaminobenzidine (0.25%) with $H_2O_2$ (0.002%) for 30 min. For electron microscopy, sections carried through this procedure were postfixed in 2% $OsO_4$ and embedded in Epon/Araldite. Sequential plastic sections were cut at 1 and 0.12 $\mu$m for correlative light and electron microscopy. Thin sections were examined without counterstain in the electron microscope.

## RESULTS

Antibody Cat-301 was one of a panel of hybridoma lines obtained from mice immunized with the fixed grey matter of cat spinal cord. The supernatants from 800 hybridoma lines were screened immunohistochemically on Vibratome sections of cat cervical and thoracic spinal cord. Twenty-nine of these lines secreted antibodies that, at the light microscopic level, bound to neuronal elements. Many of these antigens appeared to be differentially expressed in different subsets of neurons. The technical details of this screening, and some general features of the cellular distribution of these antigens at the light microscope level, have been reported elsewhere (9). Cat-301 is one of the hybrid cell lines that has been stabilized by cloning, and the specific immunohistochemical binding to a subset of neurons has been demonstrated. The specific binding of this antibody does not change over a 1,000-fold dilution of an ascites fluid, suggesting that the antigen is expressed at much greater levels in some neurons than in others. This antibody was chosen for further study because of the specificity of its binding to a subset of neurons and because, at the resolution of the light microscope, it appeared to be associated with the cell surface.

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Abbreviation: rER, rough endoplasmic reticulum.

Antibody Cat-301, an IgG1, binds to neurons in many areas of the cat central nervous system. Fig. 1 shows the distribution and staining characteristics of Cat-301 in a 50-$\mu$m-thick section of the spinal cord. In the spinal cord, the location and morphology of Cat-301-positive neurons are often similar to those of projection neurons (neurons that send axons outside of the nucleus of origin). The cell bodies and proximal dendrites of many medium- and large-diameter neurons in both the dorsal and ventral horns are recognized by Cat-301 (Fig. 1*A*). In the dorsal horn all layers except layer II (the substantia gelatinosa) contain Cat-301-positive neurons. All the neurons in a given layer are not Cat-301-positive; for example, only some of the neurons in layer I are recognized by Cat-301. At the light microscopic level in 50-$\mu$m-thick sections, the antigen recognized by Cat-301 has a granular, patchy distribution over the surface of cell bodies and dendrites (Fig. 1 *B* and *C*). Cat-301-positive neurons have the same granular surface distribution of antigen over cell bodies and proximal dendrites in all areas of the central nervous system we have examined. Axons are not recognized by Cat-301 in the spinal cord or in any other area of the central nervous system.

Electron microscopic immunocytochemistry with Cat-301 demonstrates conclusively that Cat-301 is associated with the surface of a subpopulation of neurons (Figs. 2 and 3). The observations described here on a spinal cord layer IV neuron are representative of those made in several other areas of the central nervous system, including the spinal cord ventral horn, the cortex, the trigeminal motor and main sensory nuclei, and the dorsal column nuclei. Fig. 2 illustrates an antibody-positive neuron in layer IV of the spinal cord. In 1-$\mu$m-thick plastic sections (Fig. 2*A*) and in low-power electron micrographs (Fig. 2*B*) antibody staining has a discontinuous, scalloped appearance along the perimeter of the cell body and dendrites. In electron micrographs at higher magnification (Figs. 2 *C* and *D* and 3), one sees that antibody staining is present over much of the cell surface but is not present in the synaptic cleft. Antibody staining frequently extends a distance of several hundred nanometers from the postsynaptic cell to surround the presynaptic element (Figs. 2 *C* and *D* and 3). This corresponds to the light microscopic observations of scallop-shaped profiles along the cell perimeter (Figs. 1*C* and 2*A*). This pattern of antibody staining is identical on cell bodies and dendrites. All the synapses along a Cat-301-positive neuron's membrane in a thin section are similarly surrounded by antibody stain. As seen in Fig. 2*A*, Cat-301 does not stain more distal dendrites as intensely as it stains proximal dendrites and cell bodies.

At the light microscopic level, the cytoplasm of all neurons (Cat-301-positive and -negative neurons) is devoid of stain (Fig. 1 *B* and *C*). At the electron microscopic level, however, the cytoplasm of Cat-301-positive neurons often contains antibody-positive granules associated with the rER (Fig. 2 *C* and *D*). Short lengths of labeled rER frequently lie near the plasma membrane; all the rER in antibody-positive cells is not labeled. Cat-301 does not recognize any cytoplasmic elements of neurons that do not carry the antigen on their surface, nor does it recognize any axonal structures. Neurons in the spinal cord of the monkey, rat and mouse are also recognized by antibody Cat-301, and their locations and the light microscopic level staining characteristics match those described here for Cat-301 in the cat central nervous system.

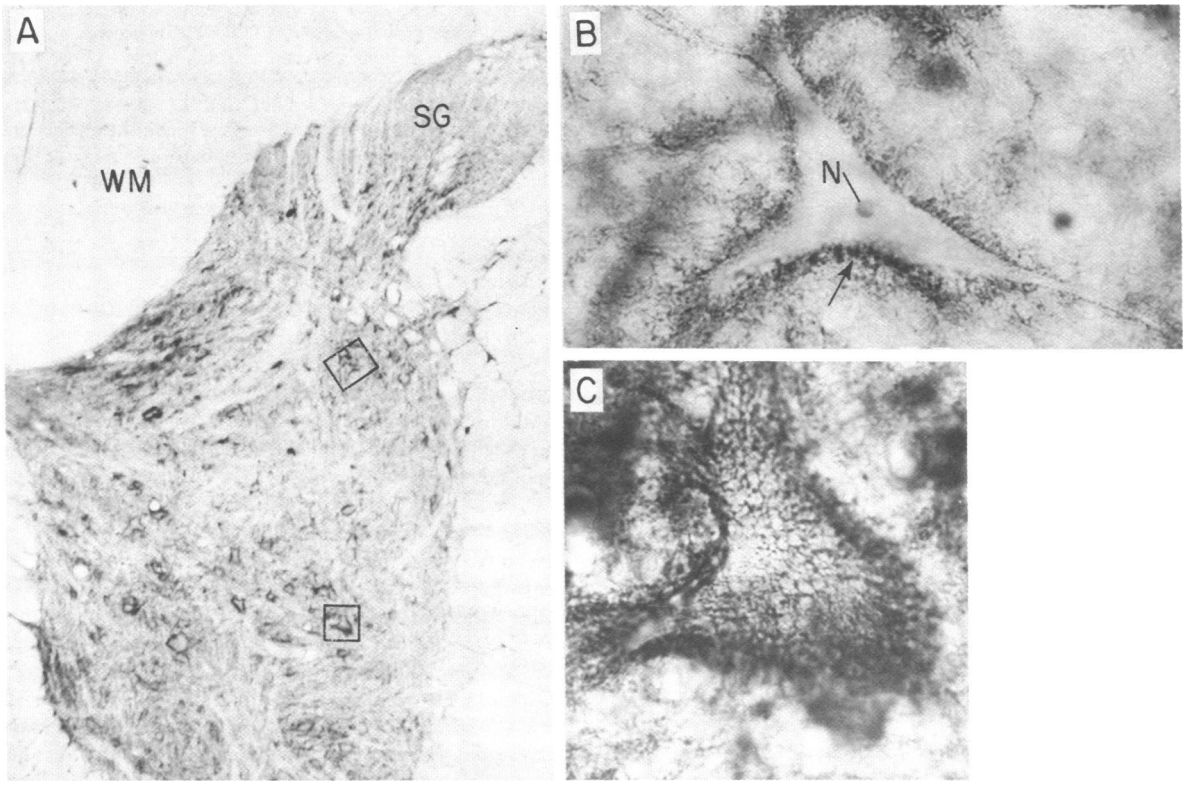


FIG. 1. Light micrograph of a Cat-301 immunohistochemically stained 50-$\mu$m-thick Vibratome section of 4% paraformaldehyde-fixed cat spinal cord. (*A*) Nissl-counterstained section of the spinal cord shows Cat-301 binding sites in the grey matter but not the white matter (WM). Labeled neurons are found in all layers of the grey matter except in the substantia gelatinosa (SG). Neurons in boxes are shown enlarged in *B* and *C*. (×37.) (*B*) Neuron in upper box in *A* has been sectioned through its cell body. Cat-301 staining is clearly seen along its surface (arrow). The staining is irregular and extends along the proximal dendrites. The cytoplasm is free of antibody-positive elements but a Nissl-stained nucleolus (N) is visible. (×525.) (*C*) The cell body of the neuron in lower box in *A* is completely contained within the section. Cat-301 staining is seen over the surface of its cell body and proximal dendrites. The staining can often be seen as an irregular lattice on the cell surface. (×525.)

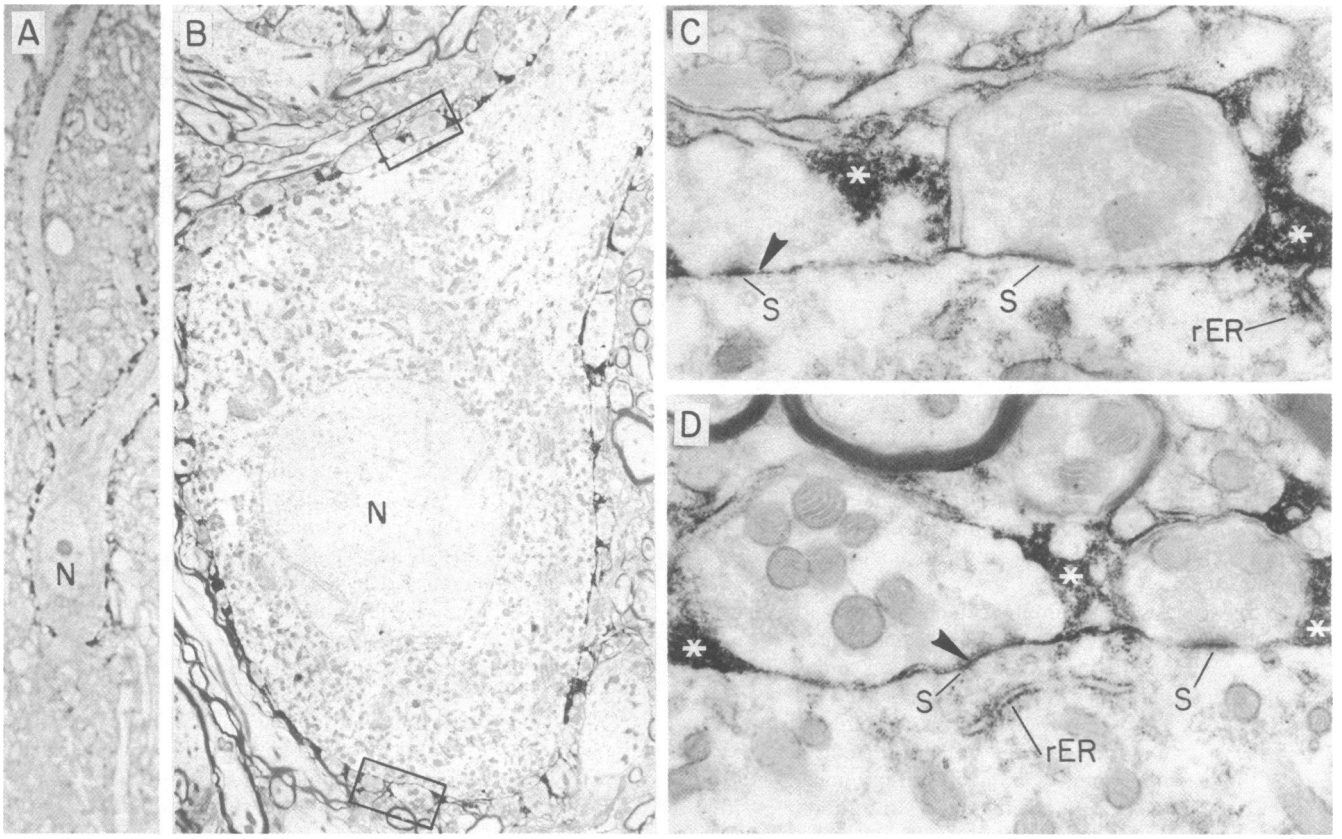


FIG. 2. Light (*A*) and electron (*B–D*) micrographs of a spinal cord layer IV neuron from a cat fixed with 1% glutaraldehyde/1% paraformaldehyde. (*A*) Cat-301 staining is visible along the cell body and proximal dendrites of a layer IV neuron whose nucleus (N) contains the nucleolus in this section. More distal areas of the dendrites are less intensely stained than are proximal dendrites. (×520.) (*B*) In a low-power electron micrograph of another section of the cell shown in *A*, Cat-301 staining along the surface of the cell body and a proximal dendrite appears interrupted by other profiles. The parts of the membrane devoid of stain are areas of synaptic contact. When a bouton's synapse occurs in another plane of section, Cat-301 staining is seen between the bouton and the postsynaptic cell. Areas in boxes are shown enlarged in *C* and *D*. (×2,800.) (*C*) Area in upper box in *B* shows two axosomatic synapses. Antibody staining (white asterisks) is present in the extracellular space along the surfaces of both the pre- and postsynaptic profiles but is excluded from the region of the synapse (S) and is absent in the synaptic cleft (arrowhead). (×23,000.) (*D*) Area in lower box in *B* also shows antibody staining (white asterisks) around pre- and postsynaptic elements but excluded from the synaptic cleft (arrowhead). A short stretch of rough endoplasmic reticulum (rER) in the postsynaptic cell stains with Cat-301. (×23,000.)

## DISCUSSION

Hybridoma technology potentially provides a new and general method for describing the distribution of different molecules in the nervous system. The ability to map the distribution of antigens could provide many opportunities for understanding the architecture of the nervous system. For example, monoclonal antibodies have been generated that distinguish different types of Müller cell in the retina (5) and that distinguish many different and complex categories of neuron in the leech nervous system (8). We and others have used hybridoma technology to detect antigenic differences between neurons in the vertebrate central nervous system (4, 9, 10). All of these studies reported light microscopic location of the antigens. In this paper, we report on the ultrastructural location of an antigen that is present on a subset of neurons. The antigen defined by the monoclonal antibody Cat-301 is present on the surface of cells and has an unusual distribution in relation to synapses. On the cell bodies and proximal dendrites of Cat-301-positive neurons, the antigen appears to be excluded from the synaptic cleft and extends from the surface of the postsynaptic cell around the presynaptic profiles. The reticulated distribution of the antigen at the light microscopic level is explained because, as the antigen outlines synapses, it forms a lattice over the cell surface. The fact that we can show that the antigen recognized by Cat-301 is on the surface and is associated with synapses is of some significance because many features of the connectivity and function of the nervous system may be explained by the selective expression of particular surface markers by subsets of neurons. This successful use of monoclonal antibodies to detect surface antigens in tissue sections, rather than in cell culture, raises the possibility that studying the ultrastructural distribution of many physiologically important surface macromolecules such as ion channels, transmitter receptors, and enzymes may now be feasible.

The ultrastructural distribution of the antigen bound by Cat-301 is reminiscent of components of the extracellular matrix at the neuromuscular junction: the antigen is apparently found outside of the synaptic cleft; it surrounds the presynaptic element; and the antigen is apparently in the extracellular space. This last point can be extended by two observations: (*i*) the antigen is found in the same locations in animals fixed by perfusion with 4% paraformaldehyde or with 1% glutaraldehyde/1% paraformaldehyde. This suggests that the extracellular location is not due to mobility of the antigen during fixation, and (*ii*) the antigen is likely to be synthesized by the postsynaptic cell as peroxidase-labeled rER is often seen close to the membrane of these labeled cells but not in other unlabeled cells, nor are labeled organelles found inside presynaptic profiles. Extracellular diaminobenzidine reaction product can be seen several hundred nanometers from the postsynaptic cell. Because of the distances involved, if the location of the reaction product

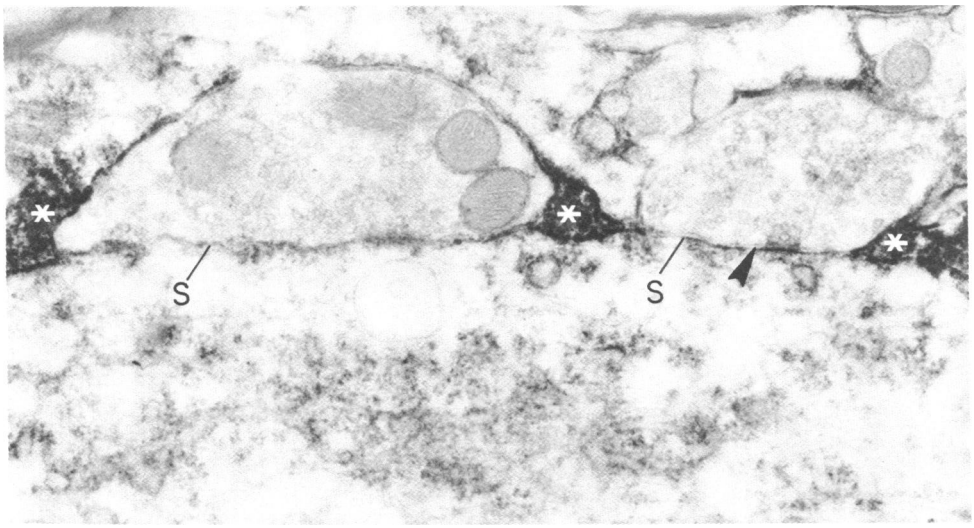


FIG. 3. Electron micrograph of two axosomatic synapses on a Cat-301-positive dorsal horn neuron from another preparation. Antibody staining (white asterisks) is seen along the external surface of the postsynaptic cell except at areas of synaptic contact(s). It extends a distance away from the postsynaptic cell around the presynaptic elements. The synaptic cleft of the bouton on the right is shown here to be free of antibody stain (arrowhead). (×29,500.)

is even a rough guide to the location of the antigen, the antigen cannot be an integral part of the membrane of the postsynaptic cell. At the neuromuscular junction, the extracellular matrix extends over the surface of the postsynaptic cell and around the presynaptic element (11, 12). Recently, monoclonal antibodies have been generated that show an unexpected complexity in the number and distribution of the molecular components of this extracellular matrix (3). Some of these components are associated with extrasynaptic extracellular matrix but excluded from the synaptic cleft (13), similar to the distribution of Cat-301 described here. The components of the extracellular matrix have been shown to play an important role in maintaining the organization of receptors in the postsynaptic membrane (14). Even though morphological similarity to the neuromuscular junction is striking here, it is clear that we need more data to definitively establish whether or not the Cat-301 antigen is an extracellular molecule synthesized by the postsynaptic cell. The main point we wish to stress here is that the antibody Cat-301 apparently recognizes a hitherto uncharacterized ultrastructural feature of a class of central synapses.

These morphological data suggest that hybridoma technology, in addition to defining neuronal cell types, will also lead us to the identification and understanding of the molecular basis of previously uncharacterized features of neuronal ultrastructure. While the presynaptic elements of the synapses described here have a common target, it is unlikely that they use a common transmitter because all the synapses along a Cat-301-positive membrane are similarly surrounded by antigen and it is likely that many different kinds of synapses are made at these locations. It is also unlikely that the postsynaptic Cat-301-positive neurons share transmitter type because of the heterogeneity of neuron classes recognized by Cat-301 and because in the cortex the morphology of Cat-301-positive neurons matches that of neurons immunologically demonstrated to be heterogenous with respect to neurotransmitter. These results will be reported in greater detail in a subsequent publication.

We have also found surface antigens that distinguish the cell bodies and processes of specific subsets of invertebrate neurons. Ultrastructural analysis shows that, in several cases, axons carrying specific surface markers travel together in fascicles in the connective, the major axon pathway in the leech. These antibody-identified groups of axons occupy stereotyped and symmetrical positions in the connective (15). We have now shown in both vertebrate and invertebrate nervous systems that surface antigens present on subsets of neurons can be identified by generating monoclonal antibodies against complex neural antigens.

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