However the δ chain that we can visualize on surface-iodinated IDP2 is clearly distinct from the TCR γ peptide on these cells. It is unreactive with antisera to TCR γ–specific peptides (*13*). Further, tryptic peptide comparisons by reversed-phase high-performance liquid chromatography (HPLC) demonstrate the patterns of iodinated TCR γ and δ peptides to be quite different (Fig. 4). Only one or two peptides at or near the flowthrough appear to comigrate. Since the gel-purified TCR δ is known to be contaminated at low levels with underglycosylated TCR γ, the TCR δ peptide at fraction 8 is probably a TCR γ contaminant that is visualized because of its heavy labeling. The overall dissimilarity between these profiles suggests that IDP2 δ could not be related to IDP2 γ simply by proteolysis or other post-translational modifications. Thus the δ peptide appears to be a distinct component of the nondisulfide-linked TCR γδ-T3 complex. Whether this component is related to the partner chain in the disulfide-linked form and whether either of these molecules displays structural properties common to TCR α, β, and γ subunits will be subjects of intensive investigation.

Note added in proof: A structure similar to that described for IDP2 TCR γ has recently been reported for TCR γ from the PEER cell line by Littman *et al.* (*21*).

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A Mab to a Unique Cerebellar Neuron Generated by Immunosuppression and Rapid Immunization

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The cerebellar cortex is perhaps the best characterized structure in the mammalian central nervous system. Although the major cerebellar cell classes are well known, a new class of cerebellar cortical neuron has now been identified with a monoclonal antibody (Mab) generated by a procedure for rapid immunization and selective immunosuppression of antibody responses. This procedure generates a high frequency of immunoglobulin G–class antibodies of desired specificity, and has allowed the generation of two antibodies that recognize subsets of cerebellar cortical neurons. One of these antibodies defines a previously unrecognized class of cerebellar neuron. The distribution and antigenic characteristics of this neuron suggest that it has a distinct role in cerebellar circuitry.

CURRENT VIEWS OF THE DERIVATION and maintenance of cell structure are that molecular differences must underlie the anatomical and physiological differences observed among neuronal classes in the mammalian central nervous system. Several recent studies suggest that the number of molecularly distinct cell classes in the central nervous system may be quite large (*1, 2*). To determine how molecular differences might give rise to or reflect neuronal heterogeneity, we and others have used hybridoma technology to immunologically dissect the biochemical constituents of the vertebrate (*3–8*) and invertebrate (*9–11*) central nervous system.

Although initially promising, the hybridoma approach has proven to have both biological and technical limitations so that few monoclonal antibodies to neuronal subsets have been generated. We have encountered three major obstacles. (i) It has been difficult to target the immune response to recognize antigens of interest; the vast majority of antibodies generated recognize a few, immunodominant species. (ii) Animals must be immunized over long periods of time to generate a hyperimmune response that will yield high-affinity, immunoglobulin G (IgG) antibodies. (iii) The large number of hybridoma colonies derived from the spleen in a successful experiment is difficult to screen immunohistochemically. For example, in our earlier experiments to obtain antibodies to molecules expressed by subsets of neurons, we immunized mice with a tissue that contained a high density of neuronal cell bodies. The spinal cord provided a convenient tissue source, because spinal gray matter contains neuronal cell bodies (intermixed with axons and glial cells) and can be dissected cleanly from the peripheral white matter, which is composed almost exclusively of axons and glial cells. After immunizations of mice with spinal cord gray matter, we screened antibodies immunocytochemically on spinal cord sections and found that, even after enrichment by dissection, only a small fraction of the antibodies generated recognized gray matter antigens, and the vast majority of antibodies recognized axons and glial cells (*3, 7*). Biochemical analyses showed that the axonal and glial antibodies recognized intermediate filaments (*5, 7*), immunodominant species in the central nervous system. Clearly then, physical separation of wanted (gray matter) from unwanted (white matter) antigens did not sufficiently attenuate the immunodominant response to axons and glia.

Here I describe techniques obviating these difficulties. These techniques have made it possible to generate antibodies that recognize restricted populations of neurons and, in particular, an antibody that identifies a novel subset of cerebellar cortical neurons.

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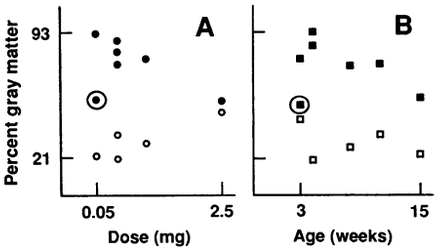

Fig. 1. The efficacy of suppression varies with (A) the dose of immunizing antigen and (B) the age at which it is administered. (**A**) With increasing dose the percentage of desired antibodies (those to gray matter antigens) decreases. Control entries are designated by open circles and tolerized entries by filled circles. A low percentage of antibodies to gray matter were generated from an animal that received a low dose (0.05 mg) of antigen at 15 weeks of age (circled). (**B**) With increasing age the percentage of desired antibodies decreases. Control entries are designated by open squares and tolerized entries by filled squares. A low percentage of antibodies to gray matter were generated from a young (3-week-old) animal that received a high dose (2.5 mg) of antigen (circled).

In order to increase the fraction of antibodies that selectively recognize neuronal subsets, this method has now been used to immunologically suppress the immune response to white matter antigens and to elicit a selective response to gray matter antigens.

Earlier studies have shown that high doses of an identified antigen presented neonatally can suppress the ability of the immune system to recognize that antigen later in life (*12, 13*). The response to a heterogeneous mixture of tissue antigens was suppressed by using a variation of one of these protocols (*13*). Newborn BALB/c mice were immunized every other day from the day of birth through day 10 with rat spinal cord white matter that had been fixed in 4% paraformaldehyde and homogenized in 0.1*M* phosphate buffer [1 mg/50 μl, intraperitoneally (IP)]. Littermate controls did not receive these early, tolerizing immunizations. At 3 to 15 weeks of age both tolerized and control animals were immunized in the hind footpads (FP) and IP with paraformaldehyde-fixed gray matter emulsified 1:1 with Freund's complete adjuvant. A short immunization schedule was followed: four immunizations were given at 4-day intervals (days 1, 5, 9, and 13). The dose of these immunizations was varied from 0.05 to 2.5 mg FP, and from 0.1 to 5.0 mg IP. On day 14 the animals were killed by cervical dislocation, and lymphocytes were harvested from popliteal and inguinal lymph nodes and spleens. The nodal lymphocytes from each animal were pooled, fused with NS-1 myelomas, and plated onto macrophages in two 96-well plates in selection medium. The splenic lymphocytes were fused separately and plated into ten 96-well plates. Both splenic and nodal lymphocytes generated hybridomas in approximately 75% of the wells. Supernatants from hybridomas were screened immunohistochemically on free-floating, 50-μm-thick sections of paraformaldehyde-fixed rat spinal cord. Equivalent percentages of positive colonies were found from splenic and nodal lymphocytes (25 to 40%). Positive supernatants were categorized as staining white matter (axonal or glial elements) or gray matter. Antibodies of interest were stabilized by three rounds of cloning by limiting dilution and then used to identify neuronal subsets in other areas of the central nervous system.

Monoclonal antibodies to both gray and white matter antigens were generated from lymphocytes from control as well as tolerized animals. However, a much greater percentage of antibodies to gray matter antigens was obtained from lymphocytes of tolerized animals. Hybridomas generated from nodal and splenic lymphocytes were scored separately, but, as equivalent results with respect to antibody specificity were obtained within each animal, the results reported here reflect pooled numbers from nodal and splenic-derived hybridomas.

Twenty-one to 47% of antibodies generated from nontolerized animals (littermate controls) recognized gray matter antigens (Table 1 and Fig. 1). In marked contrast, 50 to 93% of antibodies generated from tolerized animals recognized gray matter antigens. In tolerized animals, the percentage of antibodies to gray matter varied with two parameters of immunization—dose and time after tolerization (animal age). The most effective immunizing strategy was low doses of immunizing antigen (the dose of tolerizing antigen was held constant) at short times after tolerization (Table 1 and Fig. 1): 93% of antibodies generated recognized gray matter antigens when the immunizing dose was 0.05 mg FP (0.1 mg IP) at 4 weeks of age. Higher immunizing doses resulted in less effective suppression: only 50% of antibodies generated recognized gray matter when tolerized animals were immunized with 2.5 mg FP (5 mg IP) at 3 weeks of age. Longer periods between tolerization and immunization also reduce suppression (even with very low doses of antigen): 54% of antibodies recognized gray matter after immunization at 15 weeks of age with 0.05 mg FP (0.1 mg IP). Other studies (*13*) that have used neonatal suppression have not reported such dose- or age-dependent responses.

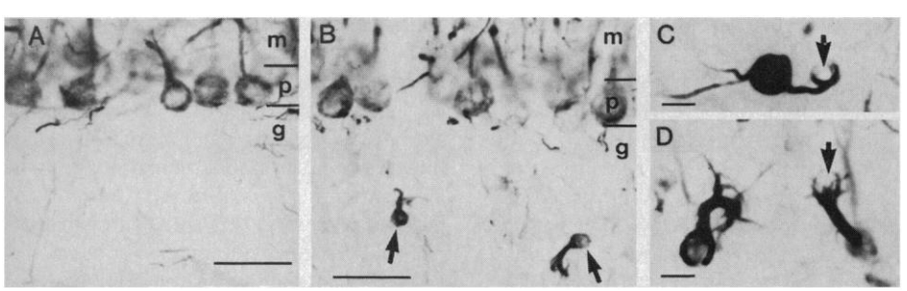

Fig. 2. Monoclonal antibody Rat-302 recognizes a subset of neurons found only in the vermis and flocculus. (**A**) Rat-302 recognizes Purkinje cells throughout the cerebellar cortex. In the granule cell layer (g) most of the cerebellar cortex is devoid of Rat-302 positive cells. Scale bar, 50 μm. Abbreviations: m, molecular layer; p, Purkinje cell layer. (**B**) Rat-302–positive cells are found in the granule cell layer of the vermis (illustrated here) and the flocculus. Scale bar, 50 μm. (**C** and **D**) The Rat-302 cell has a round cell body and short dendrites ending in a spray of appendages (arrows). Scale bar, 10 μm. Vibratome sections (50 μm thick) of 4% paraformaldehyde-fixed adult rat cerebellum were incubated in monoclonal antibody Rat-302 (full-strength supernatant) with 2% Triton X-100 overnight. After two washes with 0.1*M*, *p*H 7.4, sodium phosphate buffer, sections were incubated in horseradish peroxidase (HRP)–conjugated antibody to mouse IgG (Cappel) with 2% Triton X-100 for 2 hours and washed again. HRP was visualized with 3,3′-diaminobenzidine (DAB) (0.03% in phosphate buffer) with 0.003% H_2O_2.

Table 1. Animals (numbers 1 to 12) received tolerizing injections (T) of white matter every other day from day of birth to day 10 or received no initial injections (C). The immunizing antigen (gray matter) was administered at different ages and at doses ranging from 0.05 to 2.5 mg per injection. The resulting antibody specificities, indicated by the percentage of antibodies generated that recognized gray matter, varied with both age and dose. The most effective strategy was a low dose of immunogen at an early age, as illustrated in this table and in Fig. 1.

Animal	T or C	Age (weeks)	Dose (mg)	Percent gray
1	T	3	0.5	78
2	T	3	2.5	50
3	C	3	2.5	47
4	T	4	0.05	93
5	T	4	0.5	84
6	C	4	0.5	21
7	T	8	1.0	74
8	C	8	1.0	28
9	T	11	0.5	74
10	C	11	0.5	34
11	T	15	0.05	54
12	C	15	0.05	23

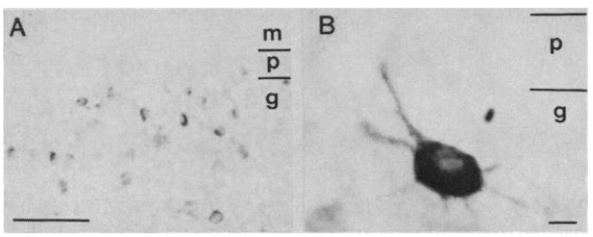

Fig. 3. (**A**) Monoclonal antibody Rat-303 recognizes neurons in the granule cell layer (g), but not in the Purkinje cell (p) or molecular (m) layers of the cerebellar cortex. Scale bar, 100 μm. (**B**) The morphology of Rat-303–positive neurons matches that described for Golgi II cells: a large cell body emitting relatively stout dendrites from many points over the cell circumference (*13*). Scale bar, 10 μm. Methods are the same as in Fig. 1 with the exception of the use of monoclonal antibody Rat-303 in the first antibody incubation.

The immunohistochemical staining patterns of antibodies derived from splenic as compared to nodal lymphocytes were identical. One difference between the two sources is that when the class of antibodies was determined (with class-specific secondary antibodies), the majority (greater than 60%) of hybridomas from nodal lymphocytes secreted antibodies of the IgG class, while the majority (greater than 90%) of splenic-derived hybridomas secreted immunoglobulin M (IgM) antibodies.

In our earlier experiments, we found that all of the antibodies we generated to spinal cord neurons also recognized neurons in other areas of the central nervous system, and in several areas they recognized extremely restricted neuronal subsets (*3, 4*). In an effort to obtain markers for cerebellar cortical neurons, the antibodies generated here were then screened on sections of rat cerebellum. In the cerebellum, two of these new antibodies, Rat-302 and Rat-303, recognized subsets of cerebellar neurons. Although Rat-302 and Rat-303 recognize overlapping sets of cells in the spinal cord, in the cerebellum they recognize completely exclusive sets of neurons.

Rat-302 stains neuronal cell bodies and dendrites and clearly demonstrates cellular morphology. On Western blots (*14*) Rat-302 recognizes a doublet of approximately 160 kD in molecular mass. In the cerebellar cortex, two classes of neurons are stained with Rat-302, Purkinje cells and an unusual class of neuron, which we call the Rat-302 cell (Fig. 2). Although some features of the morphology of the Rat-302 cell might suggest that it is a granule cell, several characteristics distinguish it from the granule cell. The position and shape of the Rat-302 cell match the classical description of granule cells (*15*): antibody-positive cells are located in the granule cell layer (Fig. 2B) and have round cell bodies with dendrites that end in a spray of neurites (Fig. 2, C and D). However, the Rat-302 cell is distinct from the granule cell in three respects: (i) the diameter of the Rat-302 cell (10 μm) is larger than that of a granule cell (5 to 6 μm). (ii) The Rat-302 cell rarely has more than a single dendrite, which can be quite long, whereas typical granule cells have three to five short dendrites. And (iii), and most significantly, the Rat-302 cell has a restricted distribution. Granule cells are found throughout the cerebellum and represent the most numerous cerebellar cell type. The Rat-302 cell is found only in the vermis, paraflocculus, and flocculus (Fig. 2B). In these regions the Rat-302 cell lies among a large number of antibody-negative granule cells of smaller diameter.

These observations suggest that the Rat-302 cell is not a granule cell. Another major cerebellar interneuron, the Golgi II cell, has its cell body in the granule cell layer. To verify that the Rat-302–positive cell is neither a granule nor a Golgi II cell, another monoclonal antibody, Rat-303 (also generated here) was used to identify Golgi II cells.

Rat-303 stains cell bodies and proximal dendrites of a set of cerebellar neurons completely distinct from the set recognized by Rat-302. Rat-303 recognizes neurons in the granule cell layer (Fig. 3A) that have multipolar cell bodies, 15 to 20 μm in diameter (Fig. 3B). The position, shape, and size of the antibody-positive neurons match those described for the Golgi II cells (*15*), which suggests that the Golgi II cells are selectively stained by Rat-303. Unlike the restricted distribution of the Rat-302 cell (Fig. 4A), the Rat-303–positive Golgi II cells are distributed throughout the cerebellar cortex (Fig. 4B). Double-labeling experiments show that these two antibodies recognize mutually exclusive sets of cerebellar cortical neurons (Fig. 5), providing further evidence that the Rat-302 cell is a novel class of cerebellar neuron.

The highly stereotyped organization of the cerebellar cortex into an almost crystalline array has allowed an unparalleled analysis of neuronal structure and connectivity (*15*). Cerebellar cortical neurons have been divided into five major, anatomically distinct cell types, which, in some cases, also have distinct molecular characteristics (*3, 7, 8, 16–18*). Other less numerous neuron classes, the Lugaro cell, for example, have been identified anatomically (*15*) and molecularly (*4*). Although the basic cellular organization of the cerebellum is consistent

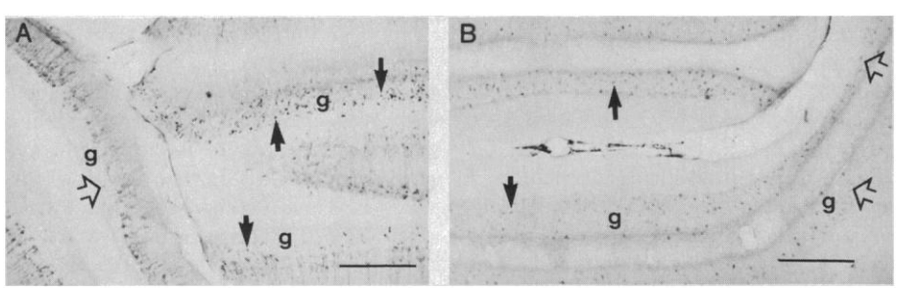

Fig. 4. The Rat-302 cell is restricted to the flocculus and vermis; the Rat-303 Golgi II cell is found throughout the cerebellar cortex. (**A**) In the vermis, the granule cell layer (g) contains many Rat-302 cells (arrows), whereas in other areas of the cerebellum no antibody-positive cells are found in this layer (open arrow). (**B**) In contrast, Rat-303 recognizes Golgi II cells in the granule cell layer (g) in both the vermis (arrows) and the more lateral lobules (open arrows). Scale bar, 500 μm in (A) and (B). Methods are the same as in the previous figures.

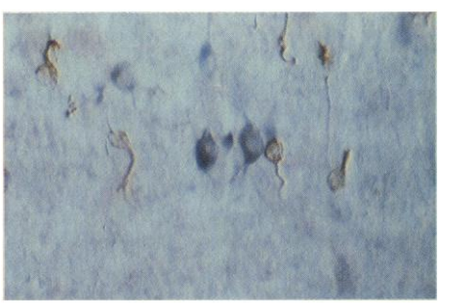

Fig. 5. Double-labeling studies show that Rat-302 (brown cells) and Rat-303 (black cells) recognize mutually exclusive sets of cerebellar cortical neurons. This photomicrograph of the granule cell layer of the vermis shows the distinct morphology of the Rat-302 cell and the Rat-303–identified Golgi II cell. Sections were stained for Rat-303 (an IgG) as described in the previous figures, with HRP-conjugated secondary antibody specific for mouse IgG. HRP was visualized with nickel sulfate and DAB to yield a black reaction product. After extensive washing, sections were stained for Rat-302 (an IgM) with an HRP-conjugated secondary antibody specific for mouse IgM. HRP was visualized with DAB alone to give a brown reaction product. Control sections showed no cross-reactivity between the class-specific secondary antibodies and the inappropriate primary antibody.

throughout the entire cortical mass, physiological and anatomical differences among cortical areas have been documented (*19, 20*). Here I have identified a molecularly distinct class of neuron with a restricted distribution in the cerebellar cortex. The areas that contain the Rat-302 cell, the flocculus, paraflocculus, and vermis, have been considered identical to the rest of the cerebellum in cytoarchitecture but distinct from other areas in connectivity. In most areas of the cerebellum, Purkinje cells project to neurons in the deep cerebellar nuclei, which, in turn, project out of the cerebellum. In the flocculus and vermis, Purkinje cells project directly out of the cerebellum (without a relay in the deep cerebellar nuclei) to the vestibular nuclei (*21, 22*). By cytoarchitectonic criteria the neuron in the granule cell layer identified by Rat-302 represents a novel cell class in the cerebellar cortex. As Rat-302 also recognizes Purkinje cells, it is possible that the Purkinje cell and the Rat-302 cell are functionally related. In other parts of the cerebellar cortex the Purkinje cell projects to the deep cerebellar nuclei, and is interposed between cortical processing and cerebellar efferent projections. In the flocculus and vermis, the Purkinje cell projects directly out of the cerebellum. The Rat-302 cell in these areas might function like the Purkinje cell, as a cell interposed between cortical processing and the cerebellar efferent projections of the floccular and vermal Purkinje cells. Indeed, some Rat-302 cell axons appear to be locally confined.

Here I have generated monoclonal antibodies to cerebellar neurons using an effective, general method to target the immune response toward antigens of interest. Neonatal tolerization reduces the immune response to the tolerizing antigens later in life. In contrast to other suppression strategies with immunosuppressive drugs in adult animals (*23*), the suppression reported here is achieved without inducing a general immunocompromised state. When combined with footpad immunization, this method provides a strategy to rapidly generate high-affinity monoclonal antibodies of desired specificity, which will permit the identification of a previously unrecognized class of cerebellar neuron.

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Early Archean (3.3-Billion to 3.5-Billion-Year-Old) Microfossils from Warrawoona Group, Australia

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Cellularly preserved filamentous and colonial fossil microorganisms have been discovered in bedded carbonaceous cherts from the Early Archean Apex Basalt and Towers Formation of northwestern Western Australia. The cell types detected suggest that cyanobacteria, and therefore oxygen-producing photosynthesis, may have been extant as early as 3.3 billion to 3.5 billion years ago. These fossils are among the oldest now known from the geologic record; their discovery substantiates previous reports of Early Archean microfossils in Warrawoona Group strata.

PUTATIVE CELLULARLY PRESERVED microfossils have been reported from at least 28 Archean (>2.5-billion-year-old) geologic units (*1–4*). However, virtually all have recently been reinterpreted (*1*) as dubiofossils or as nonfossils: pseudofossils, artifacts, or contaminants. Thus, in contrast with the relatively well-known microbial (*5, 6*) and stromatolitic (*7*) fossil records of the younger (Proterozoic) Precambrian, the Archean record remains poorly documented. The composition of the Archean biosphere and time of origin of such evolutionary innovations as oxygen-producing photosynthesis have yet to be determined.

The oldest apparently authentic (*1*) cellularly preserved microbiota known is that reported (*1, 2*) from cherts of the 3.3-billion to 3.5-billion-year-old Towers Formation of the Warrawoona Group of Western Australia. However, the precise collecting site of these microfossiliferous cherts is unknown (*1*, p. 234; *2*). For this and related reasons, the significance of this microbiota has been open to question (*8*). We now describe additional fossils recently discovered (*4*) in two formations of the Warrawoona Group. This discovery confirms the occurrence of microfossils in this sequence; the cell types detected suggest that oxygen-producing photoautotrophic cyanobacteria may have been extant as early as 3.3-billion to 3.5-billion years ago.

The Warrawoona Group, stratigraphically the lowest group of the Pilbara Supergroup, is a 14-km-thick sequence of volcanics containing extensive cherty sedimentary units less than 50 m thick (*9*). The stratigraphy (*9*) and geochronology (*10*) of the sequence are summarized in Table 1.

The microfossils here reported occur in petrographic thin sections of carbonaceous cherts from the Towers Formation and the Apex Basalt (Table 1). The former is a stratigraphic marker unit, generally about 0.5 km thick (*9*), in part deposited in a shallow subaqueous to intermittently subaerial environment (*11*). The directly overlying Apex Basalt is composed of minor chert units interbedded with pillow lavas (*9*). In metamorphic grade, the Warrawoona Group ranges from prehnite-pumpellyite to green schist facies (*12*).

The age of the microfossiliferous forma-

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