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Biochemically Differentiated Neoplastic Clone of Schwann Cells

(S-100 protein/2′,3′-cyclic nucleotide-3′-phosphohydrolase/myelin basic protein/peripheral nervous system)

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ABSTRACT **Four clonal lines have been established in tissue culture from a transplantable tumor of cervical nerve-root induced transplacentally with ethylnitrosourea in an inbred BD-IX rat strain. One clone (RN-2) has several biochemical properties of the nervous system that led to its classification as a Schwann cell line, namely (*a*) the synthesis of the nervous system specific protein S-100, (*b*) a high specific activity of 2′,3′-cyclic nucleotide-3′-phosphohydrolase, an enzyme present in high specific activity only in glial cells, and (*c*) the presence of a basic protein related by immunological crossreaction, molecular size, and amino-acid composition to the encephalitogenic protein from beef brain myelin. Clone RN-2 has a high plating efficiency, a doubling time of about 20 hr, and a mean of 43 chromosomes of normal morphology. Clone RN-2 has also been inoculated subcutaneously into syngeneic rats and grown as a clonal tumor with good growth properties.**

Established clonal cell lines that retain differentiated biochemical functions have provided material for many cell-biology studies. With such lines, one can work with functionally and genetically homogeneous, viable cell populations in quantities sufficient for most biochemical work. For example, clonal cell lines have proved invaluable for studies of such diverse organs as adrenal (1) or pituitary glands (2), and more recently cell lines with biochemical characteristics of differentiated glia (3, 4) and neuronal cells (5) have been developed. Since similar cell lines of peripheral nervous system were not available, we concentrated on the development of clonal lines of this type. Here we report the isolation of a cell line from a chemically-induced tumor of spinal-nerve root. Evidence is presented that this cell line is capable of performing functions unique to the nervous system and its myelin production, leading to its classification as a clonal line of Schwann cells.

METHODS

Tumor Induction. Tumors were induced transplacentally (6). After a single intravenous injection of 80 mg of ethylnitrosourea per kg of body weight into a pregnant inbred BD-IX rat (7) at the 15th day of gestation, all offspring developed neurogenic tumors. The tumor used in this study was a second-passage transplant of a primary tumor of cervical spinal-nerve root, characterized by light and electron microscopy as a malignant neurinoma with some anaplastic features (8). Electron-microscope specimens were fixed in buffered 2% OsO_4, embedded in Araldit, and examined with a Zeiss electron microscope 9A.

Tissue Culture. Tumor material was partially dispersed with a 0.25% solution of Viokase (Viobin Corp., Monticello, Ill.) in physiological saline and plated into plastic tissue-culture dishes (Falcon) containing Ham's Medium F12 (9) supplemented with 2.5% fetal-calf serum (Reheis Chemical Co.) and 10% horse serum (Grand Island Biological Co.) without antibiotics. Cultures were incubated in a humidified atmosphere of 5% CO_2–95% air. Morphologically distinct cell strains were separated by making use of their varying rates of detachment in the presence of Viokase, and by the use of stainless steel cylinders. Clonal cell lines were isolated by repeated serial single-cell platings and selection of colonies with stainless steel cylinders. Cells for photographic study were grown on glass cover slips, fixed with absolute methanol, and stained with 0.5% toluidine blue or hematoxylin–eosin.

Chromosome Analysis. Chromosome spreads were prepared from logarithmic phase cultures without drug treatment. Washed cells were swollen for 5 min at 37° in phosphate-buffered physiological saline (pH 7.2) diluted 1:1 with distilled water. Swollen cells were fixed in a freshly-prepared mixture of acetic acid and ethanol (1:3), dried onto microscope slides, stained with Giemsa stain, and photographed.

Protein Determination. The protein concentrations of the soluble extracts were determined by the method of Lowry *et al.* (10) with bovine-serum albumin as the standard.

S-100 Protein. Tumor samples or cell cultures were washed by centrifugation in phosphate-buffered physiological saline and suspended in Tris-buffered saline (pH 7.4) used for complement fixation (see below). Cells were disrupted by Dounce homogenization and centrifuged at 35,000 × *g* for 20 min. Supernatant fractions were analyzed for S-100 protein by microcomplement fixation (11). Antiserum No. 332-C7 prepared against bovine S-100 protein, purified by Dr. Harvey Herschman, was kindly provided by Dr. Lawrence Levine (Brandeis University) and used at a dilution of 1/1200. The sensitivity of the assay is 5 ng/ml S-100 protein.

2′,3′-Cyclic Nucleotide-3′-phosphohydrolase (CNPase) Assay. This enzyme was assayed essentially by the method of Drummond and coworkers (12) as described by Glastris and Pfeiffer (13), with adenosine 2′,3′-cyclic monophosphate as substrate. Cells or tissues were prepared for assay by Dounce homogenization in the presence of 0.4% w/v sodium deoxy-

Abbreviations: SDS, sodium dodecyl sulfate; CNPase, 2′,3′-cyclic nucleotide-3′-phosphohydrolase.

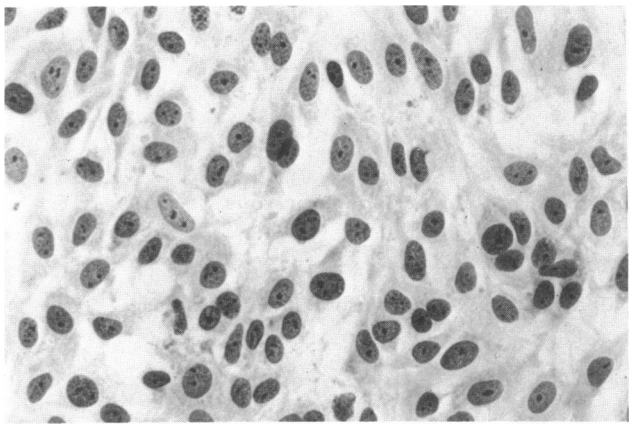

FIG. 1. Light photomicrograph of cell line RN-2 in tissue culture (toluidine blue, X250).

cholate and used without centrifugation. The reaction was run at pH 6.2 and 37° for 20 min. Specific activities are given in units of μmol of substrate converted per min per mg of protein. The sensitivity of the assay is 7.5 nmol/min.

Purification of Basic Proteins. Myelin basic proteins were purified essentially by the method of Oshiro and Eylar (14). Briefly, the method involves lipid extraction at −20°, acid extraction of the basic protein at pH 1.6, precipitation of some contaminating proteins by titration to pH 7, lyophilization, batch-wise treatment with DEAE-cellulose, and finally precipitation of the basic protein by adjustment to 45% saturation with ammonium sulfate.

Sodium Dodecyl Sulfate (SDS)–Acrylamide Gel Electrophoresis. Samples of basic protein were denatured with 1% SDS, reduced with 0.01 M dithiothreitol, heated at 100° for 3 min, electrophoresed in 5.7% polyacrylamide and 0.2% bis-acrylamide containing 1% SDS and 0.01 M 2-mercaptoethanol, and stained according to the method of Fairbanks *et al.* (15).

Amino-Acid Analysis. Amino-acid compositions were determined on samples acid-hydrolyzed in the presence of 0.05% 2-mercaptoethanol, by a Spinco model 120C amino-acid analyzer (16). Values represent the results of single hydrolysates. No corrections were made for destruction of serine, threonine, or tyrosine.

RESULTS

Rat nervous system tumors and S-100 protein

S-100 protein is a soluble, acidic protein that is restricted to the nervous system (17, 18); its presence in either normal or tumor tissue of the central or peripheral nervous system indicates the presence of neuroectodermal cells (19, 20). Previous studies have identified S-100 protein in benign human acoustic neurinomas (21, 22) and in chemically-induced malignant rat tumors of the peripheral nervous system (19, 20). Its value as a marker protein for Schwann cells is therefore highly suggestive.

A transplantable nerve-root tumor induced by ethylnitrosourea in a BD-IX rat was chosen for the present cell-culture study on the basis of its classification as a malignant neurinoma (8), and the presence of S-100 protein. The primary tumor, the first and second passages of the transplantable tumor derived from this primary tumor, and the uncloned strain established from the second transplantation tumor, all syn-

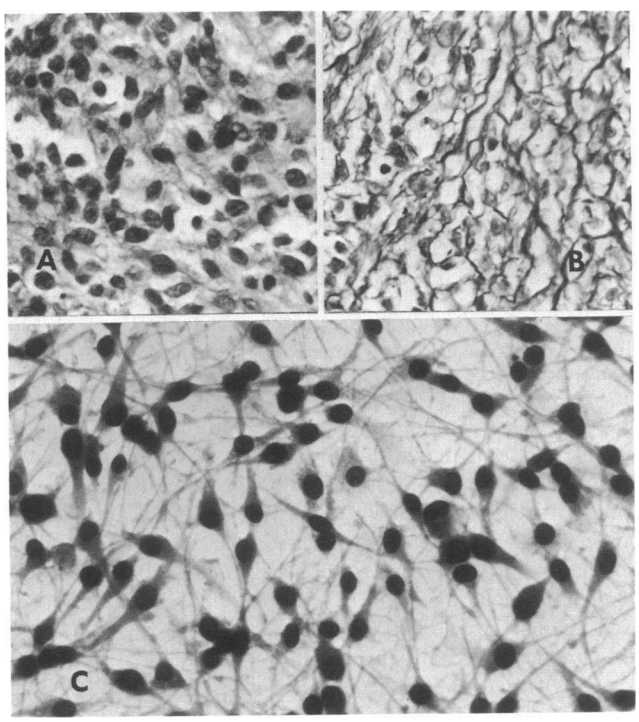

FIG. 2. Isotransplantation experiments with clone RN-2 in syngeneic BD-IX rats. (*A*,*B*) Morphology of a solid subcutaneous tumor producing reticulin fibers after (*A*) hematoxylin and eosin, X200, or (*B*), Tibor Pap reticulin fiber impregnation, ×200. (*C*) Homogeneous cell morphology of RN-2 tumor cells grown as monolayers in tissue culture after repetitive subcutaneous *in vivo* growth (hematoxylin and eosin, ×200).

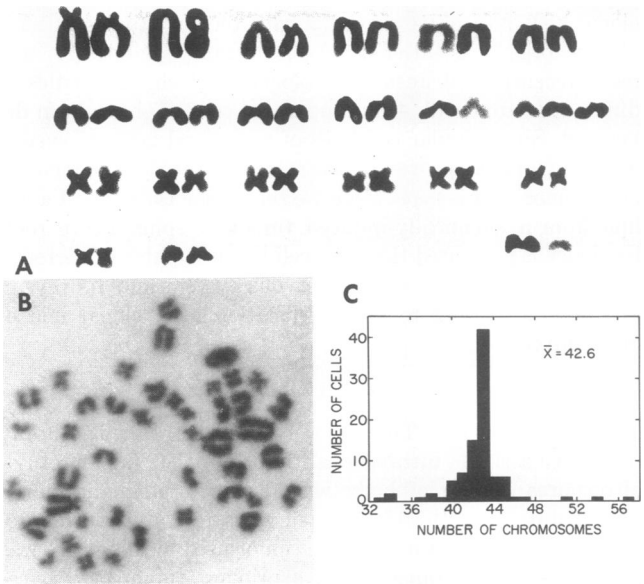

FIG. 3. (*A*) Chromosome analysis of an RN-2 cell with 43 chromosomes. The folded condition of the one chromosome in pair 2 (*top left*) is a preparation artifact; it is normal in other chromosome spreads. (*B*) Chromosome spread from which karyotype in (*A*) was made. (*C*) Histogram of chromosome number in RN-2 cells.

thesize S-100 protein to the extent of 0.02–0.3% of the total water soluble protein. Normal control values for the sciatic nerve and spinal nerve roots ranged from 0.5 to 0.8% respectively (Table 1).

Clonal lines of peripheral nervous system tumors

Four morphologically different clones were isolated from the cell strain, derived from the rat nerve-root tumor A204 (RN-A204-Cl,2,3,4), and named RN-1, RN-2, RN-3, and RN-4. Since only clone RN-2 synthesizes S-100 protein (Table 1), the majority of our subsequent studies have concentrated on this line.

General characteristics of clone RN-2

RN-2 grows in monolayer with a doubling time of about 20 hr, and a plating efficiency at low density of about 50%. The cell population is rather homogeneous, and the cell shape is bipolar or stellate with long unbranching processes (Figs. 1, 2*C*). The mean chromosome number in RN-2 is 43; cells with the mean number have an additional small telocentric chromosome (Fig. 3). The chromosome morphologies appear normal for rat.

When tumor-cell suspensions of RN-2, ranging from 10^3 to 10^6 cells per inoculum, have been injected again subcutaneously into syngeneic BD-IX rats of both sexes and various ages, tumors developed consistently after a period of about 1 week to 2 months. No changes in morphology of cells have been observed after 3 successive passages. The solid tumors are composed of spindle cells, which produce delicate reticulin fibers (Fig. 2*a* and *b*). Ultrastructurally, external basement membrane is absent; collagen fibrils are irregularly distributed in the extracellular spaces.

S-100 protein crossreaction

A comparison of the maximum percent complement fixation (vertical shift) attained at a fixed antibody concentration by a group of antigens is a measure of the degree of immunological similarity (crossreaction) among these antigens (11). Fig. 4 shows such a comparison for S-100 protein purified from beef brain, and in crude homogenates of rat brain and cell-line RN-2, with antiserum prepared against S-100 protein from beef brain. S-100 proteins from rat brain and RN-2 cells display nearly equal maxima in their complement-fixation curves, indicating a high degree of serological homology. The amount of S-100 protein in these preparations of rat brain and RN-2 cells calculated from Fig. 4 is 0.4 and 0.1% of the total soluble protein, respectively.

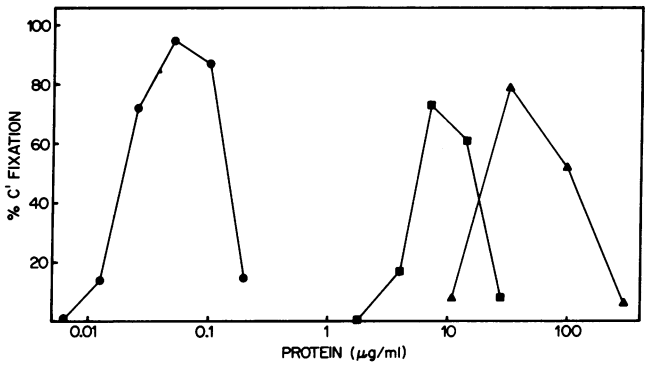

FIG. 4. Complement (C') fixation assay for S-100 protein. Symbols: purified bovine S-100 protein (●); crude rat-brain homogenate (■); crude RN-2 cell homogenate (▲).

TABLE 1. *S-100 protein in primary and secondary nerve-root tumors, in tumor-cell cultures, and in normal control nerve of adult BD-IX rats*

Material	S-100 Protein*
Primary tumor (T1637)	0.3
First transplant (A12)	0.07–0.1
Second transplant (A204)	0.02–0.06
RN-cell strain, uncloned	0.2
Clone RN-1	n.d.†
Clone RN-2	0.1
Clone RN-3	n.d.
Clone RN-4	n.d.
Spinal-nerve roots	0.8
Sciatic nerve	0.5

* Expressed as the percent of total water-soluble protein in 700,000 g-min supernatant fractions.

† n.d., not detectable.

2',3'-Cyclic nucleotide-3'-phosphohydrolase

Several lines of evidence have suggested that CNPase is confined largely to myelin. It is present in much higher activity in white than gray matter, fractionates with myelin, appears during development in parallel with myelin, and is deficient in mutant mice exhibiting subnormal myelin formation (12, 23). In contrast, other studies have identified this enzyme activity in apparently myelin-free neuronal preparations, astrocytoma cells in culture, and human erythrocyte membranes (24, 25). Table 2 shows that, in fact, CNPase activity is present in cells derived from a wide variety of tissues, but that non-neurogenic tissues have a significantly lower CNPase activity than normal nervous tissue or clonal lines of glial-tumor cells. Thus, it appears that CNPase is a general glial-cell marker, and opens the way for speculation as to a possible role of glia other than oligodendroglia in myelin formation. Of the four nerve-root clones, only RN-2 exhibits the high CNPase activity. It may be significant that there is some correlation between the capacity of a cell to make S-100 protein and the high CNPase activity.

Myelin basic protein

Of the three major classes of protein components of nervous-system myelin (28), the so-called "basic proteins" are of particular current interest because of their roles as causative agents of experimental allergic encephalomyelitis and experimental allergic neuritis, demyelinating diseases of the central and peripheral nervous systems, respectively (29). Generally, one basic protein has been isolated from myelin of the central nervous system (30). In contrast, two basic proteins have generally been purified from myelin of the peripheral nervous system, of which one may be similar to the basic protein from the central nervous system, while the other is smaller and has a different amino-acid composition (31). The specificity of these basic proteins for myelin makes their presence in cells excellent biochemical indicators of myelin synthesis.

A basic protein was purified from RN-2 cells that appears

TABLE 2. *2',3'-Cyclic nucleotide-3'-phosphohydrolase in various tumor cell lines and normal tissues*

Tissue	Cell line*	Species	CNPase specific activity†	S-100 protein synthesis
Peripheral nervous system:				
Spinal roots	—	Rat	1.7	+
Sciatic nerve	—	Rat	0.5	+
PNS tumor	RN-1	Rat	0.04	−
PNS tumor	RN-2	Rat	1.2	+
PNS tumor	RN-3	Rat	0.06	−
PNS tumor	RN-4	Rat	0.05	−
Central nervous system:				
Cerebrum	—	Rat	3.4	+
Cerebellum	—	Rat	1.2	+
Brain stem	—	Rat	9.0	+
Spinal cord	—	Rat	8.3	+
Astrocytoma	C6 (3)	Rat	0.9	+
Astrocytoma	CHB (4)	Rat	0.1	+
Glioma	C21 (3)	Rat	1.4	+
Glioma	C3 (3)	Rat	0.07	−
Neuroblastoma	NB41A (5)	Mouse	0.03	−
Other organs:				
Liver	—	Rat	0.03	−
Spleen	—	Rat	0.1	−
Testis	—	Rat	0.03	−
Leydig tumor cell	I10A (2)	Rat	0.06	−
Pituitary tumor	AT-20 (2)	Mouse	0.05	−
Adrenal tumor	Y1 (1)	Mouse	0.02	−
Melanoma	Mel (2)	Mouse	0.05	−
Fibroblast	L (26)	Mouse	0.03	−
Fibroblast	CHO (27)	Hamster	n.d.	−

* References for cell lines in parentheses.

† Specific activity in μmol of substrate converted per min per mg of protein. The sensitivity of the assay as used here is 7.5 nmol per min. n.d., not detectable.

by biochemical criteria to be closely related to the basic protein(s) of central nervous system purified from bovine and rat brains, and by inference, to one of the basic proteins from myelin of peripheral nervous system. Fig. 5 shows that purified RN-2 basic protein co-electrophoreses in SDS–acrylamide gels with both the basic protein from bovine brain and the slower of the two basic proteins found in rat-brain myelin (ref. 28, p. 457). Attempts to purify a similar basic protein from 3T6 fibroblast cultures (by procedures identical to those used for bovine and rat brain, and RN-2 cells) were unsuccessful (Fig. 5). The molecular weight of RN-2 basic protein is estimated to be about 18,000 by both SDS–acrylamide gel electrophoresis and Sephadex G-100 chromatography, a value close to the theoretical 18,400 for basic protein from bovine brain (32). The amino-acid composition of RN-2 basic protein has also been compared with that of bovine-brain basic protein and found to be similar (Table 3).

Preliminary immunological studies by passive cutaneous anaphalaxis were performed with antisera prepared against

TABLE 3. *Amino-acid compositions* of the encephalitogenic proteins from bovine brain and of basic protein from RN-2 cells*

Amino acid	Bovine basic protein		RN-2 basic protein
	Theoretical†	Observed	
Lys	7.6	7.6	7.7
His	5.9	5.5	3.9
Arg	10.6	10.1	6.7
Asp	6.5	7.0	7.1
Thr	4.1	4.3	4.6
Ser	11.2	9.8	10.4
Glu	5.9	7.1	10.4
Pro	7.1	8.0	9.6
Gly	14.7	15.2	15.8
Ala	8.2	9.2	8.3
Val	1.8	1.0	1.5
Met	1.2	1.5	2.2
Isoleu	1.8	1.4	1.8
Leu	5.9	6.0	4.9
Tyr	2.4	2.4	1.9
Phe	4.7	4.7	3.2

* Data in mole %.

† From Brostoff *et al.* (31).

bovine basic protein (kindly provided by Dr. Helene Rausch, Stanford University). Acid extracts of RN-2 cells gave rather weak but positive skin reactions, indicating the presence of a protein immunologically similar to the myelin encephalitogenic protein from bovine brain (Pfeiffer, unpublished results).

DISCUSSION

The tumors of the peripheral nervous system induced transplacentally with ethylnitrosourea consist ultrastructurally of at least 2 cell types (19, 33). It is, however, not possible by light- or electron microscopy to decide with accuracy whether Schwann cells are involved in this neoplastic process. Thus, measurements of biochemical functions characteristic for such cell types are superior to purely morphological criteria. The tissue culture and biochemical data presented above demonstrate that at least one cell type in the tumor exhibits properties characteristic of the nervous system, namely the synthesis of S-100 protein, a high specific activity of CNPase, and the production of a basic protein. On the basis of these parameters RN-2 may be classified as a Schwann-cell clone. In addition, ultrastructural and biochemical studies have demonstrated that RN-2 produces collagen (Church *et al.*, manuscript in preparation), suggesting that the collagen fibrils found in many human (22) and rat (19) peripheral neurinomas may be formed by the Schwann cells themselves.

Schwann cells are generally considered to be of neuroectodermal origin. Of particular interest is their role in peripheral myelin formation, which involves an elaboration of the Schwann-cell plasma membrane about an axon (ref. 28, pp. 520–522). Thus, the availability of a clonal line of Schwann cells is expected to provide a useful *in vitro* system for the study of many aspects of myelin formation and demyelinating disease.

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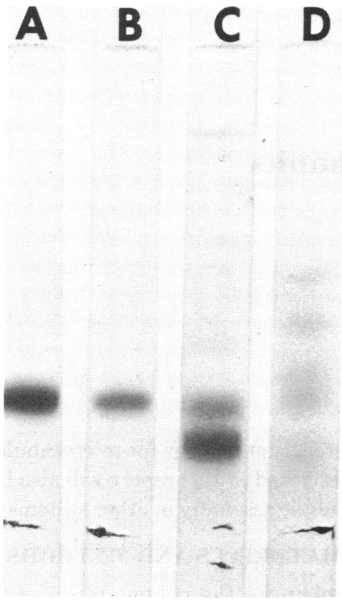

FIG. 5. SDS–acrylamide gel electrophoresis of myelin basic protein(s) from (*A*) beef brain, (*B*) RN-2 cells, and (*C*) rat brain, or (*D*) the products isolated from an attempt to purify a similar protein from 3T6 fibroblasts by the same method.

protein, and Dr. J. Ozols for amino-acid analysis. The authors are also grateful for stimulating influences offered by the activities of the Neurosciences Research Program under the guidance of Prof. F. O. Schmitt. This work was supported by grants from the National Science Foundation, No. GB-23744; the American Cancer Society, No. E-652; and the Deutsche Forschungsgemeinschaft.

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