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In vitro segregation of different cell lines with neuronal and glial properties from a stem cell line of rat neurotumor RT4

(cell type conversion/neural stem cell line/multipotential)

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ABSTRACT **A clonal stem cell line, RT4-AC, of the rat peripheral neurotumor RT4 differentiates in culture into morphologically distinct cell types RT4-B, RT4-D, and RT4-E (cell type conversion). The multipotential stem cell type RT4-AC and cell type RT4-D produce a glial marker, S100 protein, but RT4-B and RT4-E do not. The stem cells also show a small but significant response to veratridine on voltage-dependent Na^+ influx. Cell types RT4-B and RT4-E show a clear response of voltage-dependent Na^+ influx to veratridine, typical of neuronal cells, whereas cell type RT4-D is completely negative. These results indicate that (*i*) the stem cell type RT4-AC shows both neuronal and glial properties, (*ii*) cell types RT4-B and RT4-E have a neuronal property, and (*iii*) cell type RT4-D has a glial property. Therefore, cell type conversion of stem cell RT4-AC to RT4-B and RT4-E cells seems to result in differentiation towards neuronal cell types, and cell type conversion of RT4-AC to RT4-D results in differentiation towards a glial type in culture.**

As reported by Imada and Sueoka (1), a clonal cell line designated RT4-AC was derived from a rat peripheral neurotumor, RT4. RT4-AC possesses properties of a stem cell by generating morphologically distinct cell types RT4-B, RT4-D, and RT4-E in culture (cell type conversion). The stem cell type (RT4-AC) and one of the morphologically distinct cell types (RT4-D) produce the nervous system-specific protein S100, which is a characteristic glial protein (2, 3). Its production, however, is completely arrested when the stem cell differentiates into the other two types, RT4-B and RT4-E. Tumorigenicity is also correlated with cell types: cell types RT4-AC and RT4-D are tumorigenic, whereas RT4-B and RT4-E are not (4). Thus, each cell type has a unique set of characteristics, which are consistently found among different isolates of the same cell type.

In this study, in addition to further analysis of S100 protein, we have examined a neuronal property (veratridine-stimulated Na^+ influx) of (*i*) several clonal cell lines of the stem cell type (RT4-AC) and (*ii*) independently isolated clonal cell lines of each derivative cell type, RT4-B, RT4-D, and RT4-E. Veratridine-stimulated Na^+ influx is responsible for the initial depolarization phase of the membrane action potential and therefore is a measure of membrane excitability. Excitable membranes are observed in neurons, muscle, and some secretory cells, but not in glial cells. Therefore, among neurocells, membrane excitability may be the most critical difference between neuronal and glial cells.

Addition of veratridine strongly stimulates Na^+ influx in cell types RT4-B and RT4-E but not in cell type RT4-D, whereas the stem cell type, RT4-AC, has intermediate levels of response without exception. Moreover, in confirmation of our previous results (1), RT4-AC and RT4-D cell lines have S100 protein and RT4-B and RT4-E cell lines do not. We thus conclude that stem cells, RT4-AC, which have both a glial and a neuronal property, generate in culture two cell types, RT4-B and RT4-E, with the neuronal property and one cell type, RT4-D, with the glial property.

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MATERIALS AND METHODS

Cell Lines. Rat neurotumor RT4 was induced by subcutaneous injection of a newborn BDIX strain rat with the neurospecific carcinogen ethylnitrosourea. Four cell types derived from RT4 are RT4-AC (a stem cell type), RT4-B, RT4-D, and RT4-E (1).

Clonal cell lines B50 and B12, provided by D. Schubert, were isolated from a central nervous system tumor induced by ethylnitrosourea injection in BDIX rats (5). The C6 clonal cell line, provided by G. Sato, was isolated from a central nervous system tumor induced by methylnitrosourea in a Wistar–Furth rat (6). A rat mammary gland tumor cell line, 64-24, was a gift of T. Kano-Sueoka (7).

Cell Culture. The cells were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 12.5% horse serum, 2.5% fetal calf serum, and 50 units each of penicillin and streptomycin per ml in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Measurement of $^{22}Na^+$ Influx. For $^{22}Na^+$-influx experiments, cells were plated in 35-mm Falcon tissue culture dishes and allowed to grow to confluency. $^{22}Na^+$-influx measurements were performed essentially as described by Catterall and Nirenberg (8) and Stallcup and Cohn (9), with some modifications as described below.

The assay medium contained 10 mM Hepes, 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 1 mM NaH_2PO_4 adjusted with NaOH to a final pH of 7.4. The washing solution was the same as the assay medium, except that the NaCl concentration was 164 mM. Measurements of $^{22}Na^+$ influx were initiated by aspirating off culture medium, washing the cells twice with 1 ml of nonradioactive assay medium, and then adding 0.7 ml of radioactive assay medium ($^{22}NaCl$, 1–1.5 μCi/ml) containing the appropriate drugs (5 mM ouabain, 0.1 mM veratridine, and tetrodotoxin) to the culture dish. The reaction was carried out in a humidified incubator at 37°C for 30 min and was terminated by removing the radioactive assay medium. The cells were washed three times with 4 ml of washing solution and then lysed by addition of 1 ml of 1 M NaOH overnight at 37°C. Protein concentrations were determined by the modified method of Lowry (10), and $^{22}Na^+$ radioactivity was assayed by a gamma counter.

Quantitative Analysis of S100 Protein. S100 protein (11) was quantitatively assayed by the rocket immunoelectrophoresis technique (12). Bovine S100 protein was purified by procedures kindly provided by H. R. Herschman. Rabbit antiserum against bovine S100 was prepared (13). For the rocket immunoelectrophoresis, 0.15 ml of undiluted antiserum was added to 12 ml of melted 1% agarose in 50 mM Veronal buffer (pH 8.6). Electrophoresis was at 2 V/cm in the gel for 20 hr at 4°C

in 50 mM Veronal buffer (pH 8.6) of reservoir. The method can quantitate up to 100 ng with linear relationship.

The cell lysate for S100 protein analysis was prepared as follows: Cells were cultured to stationary phase by changing the medium twice a week and keeping them for 3 days after confluency without changing the medium. Cells were washed three times with 10 ml of phosphate-buffered saline (pH 7.4; 137 mM NaCl/2.68 mM KCl/8.14 mM Na_2HPO_4/1.47 mM KH_2PO_4), and then collected by scraping with a policeman and by centrifugation. The cells were suspended in 50 mM phosphate buffer/0.25 mM EDTA (pH 7.0), then sonicated on ice for 30 sec. The sonicate was centrifuged for 30 min at 12,000 rpm at 4°C. The supernatant, containing 250 μg of soluble proteins, was lyophilized and dissolved in 10 μl of water. Four microliters of the solution (corresponding to 100 μg of soluble proteins) were analyzed by rocket immunoelectrophoresis.

Chemicals. ^{22}NaCl was obtained from Amersham/Searle. Ouabain, veratridine, and tetrodotoxin were obtained from Sigma.

RESULTS

Voltage-dependent Na^+ influx: A neuronal property

The initial depolarization phase of an action potential is due to passive, voltage-dependent Na^+ influx, which can be stimulated by veratridine and inhibited by tetrodotoxin. Recently, Catterall and Nirenberg (8) showed that veratridine-stimulated Na^+ influx in the presence of ouabain, which prevents Na^+ efflux by inhibiting Na^+/K^+-activated ATPase, provides a specific and convenient biochemical assay method for determining the presence of a membrane action potential of cultured cells. Excellent correlation exists between this method and electrophysiological measurements (8, 14).

The extent of voltage-dependent Na^+ influx can be expressed as the response ratio (R) of Na^+ influx with and without veratridine, as follows:

$$R_x = \frac{V^+(X)}{V^-(X)}, \quad [1]$$

where $V^+(X)$ is the $^{22}Na^+$ influx with veratridine of cell line X and $V^-(X)$ is the $^{22}Na^+$ influx without veratridine of cell line X.

Due to the limited solubility of veratridine in aqueous solution, reproducible concentrations of veratridine were difficult to obtain. Therefore, the neuronal cell line B50 was included as the standard in each group of assays. This standardization to B50 values gives highly reproducible results and makes comparison among cell lines meaningful. The standardized value, r, is derived as follows:

$$r_x = \frac{V^+(X) - V^-(X)}{V^-(X)} \Big/ \frac{V^+(B50) - V^-(B50)}{V^-(B50)} = \frac{R_x - 1}{R_{B50} - 1}, \quad [2]$$

where $V^+(B50)$ is the $^{22}Na^+$ influx with veratridine of neuronal cell line B50 and $V^-(B50)$ is the $^{22}Na^+$ influx without veratridine of cell line B50.

The clonal cell lines B50 and B12, originally isolated from the central nervous system by Schubert *et al.* (5), were used as neural cell standards. B50 is a neuronal cell line in the sense that it has an excitable membrane potential even if it produces S100, a protein observed in glial cells *in vivo* (2, 3) (see Tables 1 and 2 and *Discussion*), and B12 is a glial cell line since it does not have an excitable membrane and it produces S100 protein. Rat mammary gland tumor cell line 64-24 (7) was used as a nonneural cell standard.

As shown in Table 1 and Fig. 1, the stem cell line RT4-AC shows a small but significant response to veratridine, indicating that the neuronal property is not fully expressed in stem cells. The clear response of cell lines RT4-B and RT4-E to veratridine suggests that they have differentiated to neuronal cells from the stem cell line RT4-AC. This is entirely based on membrane excitability, measured by Na^+ influx, in comparison with that of the neuronal standard cell line B50. In contrast to these cell lines, RT4-D cell lines do not respond to veratridine for Na^+ influx and cannot, therefore, be considered neuronal. Similar tendencies of differentiation to either neuronal or nonneuronal cells are also observed in cell lines derived from the primary cultures of the RT4 tumor (Table 1). Almost complete inhibition of veratridine-stimulated Na^+ influx by tetrodotoxin (Fig. 2) indicates that this Na^+ influx must occur through voltage-dependent Na^+ ionophores (15, 16). The concentration required for 50% inhibition is 1 μM under standard conditions.

Table 1. Response of Na^+ influx to veratridine

Cell line	R	r
B50		
(neuronal)	1.70–3.09	1.0
B12 (glial)	1.00, 1.00	0.00, 0.00
C6 (glial)	1.02	0.02
64-24		
(nonneural)	1.00, 1.02	0.00, 0.04
RT4-51-AC14	1.34, 1.28, 1.18	0.43, 0.37, 0.34
RT4-51-AC23	1.31, 1.15	0.34, 0.28
RT4-51-AC24	1.37, 1.28	0.36, 0.35
RT4-32-B1	1.57, 1.65	0.65, 0.71
RT4-32-B2	1.63, 1.70	0.65, 0.71
RT4-42-B3	1.70, 1.44	0.57, 0.51
RT4-42-B4	1.73, 2.11, 1.72, 2.35	0.71, 0.60, 0.65, 0.64
RT4-B5*	1.80, 2.69, 1.82	0.90, 0.91, 0.91
RT4-B6*	1.76, 2.72	1.00, 0.94
RT4-B7*	1.62, 3.05	0.93, 1.03
RT4-B8*	1.75, 4.00	1.02, 1.17
RT4-B9*	1.62, 2.56	1.00, 0.82
RT4-52-D1	0.93, 1.00, 0.99	−0.07, 0.00, 0.00
RT4-71-D2	0.98, 1.00, 1.17, 1.01	−0.01, 0.00, 0.08, 0.02
RT4-D3*	0.94, 1.00	−0.04, 0.00
RT4-D4*	0.99, 0.92	−0.01, −0.08
RT4-D5*	0.94, 1.00, 1.05	−0.06, 0.00, 0.02
RT4-D6*	0.96, 1.00	−0.02, 0.00
RT4-D7*	1.00, 1.06	0.00, 0.02
RT4-73-E1	1.58, 2.48	0.67, 0.71
RT4-73-E2	1.42, 1.54	0.50, 0.59
RT4-E3*	2.37, 2.38, 4.32	1.55, 1.58, 1.68
RT4-E4*	2.38, 2.15, 2.37	1.44, 1.10, 1.55
RT4-E5*	3.16, 4.08	1.02, 1.28

Cells were cultured in 35-mm Falcon culture plates until confluency under standard conditions. $^{22}Na^+$ influx was measured at 37°C for 30 min in the presence of 5 mM ouabain or 5 mM ouabain with 0.1 mM veratridine. Values of the veratridine response ratio (R) and the standardized veratridine response ratio to the neuronal standard cell line B50 (r) were calculated from Eqs. 1 and 2. Each value represents the average of triplicate cultures. $R > 1.0$ or $r > 0$ represents neuronal response to veratridine; $R = 1.0$ or $r = 0$ represents no response to veratridine.

* Differentiated clonal cell lines originating from clonal stem cell lines RT4-51-AC14, RT4-51-AC23, and RT4-51-AC24 (1). Other RT4 clonal lines listed were originally isolated from primary culture of RT4 tumor and classified as "tumor-derived" cell lines (1).

S100 protein: A glial protein

S100 protein, reported by Moore (11), is a nervous system-specific acidic protein and is considered a glial marker since it is seen predominantly in glial cells both *in vivo* and *in vitro* (2, 3, 6, 17). As shown in Table 2, S100 protein is produced in glial

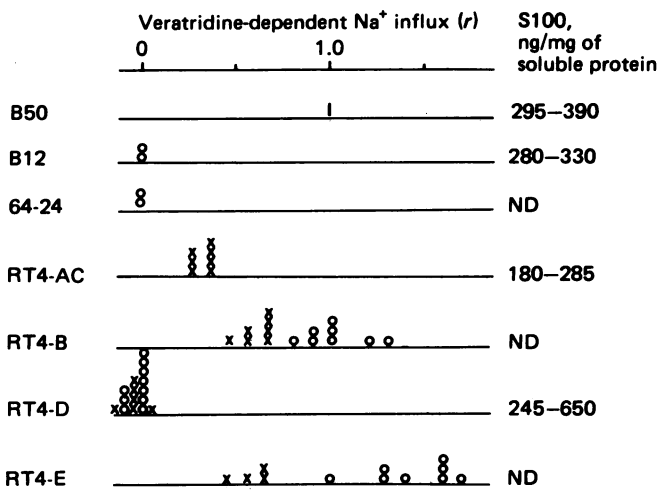
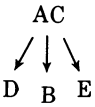


FIG. 1. Summary of Na^+ influx and S100 protein content of standard cell lines and clonal cell lines of RT4 family. The standardized veratridine response ratio (r) of Na^+ influx was calculated from Eq. 2. All clonal cell lines of each "stem-cell derived" cell type of RT4 family shown originated from clonal RT4-AC cell lines shown with asterisk in Table 1; O, their r values. X, r values of "tumor-derived" cell lines. Cell line B50 has both neuronal and glial properties (5). We have used the Na^+ influx value of B50 as the reference; its value is designated as 1.0. The positive Na^+ influx was 200–1200 nmol of Na^+ per 30 min per mg of cell protein, or 500–20,000 cpm of $^{22}Na^+$ and 0.2–0.7 mg of cell protein per sample. ND, not detectable.

cell lines C6 and B12 and in cell lines of RT4-AC and RT4-D in the range of 180–650 ng/mg of soluble protein under standard conditions. However, it is not detectable in cell lines of RT4-B and RT4-E, nor in the nonneural cell line 64-24. Therefore, the stem cell line RT4-AC and its derivative RT4-D, which produces significant amounts of S100 protein, have a glial property, while the derivatives RT4-B and RT4-E cannot be classified as glial because of lack of this protein.

DISCUSSION

Four cell types derived from the RT4 neurotumor are established as RT4-AC, RT4-B, RT4-D, and RT4-E (1). Of the four cell types, RT4-D and RT4-E were stable for at least 2 months of continuous culturing, whereas RT4-B gave still another cell type (which has not been studied in detail). However, a single-cell clone of RT4-AC can generate all of the other cell types; i.e., RT4-B, RT4-D, and RT4-E. Therefore, RT4-AC is regarded as the multipotential stem cell type of the RT4 family (1). All of these cells continue to proliferate in culture and have an apparently normal chromosome number. The unidirectional cell type conversion shown below is a conclusion based on the fact that we have not seen RT4-AC cells in RT4-B, RT4-D, and RT4-E cell cultures.



From the results of both veratridine-stimulated Na^+ influx and S100 protein production in the RT4 family, it is clear that the stem cell type RT4-AC shows both neuronal and glial properties, RT4-B and RT4-E have a property of neuronal cells, and RT4-D has a property of glial cells. Therefore, it is possible that the cell type conversion of RT4-AC to RT4-B and RT4-E is, in fact, a stem cell differentiation to neuronal cells and that the conversion to RT4-D is differentiation to a glial cell. Combining our previous (1, 4) and present results, it is clear that

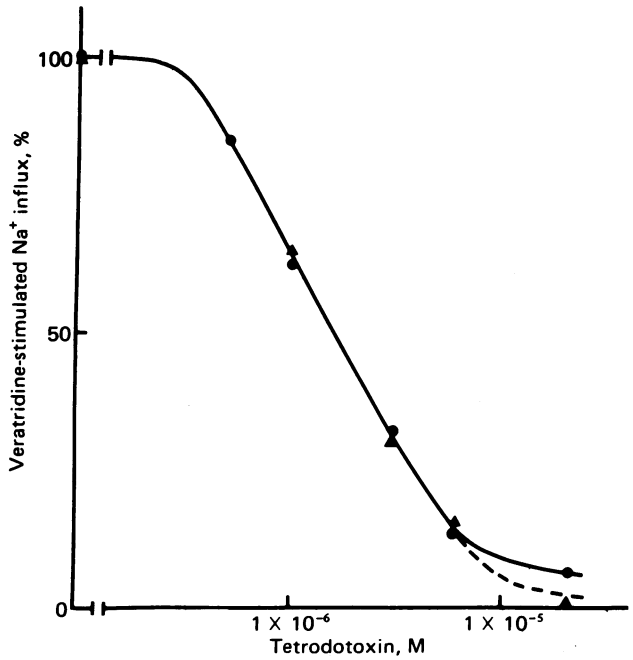


FIG. 2. Inhibitory effect of tetrodotoxin on veratridine-stimulated $^{22}Na^+$ influx. Cells were cultured to confluency under standard conditions. $^{22}Na^+$ influx (nmol of Na^+ per 30 min per mg of protein) was measured at 37°C in the presence of 5 mM ouabain (I) and 5 mM ouabain plus 0.1 mM veratridine plus varied concentrations of tetrodotoxin (II). Each point represents the average value of triplicate cultures. Veratridine-stimulated $^{22}Na^+$ influx is represented as the difference of (II) − (I). ●, RT4-E5; ▲, B50. Veratridine-stimulated Na^+ influx without tetrodotoxin (100% value) was 826 nmol of Na^+ per 30 min per mg of cell protein, or 3700 cpm of $^{22}Na^+$ and 0.3 mg of protein per sample.

the characteristics—S100 protein, veratridine response on Na^+ influx, and tumorigenicity—have never segregated from morphology; i.e., all of the different isolates of each cell type so far tested give the same set of characteristics. This observation of conversion coupling is a significant feature of the RT4 system (Fig. 3).

The Na^+ influx observed here is effected by the voltage-dependent Na^+ ionophore because of the veratridine response

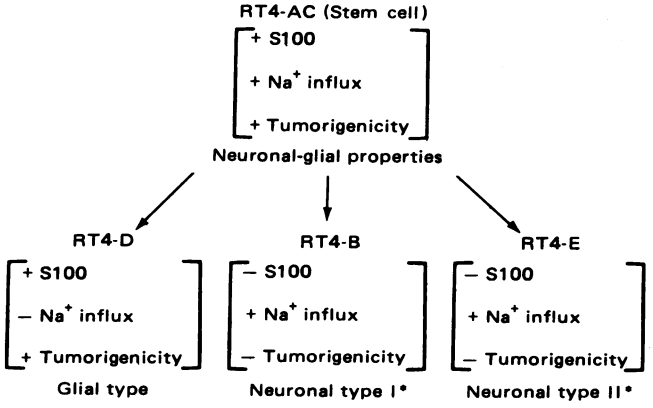


FIG. 3. Conversion coupling in the RT4 family. In cell type conversion, tumorigenicity is coupled with the expression of S100 protein, while cells with positive Na^+ influx are not tumorigenic. Thus, the expression of these and morphological characteristics are "coupled" in that whenever one characteristic (e.g., the presence of S100 protein) is observed, tumorigenicity is observed. In all cases so far examined, when cells converted to neuronal (i.e., absence of S100 and positive Na^+ influx), tumorigenicity is negative. The coupling feature is consistent in all cell lines so far examined. This diagram is based on the present results and our previous results (1, 4).

Table 2. Production of nervous system-specific protein S100

Cell line	S100, ng/mg of soluble protein
B50 (neuronal)	295, 390
B12 (glial)	280, 330
C6 (glial)	130, 110
64-24 (nonneural)	ND, ND
RT4-51-AC14	180, 240
RT4-51-AC23	180†
RT4-51-AC24	220,† 285
RT4-32-B1	ND†
RT4-32-B2	ND†
RT4-42-B3	ND†
RT4-42-B4	ND,† ND
RT4-B5*	ND,† ND
RT4-B6*	ND, ND
RT4-B7*	ND, ND
RT4-B8*	ND, ND
RT4-B9*	ND, ND
RT4-52-D1	270,† 500, 540
RT4-71-D2	245,† 370, 340
RT4-D3*	275,† 500
RT4-D4*	256, 300, 470
RT4-D5*	270, 310, 640
RT4-D6*	410, 430, 475
RT4-D7*	520, 550, 650
RT4-73-E1	ND,† ND
RT4-73-E2	ND,† ND
RT4-E3*	ND,† ND
RT4-E4*	ND,† ND
RT4-E5*	ND, ND

ND, not detectable. Amount less than 40 ng/mg of soluble protein.
* Same as in Table 1.
† Values are from Imada and Sueoka (1).

and inhibition by tetrodotoxin. However, the concentration of tetrodotoxin required for 50% inhibition (10^{-6} M) is considerably higher than that required (10^{-8} M) for mouse neuroblastoma clonal cell lines C1300 (8) or that required ($<10^{-8}$ M) for lobster giant axon *in vivo* by electrophysiological measurement (16). The concentration is, however, similar to those measured in rat clonal neuronal cell lines by both Na^+ influx and electrophysiologically (9, 14). The low sensitivity to tetrodotoxin, however, may be analogous to cases seen in neurons and muscles in the early developmental and newborn stages. These neurons and muscles increase their sensitivity to tetrodotoxin according to maturation, which coincides with the appearance of fast Na^+ ionophore (fast action potential) (18–22). The same insensitivity to tetrodotoxin and slow action potential of excitable membrane are observed in cultured monolayer cells (23) and in denervated muscle (24).

Different cell lines of cell types RT4-E and RT4-B tend to show reproducibly different levels of veratridine-dependent Na^+ influx. This may indicate some heritable difference between cell lines. Particularly noticeable is lower r values of "tumor-derived" cell lines (Table 1 and Fig. 1). Variability of S100 protein content is wide even within a cell line. This probably comes from the fact that S100 protein is produced towards the late stages of cell growth in culture (25). On the contrary, veratridine-dependent Na^+ influx is reported to be similar in different stages of cell growth, which we have confirmed with the B50 cell line (data not shown).

With regard to the small but significant response to veratridine of voltage-dependent Na^+ influx in the stem cell population (RT4-AC), we cannot rule out the possibility that, although morphologically no cell type conversion was apparent when cells were harvested, some cells may already have converted to neuronal cell types RT4-B and RT4-E in culture and that it is these latter cells that are responding to veratridine within the population. A similar possibility also exists for S100 production by the AC cell population. To clarify this point, we are examining AC populations with the fluorescent dye merocyanine 540, which is taken up by excitable membranes (26), and by immunofluorescent staining by using rabbit antiserum against S100 protein.

The well-studied glial cell line C6 produces S100 protein, but does not have veratridine-stimulated Na^+ influx (6, 14). Mouse neuroblastoma cell line C1300 has the veratridine-stimulated Na^+ influx but does not produce S100 protein (5, 8). On the other hand, most of Schubert's clonal cell lines derived from central nervous system tumors of rats produce S100 protein and also have excitable membrane, as in the case of B50. In this sense, they are similar to our RT4-AC cell type. The results of our studies raise the interesting possibility that the cell lines of Schubert *et al.* (5), with both properties, still are stem cells which, under certain conditions, may undergo cell type conversion, which may separate the two properties in different cell types, as observed in the RT4 system. The presence of S100 protein in rat-brain neuronal cells has been made unlikely (3). It is possible, however, that neuronal cells may have S100 protein in specific locations of the plasma membrane. Haglid *et al.* (27) reported immunocytochemical evidence for the presence of S100 protein in most synaptic regions of rabbit cerebral and cerebellar cortex. Although our previous results (1) and present results unequivocally show that there is no detectable amount of S100 protein in the soluble fraction of RT4-B and RT4-E cells, the existence of S100 protein in the membrane fraction has not been eliminated.

In the RT4 system, we have yet to determine whether individual stem cells (RT4-AC) simultaneously possess both neuronal and glial properties. There are several possibilities: (*i*) all of the cells express both properties prior to morphological differentiation; (*ii*) each cell expresses one or the other, not both, prior to morphological differentiation; (*iii*) the two properties are separately expressed prior to morphological changes in only some of the cells of the AC population, while the rest of the population remains as stem cells without either of the two properties expressed; and (*iv*) the population includes some RT4-D, RT4-B, and RT4-E type cells that have already converted functionally as well as morphologically. Detailed cytochemical analyses of the RT4-AC population should help us determine which of these possibilities is true. Another important question to resolve is whether cell types RT4-E and RT4-B possess other neuronal properties but no glial properties and, conversely, whether cell type RT4-D has other glial properties but no neuronal properties.

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