Clonal cell lines from the rat central nervous system

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Five neuronal and a large collection of putative glial cell lines from the rat central nervous system have been established in clonal cell culture and partially characterised. These cells shed new light on the distribution of neurotransmitter synthesis and brain-specific antigens among nerve and glia.

ALTHOUGH the C1300 mouse neuroblastoma[1,2] has been useful for studies of the differentiation and trophic interactions of nerve cells, many more neuronal cell lines will be required before generalisations can be made about the behaviour of these cells in culture and maximum use made of the clonal culture system. This also applies to the glia and other satellite cells, whose role in the metabolism and physiology of nervous tissue is poorly understood; only a few clonal cell lines of glial origin have been described[3-5].

To establish defined cell lines from the central nervous system (CNS), cells must first be adapted to permanent culture and cloned; at present neoplasms are the only efficient source of dividing cells for this purpose. The established clones must then be sorted into cell types, for example, neurones must be distinguished from glia, and, among the glial cells, astrocytes from oligodendrocytes. We describe here an attempt to establish and characterise several clonal cell lines from the rat CNS. Of approximately 120 cell lines isolated from independently arising tumours so far, some properties of 22 clones from independently arising tumours are described.

Establishment of cell lines

Neoplasms were induced transplacentally with nitrosoethylurea (NEU)[6]. BDIX rats were injected 15 d after conception with 4.0 mg of NEU per 100 g body weight, which reduced the litter size by 50%. Between 4 and 10 months after birth, approximately half the offspring had symptoms of extreme nervous system disorders and were examined for tumours. In 93% of the animals, tumours were found in the CNS; the others had tumours in other areas. Excised tumours were minced finely in modified Eagle's medium containing 20% foetal calf serum[7], cells (including pieces of tissue) were diluted and 1×10^4–1×10^6 cells were plated on Falcon 60 mm tissue culture dishes. Each dish was examined periodically for cell proliferation, and as areas of extensive growth were found, cells were isolated by cloning rings and transferred to another dish. Since the initial explants contained cells of widely different morphologies, the most complex cell types were selected. When these cells reached confluency, they were either cloned or passaged for further study. Only one clonal cell line was initially obtained from each tumour. Of the fourteen cell lines examined, all were near diploid.

Excitability and morphology

Mammalian nerve or muscle can be distinguished from glia and most other cells, as the former have electrically excitable membranes[8]. To assay for membrane excitability, cells were impaled with a microelectrode as described elsewhere[9]. When penetration was achieved, defined as a stable resting potential greater than −30mV, the cells were monitored for electrical excitability by anode-break stimulation. Table 1 shows that at least five of the cell lines (B35, B50, B65, B103 and B104) could produce a regenerative action potential. Cells were scored as negative if there was no evidence of a regenerative response after at least twenty penetrations with resting potentials greater than −30mV. Negative data, however, can be false due to technical difficulties[10]. Although electrical excitability indicates that a minimum of five of the cell lines are of neuronal or muscle origin, on the basis of other evidence discussed later, it is unlikely that any are of muscle origin.

In some nerve and muscle cells the presence of acetylcholine (ACh) receptors is correlated with electrical excitability, and the binding of ^{125}I-α-neurotoxin can be used to detect these receptors. Using established techniques[11], the number of curare-protectable α-neurotoxin-binding sites was determined in several cell lines (Table 1). Of the twelve new lines examined, three cell lines with excitable membranes have detectable ACh receptors. Clones of the C1300 neuroblastoma and the L6 myoblast line also bind toxin, while 3T3 fibroblasts do not.

After cloning, the cells could be classified into six morphological groups (Table 1 and Fig. 1). Four of the five neuronal lines—B35, B50, B65 and B103—fall into a class with clones of C1300 neuroblastoma (group 2); they all

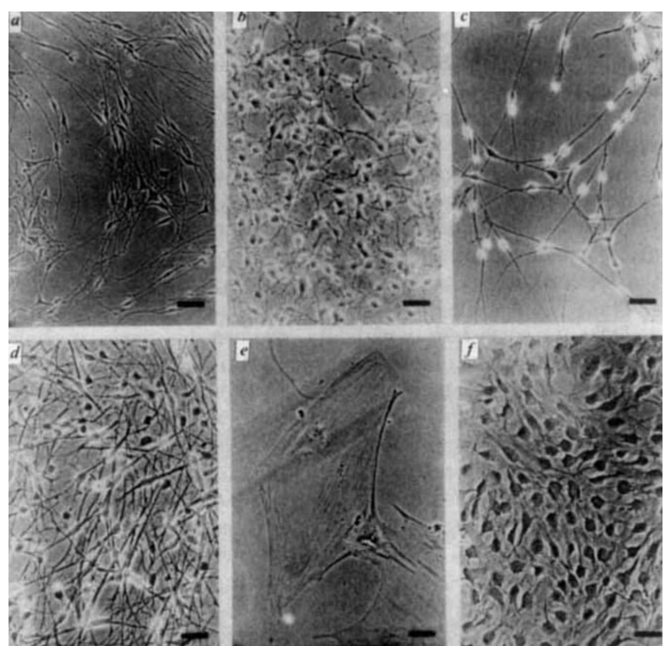

FIG. 1 Phase contrast photomicrographs of representative cell lines. The bar in each photograph represents 50 μm. *a*, B6 (group 1); *b*, B35 (group 2); *c*, B103 (group 2); *d*, B111 (group 3); *e*, B25 (group 5); *f*, B12 (group 6).

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Table 1 Characteristics of cell lines

Line	Group	Excitable membrane	α-Neuro-toxin	O-Serum	DBcAMP	ChA	TH	GAD	GABA	AChE	BChE	AChE/ BChE	%HP	S100	14-3-2
B1	IV	−		−	−	16				<1	<1	—	4.5	<5	<50
B6	I	−		−	++	31			6.3	16	1	16		41	
B9	III			−	+++	30							4.1	21	230
B11	VI			−	++	15		<20	13	1,200	880	1.4		16	780
B12	VI	−		−	+	12	7.		2.1	540	380	1.4	2.0	49	560
B15	I	−	<1	−	++	41		<20	1.5	100	60	1.7		32	930
B19	I	−		−	±	5	19			51	15	3.4		66	<50
B23	VI		<1	−	+++	36	7	<20	5.6	1.200	860	1.4	2.6	42	330
B25	V			−	±									15	
B27	I			−	±	33				540	320	1.7	4.7	<5	80
B35	II	+	<1	++	++	40	<2	330	7.1	100	60	1.7	0.7	<5	520
B49	IV	−	<1	−	+	<4	<2	<20		1,200	830	1.4	8.2	24	500
B50	II	+	<1	++	++	<4	7	1,350	30	2,300	1,800	1.3	1.8	45	560
B56	I			−	±	<4				38	38	1	2.3	<5	<50
B65	II	+	5.4	++	++	25*	50	1,200	84	130	90	1.4	1.5	28	610
B82	II	−	<1	−	+++	24	11	<20	1.1	310	280	1.1	1.8	86	3,300
B90	V		<1	−	++	38	10	<20	1.4	70	70	1		20	
B92	III	−	<1	−	++	<4	6		2.1	5,300	4,300	1.2	3.6	21	1,120
B103	II	+	7.9	+++	+++	33	<2	1,180	29	270	130	2.1	2.2	10	450
B104	I	+	82	+++	+++	77	6	<20	6.3	160	50	3.2	4.2	17	570
B108	VI	−	<1	−	+++	<4	22	<20	3.4	3	3	1	0.7	20	1,250
B111	III			−	−	<4	8	<20		290	190	1.5	2.6	33	450
C1A	II	+	5	+++	+	15			4.2	1,100	15	76	1.4	<5	500
S20	II	+	1			400			9.4						<50
L6†	IV	+	400	−	−	10	<2	<20	<0.1	12	5	2.4	4.9	<5	<50
Brain						500	450	6,000						350	890
3T3	IV		<1	−	+	<4	<2	<20	<0.1	17	3	5.7	18	<5	<50
Liver						<4									<50
P3				−	−	<4			<0.1	4	4	1	<0.01		

With noted exceptions, stationary phase cells from passages 5 to 10 after cloning were assayed for enzyme activities, proteins and morphologies as described in the text. The amount of S100 and 14-3-2 is expressed as ng specific protein per 100 ng of total soluble protein. Bovine antigens were used in both cases; these cross react extensively with the respective rat proteins (H. Herschman, personal communication). The enzyme-specific activities are expressed as follows: esterase, using either acetylthiocholine or butyrylthiocholine as substrates, ΔA_{412} per min per mg protein; choline acetyltransferase, pmol ACh formed per min per mg protein; tyrosine hydroxylase, pmol $^{14}CO_2$-released per 20 min per mg protein; glutamic acid decarboxylase, pmol $^{14}CO_2$ released per 20 min per mg protein inhibitable by 1 mM aminooxyacetic acid. The neurotransmitter products were identified chromatographically. GABA assays were done on tricholoroacetic acid soluble cell supernatants using an amino acid analyser with expanded resolution (D.S. and W.C., in preparation). The results are expressed as residues of GABA per 1,000 residues of free amino acids. The effects of serum-free medium and 1 mM dibutyryl cyclic AMP on cell morphology were assayed on exponentially dividing cells and range from no change (−) to the greatest change (+++); an effect greater than (+) is considerable. The percentage hydroxyproline is expressed as the percentage of proline found in secreted protein which is hydroxylated (see ref. 33 for discussion and assay procedures). The binding of ^{125}I-α-neutrotoxin is expressed in 10^{-15} mol toxin bound per mg protein. The morphological groups indicate the general morphologies of the cells (see Fig. 1 and text). Electric excitability is scored as positive if regenerative action potentials are observed, and negative if there was no evidence for this event in at least 20 cells examined with resting potentials more negative than −30mV. C1A and S20 were clones of the C1300 mouse neuroblastoma[12,34], L6 was a clonal rat myoblast line[33], 3T3 was a fibroblast cell line, and P3 an immunoglobulin-secreting plasmacytoma. O-serum, the effect of serum-free medium on cell morphology; DBcAMP, the effect of dibutyryl cyclic AMP on cell morphology; ChA, choline acetyltransferase activity; TH, tyrosine hydroxylase activity; GAD, glutamic acid decarboxylase activity; GABA, residues of GABA per 1000 residues of free intracellular amino acids; AChE, esterase activity using acetylthiocholine as substrate; BChE, esterase activity using butyrylthiocholine as substrate; AChE/BChE, the numerical ratio of the two esterase activities; % HP, percentage of the total secreted proline found as 4-hydroxyproline; S100 and 14-3-2, concentrations of these proteins in cell lysates.

* Exponentially growing cells.

† All assays were done in fused, multinucleate myotubes, except for the effects of dibutyryl cyclic AMP and O-serum medium on cell morphology. Myoblasts are of group 4 morphology.

grow loosely associated with the surface of the culture dish and have relatively spherical, phase-bright cell bodies. A fifth neuronal cell line, B104, has a group 1 morphology, with very long, thin and usually bipolar cells. The other groups consist of cells with short, highly branched processes (group 6), extremely large flat cells (group 5), cells which are large and flat during the exponential phase of growth, but which branch profusely in the stationary phase (group 3), and the fibroblast-like cell (group 4). Whenever more than one cellular morphology was observed during the growth cycle, stationary phase cells were the most complex. This resembles the situation with C1300 neuroblastoma, where under normal conditions cells with long neurites are rare except in stationary phase cultures[2,10].

The C1300 neuroblastoma characteristically responds to the removal of serum and the presence of dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) by rapidly extending neurites[12–14]. Although dibutyryl cyclic AMP induces morphological transformations in most cells, the effect of serum-free medium on the mouse neuroblastoma seems to be specific. If this response is restricted to cultured nerve cells, only nerve lines should respond to the removal of serum with similar morphological transformations. Table 1 shows that this was the case. Thus the morphological response to low serum concentrations seems to distinguish neuronal from non-neuronal cells in this system. As expected from work on the mouse neuroblastoma and primary cultures of nervous tissue[15], all the neuronal and most of the other cell lines were transformed morphologically in the presence of dibutyryl cyclic AMP (Table 1). A more detailed account of these changes will be presented elsewhere.

Neurotransmitter synthesis

Another distinction between the classes of cells in the nervous system is provided by the enzymes involved in neurotransmitter metabolism. The activities of choline acetyltransferase, tyrosine hydroxylase, glutamic acid decarboxylase, and both acetyl and butyrylcholine esterase were measured in stationary phase cultures as detailed elsewhere[16–19]. Table 1 shows that esterase activity was widely distributed among the cell lines, varying greatly with respect to both absolute amount and the ratio of activities on acetylthiocholine and butyrylthiocholine substrates. Al-

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though B6, B19 and B104 have relatively high ratios, demonstrating true acetylcholine esterase, no cell lines approached the activities of C1300 neuroblastoma clones. Choline acetyltransferase is also widely distributed among the cell lines. It has been suggested, on the basis of indirect evidence, that glia synthesise acetylcholine[20]; the data in Table 1 indicate that some of the non-neuronal cell lines in our collection have the requisite enzyme. Amino-oxyacetic-acid-inhibitable glutamic acid decarboxylase activity was restricted to the neuronal cell lines, while γ-aminobutyric acid (GABA) was found in all of the putative nerve and glial lines. Although there was no strong correlation between glutamic acid decarboxylase activity and the ability of the cells to retain GABA, these data again suggest that glial cells can synthesise neurotransmitters. Tyrosine hydroxylase was found in B65 and at least two of the non-neuronal cell lines. With the exception of choline acetyltransferase activity in B65, the data indicate the enzyme specific activities for stationary phase cells only. These activities and the concentrations of the putative neurotransmitters vary as a function of the growth cycle (B. K., H. T., D. S., manuscript in preparation).

As Table 1 shows, several neuronal cell lines contain more than one neurotransmitter synthetic enzyme. For example, B65 contains choline acetyltransferase, tyrosine hydroxylase and glutamic acid decarboxylase activities; B35 and B103 both contain choline acetyltransferase and glutamic acid decarboxylase activities. Although it is generally assumed that an individual nerve cell makes but one transmitter, the evidence for this is not complete. In particular, it is not known if the newly formed nerve cell can synthesise a spectrum of neurotransmitters before it becomes functionally coupled with another cell. Possibly the shift toward predominantly one transmitter is a result of this type of cell-cell interaction, an event not documented in these clonal cultures. Finally, it should be pointed out that the specific activities of the enzymes involved in neurotransmitter metabolism in the cell lines are lower than those observed *in vivo* (Table 1). Since transmitter enzymes may be concentrated at functional nerve endings, and their levels regulated by trophic interactions between cells, the relatively low activities presented here may again reflect a lack of such interactions in these cultures.

Proteins unique to the nervous system

Of several proteins which seem to be localised in the nervous system, S100 and 14-3-2 are thought to be associated with glia and nerve, respectively[21,22], in spite of exceptions[23-26]. Since stationary phase cells exhibited the greatest morphological complexity seen during the growth cycle, they were used for the quantitation of these proteins. (It should be pointed out, however, that the intracellular concentrations of some enzymes and other proteins depend on the stage of the growth cycle both in these and in other cell lines[27,28].) To assay for S100 and 14-3-2, cells were homogenised and the soluble cell fraction was assayed by an indirect radioimmune procedure using iodinated antigen and antisera prepared against the pure antigen[29]. Assays were routinely linearly sensitive between 1 ng and 100 ng for S100 and between 10 ng and 1000 ng for 14-3-2. (Concentrations of 14-3-2 and S100 in whole brain determined by this procedure were similar to those reported using other procedures[30,31].) Table 1 indicates that these proteins are not uniquely associated with either neuronal or non-neuronal cells. Although all the neuronal lines contain detectable 14-3-2, both proteins seem to be distributed independently among the remaining lines. Cells with undetectable levels of both proteins may not be of nerve or glial origin, an observation compatible with the fibroblast-like morphology of most cell lines in this group. These cells, which represent approximately 25% of the induced tumours, have not (with the exceptions of B1 and B56) been included in Table 1. However, cells which contain S100 or 14-3-2 and are not electrically excitable are of probable glial origin[21-26], and the presence of either protein cannot distinguish nerve from glia. Finally, all the cell lines of Table 1 were screened for neurophysin by the methods described for S100 and 14-3-2; none was found.

Some of the cell lines were also screened for secreted hydroxyproline, a marker for collagen[32]. Cells were labelled with ^{14}C-proline and the secreted protein assayed for 4-hydroxyproline[33]. Table 1 shows that all the cell lines examined secreted hydroxyproline. Although there was no absolute correlation with cell groups, these data suggest that more nerve-like group 2 cell lines secrete relatively less hydroxyproline-containing protein than the other morphological groups. This may explain the characteristic loose adhesion of the group 2 cells to the culture dish.

Conclusions from these data

We have drawn the following conclusions from our data. (1) Neoplasms of both neuronal and glial origin can be induced by established procedures in BDIX rats, and the resultant cells can be adapted to clonal cell culture. (2) At least 4% of our neoplasms yielded permanent nerve cell lines. (3) Nerve and glial cells can apparently be distinguished in cell culture on the basis of the effect of serum-free medium on cell morphology. All nerve and most glial cells undergo morphological changes in the presence of dibutyryl cyclic AMP, but only the nerve cells extend processes in the absence of serum. (4) Detectable glutamic acid decarboxylase activity is restricted to nerve lines, while low levels of GABA are also found in glial lines. Choline acetyltransferase and tyrosine hydroxylase activities are found in both nerve and glial cells. These results suggest that glia can synthesise neurotransmitters. (5) Some clonal nerve cell lines contain the enzymatic activities required to synthesise more than one neurotransmitter. (6) The nervous system proteins S100 and 14-3-2 are found in various combinations in both nerve and glial cell lines. All nerve cells contain detectable 14-3-2 while not all putative glial cells contain detectable S100.

Neuronal and non-neuronal lines can be distinguished on the basis of membrane excitability and neurotransmitter synthesis, but the sorting of non-neuronal lines has been difficult. On the basis of S100 and 14-3-2 protein synthesis, it can be argued that most of the cell lines presented in Table 1 are of either nerve or glial origin. It has been impossible, however, to group the putative glial cells on any basis except morphology. More specific criteria are needed.

Because all the cell lines in Table 1 derive from independently arising CNS tumours, they have greater functional diversity than is possible using one neoplasm. For example, clones of C1300 neuroblastoma have many different properties[34] but probably the original tumour was derived from a single cell, and the various stem cells resulted from later mutation and chromosome rearrangement[10]. In contrast, the rat nerve cell lines are not far removed from the original neoplastic cell, their karyotype approaches normal, and the clonal lines seem stable. Functional diversity and genetic stability are required if clonal lines are to be used with maximum effectiveness in the study of the mechanisms underlying the function of the mammalian nervous system.

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Lunar magnetism and an early cold Moon

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Models of lunar magnetism have involved dynamo action in a fluid core in an early hot Moon; an early cold Moon magnetised some time before 4.0×10^9 *yr ago, which has subsequently heated up; and local field sources which, in some models, are related to impact. Here we examine the second possibility and show that provided the Moon contained a few percent of metallic iron and was exposed to an extra-Lunar field of about 10 or 20 oersted while much of it was still below the Curie point of iron, a restricted class of thermal evolution models, which satisfy the known constraints, can be derived.*

One model of the thermal history of the Moon (see ref. 1–6) which has gained wide acceptance, involves a Moon which accreted cold but which was heated from the outside during the final stages of accretion. This would have given rise to a molten shell very early on in lunar history, and formed the crust which is indicated by seismic studies[7-9]. The interior of the Moon, however, would have been cool during its early history and would have warmed up because of radioactive heating only relatively recently, thus forming the molten or partially molten core which is indicated seismically [7-9]. Early papers accounting for the magnetic field of the Moon suggested an initially hot Moon in which an iron core formed. Although moment of inertia constraints require that the core is small, such a core would at least behave as a magnetohydrodynamic fluid even at the present slow rotation rates[10]. There are, however, many difficulties[11] and here we consider whether an early hot outside, cold inside Moon, which evolved into the present cold outside, hot inside Moon, could quantitatively account for the evidence of an ancient magnetic field.

Origin of early magnetisation

If the Moon accreted cold then any metallic iron which was present may have acquired a remanent magnetisation from any of several mechanisms, provided there was an ambient field[12]. We consider a mechanism in which the Moon was magnetised by an external steady field, thus acquiring isothermal remanent magnetisation (IRM). If the Moon were uniformly magnetised, it would need to have had a dipole moment of about 10^{23} gauss cm^3 to give an ancient surface field of 2,000 gamma (γ) which is typical of several palaeointensity studies, although a much higher value has been reported[13-16]. This is well above the present value of less than 10^{20} gauss cm^3 (ref. 17). We have measured the IRM of iron-bearing, igneous rocks from the Moon,

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