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Biochemically Differentiated Mouse Glial Lines Carrying a Nervous System Specific Cell Surface Antigen (NS-1)

(S-100 protein/2':3'-cyclic nucleotide 3'-phosphohydrolase/ 3':5'-cyclic AMP stimulation by catecholamines)

NIRMALA SUNDARRAJ*, MELITTA SCHACHNER†, AND S. E. PFEIFFER*

* Department of Microbiology, University of Connecticut Health Center, Farmington, Conn. 06032; and † Department of Neuropathology, Harvard Medical School and Department of Neuroscience, Children's Hospital Medical Center, Boston, Massachusetts 02115

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ABSTRACT Six biochemically differentiated clonal lines have been established from a transplantable glioma (G26) of the C57BL/6 inbred mouse strain. Antibodies have been previously raised against G26 tumor cells, which define a cell surface component(s), NS-1 (nervous system antigen-1), found exclusively in the nervous system. NS-1 concentrations approximate the levels of the original G26 tumor when the clonal lines are grown as clonal tumors *in vivo*, but are reduced when the cells are grown *in vitro*. NS-1 concentrations are further reduced *in vitro* upon incubation of the cells with 1 mM dibutyryl 3':5'-cyclic AMP. H-2 histocompatibility antigen concentration, in contrast, is unaffected by dibutyryl cAMP. In addition to expressing NS-1, the neuroectodermal origin of these cell lines is further confirmed by their synthesis of the nervous system specific acidic protein S-100 and by the high specific activity of the enzyme 2':3'-cyclic nucleotide 3'-phosphohydrolase. In addition, they respond to catecholamines by the elevation of intracellular 3':5'-cyclic AMP levels. Whereas expression of S-100 protein is high under *in vitro* conditions but negligible after one passage *in vivo*, 2':3'-cyclic nucleotide 3'-phosphohydrolase is not detectable *in vitro* but becomes detectable again *in vivo*. The two membrane-bound constituents, NS-1 and 2':3'-cyclic nucleotide 3'-phosphohydrolase, therefore seem to be subjected to different regulatory mechanisms from that of the soluble, intracellular S-100 protein.

Until now, the only established mouse clonal cell lines described that express biochemical functions unique to the nervous system were those derived from mouse neuroblastoma C1300 (1–3). Here we describe clonal lines from a mouse glioma (G26) (4) that express several nervous system specific biochemical properties. These include a glial specific surface antigen(s) called NS-1 (nervous system antigen-1) (5), the presence of S100 protein (6, 7), and high 2':3'-cyclic nucleotide 3'-phosphohydrolase activity (8, 9). In addition, several of the lines exhibit marked increases in intracellular 3':5'-cyclic AMP levels in response to stimulation by catecholamines (10).

MATERIALS AND METHODS

Tumor. Glioma G26 was induced by methylcholanthrene treatment in the C57Bl/6 inbred mouse by Zimmerman thirty years ago (4) and has been classified as an immature glial cell with oligodendroglial and some astrocytic features (Schachner *et al.*, manuscript in preparation). It is maintained subcutaneously as a homogeneous cell mass by passage in C57Bl/6 mice at intervals of 2–3 weeks (5).

Abbreviation: NS-1, nervous system antigen-1.

Tissue Culture. Tumor cells were dissociated and plated in Ham's medium F12 (11) supplemented with 2.5% fetal calf serum (Reheis Chemical Co.) and 10% horse serum (Grand Island Biological Co.) as described (9). Clonal lines were isolated by repeated single cell plating and selection of clonal colonies using glass cylinders. Cells for morphological studies were grown in glass slide chambers, fixed with absolute methanol, and stained with hematoxylin and eosin.

Chromosome Analysis. Cells were accumulated in mitosis by exposure to colchicine (1.0 μg/ml) for 1–2 hr, and chromosome spreads prepared as described (9).

Cytotoxicity Test. The presence of surface antigen NS-1 was examined either by the direct cytotoxicity test or by the absorption method, as described in detail elsewhere (5). In the latter approach, antiserum against NS-1 is first incubated with washed and packed cells or with particulate fraction of tissue homogenate at a ratio of 1:1 of packed particulate material to antiserum, used at antibody excess (three to four serial dilutions below the titer endpoint; see legend for Fig. 3). After absorption, the supernatant of the particulate material is assayed by the cytotoxicity test for remaining cytotoxic activity on single cell suspensions prepared from the G26 tumor grown *in vivo*. Target cells are first incubated with antiserum and then washed before addition of complement to avoid anticomplementary effects. Low residual cytotoxic activity after absorption is indicative of high levels of antigen on the absorbing tissue, whereas high residual cytotoxic activity is due to low expression of antigen on the tissue. The data presented in the tables are the averages of values obtained from repeated assays. Cytotoxic activity is presented as the cytotoxicity index, C.I., calculated as follows:

$$\text{C.I.} = \frac{\begin{matrix}\%\text{ dead cells} \\ \text{with antiserum}\end{matrix} - \begin{matrix}\%\text{ dead cells} \\ \text{without antiserum}\end{matrix}}{(100) - \begin{matrix}\%\text{ dead cells} \\ \text{without antiserum}\end{matrix}} = \frac{\begin{matrix}\%\text{ killed} \\ \text{by test}\end{matrix}}{\begin{matrix}\%\text{ available} \\ \text{for killing}\end{matrix}}.$$

The percent of dead cells without antiserum was generally less than 15%.

Elevation of 3':5'-cyclic AMP Levels in Response to Catecholamines. Intracellular levels of cAMP in untreated, norepinephrine-treated, or isoproterenol-treated cells were estimated by the binding protein assay of Gilman (10, 12). In some cases, the level of cAMP in the acid extracts was over-

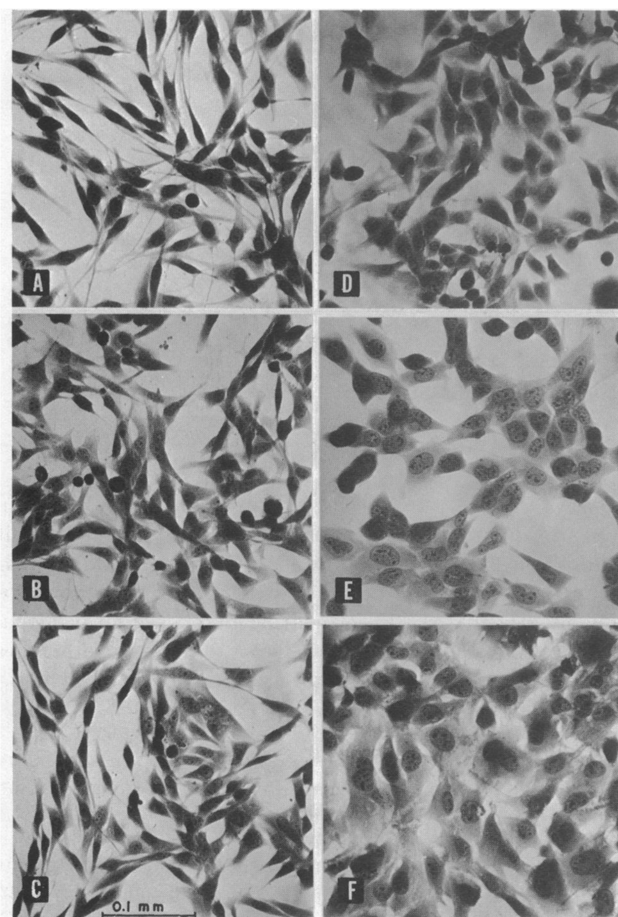

FIG. 1. Clonal lines grown in monolayers in culture stained with hematoxylin and eosin. (A) G26-15, (B) G26-19, (C) G26-20, (D) G26-24, (E) G26-28, and (F) G26-29.

estimated by the binding protein assay due to the presence of other unidentified acid-extractable substances that interfered with cAMP binding (either by direct competition or indirect inhibition). Thus, in later experiments, cAMP in acid extracts was routinely partially purified prior to assay by chromatography on AG50H$^+$ (Biorad 200–400 mesh) followed by $ZnSO_4$ and $Ba(OH)_2$ precipitation (13).

Radioimmunoassay for Myelin Basic Protein. Beef brain myelin basic protein was purified by a combination of procedures described by Oshiro and Eylar (14) and Deibler et al. (15). Antisera were prepared in female New Zealand white rabbits. The animals received intradermally four times, at weekly intervals, an emulsion containing 1 mg of the basic protein (in 0.5 ml of water) flocculated with phosphorylated bovine serum albumin (16) and 0.5 ml of Freund's (H37Ra, DIFCO) complete adjuvant (first injection) or Freund's incomplete adjuvant (subsequent injections). Rabbits were bled 1 week after the last injection. They were hyperimmunized by injecting the same dose of the basic protein 5 weeks later and bled after a further 5, 6, and 7 days.

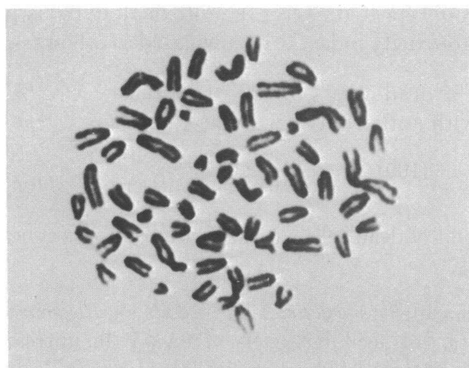

FIG. 2. Chromosome spread of a single cell from clonal line G26-24.

TABLE 1. *Influence of dibutyryl cAMP on the expression of NS-1*

Exp. no.	Clone	Cytotoxicity index: Control	Cytotoxicity index: Dibutyryl cAMP
1	G26–15	52	88
2		45	74
1	G26–19	58	83
2		70	82
3		54	86
1	G26–20	62	81
2		54	71

The difference between "control" and "dibutyryl cAMP-treated" values of the cytotoxicity index are significant at the 0.001 level, as examined by a two-tailed matched-pair analysis. The experiment was initiated by replacing the culture medium with medium without (control cultures) or with (treated cultures) 1 mM dibutyryl cAMP. Treatment was continued for 3 hr in Exp. 3, and 12 hr in Exps. 1 and 2. Similar results were obtained in medium with and without serum.

Antisera were titrated by the following radioimmunoassay: Basic protein was labeled with ^{125}I by a modification of the method of McFarlane (17) as described by Vogel et al. (18) to a specific activity of approximately 0.3 $\mu Ci/\mu g$, stored at 0° in buffer (0.5% protamine sulfate, Sigma Chemical Co., in 0.1 M borate buffer, pH 8.4), and used within a week after labeling. All the dilutions of basic protein and the assays were carried out in Eppendorf microtubes (polypropylene) since the basic protein binds to glass surfaces. Two-fold dilutions of unlabeled basic protein were prepared in buffer, starting with 20 $\mu g/ml$. Fifty microliters of an appropriate dilution of the antiserum (that which precipitates 50% of the labeled basic protein in the absence of unlabeled basic protein) were incubated with 30 μl of unlabeled basic protein or acid extracts of brain, tumor, or cells at 0–4° for 16–20 hr. Acid extracts were prepared by extracting the tissue homogenates first with chloroform/methanol and then with acetone, and finally extracting basic proteins with 10 mM HCl, and centrifuging at 15,000 rpm for 40 min (14, 15). ^{125}I-Labeled basic protein (2 $\mu g/ml$) was added to all the tubes in 20 μl of buffer. After the samples were mixed and incubated at 37° for 2 hr, 100 μl of 95% ethanol was added to each tube. Tubes were then incubated for an additional 10 min at 37°. They were centrifuged at 12,000 rpm for 4 min in an Eppendorf microcentrifuge. The precipitates were washed twice with 50% ethanol and dissolved in 100 μl of Protosol (New England Nuclear). The tubes with the dissolved precipitates were dropped into scintillation vials and radioactivity was determined in toluene/Liquifluor/Biosolve (134:16:50).

TABLE 2. *Influence of dibutyryl cAMP on expression of H-2 antigen in G26-19 cells*

Treatment	Cytotoxicity index	
	Exp. 3	Exp. 4
Control	54	55, 54
Dibutyryl cAMP	57	53

The differences between "control" and "treated" values of the cytotoxicity test are not significant, as examined by a two-tailed standard error analysis of grouped (control compared with treated) data. Experiments were initiated and carried out as described for Table 1. Treatments were continued for 12 hr in Exp. 3, and 3 hr in Exp. 4. Experiment numbers correspond to those in Table 1.

Other Differentiated Biochemical Properties. S-100 protein was assayed by microcomplement fixation (9, 19). The activity of *2':3'-cyclic nucleotide 3'-phosphohydrolase* was assayed by the method of Olafson *et al.* (20) as described by Glastris and Pfeiffer (21). *Glycerol phosphate dehydrogenase* (EC 1.1.99.5) activity was assayed in cell extracts as described by de Vellis and Inglish (22). *Protein* was estimated according to Lowry *et al.* (23), with bovine serum albumin as the standard.

RESULTS

Clonal Lines of Glioma G26. Six clones were isolated from glioma G26. The clonal lines G26-15, G26-19, and G26-20 consist mostly of long bipolar cells (Fig. 1A, B, and C). G26-24 also resembles these lines, but many cells are rounded and are either loosely attached or unattached to the plates when grown as monolayers (Fig. 1D). In contrast, G26-28 and G26-29 grow as larger flat cells (Fig. 1E and F). The mean chromosome number of G26-15, G26-19, G26-20, and G26-24 is between 60 and 62, i.e., near triploid. In contrast, G26-28 and G26-29 are heteroploid, the chromosome number varying widely from 52 to 110 among cells even at one passage. In addition, whereas normal mouse cells have all telocentric chromosomes, all of these G26 derived cell lines have two to four metacentric chromosomes (Fig. 2).

The Glial Cell Specific Surface Antigen, NS-1. The levels of NS-1 were lower in clonal cells grown *in vitro* than in the uncloned G-26 tumor grown *in vivo* (Fig. 3). However, NS-1 levels returned to approximately those found in the uncloned tumor when the *in vitro* adapted lines were repassaged for one passage of 2–3 weeks *in vivo* as clonal tumors in C57BL/6J mice (Fig. 3).

When G26-15, -19, and -20 were grown *in vitro* in the presence of 1 mM dibutyryl cAMP for 3–12 hr, the concentration of NS-1 per cell was reduced further (Fig. 3 and Table 1). In contrast, the expression of the major histocompatibility antigen, H-2, was unaffected by dibutyryl cAMP treatment (Table 2). Dibutyryl cAMP treatment promoted cell elongation and process formation, a phenomenon not observed by the removal of serum. However, the size and the number of cells per packed volume of scraped, rounded cells were not grossly altered by this drug treatment.

2':3'-Cyclic Nucleotide 3'-Phosphohydrolase. This membrane-bound phosphohydrolase is present in significantly higher activity in cells derived from neural tissues than in non-neural ones (9). The specific activity of the phosphohydrolase in the

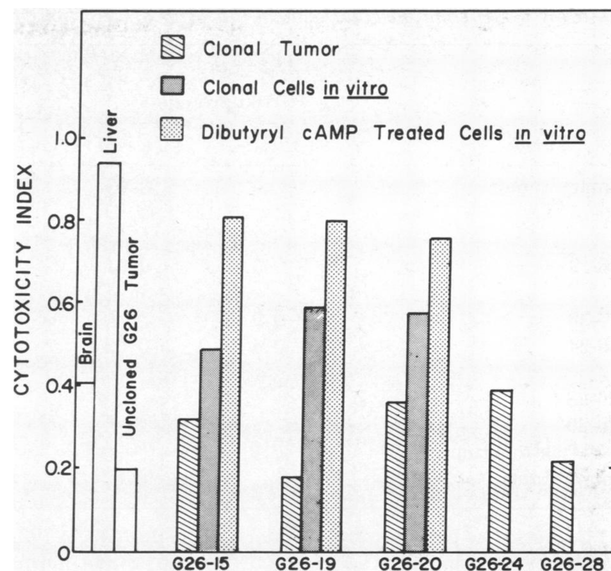

FIG. 3. Expression of NS-1 on G26 clonal lines under different growth conditions. NS-1 was measured by absorption of antibody against NS-1 with packed pellets of tissue culture cells (containing 2.25×10^7 cells) and subsequent assay of residual cytotoxic activity, with the glioma G26 grown *in vivo* as target cell, as described briefly in *Materials and Methods* and in detail before (5). The cytotoxicity index was calculated as shown in *Materials and Methods*. Antiserum against NS-1 is not cytotoxic when clones G26-19, -20, -24, and -28, grown *in vitro*, are taken as target cells; clone G26-15 carried barely detectable levels of NS-1 when assayed by this method. However, NS-1 becomes clearly demonstrable by the more sensitive absorption method. The standard error for combined cytotoxicity indices was ±0.062 for the experiments with untreated clonal lines and ±0.085 for experiments with dibutyryl cAMP-treated cell lines. The antiserum had a titer endpoint (cytotoxicity index = 0.5) of 1:64 when tested on the original G26 tumor; it was used at a dilution of 1:8 for the absorptions. G26-24 and G26-28 were not studied *in vitro*.

uncloned glioma G26 (Table 3) was comparable to the activities of 0.1–1.4 previously reported for a rat glial line (9). Although no phosphohydrolase activity was detected in cells grown *in vitro*, the tissue culture cells regained the ability to express phosphohydrolase activities ranging from 0.1 to 0.6 when grown *in vivo* as clonal subcutaneous tumors (Table 3).

Effect of Isoproterenol and Norepinephrine on 3':5'-cyclic AMP Levels. Gilman and Nirenberg (10) have demonstrated the elevation of intracellular levels of cAMP in glial cells upon addition of norepinephrine or isoproterenol. When monolayers of the G26 clonal lines were incubated for 15 min with 0.1 mM norepinephrine or isoproterenol in serum-free medium, stimulations in the intracellular levels of cAMP resulted which were between 15- and 380-fold in G26-19, -20, -24, and -29, but relatively less in G26-15 and -28 (Table 3).

Presence of S-100 Protein. S-100 protein is a soluble acidic protein that is restricted to the nervous system (6, 7). Of the six lines growing in culture, G26-15, -19, -20, and -24 had detectable amounts of S-100 ranging from 0.1 to 0.9% of total soluble protein (Table 3). However, S-100 protein was negligible in cells grown *in vivo* as clonal subcutaneous tumors, even though the uncloned tumor did have very low amounts of S-100 protein (Table 3).

TABLE 3. *Differentiated biochemical functions of G26 clonal lines grown in culture and of clonal tumors grown subcutaneously in mice*

Tissue	S-100 protein*		Phosphohydrolase†		Intracellular cAMP levels‡		
	In vitro	*In vivo*	*In vitro*	*In vivo*	Untreated control	Isoproterenol-treated	Norepinephrine-treated
Primary tumor	—	0.03	—	0.4	—	—	—
G26-15	0.2	<	<	0.1	33	68	—
					8	—	23
G26-19	0.2–0.5	0.01	<	0.4	20	—	486
					22	340	—
G26-20	0.2–0.9	<	<	0.3	14	1740	2000
G26-24	0.1–0.4	0.02	<	0.4	17	6420	—
					55	3160	—
G26-28	<	<	<	0.7	38§	113§	111§
G26-29	<	—	<	—	28§	216§	646§
C_6 (rat astrocytoma)	0.1–0.3	—	0.9	—	33§	9150§	7420§
Mouse brain	—	0.65	—	4.5	—	—	—

* Expressed as percent of total water-soluble proteins in 700,000 *g*-min supernatant fraction; sensitivity of the assay is 0.01 μg of S-100 protein per ml. < indicates less than 0.01% S-100 protein of total soluble proteins.

† 2′:3′-Cyclic nucleotide 3′-phosphohydrolase; μmol of substrate converted per min/mg of protein. Sensitivity of the assay is 7.5 nmol/min. < indicates no phosphohydrolase activity with samples of 1–3 mg of total protein per ml.

‡ pmol/mg of total protein.

§ Assayed directly from acid extracts without prior partial purification (see *Materials and Methods*). The variations in the intracellular 3′:5′-cyclic AMP concentrations for the same cell line among experiments appear to be partly due to differences in cell density and growth phase at the time of catecholamine treatment. Except for G26-19 and G26-28, the approximate magnitude of the stimulations were reinforced for each clone by several additional earlier assays performed without prior purification (see *Materials and Methods*).

Induction of Glycerol Phosphate Dehydrogenase Activity by Cortisol and Hydrocortisol. De Vellis and Inglish (22) have presented evidence that the induction of glycerol phosphate dehydrogenase by cortisol is brain specific, providing another useful glial marker function. However, basal levels of glycerol phosphate dehydrogenase activity in G26 lines were less than 0.75 nmol/mg per min, and there was no induction of this enzyme activity by either cortisone or hydrocortisone in any of the G26 lines. We observed a 4-fold stimulation in the C6 rat astrocytoma (24), as described by de Vellis and Inglish (22).

Myelin Basic Protein. Central nervous system myelin is an elaboration of oligodendroglial plasma membranes (25). Since G26 tumor carries some traits of an oligodendroglioma, the presence of the myelin-specific basic protein (26) in the uncloned and clonal tumors was investigated, using antiserum to myelin basic protein of bovine central nervous system. Although the assay detected 130 μg of myelin basic protein per mg of acid-extractable protein in mouse brain, none was detected in either the uncloned G26 tumor or in cell lines grown either *in vitro* or *in vivo*.

DISCUSSION

Clonal lines derived from neural tissues provide functionally and genetically homogeneous, viable cell populations in quantities sufficient for most biochemical and cell biological studies (27). Studies on mouse lines offer some particular advantages in view of the relatively advanced state of genetic studies in this species. The neuroectodermal origin of the G26 clonal cell lines is confirmed by the presence of nervous system specific properties, including the S-100 protein, the elevation of 3′:5′-cyclic-AMP levels in response to norepinephrine and isoproterenol, high levels of 2′:3′-cyclic nucleotide 3′-phosphohydrolase activity, and the surface antigen NS-1. The stable phenotypic variations among the clonal lines are interesting in view of the apparent homogeneity of the parent tumor, a homogeneity expected in view of the long-term growth and many passages of this tumor *in vivo* prior to its introduction into tissue culture. Whether all of the various phenotypes represented in the six clonal lines are also present in the uncloned tumor or, if instead, they occur only after the tumor cells are introduced into tissue culture, cannot be ascertained at this time. However, the phenotypes of clonal lines seem to have remained stable over at least a 6-month period through growth both *in vivo* and *in vitro*.

In vitro the level of cell surface antigen NS-1 was reduced relative to growth *in vivo*, and membrane-bound 2′:3′-cyclic nucleotide 3′-phosphohydrolase activity was not detectable. Nevertheless, the latent genetic potential for the expression of these two properties became fully manifest again when the *in vitro* adapted clones were reintroduced into the animal to produce clonal tumors (Table 3 and Fig. 3). Reexpression of properties characteristic of the original tumor has also been observed for tissue culture lines of neuroblastoma C1300 (28) and adrenal tumor (29), upon return of the cells to *in vivo* conditions. Conversely, although S-100 protein was not detectable in cells grown *in vivo* as subcutaneous clonal tumors, this protein was present at significant levels *in vitro* (Table 3). The two membrane-bound constituents, NS-1 and 2′:3′-cyclic nucleotide 3′-phosphohydrolase, therefore seem to be under different regulatory control than the soluble intracellular S-100 protein.

When clonal cell lines growing *in vitro* were treated with dibutyryl cAMP, concentrations of NS-1 as detected by absorption in the cytotoxicity assay but not of the histocompatibility H-2 antigen, underwent a small further decrease on a per cell basis. Kurth and Bauer (30, 31) have reported that after treatment of a mouse tumor cell line with dibutyryl cAMP, the concentrations of normal xenogeneic antigens decreased, but expression of tumor specific and embryonic sur-

face antigens was enhanced. Decreases in the concentrations of normal surface antigens after exposure to dibutyryl cAMP were somewhat unexpected, since under these conditions the expression of other differentiated properties of neoplastic cells often revert to a more normal state (32–34).

From these experiments it cannot be concluded whether dibutyryl cAMP influences NS-1 levels per cell quantitatively or qualitatively; i.e., in the total number of accessible antigenic sites or in their distribution on the cell surface. The topographical distribution of antigenic sites on a given cell surface area may influence antibody binding; for example, one antibody molecule may sterically block the binding of another if their antigenic targets are separated by less than the radius of the antibody molecule, or the antigens may be expressed on the cell surface, but not exposed in a manner so as to be accessible to antibody.

Dibutyryl cAMP is often found to be more effective than cAMP in producing these effects when added exogenously, presumably because it penetrates the cell better and is resistant to attack by intracellular nucleotide phosphodiesterase (35). It could act by raising the effective intracellular level of cAMP either directly (36) or indirectly by inhibiting intracellular phosphodiesterase (37).

The treatment of C_6 astrocytoma cells has been reported to produce striking β-adrenergic receptor mediated elevations in intracellular levels of cAMP (10) and enhanced glycogen phosphorylase *b* to *a* transformation, leading in turn to increased glycogenolysis (38). Gilman and Nirenberg (10) suggested that the elevation of cAMP in response to catecholamines might represent a molecular mechanism for neuronal-glial communication.

Note Added in Proof. Quantitative estimations of NS-1 on G26-19 cells by an indirect radioimmunobinding assay, using ^{125}I-labeled F(ab′)$_2$ fragments (kindly supplied by A. Williams & R. Morris, Oxford) of purified rabbit anti-mouse IgG antibody, indicated that the levels of NS-1 were 20% lower in clonal cells grown *in vitro* than in the clonal tumors grown *in vivo*. When G26-19 cells were grown *in vitro* in the presence of 1 mM dibutyryl cAMP for 3–12 hr, the NS-1 concentration was further reduced by 10% as detected by the binding assay. Similar decreases in the NS-1 concentrations were detected both by the cytotoxicity test and the binding assay when the cells were treated with 0.1 mM norepinephrine or isoproterenol.

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