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Growth Factors and Gangliosides: A Possible New Perspective In Neuronal Growth Control

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For many permanent cell lines the transition from a growing (P) to a resting (R) state is reversibly controlled by growth factors present in serum. This P-to-R transition was studied in a neuronal cell line (B104) with respect to the action of serum, dibutyryl cyclic AMP (DBcAMP), gangliosides, and a glioma cell-produced growth factor GGF. In this cell system gangliosides seem to act as differentiation and survival factors. The kinetics of uptake of radioactively labeled gangliosides and survival experiments both support the idea of the stable incorporation of exogenously added gangliosides into the cells. Based on the experimental evidence a new model of cell development is proposed. Thus in addition to the R or G_0 state, which in this cell system is rather unstable and probably regulated by cyclic nucleotides, we postulate a differentiated D state, which is controlled by gangliosides and which is characterized by its stability (survival time). This D compartment seems to be closer to the in vivo differentiated neuron than does the R or P state. The possible mechanisms for the action of gangliosides are discussed.

Key words: growth factors, gangliosides, neurogenesis, cell developmental program, neuronal cell lines

Permanent cell lines maintained in vitro have yielded much potentially valuable information concerning the molecular control of cell development. One promising concept from such culture systems is the notion that cell proliferation may be sustained by growth factors which are normally present in serum and various tissue extracts. In this context we would like to present evidence concerning the control of growth and differentiation of nerve cells in culture.

B104 cells are a neuronal cell line derived from a chemically induced rat brain tumor [1–4]. These neuroblastoma cells have a number of properties which make them a particularly useful model for studying the in vitro regulation of neuronal development. Thus, as observed with fibroblast cultures, B104 cells may exist in either a proliferating (P) state or in a state of relative quiescence (G_0 or R state), as is depicted diagrammatically in Fig. 1. In this respect B104 cells behave more like the classical 3T3 cells, since most tumor cell

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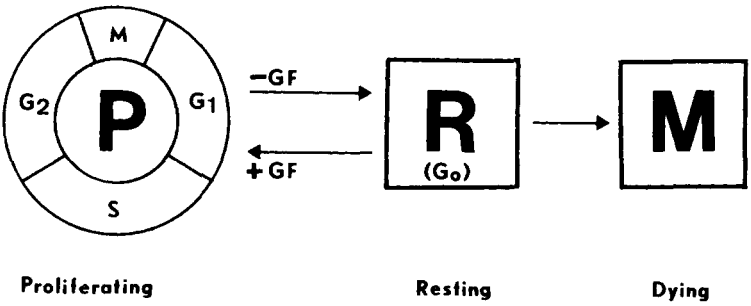


Fig. 1. Schematic representation of cell development. Cells may exist in three developmental compartments: actively proliferating (P), reversibly quiescent (R or G_0), or dying (M). For further details consult text.

lines lack the typical G_0 state of quiescence. The transition between these two cell programs is reversible and may be controlled in part by growth factors present in serum. Thus, serum starvation causes these cells to cease division and to accumulate in the G_1 (G_0) phase of their cell cycle. Subsequent addition of serum to such starved cultures elicits the induction of DNA synthesis and the resumption of normal mitotic cycling. The G_1 (G_0) arrest of B104 cells under serum starvation has been shown by flowmicrofluorimetric studies and by the kinetics of DNA synthesis after serum addition, which shows a characteristic lag period of 12–14 h (W. Seifert, unpublished data).

The transition from the P to the R state is also accompanied in this cell type by marked morphologic alterations, the cells developing long processes or neurites. Likewise there are changes in neurotransmitter synthesis, which would suggest that the movement from the P to the R program is associated with an increase in the degree of differentiation of the B104 cell.

An alternative method of provoking B104 cells to move from a proliferative to a resting state with all the accompanying phenotypic alterations is to add dibutyryl cyclic AMP (DBcAMP) to the cultures. Indeed many authors have suggested that it is an elevation of the intracellular cyclic AMP concentration which ultimately triggers the P-to-R transition whatever the method of arrest employed [5–7]. However, whether one induces the R state in B104 cultures by serum deprivation or DBcAMP addition, such cells rapidly enter into a death (M) compartment within 7–10 days of culture.

This unstable state is of course not typical of neurons in vivo, which are characterized by being very long-lived in a nonproliferative differentiated form. Therefore we describe here some results which suggest that serum-arrested cells are not in a true differentiated state, but in some intermediate and unstable condition which requires the presence of other factors to induce the complete transition from this R state to a stable differentiated compartment (D state). Specifically we propose that gangliosides play a role in this transition since they induce morphologic changes and increase the survival time of our neuronal cells, both phenomena being characteristics of a stable differentiated state.

METHODS

Growth and Handling of Cells

B104 and B12 cells were routinely cultured on 10-cm plastic Petri dishes in Eagle's medium supplemented with 10% calf serum according to standard techniques. The cells

were maintained at 37°C under an atmosphere of 5% CO_2/95% air until required for experimentation, whereupon they were plated onto 6-cm plates in the presence of 1% serum. Subsequently this medium was either adjusted to the final serum concentration, or in the case of serum-arrest conditions, the medium was removed and replaced by fresh medium containing only 0.1% serum. Cells were deemed to be serum-arrested after exposure to this low-serum regime for three days; thus in the experiments described, day 0, on which additions were made, represents this third day of starvation.

Preparation of Glial Growth Factor (GGF)

The growth activity employed in this study was merely the conditioned medium obtained from B12 glioma cells grown in the absence of serum and diluted 1:1 with serum-free Eagle's medium. In practice this involved growing B12 cells under standard conditions. At subconfluency the cells were washed three times with serum-free Eagle's medium and then left for a further 24 h in serum-free medium. Subsequently this medium was replaced by fresh serum-free medium, which 24 h later was used as a source of GGF (B12-conditioned medium).

Cell Cycle Analysis

This was performed with a Biophysics 4801 cytofluorograph. The cells were pretreated with RNAase and pepsin and stained with ethidium bromide.

DNA Synthesis

Cells were labeled for a 4-h period with tritiated thymidine (Amersham) and thymidine cold carrier to give a final radioactivity level of 2.5 μCi/ml and cold carrier concentration of 2×10^{-6}M. At the appropriate time the supernatant medium was decanted and cells removed from the plate by trypsinization. DNA was precipitated in 5% trichloroacetic acid (TCA) and harvested onto a Whatman GF/A fiberglass filter. The filter was washed three times with 5% TCA and once with absolute ethanol, dried, and counted in a suitable scintillation fluid in an Intertechnique scintillation counter.

Survival Experiments

B104 cultures were maintained in serum-free conditions for three days, at which time the appropriate additions were made. Cell viability was then monitored over subsequent days, and a culture was deemed dead when cell numbers had decreased to 10% of that observed on the day of additions (ie, day o). Surprisingly, cell death appeared to be very consistent in triplicate culture plates. Cell numbers were normally estimated by hemocytometry since Coulter counting was found to be very unreliable for this cell type under arrest conditions (Table I, Fig. 4). In another set of survival experiments the attached and floating cells were combined and the plating efficiency was determined by a count of the numbers of colonies formed in appropriate dilutions into Eagle's medium + 10% serum after growth for 3–4 days (Table II).

Ganglioside Uptake

Gangliosides (Sigma type III) were tritiated in their sialic acid moiety by sodium metaperiodate oxidation and subsequent tritiated sodium borohydride reduction (Amersham) according to an established procedure [8]. Authenticity of labeling was established by chromatographing the product of silica gel F_{254} thin-layer chromatography plates (Merck) in a solvent system made up of n-butanol : pyridine : water containing 1 mg/ml KCl

TABLE I. Influence of Various Treatments of DNA Synthesis and Survival of B104 Cells

Treatment	Relative DNA synthesis	Days of survival
No additions	1.0	6
1% Calf serum	10.2	9
GGF	6.7	6
Gangliosides (100 $\mu g \cdot ml^{-1}$)	2.1	> 30
DBcAMP (5×10^{-4}M)	0.5	5
DBcAMP + gangliosides (100 $\mu g \cdot ml^{-1}$)	2.1	18
Isobutyrylmethylxanthine (10^{-4}M)	0.7	6
Isoproterenol (10^{-4}M)	0.2	4
Serotonin (5×10^{-3}M)	0.3	5

B104 cultures were serum-arrested for three days as described in Methods. Subsequently the cultures were exposed to various treatments, and DNA synthesis and cell survival time were monitored. DNA synthesis was assessed by tritiated thymidine incorporation into DNA 20 h after additions were made. In order to compare values obtained over a period of time, the data have been normalized to the no-additions control; that is, counts observed in any given regimen have been divided by the no-additions control value for that particular experiment. Survival times were assessed as described in Methods. All data points are the mean value derived from at least three determinations.

TABLE II. Colony-Forming Capacity of Serum-Arrested B104 Cells With and Without Gangliosides (number of colonies formed after 3 days from combined attached and floating cells)

	No additions	Addition of gangliosides
Day 0	1.70×10^5	1.70×10^5
Day 4	0.73×10^5	3.00×10^5
Day 7	0.17×10^5	3.60×10^5
Day 17	$< 0.03 \times 10^5$	3.92×10^5

The numbers are the colonies formed after 3–4 days from the combined attached and floating cells. To plates of B104 cells serum-arrested for three days were added either 100 μl of a 5 mg/ml ganglioside solution (ganglioside-treated) or 100 μl of Eagle's medium (no-addition control). After various numbers of days, a proportion (2%) of both those cells floating and those cells attached were added to 5 ml of Eagle's medium + 10% calf serum on a fresh plate. The cells were left 3–4 days to attach and form colonies. The number of colonies per plate was then estimated by counting in a random fashion until 50 colonies had been scored. The number of viewed microscope fields (1 mm^2) required to reach this total was noted. This value was then converted into number of colony-forming cells per original plate. The table shows the average value from triplicate cultures for both ganglioside-treated and control plates on various days after additions were made.

9 : 6 : 4 v/v). Radioactive bands were located by means of a Beta Camera (Berthold 290 A), and tritiated gangliosides and pure ganglioside markers were identified by the resorcinol method [9]. The vast majority of the radioactivity was associated with resorcinol-positive bands detectable in the original ganglioside preparation. Traces were also identified in bands which migrated as G_{M2} and G_{M3}, and which were presumably present in the Sigma preparation at concentrations too low to resolve using the resorcinol method of staining.

For ganglioside uptake experiments, cells were exposed to tritiated gangliosides at a final concentration of 50 μg/ml (approximately 2×10^6 cpm). At the desired times the supernatant medium was removed and the cells were washed twice with 5 ml of phosphate

buffered saline (PBS) at room temperature..The cells were then trypsinized and counted directly in Unisolve scintillant for their radioactive content.

RESULTS

The results depicted in Fig. 2 and Table I indicate that serum arrest results in the reduction of DNA synthesis in B104 cultures and an accumulation of the cells in G_1/G_0. As observed in many cell types serum addition to arrested cultures provokes the induction of DNA synthesis and resumption of mitotic cycling (Fig. 2, Table I). We also find that the conditioned medium from the B12 glioma cell line [1] like serum has the ability to restart DNA synthesis (Fig. 2, Table I). This activity is also present to a lesser extent in the conditioned medium of other glioma cell lines (C_6, B 23), but does not seem to be present in the conditioned medium of fibroblasts (3T3) or C1300 neuroblastoma cells (W. Seifert, unpublished data). In two previous abstracts [10, 11] we have referred to this growth activity as glial growth factor (GGF). Thus, it may be that glial cells in vivo have some role to play in neurogenesis by the secretion of factor(s) which influence the development, maintenance, or regeneration of neurons. Thus serum and GGF tend to support an R-to-P transition which is characterized by a resumption of mitotic cycling and a morphologic change to the fibroblastic phenotype typical of growing B104 cells (Fig. 3).

In contrast to serum and GGF, dibutyryl cyclic AMP and agents such as the phosphodiesterase inhibitor isobutyrylemethylxanthine (IMX), as well as adenylate cyclase stimulants such as isoproterenol and serotonin, all suppress DNA synthesis and induce morphologic changes indicative of the R state (Table I). However, the resting state of the B104 cell elicited by serum deprivation and the various cyclic AMP-elevating regimens is relatively unstable, the cells dying in culture after about seven days (Table I). We have previously established [11] that mixed brain gangliosides induce, like GGF, a modest stimulation of DNA synthesis (Table I). As assessed by Flowmicrofluorimetric analysis (FMF), however, they do not markedly induce G_0 cells to reenter their DNA synthetic S phase, that is, they are not mitogenic in the same sense that serum and GGF are mitogenic (Fig. 2). In fact the apparent stimulation of DNA synthesis by gangliosides appears to be attributable to a powerful survival action on serum-starved B104 cells (Table I), such that cell numbers do actually increase slowly over a period of time (Fig. 4) because of the low level of residual proliferation observed even in serum-arrested cultures. In addition the small increment in DNA synthesis evident in ganglioside-treated cultures is insensitive to treatment with DBcAMP at concentrations which markedly impair DNA synthesis in normal control cultures (Table I).

Furthermore gangliosides can enhance the survival time of serum-starved cultures and of cultures which have been arrested by cyclic AMP (Table I). Thus gangliosides would seem to act at a different locus than do agents which either simply stimulate an R-to-P transition (ie, GGF and serum growth factors) or agents and treatments which cause arrest of division (ie, serum-deprivation or cAMP-elevating regimens). This is perhaps emphasized by the observation that after some 14–30 days of ganglioside treatment serum-arrested B104 cultures attain another morphology characterized by numerous radial neurites around the central cell body, quite different from the predominantly bipolar shape of the serum-arrested cells (Fig. 3). Remarkably, even if the medium is removed from these 30-day-old cultures and replaced with serum-free medium containing no gangliosides, the survival effect seems to persist. In fact the action is only lost following two subsequent passages of the cells (unpublished data).

This survival effect of gangliosides is also demonstrated in Fig. 4 and in Table II.

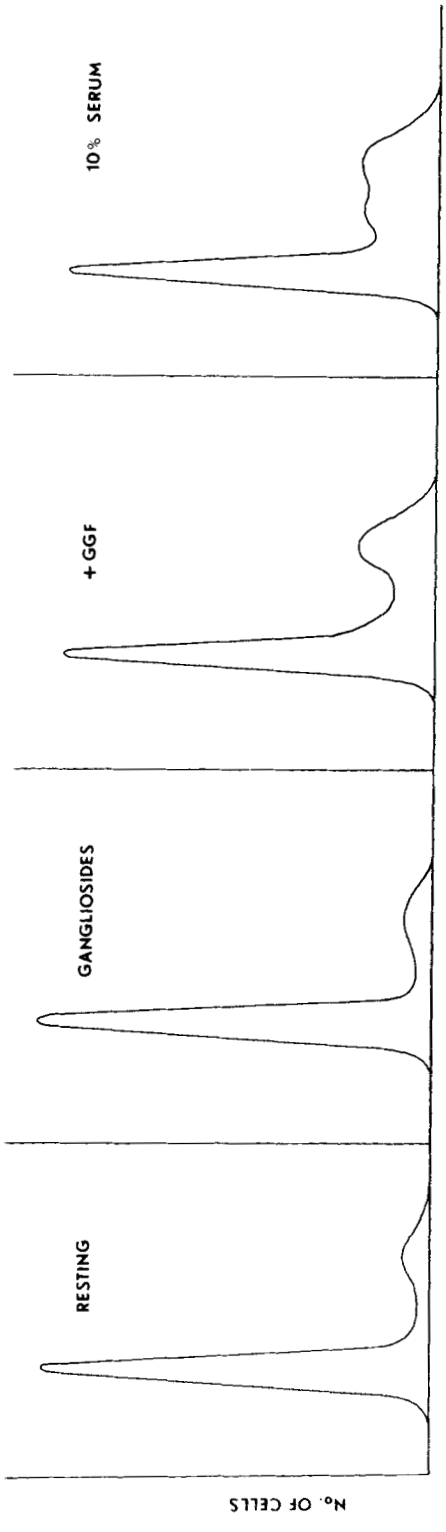


Fig. 2. Action of growth factors and gangliosides on the distribution of B104 cells in the cell cycle. B104 cells were serum-arrested for three days and then treated with addition of 10% serum, GGF, gangliosides (0.1 mg/ml, Sigma type III), or left without further additions. Twenty-four hours later the cells were harvested and processed for flowmicrofluorimetry as described in Methods. The results are typical traces obtained from one series of cultures.

In control cultures of serum-starved B104 cells (no additions) the number of attached and viable cells (Fig. 4) or of colony-forming cells (Table II) is rapidly decreasing, so that only about 10% viable cells are left after seven days and practically none after 17 days. In contrast, ganglioside addition to these cultures leads to survival and even to a small but significant increase, in cell numbers (Fig. 4, Table II). This is in good agreement with the observed small increment in DNA synthesis (Table I) which most likely reflects the small percentage of cells which under these conditions were not arrested in the G_0 state. It should be noted here that in contrast with the neuroblastoma C1300 cells, which may be attached or grow in suspension depending on serum conditions and growth state, the B104 cells are only viable when attached. Floating cells are no longer able to form colonies. Nevertheless the results in Table II were obtained by combining attached and floating cells and counting the numbers of colonies formed after replating. The results substantiate the observations demonstrated in Figure 4, where only the attached and viable cells were counted.

Preliminary experiments would further indicate that resting B104 cells do take up gangliosides in a time-dependent fashion (Fig. 5). In contrast, incorporation into growing cultures is markedly lower than in resting cultures. However, simply replacing the serum-containing medium by serum-free medium very rapidly leads to a large increase in uptake, thus suggesting that serum is binding gangliosides and simply making them unavailable to the cells (Fig. 5). Such an idea is corroborated by trapping labeled gangliosides bound to serum on millipore filters (unpublished). However, the time course of incorporation into resting cultures is in good biologic agreement with the kinetics of DNA synthesis induction and reversibility studies which indicate that cells require about 8 h of prior exposure to gangliosides before their action becomes irreversible.

DISCUSSION

Serum and GGF both have the ability to stimulate serum-arrested B104 cells to initiate DNA synthesis (Fig. 2, Table I). This involves a morphologic change to the fibroblastic P-state phenotype (Fig. 3). On the other hand serum deprivation or exhaustion of the serum growth factors in P-state cultures results in a cessation of DNA synthesis, an accumulation of the cells in G_1, and an induction of neurite formation (Fig. 2, Table I). A similar P-to-R transition may be attained by addition of DBcAMP or agents which would be expected to elevate endogenous cAMP (Table I). However, despite this apparent differentiation, which can be morphologically quite extreme in the case of DBcAMP-induced arrest, this R state is unstable and the cells die (Tables I and II, Fig. 4). Gangliosides appear to act as a third type of effector molecules, since their mode of action is different from that of true mitogens or arresting agents. Thus it would appear that gangliosides do not depress DNA synthesis as does DBcAMP; indeed they appear to slightly enhance thymidine incorporation in resting cultures (Table I). This elevation of DNA synthesis would seem to be attributable to the presence of more viable cells in ganglioside-treated cultures (Tables I and II), this interpretation being further supported by the fact that FMF analysis shows no marked recruitment of G_0 cells into S phase. In this context gangliosides do not act as a mitogenic factor like GGF or serum factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet factor. It might also be noted that gangliosides possess far more survival activity for B104 cells than does serum itself (Table I).

In this respect the survival activity of gangliosides on B104 cells superficially resembles the stabilizing effect of factors present in platelet-poor plasma on quiescent G_0-arrested fibroblasts [12]. However gangliosides do not stabilize resting 3T3 or W138 fibroblasts (unpublished results). In addition ganglioside-treated B104 cells apparently progress into a

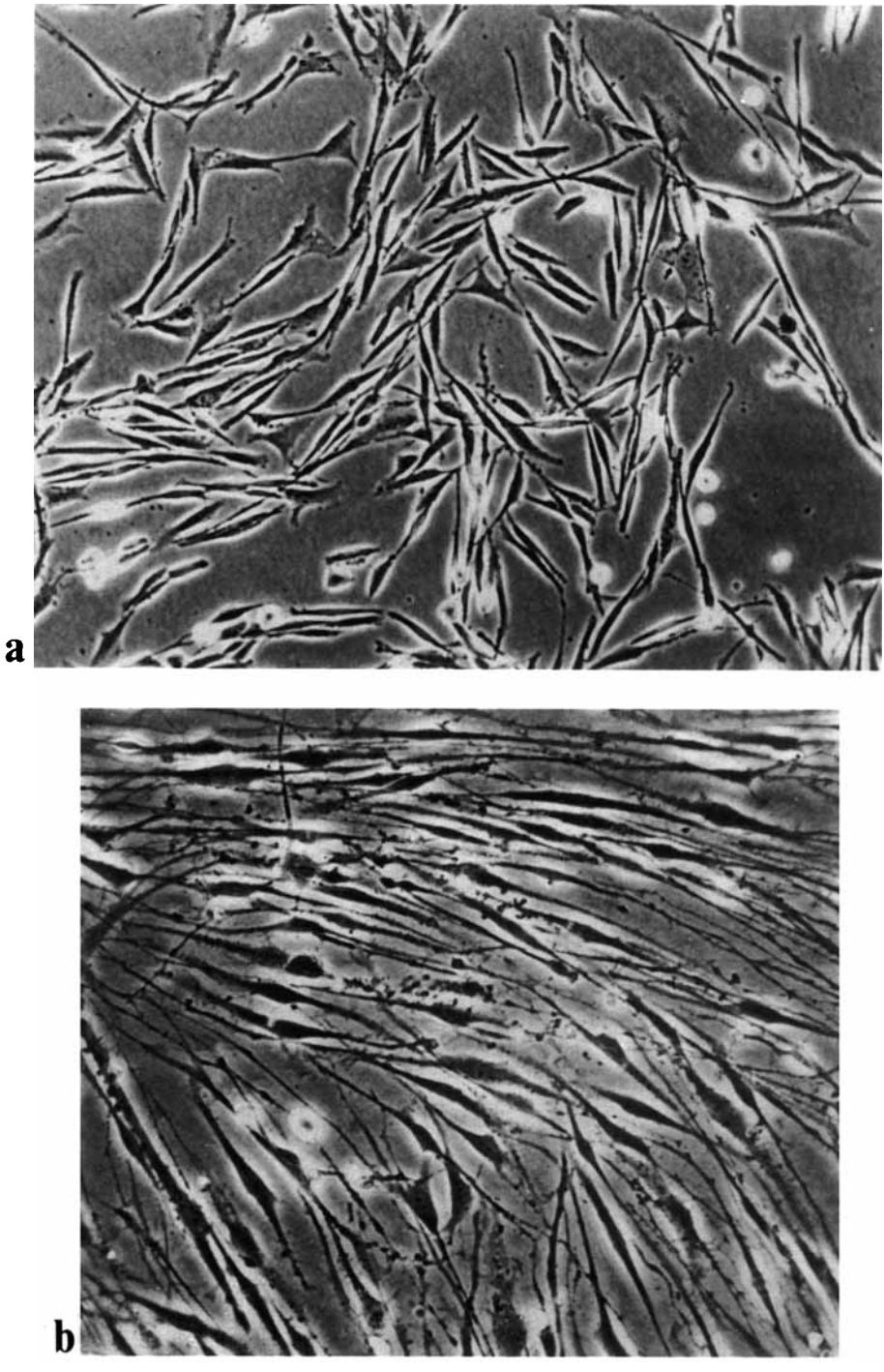
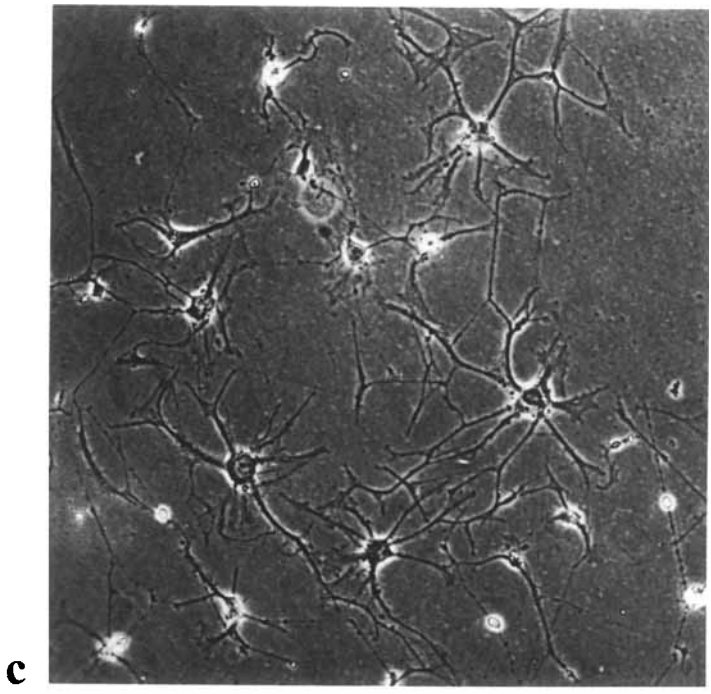


Fig. 3. Photomicrographs of B104 cells. a) B104 cells actively growing in Eagle's medium in presence of 10% calf serum(P compartment). b) B104 cells arrested in the G_0 or R state by serum starvation



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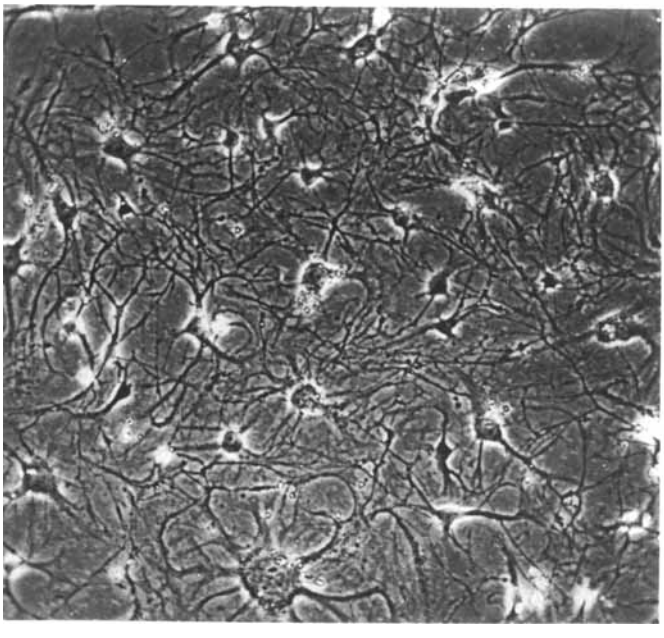

d

(R compartment). c,d) B104 cells after serum arrest and treatment with gangliosides for 10 days: c) sparse culture; d) dense culture. (D compartment, see model in Fig. 6.)

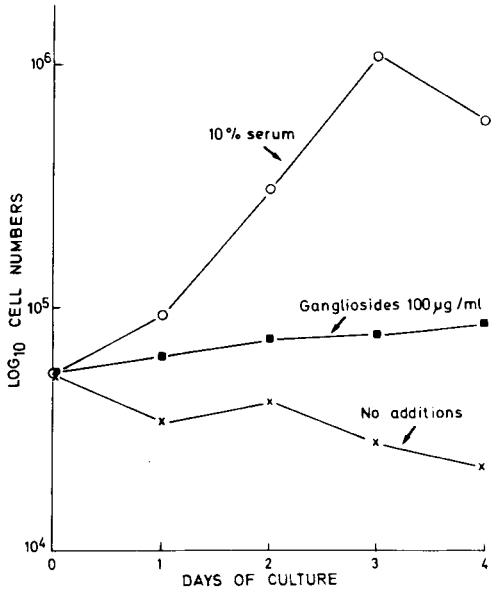

Fig. 4. Growth or survival of serum-arrested B104 cells. B104 cells were serum-starved for three days and then treated with 10% serum or gangliosides (0.1 mg/ml) or no additions. The number of cells were counted each day in triplicate culture plates by hemocytometry. Further details in Methods on survival experiments.

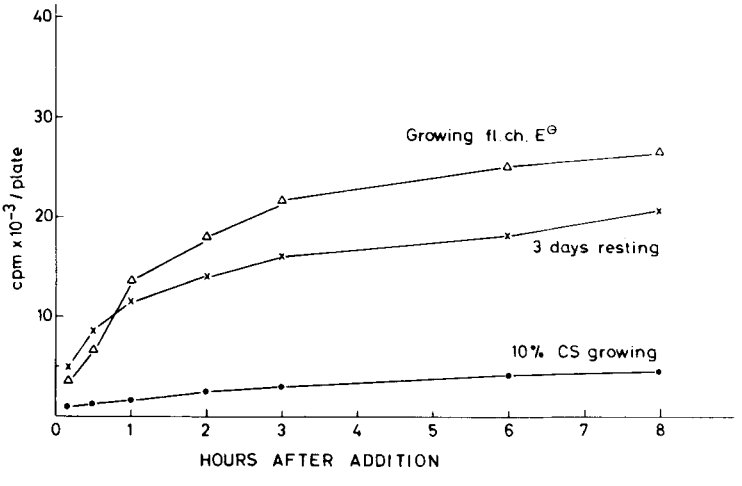

Fig. 5. Uptake kinetics of ^{3}H-gangliosides by B104 cells. Gangliosides (Sigma type III) were tritiated and added to cultures at a final concentration of 50 µg/ml (~ 1.6 X 10^6 cpm) as described in Methods. Three culture conditions were employed: One group of cells had been serum-arrested for three days prior to addition of the radioactive gangliosides (X). A second large batch of cells were actively proliferating in 10% serum (•); however, some of these cultures (fl. ch. E^-) had been transfered one hour prior to labeling into a medium devoid of serum (Δ). Cells were harvested from the plates at the times indicated as described in Methods. The points are the mean of duplicate determinations.

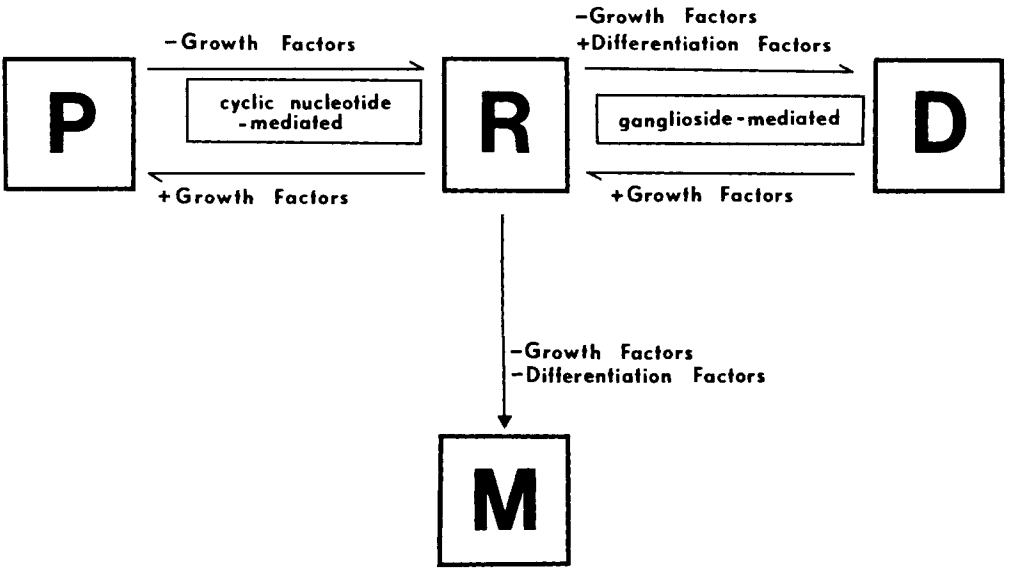

Fig. 6. Proposed model of B104 developmental programs. The details of this model are largely as described in the text. P) Proliferative cell program, R) resting cell compartment, M) death or moribund phase, D) stably differentiated developmental program. The various program transitions are indicated by arrows, all being reversible with the exception of the M state. The transitions are mediated by either cAMP or gangliosides as indicated, there being a cAMP-regulated morphologic differentiation intrinsic in the P-to-R transition.

further cell compartment as indicated by morphologic changes (Fig. 3). This new morphology may be a consequence of time in culture and not directly caused by gangliosides. Thus gangliosides would appear to mediate some process which permits the progression of these cells from the classical G_0 state into a more stable and more differentiated state.

One interpretation of the observations concerning the interaction between gangliosides and growth-arresting regimes is shown in Fig. 6. Thus, we propose that the initial events involved in the movement of a cell out of the P state involves the elevation of endogenous cyclic AMP. This directly induces morphologic and biochemical changes, and such cells arrest division at some restriction point in G_1, thus entering the resting G_0 or R state. Since gangliosides appear to be able to influence cAMP effects whilst cAMP does not markedly affect ganglioside action, it might be proposed that gangliosides act on a process subsequent to that controlled by this cyclic nucleotide. Therefore gangliosides may stabilize the R state and perhaps induce further differentiation, as might be evidenced by the new cell morphology attained in such cultures (Fig. 3) and by the increased survival time (Table II, Fig. 4). This might then be deemed a fully differentiated (D) state. Such a compartment might only lose cells very slowly into the death (M) phase, whereas the R state has a high rate constant into the moribund compartment. Furthermore, in our hands gangliosides, when added to serum-stimulated cultures, do not induce differentiation, and indeed they appear to synergize with serum and promote proliferation (unpublished data). Thus, to exhibit their survival action gangliosides may require cells to have first undergone the presumptively cyclic nucleotide-dependent transition into the R state.

Mechanistically we have not isolated the site of action of gangliosides. However, it does appear that gangliosides do enter B104 cells in quite appreciable amounts (Fig. 5).

In addition we have determined that both growing and serum-arrested B104 cells contain only detectable levels of G_{M_2} (unpublished), a ganglioside not normally present in high amounts in brain or in our commercial ganglioside preparations. Thus B104 cells, like many transformed brain cells, do not contain the higher polysialogangliosides thought to be present in neurons [13, 14]. Therefore, our cells are biochemically undifferentiated with respect to their ganglioside content. They become differentiated with respect to survival, morphology, and ganglioside pattern by exogenous addition and subsequent incorporation of gangliosides. Another observation indicates a direct correlation between endogenous ganglioside pattern and survival: B104 variant clones (mutants) which show increased survival in the serum-arrested state without exogenous ganglioside addition seem to have additional ganglioside species not found in the B104 wild type (unpublished data).

We propose three possible mechanisms by which gangliosides may act (Fig. 7).

In Fig. 7a we depict the first possibility and this may be a function of transformation. We suggest that removal of serum growth factors normally leads to growth arrest (via cAMP) and the triggering of an unknown endogenous process which leads to various cellular changes, one of which is alteration in ganglioside metabolism. Such ganglioside changes must be essential for survival of normal nonproliferating cells. In the tumor cell the cyclic nucleotide changes may still occur, but there may be a defect in the mechanism which causes ganglioside pattern alterations. Thus by adding gangliosides we bypass this defect and artificially stabilize and differentiate the cell.

Figures 7b and 7c are related. This model proposes that as well as growth factors there are also differentiation or survival factors (DF) present in the system. Such agents may be contained in serum or may be secreted by the cells themselves (an endogenous survival factor) or by other cell types. Figure 7b then proposes that one of the gangliosides added enters the cell membrane, where it acts as a receptor for such a putative molecule (DF); that is it might sensitize the cell to the low levels of such factors, which might still be present in the medium or which might be secreted by the cell. In mechanism 7c it is proposed that the survival factors (DF) trigger an intracellular signal, which results in a subsequent change in ganglioside metabolism. Thus, as in 7a, addition of gangliosides bypasses the survival factor/cell interaction. Certainly there are precedents for two cellular axes in some cell types which encompass the possibility of a new class of differentiation/survival factors [15]. Indeed one such differentiation factor for nerve cells of sympathetic or sensory type that combines differentiating and survival properties is the well-established nerve growth factor NGF [16, 17].

At present we are attempting to unravel which of these mechanisms is the one responsible for gangliosides producing a stable differentiated state.

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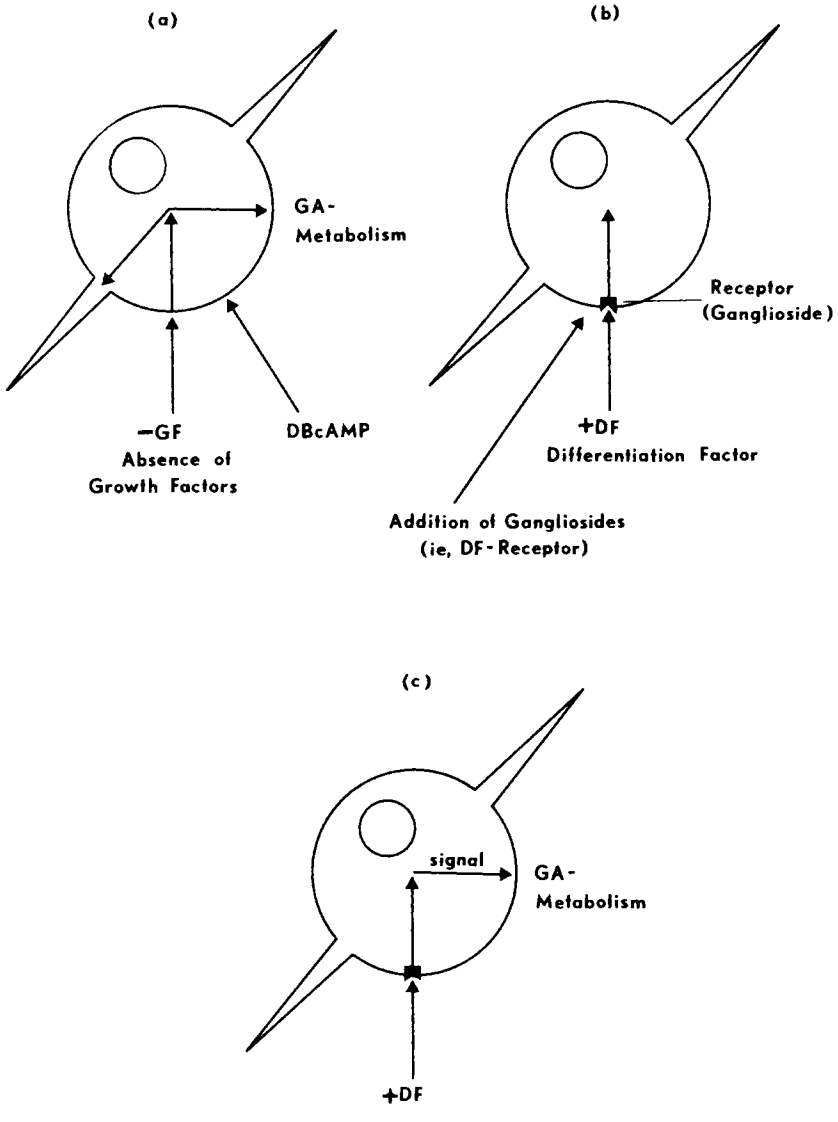

Fig. 7. Three possible mechanisms of action of gangliosides. Mechanisms illustrated are as described in Discussion. a) The absence of growth factors (−GF) or the presence of DBcAMP bring about changes in endogenous ganglioside (GA) metabolism, which promotes an R-to-D transition. b) Gangliosides are represented as putative receptors for exogenous survival or differentiation (DF) factors. c) Putative differentiation factor(s) act via a nonganglioside receptor to institute a signal which subsequently alters ganglioside (GA) metabolism.

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