Factors released by rat type 1 astrocytes exert different effects on the proliferation of human neuroblastoma cells (SH-SY5Y) in vitro

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Abstract

Brain metastases derived from abdominal neuroblastoma are an uncommon complication of this tumour; however, an increase in their occurrence has recently been reported. In the present study, we have investigated the influence of factors derived from central nervous system glial cells on the proliferation of human neuroblastoma cells (SH-SY5Y) in vitro. Co-culture experiments show that a 24-h exposure to factors released by type 1 astrocytes (A1) may induce a significant decrease in [3H]thymidine ([3H]TdR) incorporation by SH-SY5Y cells. This effect was not duplicated by fresh A1-conditioned medium (A1-CM); A1-CM became active only when it was heated or frozen. In contrast to this short-lived inhibitory effect, long-term treatment (3, 6 and 9 days) with A1-CM produced a significant and dose-dependent increase in SH-SY5Y cell number. Immunoneutralisation of A1-CM with an anti-transforming growth factor-β antibody eliminated the inhibitory effect on [3H]TdR uptake in SH-SY5Y cells, but did not affect the increased number of viable cells observed after long-term treatments.

In conclusion, these results showed that factor(s) released by A1 may affect the proliferation/survival of a human neuroblastoma cell line in vitro inducing: (a) a short transient negative effect on DNA synthesis and (b) an overall sustained trophic action. These results are suggestive of a possible role of glial cells in the establishment of brain metastases of neuroblastomas.

Introduction

Neuroblastomas are solid tumours of childhood characterised by undifferentiated cell phenotypes and a poor prognosis. Occasionally, these tumours show spontaneous regression because of their differentiation into benign ganglioneuromas. Unfortunately, the mechanisms which drive this differentiation are still poorly understood. The neuroblastoma is usually detected as a solid abdominal mass with diffuse metastasis, although invasion of brain tissue was believed to be a rare manifestation of extra- or intracranial neuroblastoma. However, it has been reported recently that 68% of patients affected by neuroblastoma or ganglioneuroblastoma suffer from neurological complications because of undiagnosed or hidden brain metastases (Weyl-Ben Arush et al. 1995, Tasdemiroglu & Patchell 1997). These lesions may appear as a relapse after chemotherapy, and they may become evident after the disappearance of tumoral tissue from the usual localisation (Shaw & Eden 1992, Astigarraga et al. 1996, Bouffet et al. 1997, Lydaki et al. 1997).

The nervous system is composed of different types of cells which include, in addition to neurones, glial elements (e.g. astrocytes, oligodendrocytes, Schwann cells etc.). In the last few years it has become evident that glial cells may play a relevant role in modulating several neuronal functions. Glia-neurone interactions were shown to control neuronal migration and differentiation, to provide spatial and metabolic support to neurones, and to modulate their synaptic activity (Banker 1980, Lindsay 1987, Hatten & Mason 1990, LoPachin & Aschner 1993). Glial cells, and astrocytes in particular, were found to release many factors (e.g. neurotransmitters, neuropeptides, cytokines, growth factors etc.) known to possess trophic or differentiating activities on...
neuronal and non-neuronal (Giulian et al. 1988, Muller et al. 1995). Consequently, the establishment of brain metastases of tumoral cells might also depend, at least in part, on the trophic support provided by glial elements.

Many studies have shown that the conditioned media (CM) in which glial cells are grown can induce neuritogenesis and a neurone-like morphological differentiation of neuroblastoma cells. Even though these results appear to be in apparent contrast with the possibility of establishment of brain metastases of these tumours, the data concerning the effects of secretory products of the glial cells on the proliferation of neuroblastoma cells (Monard et al. 1973, Kato et al. 1983, Sakazaki et al. 1983, Lim et al. 1990, Amano et al. 1994) sometimes appear to be incomplete. The reasons for such discrepancies may reside in the different experimental conditions adopted. For instance, a number of these studies have been performed using the CM obtained from tumoral glial cells (e.g. glioma cells) rather than from untransformed astrocytes; moreover, it has recently been reported that the glial factors involved in the differentiation and neuritogenesis of neuroblastoma cells are apparently released only from young and subconfluent astrocytes (Shea et al. 1994). On the other hand, the presence of brain metastases may suggest a possible role for the glial elements on the survival and/or proliferation of neuroblastoma cells. Also, considering that astrocytes in vitro may behave differently from those in vivo, the utilisation of cells obtained from primary cultures may provide useful information on the trophic factors physiologically produced and released by these glial cells. The aim of the present study was therefore to investigate whether cultures of neonatal type 1 astrocytes might produce and release in vitro some factor(s) which might exert trophic activity on neuroblastoma cells.

The experiments were conducted using the human neuroblastoma cell line SH-SY5Y (Biedler et al. 1973); these cells were subcloned from the parental SK-N-SH cell line, obtained from a bone marrow metastasis of a neuroblastoma, and have been extensively characterised (Pahlman et al. 1990).

Materials and methods

Cell cultures

Primary cultures of type 1 astrocytes (A1) and type 2 astrocytes were obtained from the cerebral cortex of 1- to 2-day-old rats as previously described (Melcangi et al. 1993). Human SH-SY5Y neuroblastoma cells (kindly provided by Dr June Biedler, Sloan–Kettering Memorial Cancer Center, New York, NY, USA) were derived from the parental SK-N-SH cell line which was established from a bone marrow metastasis of a 4-year-old girl (Biedler et al. 1973). The cells were grown at 37°C in a humidified CO₂ incubator in minimum essential medium (MEM) (Biochrom, KG, Berlin, Germany) supplemented with non-essential amino acids, 1 mM sodium pyruvate, 100 µg streptomycin/ml, 100 IU penicillin/ml, 10 mg phenol red/l (Biochrom KG, Berlin, Germany) and 10% foetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Confluent cells were harvested with 0.05/0.02% trypsin/EDTA and 1.10⁶ cells were seeded in 57 cm² dishes. The medium was replaced at 2-day intervals.

The neutralising pan-specific anti-transforming growth factor-β (TGFβ) antibody (directed to rat(human)TGFβ1, 1, 2, 3 and 5; R&D Systems, Minneapolis, MN, USA; catalogue no. AB-100-NA) was diluted according to the manufacturer’s instructions.

Collection of CM

Glial cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Biochrom, KG, Berlin, Germany), supplemented with 10% FBS, 100 µg streptomycin/ml and 100 IU penicillin/ml (Melcangi et al. 1993), until confluence was reached. To avoid interference because of factors present in the FBS, the culture medium was replaced with Sato’s chemically defined medium (DMEM supplemented with 5 µg bovine insulin/ml, 50 µg human transferrin/ml, 20 nM progesterone, 100 µM putrescine dihydrochloride, 30 nM sodium selenite (Sigma Chemical Co., St Louis, MO, USA) and the cell cultures were returned to the incubator; the medium (CM) was collected 24 h later and used immediately or stored at –20°C as described in the Results section. Sato’s medium alone left in the incubator for 24 h was stored as a control.

Co-culture experiments

Neuroblastoma cells were plated in six-well tissue culture plates, while A1 were plated in 25 mm cell culture inserts (Falcon; Becton Dickinson Labware, High Wycombe, Bucks, UK). The bottom of the insert is an opaque membrane with a pore size of 0.45 µm which is specifically designed to provide independent access of the medium to both sides of the membrane. Cell culture inserts containing confluent purified A1 at 95% confluence were transferred to wells in which neuroblastoma cells had been plated 24 h previously. At the same time the medium was replaced with fresh Sato’s defined medium.

Neuroblastoma cells cultured alone in six-well plates and pure A1 cultured alone on the well inserts were utilised as controls. Four hours before the end of the co-culture period, the cultures were processed for [³H]thymidine incorporation and cell counting performed as described below.

[³H]Thymidine incorporation

DNA synthesis was evaluated by measuring the incorporation of radiolabelled nucleotides in trichloroacetic acid-
precipitable fractions of the cells, after a short period of exposure of the cells (less than half of their doubling time) to the factors under investigation. This allows evaluation of the rate of DNA synthesis in the absence of significant modifications of cell number which, however, was verified at the end of the incubation period. For evaluation of the incorporation of [methyl-3H]thymidine ([3H]TdR) (Amersham, Milan, Italy), the cells were seeded onto 24 wells/plate (50 000 cells/well). Three days later the media were replaced with fresh medium with or without FBS; 48 h later the medium was replaced with control medium or glial cell-CM. A pulse of [3H]TdR (10 µCi/ml) was added 20 h after the beginning of the various treatments and left in the culture medium for 4 h. The cells were then washed with 0.5 ml Dulbecco’s phosphate-buffered saline prewarmed to 37°C, fixed with 1 ml ice-cold 10% trichloroacetic acid and 10 min later lysed with 0.1 ml 6M NaOH. A parallel set of wells from the same plate was processed as for [3H]TdR incorporation and the cell number evaluated at the end of the washing procedure. The cell lysates were then collected and mixed with 7 ml scintillation cocktail (Instagel; Packard, Milan, Italy) and counted in a scintillator for beta emitters (TriCarb 6000; Packard) with 60% efficiency.

**Evaluation of cell viability**

The number of cells, counted using a haemocytometer, and the cell viability were taken as the measure of cell proliferation and survival in long-term experiments. To this purpose, SH-SY5Y cells were seeded in 24 wells/plate (30 000 cells/well) and 3 days later the media were replaced with control medium or type 1 astrocyte-conditioned medium (A1-CM). The media were changed every 3 days throughout the period of treatment. The viable cells were measured by counting the cells with a haemocytometer after trypan blue staining or by using the diphenyl-tetrazolium salt (MTT) procedure (Manthorpe et al. 1986). Briefly, cells exposed to the various treatments were washed with warm culture medium and incubated for 1 h at 37°C with a solution of MTT (1 mg/ml; Sigma Chemical Co.) dissolved in culture medium without FBS and phenol red. The solution was then decanted and 1 ml/well isopropanol was added to dissolve formazan crystals. The absorbance at 560 nm was measured by a microplate spectrophotometer; the absorbance of blank well and that measured at 630 nm were then subtracted; the remaining signal was taken as an index of the number of living cells in the culture. The linearity of the response (r > 0.98) of the MTT assay ranged from 0.001 to 0.3 units of absorbance, corresponding to 5000 and 250 000 cells/well respectively. The assay was run in triplicate (the coefficient of variation among replicates was less than 10%).

Every other result presented in this study was obtained from three to four independent experiments run in four replicates.

**Statistical analysis**

The results were analysed by ANOVA and adequate post-hoc tests (Student’s t-test, Dunnett). The dose–response curves were analysed by means of a Macintosh version of the program ALLFIT (De Lean et al. 1978).

**Results**

The study was initially conducted using confluent rat A1 and SH-SY5Y neuroblastoma cells maintained in co-culture in a two-chamber device. To ensure that the effects observed were due directly to factor(s) released from the astrocytes, and not mediated by principles present in FBS, a chemically defined medium (Sato’s medium) was used throughout the experiments.

The results of the co-culture experiments are illustrated in Fig. 1. For simplicity, the data are presented as per cent variations vs the respective controls. All necessary statistical analyses were performed, however, on absolute values. Figure 1a shows that a 24-h co-culture with A1 significantly inhibited the rate of incorporation of [3H]TdR in SH-SY5Y cells when compared with that occurring in neuroblastoma cells cultured alone in chemically defined medium. There were no significant modifications of the number of living neuroblastoma cells, evaluated by counting the cells at the end of the 24 h of co-culture, using the trypan blue exclusion method (Fig. 1b). This suggested that the decrease observed in the incorporation of [3H]TdR is linked to a decrease in DNA synthesis, rather than to a decrease in the cell viability caused by toxic factors released by astrocytes. A more prolonged period of co-culture (48 h) with A1 allowed [3H]TdR incorporation in SH-SY5Y cells to return to control levels (Fig. 1a). After 48 h of co-culture with A1, the number of living neuroblastoma cells was significantly increased vs controls (Fig. 1b). Apparently, DNA synthesis returns to normal levels allowing cell duplication.

To reduce the complexity of the experimental model, subsequent experiments were performed by evaluating the incorporation of [3H]TdR in SH-SY5Y cells exposed for 24 h to media in which A1 had been cultured for 1 day (A1-CM; see Materials and methods). As shown in Fig. 2a, 24 h of treatment of SH-SY5Y cells with fresh A1-CM, used immediately after collection, surprisingly did not induce any modification of the incorporation of [3H]TdR.

The effects of the same A1-CM on the viability of neuroblastoma cells were subsequently evaluated at much longer time-intervals (3, 6 and 9 days). It was observed that control SH-SY5Y neuroblastoma cells grew up to 6 days after transfer to a chemically defined medium; afterwards, the number...
Effects of co-culture between SH-SY5Y cells and A1 in chemically defined medium on (a) \[ \text{H}\text{TdR} \] uptake and (b) number of SH-SY5Y. A1 were cultured on 0.45 µm membrane inserts and were in contact with the culture medium present in culture wells containing SH-SY5Y cells adhering to the plastic surface. In this system only the exchange of soluble signals is possible. The number of living cells was evaluated with a haemocytometer and the trypan blue exclusion test. The values represent the means±S.D. of three independent experiments run in triplicate. \(* P<0.05\) vs control (C).

Of viable cells decreased to reach, at the longest time of observation (9 days), a value significantly lower than that found at 6 days and close to that measured at the beginning of the experiment (time 0) (Fig. 2b). Visual inspection of the cultures showed that this effect was linked to a morphological differentiation of SH-SY5Y cells, which was then followed by cell death (data not shown), probably caused by the absence, in the chemically defined medium, of trophic factors which support the growth of SH-SY5Y cells. When the neuroblastoma cells were incubated in freshly collected A1-CM, a significantly greater number of viable SH-SY5Y cells was observed at any time-interval when compared with control cells; this effect, already apparent at day 3 of incubation, became much more evident at 6 and 9 days (Fig. 2b).

In preliminary experiments, we had observed that A1-CM still showed its stimulatory activity when applied to SH-SY5Y cells in the presence of FBS (after 9 days of treatment: control, 0.21±0.03; A1-CM, 0.47±0.03, units of absorbance at 560 nm±S.D. \( P<0.05 \)), indicating either the presence of growth-stimulatory factors in the A1-CM differing from those present in FBS, or the possibility of addictive effects between the growth-stimulatory principles present in A1-CM and in FBS.

Figure 3 shows that the effect of A1-CM was dependent on its concentration in the culture medium. No morphological differentiation of SH-SY5Y cells treated with A1-CM was observed at 3, 6 and 9 days of observation.

In order to verify the physico-chemical stability of the factor(s) responsible for the effects exerted by A1-CM, this CM was heated to 70°C for 20 min, a treatment known to induce denaturation of protein secondary structures.

The results showed that this treatment did not decrease the activity of A1-CM on SH-SY5Y cell viability; a growth curve similar to the one represented in Fig. 2b was actually obtained (data not shown). However, a significant inhibitory effect on \[ \text{H}\text{TdR} \] incorporation, similar to that observed in co-culture experiments (see Fig. 1), was found using heat-denatured A1-CM (Fig. 4). Serendipitously, similar effects on both cell viability and \[ \text{H}\text{TdR} \] incorporation were obtained using A1-CM stored at −20°C for a prolonged time (30 days) (Fig. 4).

On the basis of these results, it was decided to use CM which had been stored for at least 30 days at −20°C in all further experiments.

It was therefore verified whether the effects of the CM on neuroblastoma cells were specific for A1, or whether other glial elements (i.e. type 2 astrocytes) might also induce them. SH-SY5Y cells were incubated with media conditioned for 24 h by cultures enriched in A1 or type 2 astrocytes. The results obtained showed, once again, that the CM obtained from A1 induced a significant decrease in \[ \text{H}\text{TdR} \] incorporation at 24 h, followed by a significant increase in the number of viable cells after 6 days of treatment (Table 1).

On the contrary, no significant effect on either \[ \text{H}\text{TdR} \] incorporation or cell viability was observed using the CM obtained from type 2 astrocytes (Table 1).

On the basis of the observation that the decrease in incorporation of \[ \text{H}\text{TdR} \] in SH-SY5Y cells treated with
A1-CM was followed by an increase in the number of living neuroblastoma cells, it was decided to perform a time-course experiment in which the incorporation of [3H]TdR in SH-SYSY cells was evaluated using A1-CM submitted to 30 days of freezing, rather than the previously described co-culture method (see Fig. 1).

In agreement with the results reported in Fig. 1, it was found that the incorporation of [3H]TdR in SH-SYSY cells treated with undiluted A1-CM was characterised by an inhibitory phase evident at 12 h and which lasted up to 24 h (Table 2); this was followed by a progressive normalisation of this parameter, which returned to control levels 48 h after the beginning of the incubation.

At this point, it was felt necessary to attempt at least an initial identification of the factor(s) involved in the opposite effects (inhibitory on the DNA synthesis and stimulatory on the cell survival/proliferation) exerted by the A1-CM on neuroblastoma cells. The physico-chemical characteristics (e.g. activation by heating and freezing) of the factor(s) contained in A1-CM, and responsible for the inhibitory effects, suggested a possible similarity with some components of the TGFβ superfamily. In fact, TGFβ, which may also be synthesised by astrocytes (Constam et al. 1992), is secreted in an inactive form complexed to a connecting glycopeptide which may be removed by physical (freezing or boiling) or chemical (extreme pH values) treatments, which liberate the free active form of the growth factor (Barnard et al. 1990).

Consequently, a set of experiments was performed to verify the possible participation of TGFβ in the inhibitory effects described above. Immunoneutralisation of the frozen A1-CM was performed, using a pan-specific antibody which recognises the major forms of TGFβs (see Materials and methods), since different isoforms of TGFβ have, so far, been characterised.

It was found that the presence of the antibody totally blocked the inhibitory effect of A1-CM on [3H]TdR incorporation (Fig. 5a) observed in SH-SYSY cells after a 24-h exposure; however, the same antibody was unable to modify the stimulatory effect of A1-CM on SH-SYSY cell number (Fig. 5b).

**Discussion**

The results reported in the present paper show that confluent cultures of rat A1 secrete some humoral factor(s) which is able to stimulate the survival/proliferation of SH-SYSY neuroblastoma cells, and that this effect is preceded by a transient decrease in DNA synthesis, detected by measuring [3H]TdR incorporation. Both effects are evident when neuroblastoma cells are maintained in co-culture with A1, or are exposed to the CM obtained from cultures enriched in A1 (A1-CM) provided this was ‘activated’ by either heating or freezing. However, the inhibitory effect on [3H]TdR incorporation appears at short time-intervals (24 h) and a normalis-
Incorporation of [3H]TdR in SH-SY5Y cells exposed to non-conditioned chemically defined medium (control), fresh A1-CM or A1-CM which had been submitted to heating (70°C/20 min) or prolonged freezing (−20°C/30 days). Values represent means ± S.D. *P<0.05 vs control.

Table 1  Effect of CM obtained from type 1 and type 2 astrocytes on the incorporation of [3H]TdR in SH-SY5Y cells (24-h exposure) and on their viability (6-day exposure). Values are expressed as means ± S.D. percent variations with respect to control cultures exposed to non-conditioned chemically defined medium.

<table>
<thead>
<tr>
<th>Glial cells</th>
<th>[3H]TdR incorporation</th>
<th>Viability (MTT assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 astrocytes</td>
<td>−40 ± 7*</td>
<td>+93 ± 3*</td>
</tr>
<tr>
<td>Type 2 astrocytes</td>
<td>+12 ± 5</td>
<td>−8 ± 1</td>
</tr>
</tbody>
</table>

*P<0.05 vs respective controls.

SH-SY5Y cell number in the presence of control values of [3H]TdR incorporation, after 48 h of co-culture, may be indicative of an effect of A1-released factors on cell duplication and/or on cell survival.

The rapid inhibitory effect on [3H]TdR incorporation by neuroblastoma cells exerted by A1 is specific and cannot be ascribed to a general toxic effect, since this effect was not evident when fresh A1-CM or the CM derived from type 2 astrocytes were used. These observations suggest that the decrease in [3H]TdR incorporation is not due to the presence, in the CM, of molecules (e.g. thymidine derivatives or metabolites) which would interfere with the uptake, rather than with DNA incorporation of [3H]TdR (Wilson 1989).

The hypothesis that the inhibitory effect exerted by A1-CM on DNA synthesis might be due to a TGFβ-like molecule was proposed on the basis that elements of the TGFβ family become fully active after heating or freezing; this was confirmed by the observation that the inhibitory effect was abolished by the presence of an anti-TGFβ antibody (Barnard et al. 1990). It is known that rat and mouse astrocytes release, in culture, various isoforms of TGFβ in a latent form (Saad et al. 1991, Constam et al. 1992); however, the wide spectrum of the antibody used did not allow us to identify which TGFβ isoform (i.e. TGFβ1, 2β, 3β etc.) was directly responsible for the effects observed. The similarity of the results obtained from co-culture experiments and from those performed using A1-CM lead us to believe that the same principle is involved. However, the observation that the medium of the co-culture is effective as such, while the A1-CM needs to be activated by heating or freezing, needs explanation. It has been found that, in heterotypic cell cultures, TGFβ may be present in free active form (Antonelli-Olridge et al. 1989, Sato & Rifkin 1989); this is easily explained by the fact that the release of TGFβ-
activating enzymes from a cell population may be influenced
by the other cell lines present in the co-culture.

On the other hand, the data here presented also show
that A1 secrete one or more factor(s) able to induce a strong
stimulatory effect on survival and/or proliferation of neuro-
blastoma cells. In fact, the results obtained from co-culture
experiments clearly show that, when neuroblastoma cells are
in humoral contact with A1, they receive a trophic stimulus.
At variance with the inhibitory effect on \([3\text{H}]\text{TdR}\)
inclusion of \( \text{A1-CM} \) is highly active and its
effects are still clearly and significantly evident at high dilu-
tion. This stimulatory effect of \( \text{A1-CM} \) did not require activa-
tion by freezing or heating, and was resistant to heat.

The results obtained from the analysis of the time-course
of \([3\text{H}]\text{TdR}\) incorporation show that the \( \text{A1-CM} \)-induced
increase in the number of viable cells was not paralleled by
an increase in thymidine uptake at the shorter time; this may
suggest an action of glial factor(s) on the survival rather than
on the proliferation rate of SH-SY5Y cells. However, in a
parallel set of experiments, it was shown that the \( \text{A1-CM} \)
was also able to increase the number of SH-SY5Y cells
during their growth in FBS-supplemented medium, sug-
gestng an additive effect of the astrocytic factor(s) on cell
proliferation.

A first obvious explanation of this effect includes the
possibility that the same TGF\( \beta \), able to induce an early
depression of DNA synthesis, may exert some stimulatory
effect at longer time-intervals. In fact, even if TGF\( \beta \s\) appear
to exert a general inhibitory effect on cell growth, stimulatory
actions of this factor have been also reported; these are prob-
ably indirect and mediated by the induction of other growth
factors by the TGF\( \beta \) target cells (Barnard et al. 1990). How-
ever, the data show that the neurotrophic effect exerted by
\( \text{A1-CM} \) was not eliminated by the presence of the antibody
directed against TGF\( \beta \s\), suggesting that the stimulatory effect
was not due to this growth factor.

On the other hand, a linkage with the presence of TGF\( \beta \)
might be foreseen, because mannose 6-phosphate, which is
contained in the glycopeptide included in the latent form of
TGF\( \beta \), is known to exert a strong proliferative action on SH-
SY5Y cells through its interaction with the insulin-like
growth factor-II receptor (Feldman & Randolph 1991); work
is currently in progress to verify this hypothesis.

It is well known that glial cells synthesise and release
neurotrophins, as well as a series of well-characterised
growth factors, cytokines and adhesion factors (Muller et al.
1995). In addition, a series of glia-derived factors active on
neuroblastoma cells has been reported. These have been
defined gliostatin (Ueki et al. 1993), glia maturation factor
(Lim et al. 1987, 1989, 1990), pleiotrophin (Wanaka et al.
1993) and human neuroblastoma growth inhibitory factor
(Eksioglu et al. 1994). In general, they exert an inhibitory
effect on neuroblastoma cell proliferation which is usually
linked to an induction of morphological differentiation
(Monard et al. 1973). In the present work, no morphological
differentiation of SH-SY5Y was observed; a result similar to
that obtained by Hirose and co-workers (1994) who found
that the treatment of SH-SY5Y cells for 3 days with embry-
onic astrocyte CM did not produce any morphological differ-
entiation of the cells. The explanation of this apparent
discrepancy in the effects of glial factors on morphological
changes may be found in Shea et al. (1994); these authors
found that the induction of neuritogenesis of neuroblastoma
cells by glial CM is dependent on the density and age of the
glial cultures; the maximal activity being observed in
younger and non-confluent cultures. As previously men-
tioned, in the present study the CM were collected from con-
fluent cultures of A1 maintained in serum-free medium and
therefore from cells with a pattern of secretory products
which may be different from that of more dispersed glial
cells. The results reported here have been obtained in experimental conditions that are different from those used in the other studies quoted above; however, the observation that brain metastases of abdominal neuroblastoma can effectively occur suggests that the protocols adopted here may be the nearest to in vivo pathological conditions.

It may be of interest to quote here the finding of humoral factors derived from glial cells (newborn rat cortical astrocytes and C6 rat glioma cells) which protect cultured mouse neuroblastoma cells against glutamate toxicity (Amano et al. 1994), as well as glia-derived neurotrophic factor (GDNF), a member of the TGFβ superfamily which has been implicated in the survival of midbrain dopaminergic neurons and motorneurones in vivo and in vitro (Lin et al. 1993). More recently, it has also been reported that rat C6 glioma cells release factors which may increase the survival of SH-SYSY cells in serum-deprived medium (Zuo & Yu 1995). However, the different methodological approaches adopted (i.e. tumoral vs normal astrocytes) and the different biochemical characteristics (i.e. heat sensitivity) of the factors involved in such effects, make a direct comparison of the results of the present study with those reported in the studies cited above very difficult, at least until the factors involved are characterised.

In conclusion, the results of the present study, even if they are limited only to the cell models adopted, indicate that trophic factors released by astrocytes may exert a stimulatory effect on human neuroblastoma cell growth in vitro; these factors may therefore exert either a permissive (by increasing survival) or a trophic (by increasing proliferation) action. The imbalance of the effects of such factors, or the acquired resistance of tumour cells to some of them (Johnson et al. 1993), may play a key role in favouring the occurrence of neuroblastoma-derived brain metastases. The future identification of the factor(s) involved in the trophic effects exerted by A1 on neuroblastoma cells in vitro will open new perspectives for the understanding of the mechanisms supporting the formation of metastases of this tumour in the central nervous system.

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