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Long Term Bone Marrow Culture in Metastatic Neuroblastoma

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Abstract—*We have developed a long term marrow culture assay for the study of advanced bone marrow metastatic neuroblastoma. In this* in vitro *system the hemopoietic growth (GM-CFU) is not affected by the presence of tumor cells. The neuroblastoma cells grow and differentiate partially when in contact with the stromal layer, arresting the culture. We present culture and histological data suggesting that the solid tumors interact specifically with the stromal layer of marrow origin.*

INTRODUCTION

SINCE disseminated neuroblastoma still has a poor prognosis, massive therapy followed by autologous bone marrow transplantation has become a current modality of consolidation treatment for these patients. In our group, patients are consolidated with intensive combination chemotherapy and total body irradiation. As a small amount of malignant cells is still present in the marrow of these children after the induction treatment, neuroblastoma cells are magnetically depleted *in vitro* with specific monoclonal antibodies before grafting [1]. In order to study the interaction between neuroblastoma cells and the hemopoietic and stromal bone marrow cells, we have developed a long term bone marrow culture (LTBMC) system with potential application for the follow up of metastatic neuroblastoma children before and after intensive therapy.

PATIENTS, MATERIALS AND METHODS

Bone marrow samples were taken from five patients with bone marrow involvement at diagnosis before any treatment (four patients with stage IV and one, patient 3, with stage III disease according to Evans' classification [2]; patients were between 2 and 4 years old). A hematologically normal patient undergoing surgery was also investigated as a control.

The sample (about 2 ml over heparin, Roche) was diluted (1/3) with Iscove's medium (Gibco) and centrifuged over ficoll d.1.119 (Sigma) 10 min at 200 ***g*** followed by 15 min at 400 ***g*** to get rid of most of the red cells. The nucleated overlaying cells were washed three times (10 min at 400 ***g***) in Iscove's medium 2% fetal calf serum (FCS, No. 60023201 Boehringer) and cultured after the viability of the cells had been assessed by the trypan blue exclusion test (2×10^6 viable cells/ml in Iscove's medium 10% FCS, 10% horse serum, No. 69556101 Boehringer, 10^{-6} M hydrocortisone hemisuccinate, Sigma, cultured in flasks at 33°C, 5% CO_2). Half of the supernatant was removed each week, the collected cells were counted (expressed in million cells per milliliter, C/ml) and the clonogenicity of granulocyte–macrophage progenitors (GM-CFU) was tested in triplicate among the non adhering cells using conditioned medium of the human 5637 cell line as a source of hemopoietic colony stimulating factors [3] (10^5 cells/ml in Iscove's medium, 0.3% agar, Difco, 20% conditioned medium of 5637 cell line, 15% FCS, cultured in Petri dishes at 5% CO_2, 37°C).

Cultures in agar without conditioned medium from 5637 cells were also set up in order to test the growth of other cell types (i.e. tumor cells). All the semi-solid cultures were scored on day 11, the colonies being defined as groups of more than 50 cells, clusters as groups of 10 to 50 cells. The mean counts of three Petri dishes containing GM-CFU colonies was expressed as the quantity of clonogenic cells to the number of viable cells harvested per milliliter of LTBMC supernatant, on the day of collection and agar culture (GM-CFU/ml).

Supernatant samples from LTBMC were cyto-

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centrifuged (10 min at 200 **g**) on glass slides. The cells were stained or assayed for neurone specific cnolase (NSE) using an enzymo-immunological assay (DAKO) to evaluate the presence of tumor cells in the culture. Three-dimensional outgrowth of tumor cells occured in the cultures established from patients with advanced bone marrow metastatic neuroblastoma. They were removed from the stroma layer in some of the flasks of each cultured sample by flushing with medium and subcultured for 4–6 weeks, in order to initiate spheroid growth (Iscove's medium, 15% FCS, 0.2% agar at 5% CO_2, 37°C). The spheroids of cells obtained were included in paraffin and processed for histological analysis and/or NSE assay (DAKO) using a routine assay.

RESULTS

Figures 1a and b show the quantity of cells and the pattern of clonogenicity of the GM-CFU among

(*a*)

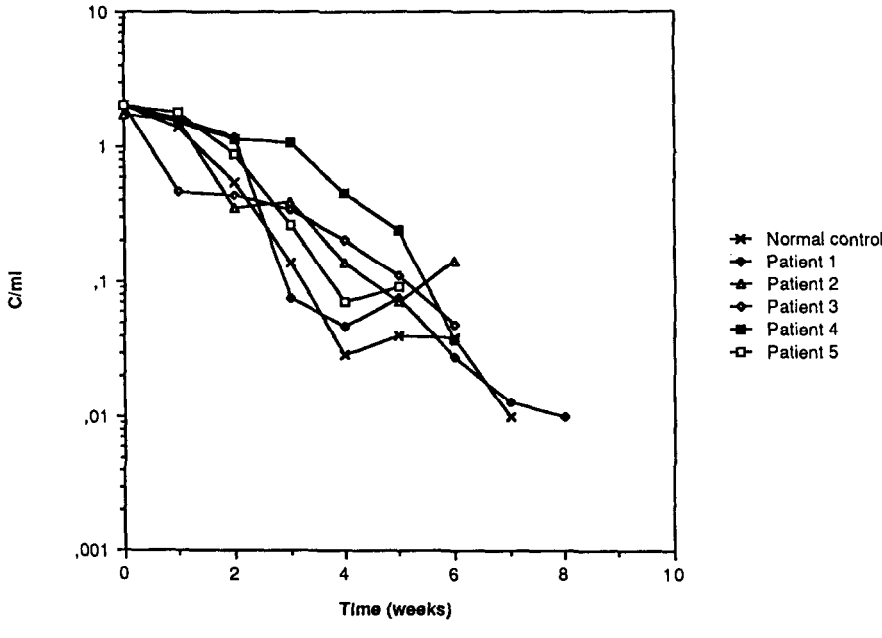

Fig. 1a. Cellularity (10^6 *cells*/ml) *of the supernatant from LTBMC.*

(*b*)

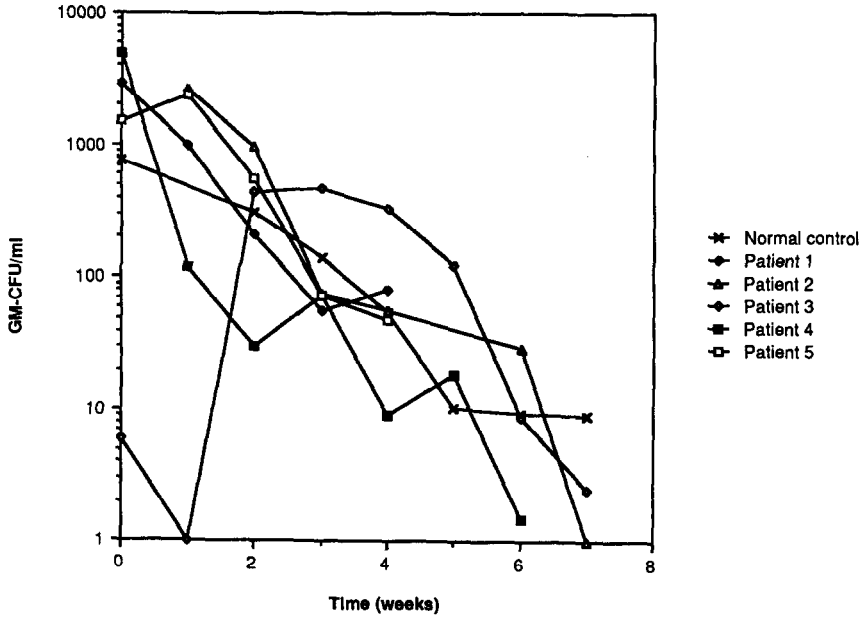

Fig. 1b. GM-CFU (*GM-CFU*.ml) *among supernatant cells from LTBMC.*

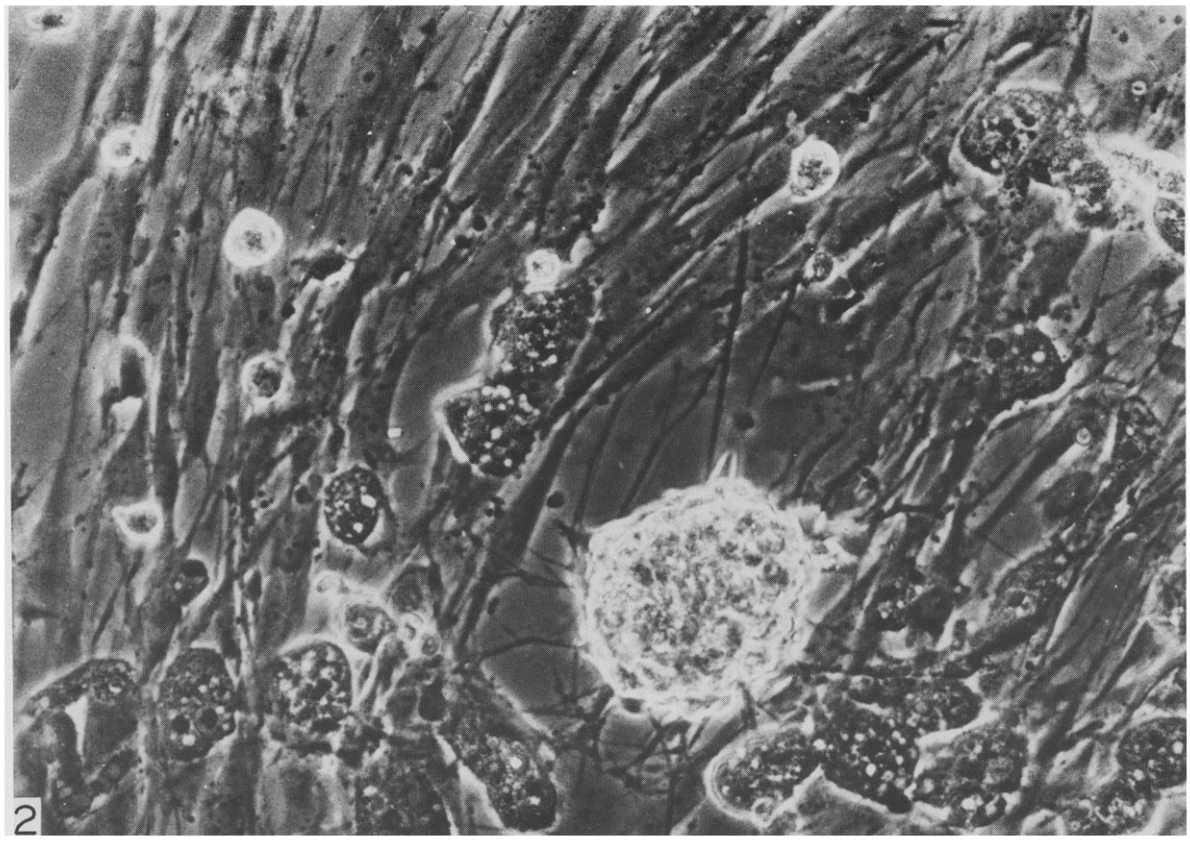

Fig. 2. Tumoral cells in LTBMC (× 150).

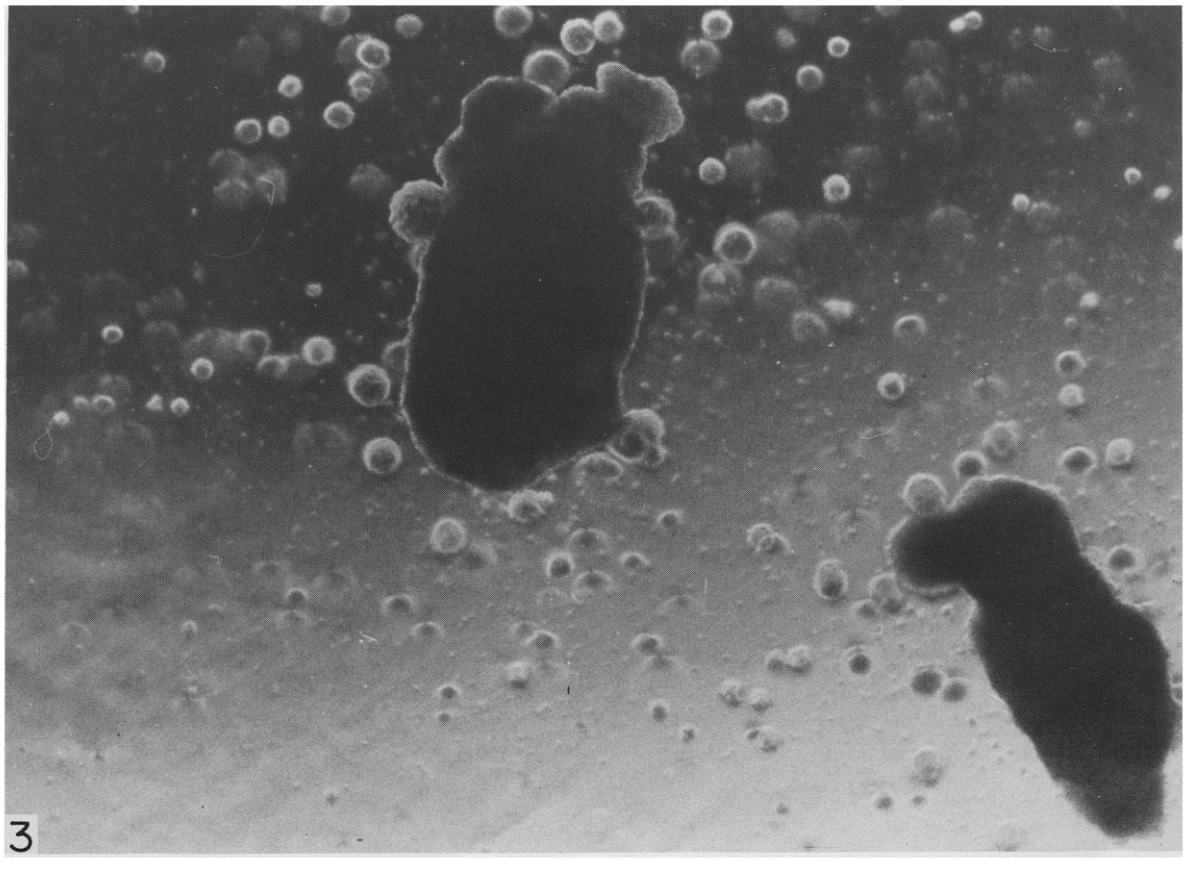

Fig. 3. Spheroids of tumoral cells subcultured in .2% agar (×60).

supernatant cells during the time course of the culture. Both parameters were normal when compared to control except for the two first points of patient 3 which were very low; this was likely due to the conditions of sampling. The coefficients of variation for patients or normal control ranged from a maximum of 23.9% for a mean initial value of 1506 GM-CFU/ml (normal control: 15.1% for 766 GM-CFU/ml), to a maximum of 33.5% for a mean final value of 48 GM-CFU/ml (normal control: 32.8% for 9 GM-CFU/ml). In most of the experiments of secondary semi-solid culture from neuroblastoma patients bone marrow cells as well as some colonies or clusters of tumor cells may be found due to their peculiar morphology.

The quantity of viable cells and the clonogenicity of the GM-CFU among supernatant cells during the LTBMC were followed for at least 1 month. During this time stromal cells grew and produced a thick extracellular matrix. When present, part of the neuroblastoma cells and rosettes settled on the stromal layer; the tumor cells grew and differentiated exhibiting neurites (Fig. 2); the surrounding matrix and stromal layer shrank, got loose of the plastic flask and, finally, the culture stopped because of this loss of anchorage. The settling tumor cells could be subcultured and characterized as spheroids (Fig. 3). The interactions between neuroblastoma cells and the bone marrow stroma could be evidenced through the culture, histological and immunocytological data (see Table 1).

DISCUSSION

Human LTBMC demonstrate the production of hemopoietic stem cells for several weeks [4]. In cultures established from patients with chronic myeloblastic leukemia, it was observed that the maintenance of leukemia cells was not favored in this system and that the culture conditions allowed the growth of normal stem cells and suppressed the development of leukemia cells. Following this work the cultured marrow was used in the treatment of acute myeloblastic leukemia for transplantation purposes [5].

The report of this successful treatment prompted us to study whether this system of culture and grafting could be used in other diseases where bone marrow transplantation is required and the culture of the invading tumor cells is known to be difficult, so that *in vitro* normal hemopoietic and stromal cell growth may be favored. Unfortunately, neuroblastoma cells persist in the culture system used and in our hands LTBMC is improper for this direct therapeutic indication.

Cloning and growth of neuroblastoma cells in semi-solid media have been reported previously [6] and the fundamental importance of the interaction between tumor cell and hemopoietic function for diagnosis, prognosis and follow up has been emphasized in a recent study [7].

However, the observed normal hemopoietic cell production already documented by these authors suggests that the interaction between bone marrow function and neuroblastoma cell growth is not a direct inhibition of the hemopoietic stem cell production.

This preliminary report demonstrates an interaction between neuroblastoma cells and stromal cells. The inhibiting effect of tumor cells over the normal cells in culture may be due to a specific factor produced by neuroblastoma cells which deserves further study. Apart from the well established prognostic significance of the clonogenicity of neuroblastoma cells, the pattern of spheroid growth and development of neuroblast cells in LTBMC experiments could also be of prognostic value. In the present study, bone marrow metastases were found at the time of experiment in all the patients, but the LTBMC system could be used to demonstrate bone marrow involvement in some histologically negative specimen, as reported previously [6] for clonogenicity experiments.

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Table 1. Assessment of the presence (+) or absence (−) of tumor cells in LTBMC

Samples	Tumor cells among cultured cells assessed by histological examination	Colonies of tumor origin in agar culture	Spheroid growth of tumor cells subcultured	NSE positivity among cultured cells
Patient 1	+	+	+	+
Patient 2	+	+	+	+
Patient 3	−	+	+	−
Patient 4	+	+	+	+
Patient 5	+	+	+	+
Normal control	−	−	−	−

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