The In Vitro *Growth, Heterotransplantation, and Differentiation of a Human Rhabdomyosarcoma Cell Line*

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A human rhabdomyosarcoma (RMS) cell line was established from a case of childhood small cell sarcoma, which *in vivo* showed no evidence of differentiation, but which demonstrated myogenic differentiation in tissue culture. In a serum-free culture medium, the tumor cells demonstrated continuous growth without ultrastructural or biochemical evidence of differentiation. Heterotransplanted RMS cells gave rise to tumors in nude mice which also showed no myogenic differentiation. However, RMS cells grown in the presence of either retinoic acid (5 μM), phorbol ester (1 nM), prostaglandin E_1 (10 ng/ml), or 2% fetal calf serum gave rise to myotubes with a biochemical shift in the creatine kinase isoenzyme pattern from the embryonic to the mature skeletal muscle form. The karyotype of the RMS cells revealed a translocation of Chromosomes 2 and 13, which may represent a nonrandom aberration unique to this morphologic subtype. In addition, the RMS cells gave evidence for gene amplification in the form of double minute chromosomes. This human RMS cell line provides a valuable *in vitro* system for study of myogenesis and factors which induce differentiation. (Am J Pathol 1986, 125:208–217)

RHABDOMYOSARCOMA (RMS) is a highly malignant soft tissue sarcoma that occurs primarily in childhood. The diagnosis of RMS depends upon the identification of skeletal muscle differentiation of the tumor cells. Routine histologic and ultrastructural examination rarely demonstrates conclusive evidence of myogenic differentiation; so a variety of ancillary techniques have been proposed to aid in their distinction from other undifferentiated tumors of childhood. These techniques include enzyme histochemistry,[1] enzyme assays,[2] tissue culture,[3] and immunohistochemistry for the cytoplasmic antigens desmin,[4] creatine kinase,[5] myoglobin,[6] skeletal muscle myosin,[7] actin,[8] and Z protein.[9] In spite of extensive data on the presence of these antigens with regard to the degree of histologic differentiation and type of RMS, little is known of the capacity of this tumor to differentiate or of factors known to influence differentiation. In a recent study, Molenaar et al[10] reported that polychemotherapy resulted in an increase in the "cytologic differentiation" of human RMS. Experimentally, the inducing agent n,n-dimethylformamide has been shown to induce morphologic differentiation in a cultured mouse RMS cell line.[11] These observations suggest that the capacity of RMS to differentiate is not lost; ie, the necessary structural genes may still be operative or accessible but may require induction for gene activation or regulation.

In a previous report[3] we described a case of childhood small cell sarcoma which *in vivo* showed no evidence of differentiation but which demonstrated myogenic differentiation in tissue culture. This report describes the establishment of a human RMS cell line from that tumor, its distinct karyotype, the heterotransplantation of the cell line into nude mice, and the *in vitro* study of agents which induce its differentiation.

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Materials and Methods

Reagents

Dulbecco's modified eagle's medium (DMEM), Ham's F-12 medium (F-12), and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY). Components for serum-free growth medium, including prostaglandin E (PGE), were obtained from Collaborative Research (Lexington, Mass). Bovine Type I collagen was obtained from Flow Laboratories (McLean, Va). Reagents for the preparation of buffers and other routine solutions were obtained from Fisher Scientific (Atlanta, Ga). Tissue culture flasks were of the Corning trademark. Triple-distilled water was utilized for the preparation of all cell culture reagents. Retinoic acid (RA), dibutyryladenosine 3,5 cyclic monophosphate dibutryl cyclic AMP (dbCAMP), 12-0-tetradecanoylphorbol-13-acetate (TPA), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, Mo).

Cell Cultures

A 14-year-old girl developed a large soft tissue mass in her right chest wall. A biopsy of the tumor showed an undifferentiated round cell sarcoma involving adjacent tissues. A PAS stain with and without diastase digestion demonstrated cytoplasmic glycogen. Immunoperoxidase stains for myoglobin and skeletal muscle myosin were negative. Ultrastructural examination of tumor profiles provided no evidence of differentiation. The tumor was diagnosed as an extraskeletal Ewing's sarcoma, and she was treated on an Intergroup Rhabdomyosarcoma Study (IRS) protocol as previously reported.[3] She relapsed 9 months later with a recurrent malignant effusion. After cytologic confirmation, 500 ml of effusion was obtained for culture. The patient died 4 months after relapse with disseminated metastasis. At autopsy, the tumor showed no evidence of differentiation except for a focus at the original biopsy site (Figure 1), which had an alveolar pattern and a rare multinucleated giant cell.

The malignant effusion demonstrated myogenic differentiation after growth in a defined serum-free medium. This medium (DMEM 15) consisted of DMEM supplemented with 5 μg/ml of insulin, 5 μg/ml transferrin, 5 ng/ml selenium sulfide, 36 ng/ml hydrocortisone, 4 pg/ml triiodothyronine, and 10 ng/ml of PGE_1. However, the medium that gave myogenic induction did not permit continuous growth of the tumor cells. A growth medium previously developed in our laboratory for the long-term growth of the skeletal muscle component of Wilms' tumors[12] gave continuous growth of the rhabdomyosarcoma cells in culture. This growth

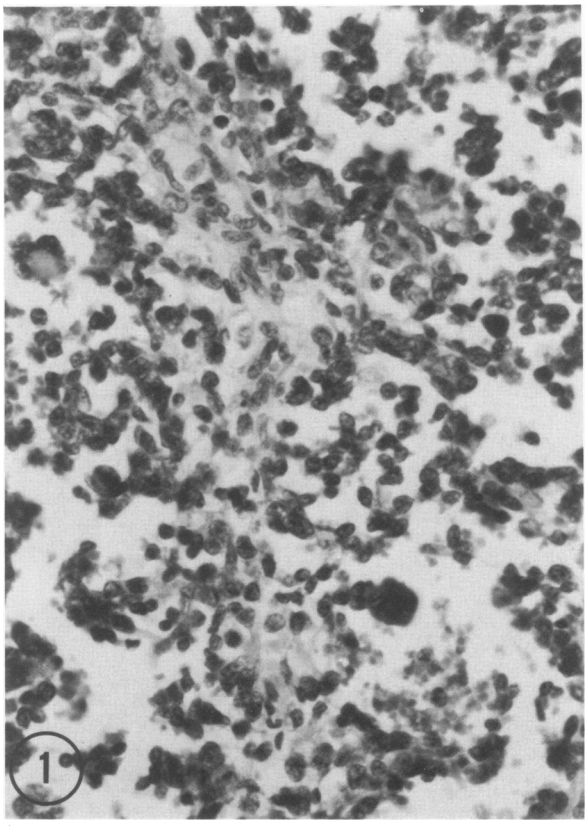


Figure 1—The microscopic appearance of the primary rhabdomyosarcoma at autopsy showed ill-defined aggregates of small undifferentiated tumor cells within irregular "alveolar" spaces and surrounded by a fibrous septa. (×250)

medium (20/12/EGF) consisted of a 1:1 mixture of DMEM and F-12 growth medium supplemented with selenium (5 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and epidermal growth factor (10 ng/ml). The growth surface of the culture flask contained a bovine collagen Type I matrix with absorbed FCS proteins as previously described.[12] Cultures were maintained at 37 C in a humidified atmosphere of 5% CO_2 in air and were fed fresh growth medium every 3 days. The cultures have been maintained through 30 passages to date.

When confluent, the cell monolayers were subcultured by rinsing the monolayer twice with phosphate buffered saline (PBS) followed by the addition of 1.0 ml of trypsin-EDTA. Cell detachment was monitored by light microscopy; and after cell detachment, further trypsin action was halted by the addition of an equal volume of FCS or 0.1% soybean trypsin inhibitor. The detached cells were transferred to a 15-ml centrifuge tube, brought to volume with PBS, and centrifuged at 800*g* for 5 minutes. The cell pellet was resuspended in PBS, recentrifuged, and resuspended into culture medium and distributed to new flasks at a 1:3 subculture ratio.

Photomicrographs of cultures were recorded on 35-mm Kodak Panatomic X film, ASA 32, with the use of an Olympus 1 M inverted microscope.

Cytogenetics

For cytogenetic analysis, tumor cells were grown in Chee's essential medium supplemented with fetal bovine serum (10%), insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml). Prior to cell harvesting, cultures were treated with colchicine (0.01 μg/ml) for 60 minutes. Trypsin-released cells were conventionally prepared by hypotonic treatment for 30 minutes in 0.075 M KCl followed by fixation in methanol/acetic acid (3:1). Slides were made by routine air-drying methods and heated at 50 C for 2 days. Chromosomes were G-banded with 0.25% trypsin in phosphate buffer, pH 7.4, and stained with 0.5% Wright's stain.

Electron Microscopy

Monolayers were fixed *in situ* with 2.5% glutaraldehyde in pH 7.4, 0.1 M cacodylate buffer for 1 hour at room temperature. The monolayer was postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at room temperature. The monolayers were dehydrated, and the flask was embedded in Epon 812. After polymerization, the plastic was removed, and ultrathin sections were cut parallel and perpendicular to the surface of the flask. The ultrathin sections were examined with uranyl acetate–lead citrate staining in a JEOL 100S electron microscope.

Heterotransplantation Into Nude Mice

Confluent 75-sq cm flasks containing approximately 2×10^6 cells each were trypsinized by the above technique. The cell pellet was suspended in 0.5 ml PBS and injected subcutaneously in BALB/C nude mice, 6–10 weeks of age, obtained from the National Cancer Institute. Approximately 2×10^6 cells were injected per site.

Electron microscopy was performed on the resulting subcutaneous tumors by mincing the tumors into 1-cu mm fragments, fixation in 2.5% glutaraldehyde overnight, and processing as mentioned previously.

Creatine Kinase (CK) Isoenzyme Determination

The cells under study were grown to confluency in 75-sq cm tissue culture flasks, the monolayer of which was rinsed twice with 10 ml of phosphate-buffered saline (PBS) and then evacuated. Two milliliters of PBS was added to each dish, and the cells were scraped off with a rubber policeman. Cultured cells and heterotransplants were disrupted in a homogenizer, and the suspension was centrifuged at 10,000 rpm for 20 minutes at 4 C. Total creatine kinase activity was measured with creatine phosphate and ADP as substrates by the modification of Szasz.[13] For isoenzyme separation, the supernatant was placed on agarose gel; and after electrophoresis, visualization was accomplished with a fluorometric substrate (Beckman's Paragon Electrophoresis System). Quantitation of isoenzymes was accomplished by densometric scanning with reference controls.

Induction Experiments

For evaluation of the induction of differentiation, 1×10^6 viable cells were transplanted onto groups of 75-sq cm tissue culture flasks in 20/12 EGF growth medium containing one of the following: 1) 5 μM *trans*-retinoic acid, 2) 2 mM dbCAMP, 3) 1 nM TPA, 4) 10 nM TPA, 5) 2% FCS; and 6) 10 ng/ml PGE_1. Retinoic acid was dissolved in 95% ethanol at a 5mM concentration and diluted in medium to a 4 μM concentration. dbCAMP was dissolved in PBS at 1000× the final concentration. TPA was dissolved in DMSO at a 1 μg/ml and 10 μg/ml concentration. Control cultures for retinoic-acid–treated cells received growth medium with 0.2% ethanol. Control cultures for TPA received growth medium with 0.1% DMSO. Control cultures for the serum containing medium were maintained on 20/12 EGF growth medium. Media in both control and drug-treated cultures were replaced every 48 hours, and the cultures were maintained for 7 days before photography, electron microscopy, or creatine kinase assay was performed by the methods mentioned previously.

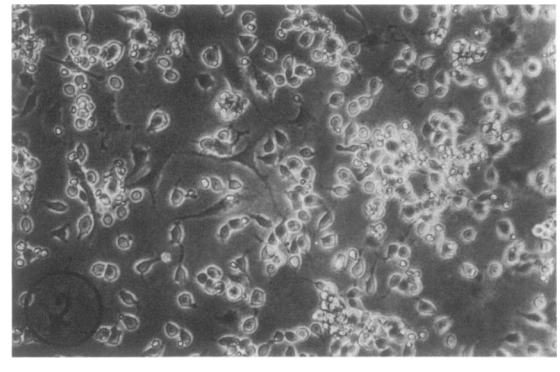


Figure 2—The rhabdomyosarcoma (RMS) cells grew in a serum-free medium (20/12 EGF) as round to polygonal cells, which were loosely attached to the matrix. Inverted phase-contrast micrograph. (×100)

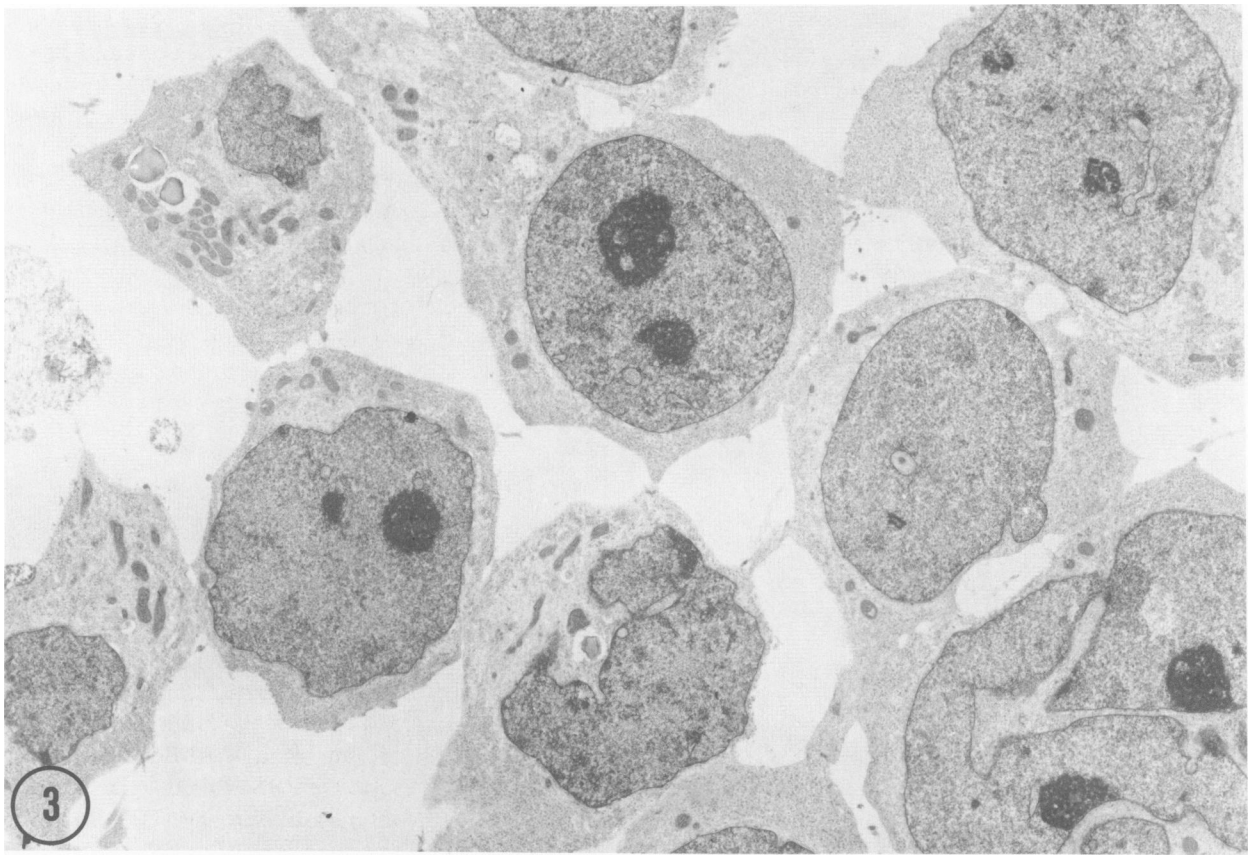


Figure 3—Electron microscopy of the RMS cultured cells demonstrated cytoplasmic glycogen deposits but no evidence of contractile filaments. (×3500)

Results

Growth in Serum-Free Medium

The untreated RMS cells grew as round to polygonal cells which were loosely attached to the matrix (Figure 2). They grew piled up on top of one another in sparse or confluent cultures. Scanty cytoplasm was observed when cultures were stained with hematoxylin and eosin (H&E).

Ultrastructurally, the tumor cells had a rounded contour, with highly irregular and indented nuclei (Figure 3). The nuclei contained multiple nucleoli, most often attached to the nuclear membrane. The cytoplasm contained elongated mitochondria and glycogen rosettes. Single lipid droplets were found in most cellular profiles. The rough endoplasmic reticulum and Golgi area were poorly developed. Adjacent cells were attached by long gaplike junctions. There was no ultrastructural evidence of actin or myosin filaments within the cytoplasm.

Karyotype

The modal chromosome number derived from 25 metaphase cells was 47 (range, 41–49), and 18 of these cells had double minute chromosomes (mean, 27; range, 4–100). Cytogenetic analysis of 10 cells revealed 5 consistent marker chromosomes: 2q+, 4p+, 13q−, and two unidentified markers, M1 and M2. The 2q+ and 13q− chromosomes resulted from a translocation t(2,13)(q37;q14) (Figure 4).

Heterotransplantation

Subcutaneous tumor nodules were noted 40 days after injection. The tumors grew rapidly and upon further passage gave rise to subcutaneous tumors in 20 days. Occasional tumors grew through the flank and produced growths within the peritoneal cavity. Mice that underwent autopsy gave no evidence of systemic metastasis. H&E-stained sections showed the tumor to have a diffuse pattern of growth. The tumor cells (Figure 5) had rounded nuclei with clumped chromatin and occasional single or multiple nucleoli. The cytoplasm had distinct borders and was clear or lightly eosinophilic. No multinucleated or "strap" cells were noted. Phosphotungstic acid stains were negative for cross-striations. Ultrastrucktural examination of the heterotransplanted tumor (Figure 6) showed rounded nuclei with clumped

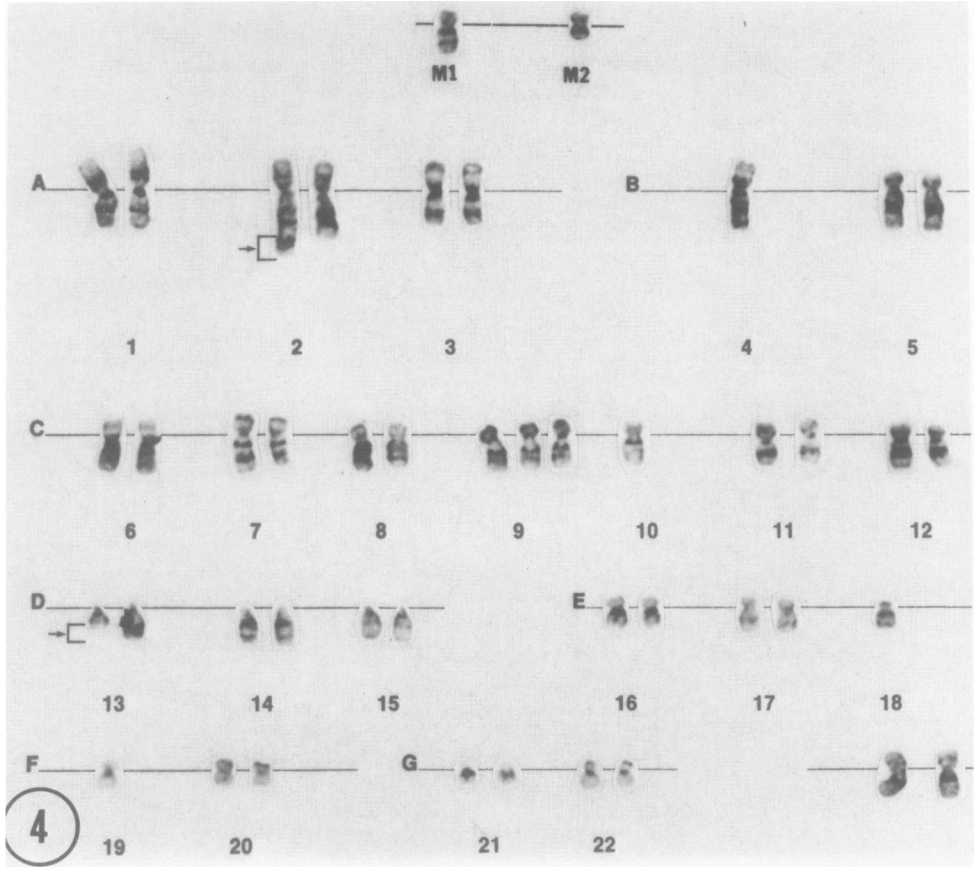


Figure 4—Cytogenetic analysis of cultured RMS cells revealed consistent marker chromosomes 2q+ (*arrow and brackets*) and 13 q− (*arrow and brackets*). The 2q+ and 13q− chromosomes resulted from a translocation t(2,13) (q37;q14).

chromatin. The cytoplasm contained oval mitochondria, glycogen rosettes, rare lipid droplets, and a poorly developed rough endoplasmic reticulum and Golgi area. Adjacent cells demonstrated sparse focal densities beneath their plasma membranes which resembled a gap junction but lacked the morphologic characteristics of well-developed gap junctions. No basement membrane material was seen surrounding the tumor cells, and the cytoplasm contained no filaments.

Table 1—Creatine Kinase Activity of RMS Cells

	Creatine kinase isoenzymes*		
	BB	MB	MM
RMS cell line	74%	15%	11%
RMS heterotransplants	70%	25%	5%
DMSO control	52%	33%	15%
2% serum	16%	39%	25%
1 nM TPA	37%	36%	27%
10 nM TPA	50%	41%	9%
Retinoic acid	36%	38%	26%
PGE_1	38%	39%	23%

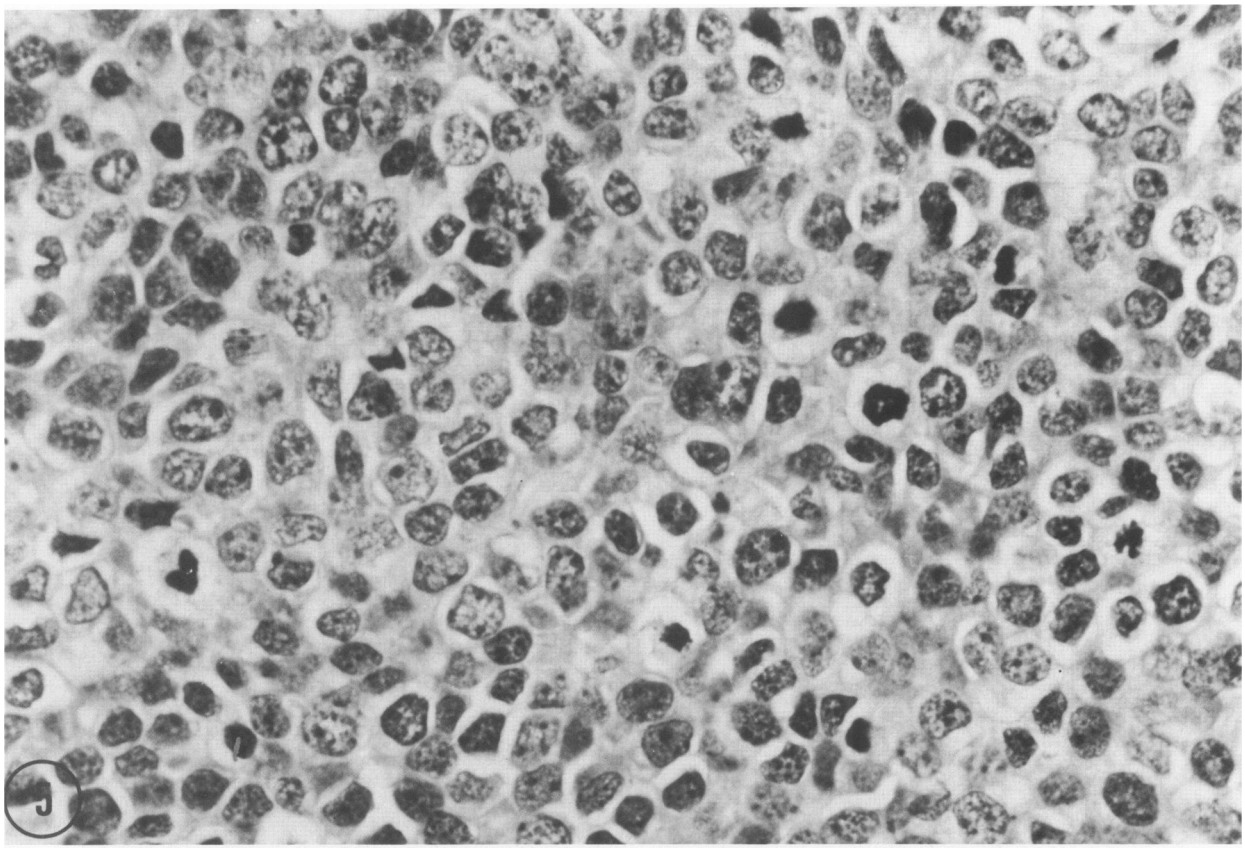
* Mean values of triplicate samples expressed as percent of total activity.

Creatine kinase isoenzymes of tumor cells grown in culture showed 74% to be of the fetal BB isoenzyme with 14.8% MB and only 11.2% of the MM type (Table 1). Similar isoenzyme profiles were seen from the heterotransplanted tumors with 70% BB, 25% MB, and 5% MM (Table 1).

Induction of Differentiation

After 4–5 days in culture, the tumor cells grown in the presence of TPA, retinoic acid, PGE_1, or 2% serum appeared elongated and frequently contained multiple nuclei (Figure 7a–d). These multinucleated cells had the characteristic appearance of myotubes. These

Figure 5—RMS heterotransplants demonstrated tumor cells with rounded nuclei, clumped chromatin, and multiple nucleoli. The cytoplasm was lightly eosinophilic, with distinct cytoplasmic borders. Mitotic figures were frequent. (×800) **Figure 6**—Ultrastructural examination of the RMS heterotransplants disclosed cytoplasmic glycogen, sparse endoplasmic reticulum, and mitochondria, but no myofilaments. Cells were attached by small gaplike junctions. (×5000)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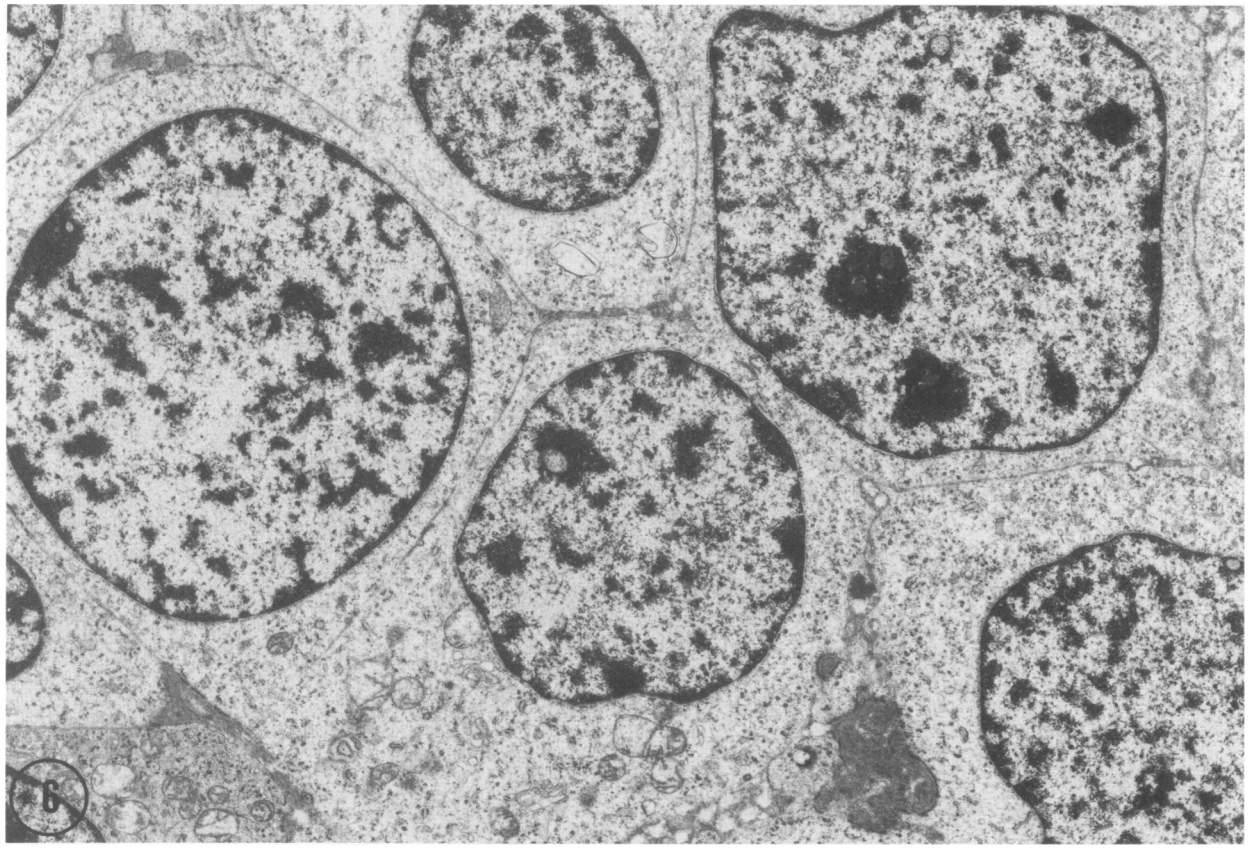
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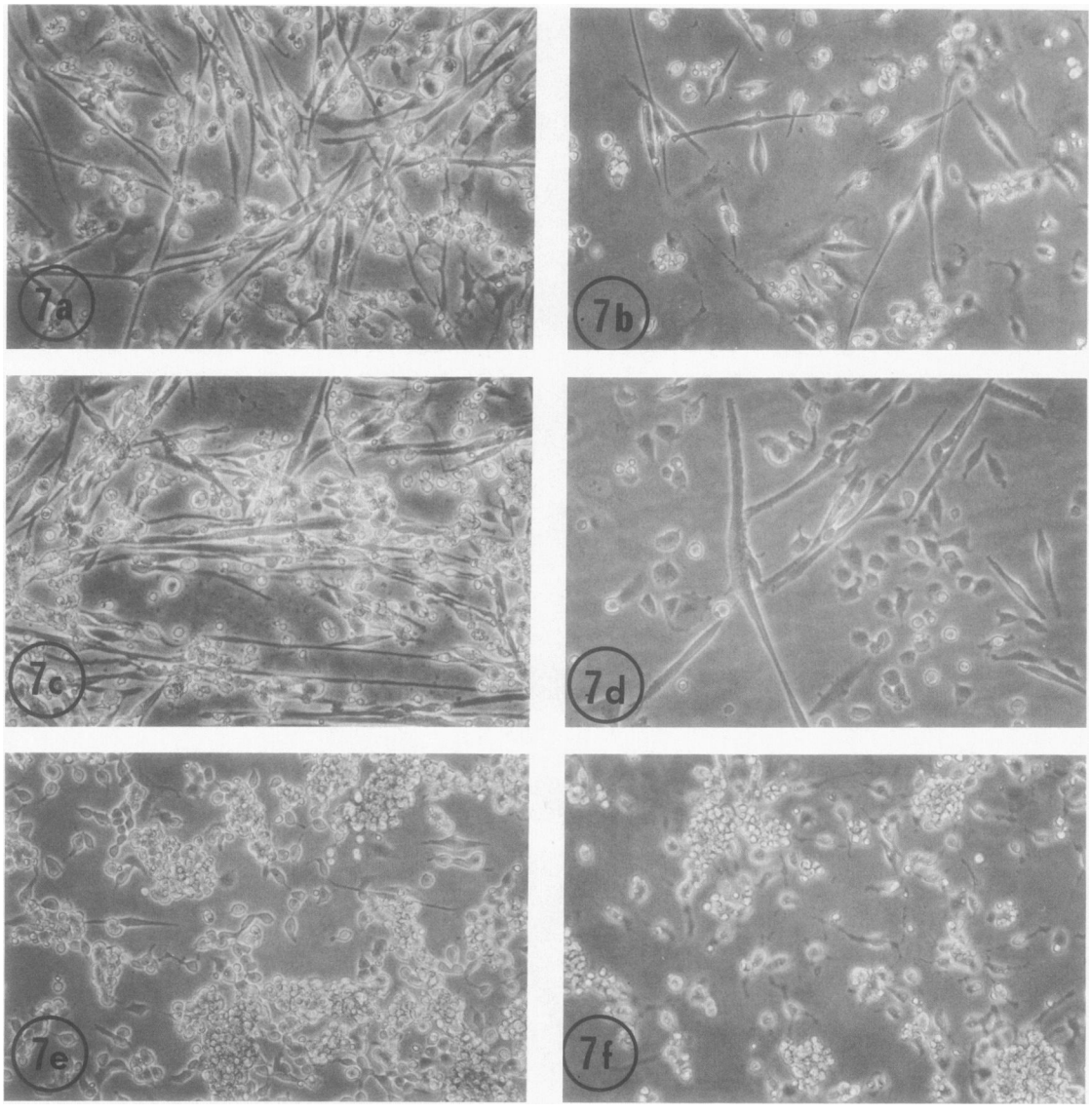
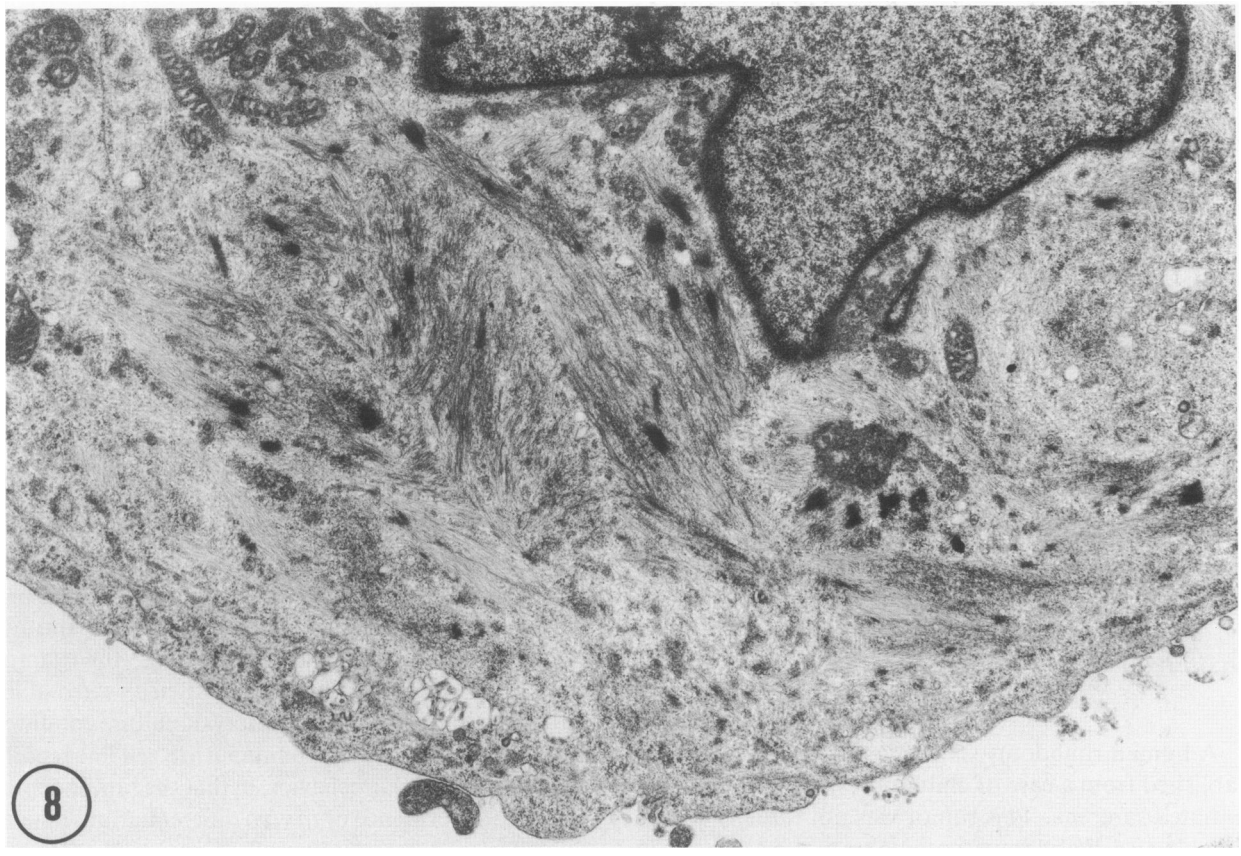


Figure 7—RMS cells grown in medium supplemented with 5 μM retinoic acid (**a**), 1 nM phorbol ester (**b**), and 2% fetal calf serum (**c** and **d**) displayed long multinucleated cells. These cells had the characteristic appearance of myotubes. RMS cells grown in 0.1% DMSO (**e**) retained the morphologic characteristics of untreated cells. Cells grown in dbCAMP (**f**) were rounded, with thin cytoplasmic extensions. Inverted phase-contrast micrographs. (×100)

cells frequently contained four or five nuclei with long thick cytoplasmic extensions. Once the cells formed myotubes, they would not be further subcultured because they failed to divide. Control cultures grown in DMSO (Figure 7e) and ethanol exhibited the same morphology as the parent untreated cell line. Cultures grown in dbCAMP were rounded and more refractile than the control cultures (Figure 7f). These cells had thin processes which often extended to two to three times the cell's diameter. Cells grown in 10 nM TPA were similar morphologically to the control cultures and showed no evidence of differentiation.

An ultrastructural examination was made of the cultures which showed evidence of myotube differentiation. These multinucleated cells (Figure 8) contained nuclei with highly irregular nuclear membranes and prominent nucleoli. The cytoplasm contained an abundance of thick and thin filaments with an orientation primarily along the long axis of the cell. Electron-dense material was seen at irregular intervals along the filaments.

Figures 8 and 9—Electron micrographs of the myotubes showed abundant cytoplasmic thick and thin filaments. Electron densities along these filaments had the characteristic appearance of Z-band material. **Figure 8**, ×10,000; Figure 9, ×21,000; **inset**, ×52,500)



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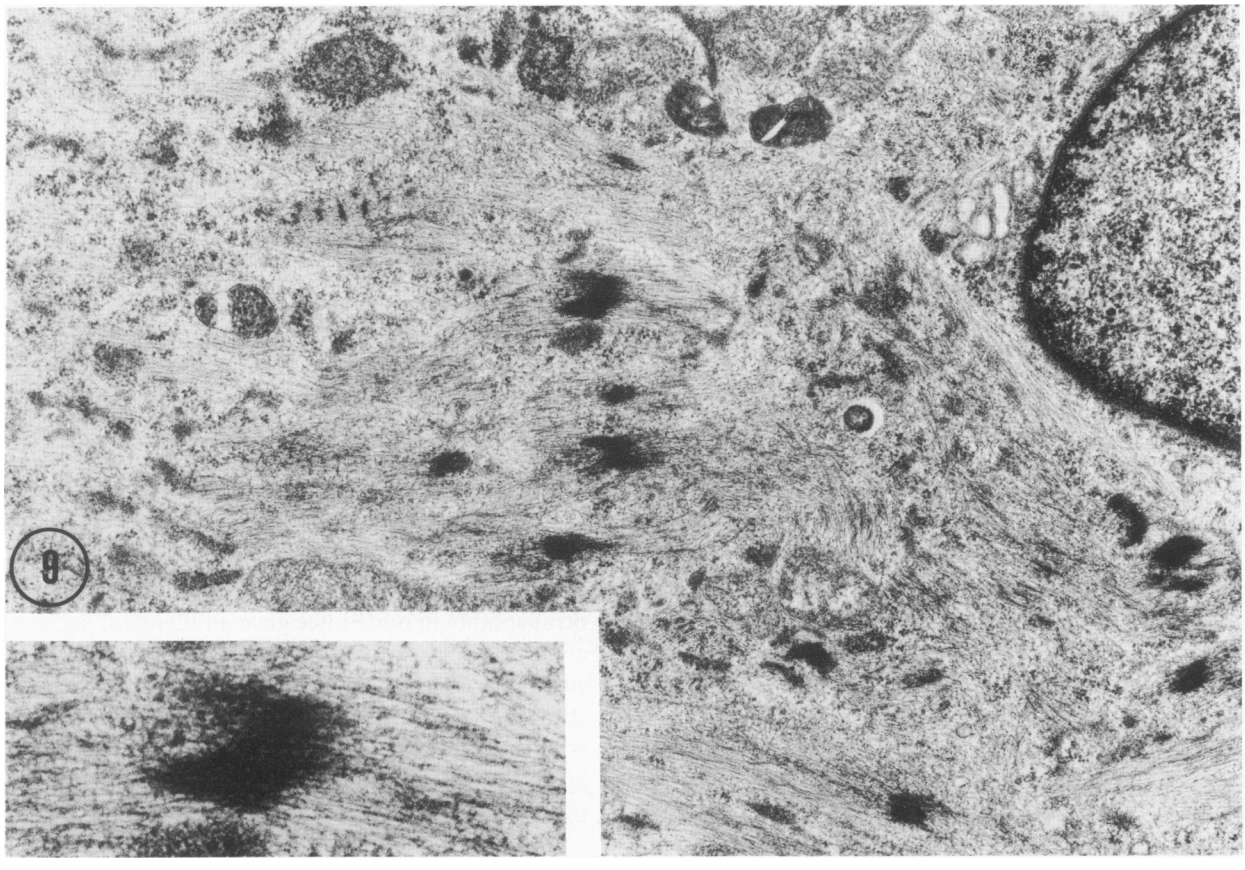

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These had the appearance of poorly formed Z bands (Figure 9). Interspersed between the filaments were oval mitochondria and glycogen deposits. Rough endoplasmic reticulum was sparse, and the Golgi area was poorly developed. The cytoplasmic membranes possessed a few short microvilli and sparse pinocytotic vesicles. No basement membrane material was seen surrounding the cells. Many cells in these cultures failed to show ultrastructural evidence of differentiation and had the same appearance as the untreated controls.

Creatine kinase isoenzymes of the cultures that showed evidence of differentiation demonstrated in general a large increase in the MM isoenzyme. Cultures grown in 1 nM TPA contained 37.3% BB, 36% MB, and 26.6% MM (Table 1), and a similar profile was obtained with 2% fetal calf serum, PGE_1, and retinoic acid. However, cultures grown in 10 nM TPA demonstrated an isoenzyme profile similar to that of control cultures.

Discussion

A human rhabdomyosarcoma cell line has been established from a case of childhood small-cell undifferentiated sarcoma. This tumor was not initially recognizable as a rhabdomyosarcoma until it was grown *in vitro*. The medium which gave initial myogenic differentiation, however, did not permit continuous growth of the cells. A serum-free medium was identified which sustained continuous growth. The study of this RMS cell line resulted in the identification of a specific cytogenetic abnormality for this tumor and the previously undescribed ability of human RMS cells to differentiate in response to a variety of inducing agents.

Cytogenetic analysis of the rhabdomyosarcoma gave a specific chromosomal translocation between Chromosomes 2 and 13 (t[2;13] [q37;q14]). This same translocation has been reported by Seidal et al[14] and Turc-Carel et al[15] in single cases of alveolar RMS. Douglas et al[16] also observed this translocation in 4 of 10 cases of RMS. The tumor was of alveolar type in 2 cases and of alveolar/embryonal type in 1 case. All 3 of these cases were of the monomorphous round type according to the classification of Palmer.[17] The tumor from which our cell line originated had an alveolar pattern at autopsy and was of the prognostically unfavorable monomorphous round cell type. In addition, cytologic evidence for gene amplification in the form of double minute chromosomes was present in our case. Douglas et al[16] also observed double minute chromosomes or homogeneously staining regions in 3 of 4 cases with the t(2;13) but in none of the cases without this translocation. Oncogene amplification has recently been suggested to be of prognostic importance in neuroblastoma,[18] and investigations are now in progress to determine the nature of any amplified genes in our RMS cell line. Recently, Trent et al[19] described cytogenetic results from 6 cases of RMS. In 4 of these there were structural aberrations of Chromosomes 1 and 3, with a consistent breakpoint at band p21 on 3. Other structural or numeric aberrations have also been described in this tumor.[20,21] In these cases, however, histologic subtypes were not identified. Thus, the t(2;13) may represent a nonrandom aberration unique or common to the alveolar or monomorphous round cell type of RMS, as suggested by Douglas et al.[16] This specific cytogenetic abnormality may serve as an objective method of diagnosing the predicting the aggressive clinical behavior of this tumor.

It has been proposed that the fundamental disorder of the neoplastic cell is its inability to differentiate without the loss of its proliferative capacity.[22] However, cells cultured from a number of undifferentiated tumors retain their ability to differentiate *in vitro*.[23] This has most commonly been observed in hematopoietic and neuroectodermal tumors. Neuroblastomas, for example, have been extensively studied for their *in vitro* ability to differentiate with a wide variety of culture conditions and inducing agents. The human RMS cell line reported here showed similar behavior, in that the tumor demonstrated little evidence of *in vivo* differentiation, but gave evidence of differentiation when grown *in vitro*.

The *in vitro* differentiation of RMS was easily demonstrable by light and electron microscopy. Myotubes were identifiable by their distinct cytoplasmic elongation and multinucleation. Ultrastructural examination confirmed the presence of myotubes by their numerous thick and thin filaments with poorly formed Z-band material.

During skeletal muscle development, the "embryonic" creatine kinase isoenzyme BB is gradually replaced by the "adult" MM type going through an intermediate MB type.[24] Creatine kinase isoenzymes of the RMS cell line verified the lack of differentiation of the tumor cells by the predominance of the "embryonic" BB isoenzyme. However, when differentiation was observed morphologically, there was a concomitant shift in the isoenzyme pattern to the "adult" MM isoenzyme type.

The inability of the RMS cells to differentiate *in vivo* was also observed in nude mouse heterotransplants, which showed no morphologic evidence of differentiation by light or electron microscopy. Heterotransplants in nude mice gave an identical pattern of creatine kinase isoenzymes as the parent cell line, thus substantiating the lack of differentiation. Previous studies of heterotransplants of various soft-tissue sarcomas in nude mice have reported increased differentiation by light microscopy evaluation in only a small percentage of tumors.[25]

In a preliminary report,[3] the RMS cells demonstrated differentiation into myotubes but failed to proliferate.

Another defined serum-free medium (20/12 EGF) was developed in which the RMS cells were able to proliferate, but gave no evidence of differentiation. Components of the original medium (DMEM 15) were tested, and PGE_1 was found to induce differentiation. Differentiation was also induced by 2% FCS, 1 nM TPA, and retinoic acid. Experiments with dbCAMP and the polar solvent DMSO gave no evidence of differentiation. This differs from the murine RMS, which is inducable by dbCAMP,[11] and suggests that the human RMS may differ from the experimental murine tumor. Because 1 nM TPA induced differentiation but 10 nM TPA failed to do so, an optimal differentiating dose may be necessary to induce differentiation. Therefore, one must test a variety of concentrations to entirely exclude a lack of induction. Morphologic and biochemical evidence suggests that not all of the tumor cells responded to the inducing agents, although combinations of agents have not been evaluated. Isoenzymes of creatine kinase gave no quantitative difference in the ability of the various agents tested to induce differentiation.

This human RMS cell line provides a valuable *in vitro* system for the study of human myogenesis and the factors that induce differentiation. It will provide comparison of myogenesis with the numerous *in vitro* studies involving other animal systems. It may also provide a system for the study of gene amplification and the effect of differentiation on that amplification.

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