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Experimental Evidence for a Neural Origin of Ewing's Sarcoma of Bone

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The histogenesis of Ewing's sarcoma remains unknown. Recent studies have suggested a relationship to an unusual form of childhood neural tumor, often termed peripheral neuroepithelioma or primitive neuroectodermal tumor. Five Ewing's sarcoma tumor cell lines were studied for evidence of a neural phenotype. Under normal culture conditions, no morphologic evidence of neural differentiation was detected. Treatment with retinoic acid, an agent known to induce marked neural differentiation in neuroblastoma, had no demonstrable effect. Treatment with either cyclic AMP or TPA, in contrast, induced pronounced morphologic evidence of neural differentiation. Cells developed elongate processes with varicosities by phase-contrast microscopy; filaments, microtubules, and uraniffin-positive dense core granules were present by electron microscopy. Three neural markers (NSE, NFTP, and cholinesterase) were absent or barely detectable in untreated cells, but became abundant after treatment. These results provide convincing evidence for a neural histogenesis of Ewing's sarcoma. They also suggest a close relationship between Ewing's sarcoma and peripheral neural tumors, including the chest wall tumor described by Askin, but only a distant relationship to neuroblastoma. (Am J Pathol 1987, 127:507–518)

EWING'S SARCOMA of bone (ESB), first described by James Ewing in 1921 as a presumed diffuse endothelioma of bone,[1] is a primary bone tumor of still uncertain histogenesis. Numerous studies to date have failed to provide definitive evidence of a common histogenesis for this tumor. Willis[2] repeatedly suggested that the entity was in fact only metastatic neuroblastoma. Many other authors have proposed a variety of origins of relationships, including endothelial,[3] vascular pericytes or smooth muscle,[4] primitive vascular mesenchyme,[5] pluripotential uncommitted mesenchyme,[6–8] osteoblastic (based on collagen matrix synthesis patterns),[9] and small cell osteosarcoma, and/or mesenchymal chondrosarcoma.[10] Ultrastructural studies have confirmed the completely undifferentiated character of most tumors classified as ESB,[11,12] however, at least three recent reports[13–15] have described a neural tumor of bone or ESB with suggestive neural features.

It has been difficult to reconcile these conflicting reports with a common histogenesis for all ESB, yet certain recent studies have strongly suggested that this is a homogeneous group of tumors. Specifically, Turc-Carel et al[16] have identified a unique, characteristic chromosomal abnormality (a reciprocal translocation of a portion of the long arm of Chromosomes 11 and 22) in 5 of 5 cases studied. Aurius et al[17] reported an identical translocation in 4 additional cases at the same time. Recently, Whang-Peng et al[18] confirmed this finding in 18 additional cases of ESB. These same authors have identified the identical abnormality in peripheral neuroepithelioma.[19] Both they[18] and De Chadarevian[20] have also found this abnormality in the small-cell tumor of thoracopulmonary region, first described by Askin, et al.[21] This so-called Askin tumor has recently been found to evince certain neural features, such as expression of neuron-specific enolase,[22] S-100 protein,[23] and neural ultrastructure (neurites, dense core granules).[22] McKeon and co-workers have found that both the Askin tumor and similar peripheral neural tumors of

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soft tissue (so-called peripheral neuroepitheliomas), along with ESB, possess an indistinguishable cytogenetic abnormality and express a similar pattern of oncogenes.[24] In particular, these tumors *always* lack N-*myc* expression, unlike nearly half of neuroblastomas.[25] This is especially apparent when cell lines are examined; all true neuroblastomas which grow *in vitro* overexpress N-*myc*; none of the peripheral neuroepitheliomas do so.

Taken together, these observations have raised the obvious possibility that classical Ewing's sarcoma represents only the most undifferentiated portion of a spectrum of neural tumors of bone and soft tissue.[26] Experimental proof of this has to date been lacking, however. Although one of two published *in vitro* studies identified β-adrenergic receptors on ESB cells,[27] this was not considered specific for any particular histogenesis, because uncoupled β-adrenergic receptors were also identified on, for example, lymphoma cells. The other *in vitro* study[7] (from this laboratory, and utilizing three of the present lines) failed to detect any evidence of neural differentiation under normal growth conditions; instead, only a primitive "mesenchymal" pattern of extracellular matrix synthesis was identified.

In the present report, we present data which strongly suggest a neural histogenesis for this tumor, based on studies of five such tumors in culture.

Materials and Methods

Tissue Culture

Five tumor cell lines established from NCI patients were evaluated in the present study. The details of each line are listed in Table 1. All lines were established and maintained in RPMI 1640 tissue culture medium (Whittaker, M.A. Bioproducts, Walkersville, Maryland) supplemented with 10–20% fetal bovine serum (GIBCO Laboratories, Grand Island, NY). Cells were grown to confluence in T75 tissue culture flasks (Nunc, Roskilde, Denmark) and passaged as necessary.

Nude Mouse Tumors

Tumorigenicity of the cell lines was also established by growth in nude mice (BALB/c nu/nu). For these studies, 5×10^6 cells were injected subcutaneously in the interscapular region. Upon visible evidence of tumor growth, the animal was sacrificed and the tumors were processed for light and electron microscopy, as detailed below.

Differentiation Experiments

Cells from all five lines were plated in 80-sq cm plastic flasks at a density of 5×10^4 cells/ml with 5 ml of medium that was either serum-free or contained 1% or 10% fetal calf serum.[28] This medium was composed of Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F12 (50:50), supplemented with L-glutamine, $NaHCO_3$ (1.2 g/l), and 15 mM HEPES. To this was added the following growth factors: insulin (5 μg/ml), transferrin (100 μg/ml), progesterone (6.3 ng/ml), selenium (30 nM), and putrescine (8.8 ng/ml).

Four agents known for their ability to induce terminal differentiation in other cell lines were tested. All-transretinoic acid (RA, Sigma Chemical Co., St. Louis, Mo) was used at a concentration of 2.5×10^{-7} M. Nerve growth factor (NGF, 7S, Collaborative Research, Inc., Lexington, Mass) was used at 10 ng/ml. N^6-O^2-dibutyryladenosine-3′:5′-cyclic monophosphate (c-AMP) (Sigma) was used at a concentration of 2.5 mM. Phorbol 12-myristate 13-acetate (TPA) (Sigma) was used at a concentration of 25 nM. The medium was changed every 3 days; and after three changes the cells were harvested and prepared for light (LM), electron (EM), or fluorescence (FITC) microscopy. Throughout the time course of the experiments, the morphologic response, if any, of the cells to differentiating agents was documented by phase-contrast microscopy of viable, unfixed cells.

Morphology

For LM, cells or tumor tissue were fixed in phosphate-buffered 10% formalin at 20 C for 4–24 hours

Table 1—Clinical Characteristics of Cell Lines in the Present Study

Case	Age/Sex	Site	Follow-up	PAS/NSE	Cytogenetics
TC-71	23/M	Forearm	DOD	+/−	*rcp* (11:22)
A4573	17/F	Clavicle	DOD	+/−	*rcp* (11:22)
5838	27/M	Forearm	DOD	+/−	*rcp* (11:22)
6647	12/F	Femur	DOD	+/−	*rcp* (11:22)
TC-106	19/M	Sacroiliac	DOD	+/−	*rcp* (11:22)

and embedded in paraffin. For EM, they were fixed in 2.5% glutaraldehyde and embedded in Maraglas (Ladd Research Industries, Inc., Burlington, Vt) epoxy resin by routine techniques.

Uraniffin Reaction

In order to confirm the neurosecretory nature of the dense core granules seen in the cellular processes of differentiated cells, we exposed the cells or tissue to uranyl salts, using the so-called uraniffin reaction.[29] Briefly, cell pellets after fixation were washed twice in Sorensen's phosphate buffer containing 0.1 M glycine for quenching any residual aldehyde groups. The pellets were then washed for 72 hours in 0.9% NaCl at 4 C (with several changes of fresh NaCl). Then the pellets were immersed in a 4% aqueous solution of uranyl acetate at 4 C for 48 hours. They were then rinsed again in NaCl and routinely processed for EM in epoxy sections. As a positive control, a neuroblastoma cell line (KCNR) with known neurosecretory granule expression was processed in parallel.

Immunofluorescence

Differentiated and undifferentiated tumor cells were grown on glass coverslip or in chamber slides (LabTech, Miles Scientific, Naperville, Ill). After 9 days the cells were rinsed in cold (4 C) phosphate-buffered saline (PBS) twice and fixed in cold (−20 C) methanol for 10 minutes. After three washes in cold PBS, the primary antibody (as listed in Table 2) was applied at the previously experimentally derived optimal dilution (Table 2), either overnight at 4 C or for 30 minutes at 37 C. After the first incubation, the cells were rinsed three times in PBS, and the secondary Ab/FITC conjugate was applied (1:10–1:20) for 1 hour at 20 C. Unbound secondary Ab was removed with three washes in PBS. The slides or coverslip was then mounted in a solution of 50% glycerol: 50% PBS and examined in a Zeiss epifluorescence microscope equipped with narrow band pass dichroic filters for fluorescein. Images were recorded on Kodak Ektachrome film exposed at ISO 800.

Table 2—Primary Antibodies

Antibody	Source	Working dilution
NSE	Dako, California	1:50
Cholinesterase	Dako, California	1:100
Neurofilaments	Labsystems, Finland	1:10
Vimentin	Boehringer-Mannheim, West Germany	1:100

Results

Original Tumors

Because it was essential to exclude at the outset any tumor with any feature suggestive of neural differentiation, both existing (A4573, 5838, and 6647) and newly established (TC-71, TC-106) cell lines were rigorously evaluated, both in the patient and *in vitro*. All patients were of typical age (12–23), nearly equally divided by sex (3 male, 2 female), and presented with a primary tumor of bone (Table 1). All have died of the disease. Cytogenetic analysis of both primary tumor (when available) and/or cultured tumor cells revealed a characteristic *rcp* (11:22) translocation in all cases (Table 1).

All tumors satisfied the most stringent light-microscopic criteria for the diagnosis of Ewing's sarcoma (Figure 1A–D), most having a characteristic large clear/small dark tumor cell pattern (Figure 1A, inset). All cases had been referred to the NCI with a diagnosis of Ewing's sarcoma, and this diagnosis had been confirmed upon internal review. Further, original tissue was obtained and examined by EM, even if the patient had not undergone biopsy at the NCI. In every case, the ultrastructure was typically bland and unremarkable (ie, regular, round to oval, blastic nuclei and surrounding primitive cytoplasm), with *no* evidence of neural differentiation (Figure 2). Glycogen was demonstrable by LM (periodic acid–Schiff [PAS] ± diastase) and EM in every case (Figure 2, inset). All cases were also negative for neuron-specific enolase on paraffin-embedded tumor sections (Table 1).

Establishment of Tumor Cell Lines

The tumorigenicity of cultured cells from the original tumors was established by growth in nude mice. The cells (Figure 3A) were isomorphous with the original tumor cells. Further, the cells exhibited a highly transformed phenotype *in vitro* (Figure 3B), and the ultrastructure of these cultured cells (Figure 3C) was also indistinguishable from both the original tumors (Figure 2) and the nude mouse tumors (Figure 3A). This was true of all five lines. Although subtle differences exist between lines, all show an appearance identical to that of Ewing's sarcoma of bone *in vivo*. Thus, by the most rigorous criteria, all cell lines employed in the present study were Ewing's sarcoma.

Differentiation

Because neuroblastoma cells are known to sometimes undergo spontaneous differentiation *in vitro* when grown in the absence of serum, we first exam-

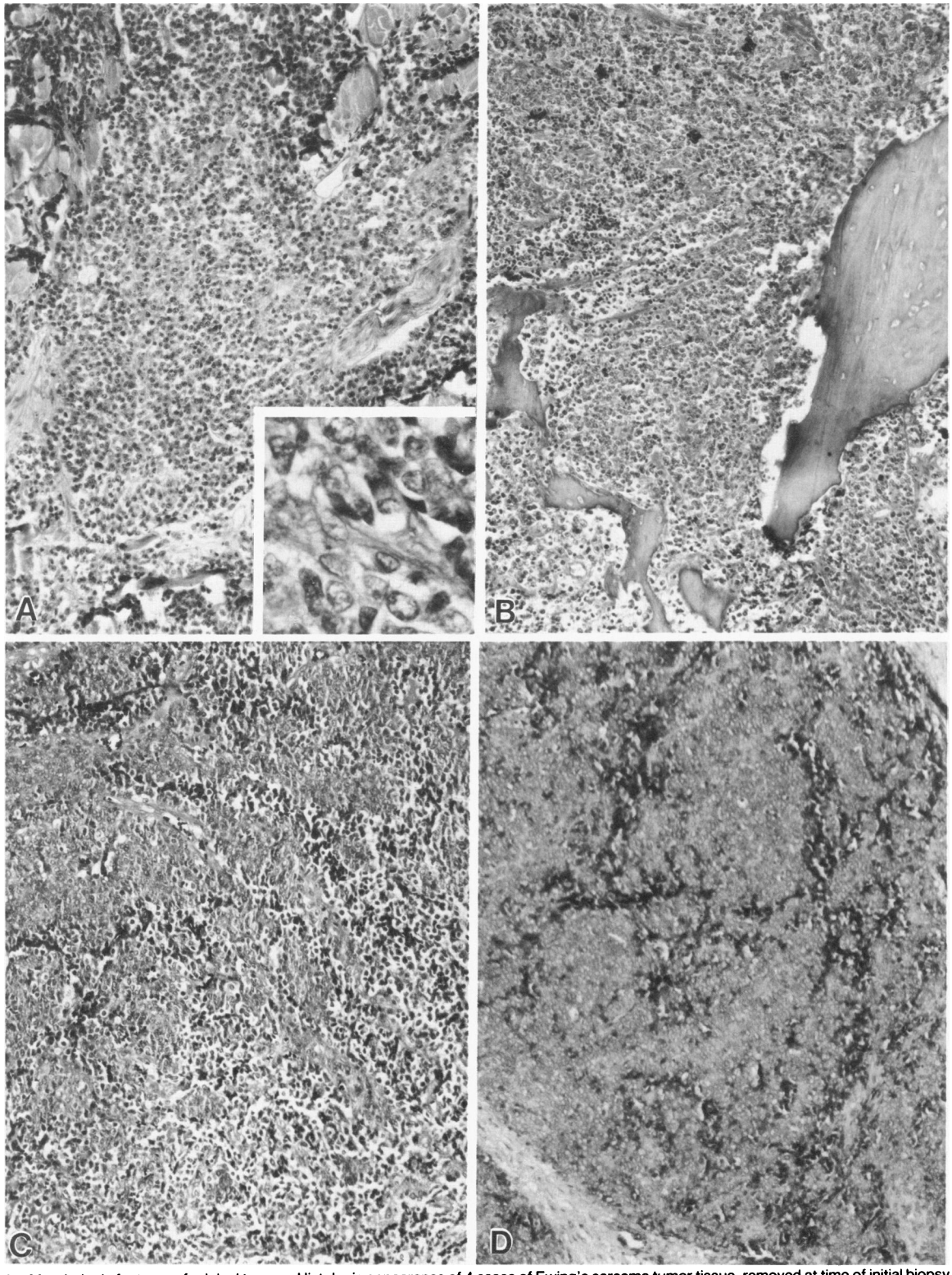

Figure 1—Morphologic features of original tumors. Histologic appearance of 4 cases of Ewing's sarcoma tumor tissue, removed at time of initial biopsy, from which tumor cell lines TC-71 (**A**), 6647 (**B**), TC-106 (**C**), and 4573 (**D**) were established. Typical large, clear and small, dark cell populations are apparent (**A, inset**). The tumors are completely undifferentiated by light microscopy.

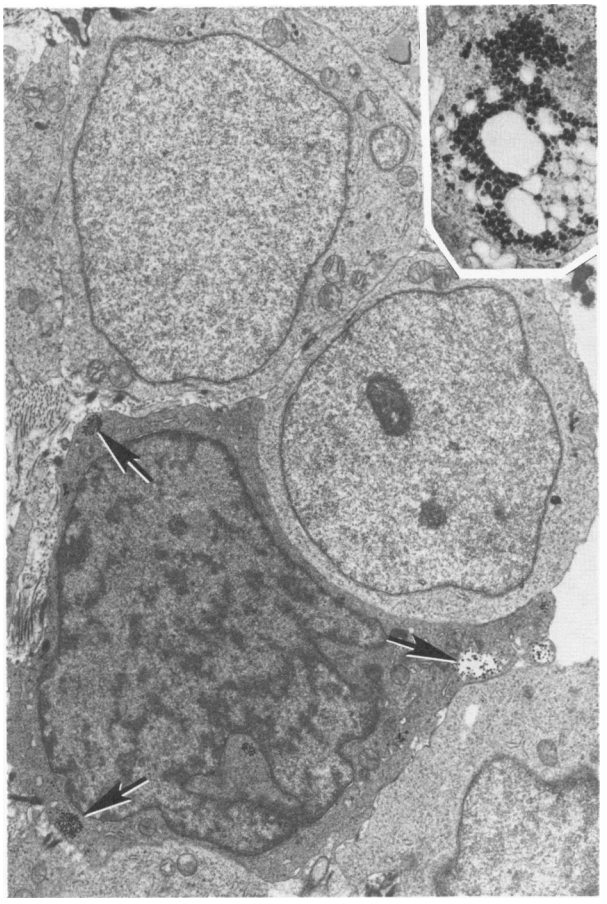

Figure 2—Ultrastructure of original tumors (example). Electron-microscopic appearance of same tumor tissue as in Figure 1C. The same light and dark tumor cell population is apparent. Again, the tumor is completely undifferentiated. The only conspicuous feature is cytoplasmic deposits of dense granular material (*arrows*), which at high magnification are identifiable as glycogen rosettes surrounding lipid droplets (**inset**).

ined the effect of serum-free growth conditions on cell morphology. In no case (in the presence or absence of serum) was any evidence of neural differentiation detected in cultured Ewing's sarcoma cells (Figure 4A), unlike neuroblastoma cells in serum-free conditions (Figure 4B).

Treatment with RA, although previously shown to be an extremely potent neural differentiating agent in neuroblastoma, had little or no effect on Ewing's sarcoma cells. This was equally true whether the cells were grown in the presence of serum or whether in defined, serum-free conditions. The only reproducible effect was marked cell toxicity, even at 10^{-7} M.

In stark contrast, treatment with c-AMP, with or without NGF, resulted in striking neural differentiation. This was true whether the cells were grown in the presence of 1% fetal calf serum (FCS) (Figure 4C) or in serum-free conditions. This was true of all cell lines tested; in some cases, the degree of differentiation obtained was similar to that seen in neuroblastoma. In these cases, neurites with varicosities and terminal boutons were readily appreciated (Figure 4D). Treatment with NGF alone resulted in minimal differentiation (data not shown).

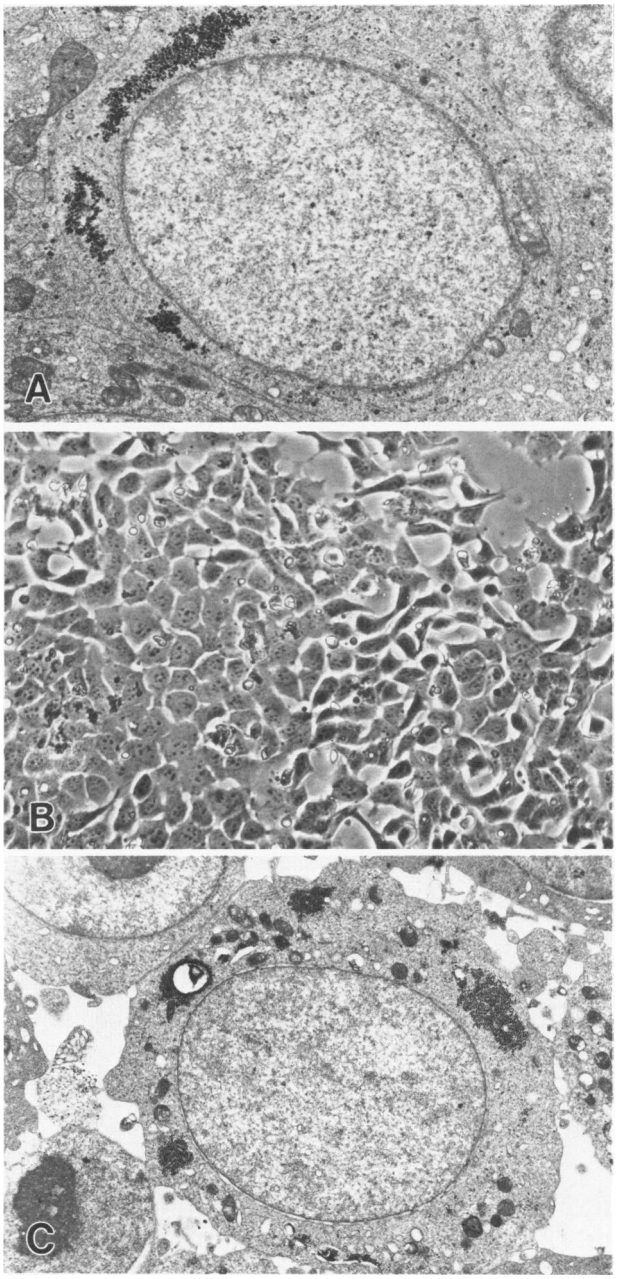

Figure 3—Characterization of cell lines established from tumor tissue. **A**—Electron-microscopic appearance of tumor cell from nude mouse tumor (TC-71). Again, no differentiation of any kind is detectable; only cytoplasmic glycogen deposits are appreciated, as in original tumor. **B**—Phase-contrast microscopic appearance of typical tumor cell line established from tumor (TC-71). A uniform appearance of substrate-adherent, polygonal, transformed cells lacking demonstrable evidence of neural or other differentiation can be seen. Spontaneous differentiation has not been observed. **C**—Electron-microscopic appearance of tumor cells from TC-71. As in original tumors (2A) and nude mouse tumors (3A), the cells are completely undifferentiated and possess only cytoplasmic pools of glycogen. No neural or other differentiation was ever observed in control cultures.

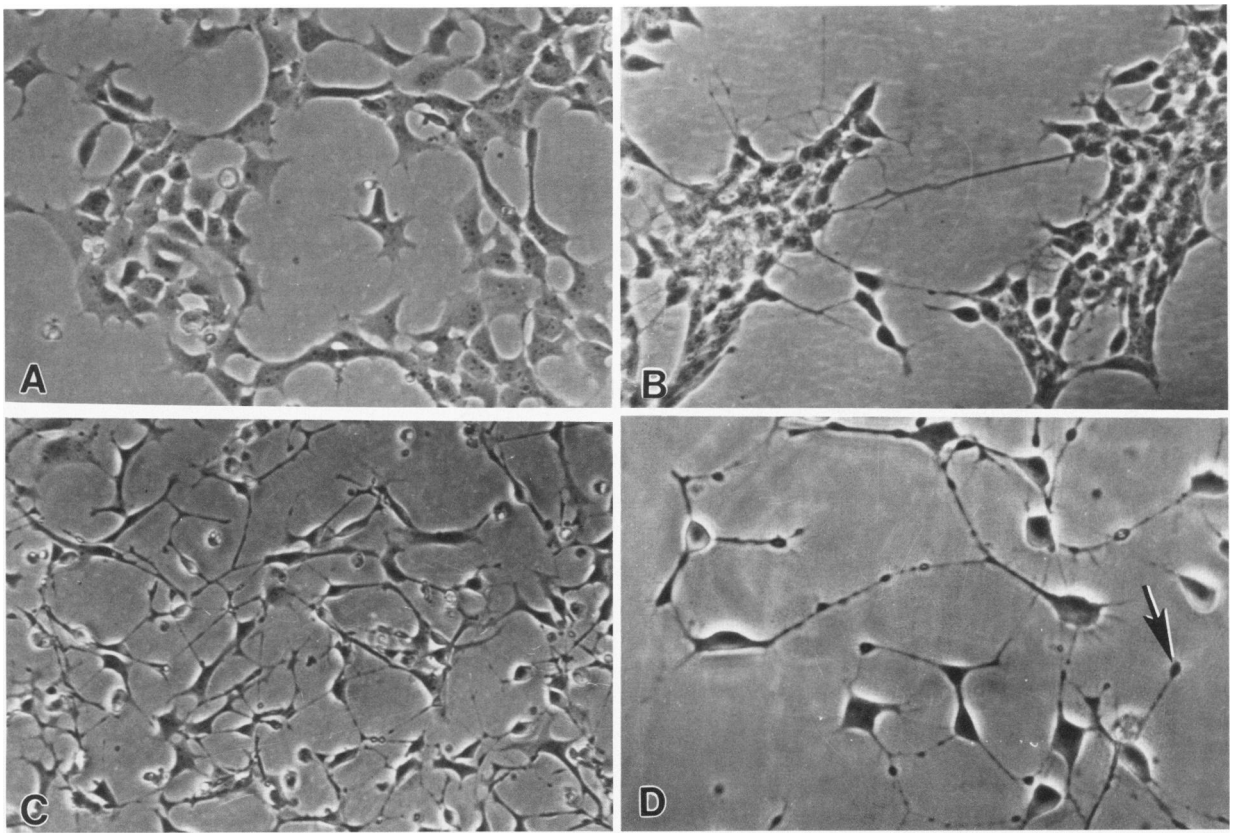

Figure 4—Tissue culture appearance of treated tumor cells. **A**—TC-71 tumor cells treated with RA in the absence of serum for 12 days. No neurites or phase-dense neuroblasts are found. Comparable results were obtained with and without serum. **B**—Typical control neuroblastoma (KCNR line), untreated with any differentiating agent. Note poor substrate adherence ("tear drop cells"), spontaneous extension of cell processes, bundle branch recruitment of processes (*center*), and rare varicosities (*bottom center*). **C**—TC-71 tumor cells treated with dibutyrylcyclic AMP under serum-free conditions. Phase-dense cell bodies, which are less substrate adherent, are interconnected by numerous neuritic processes. **D**—Detail of AMP-induced differentiation. The slender, generally unbranched neurites typically possess one or more varicosities and terminate on other neurites, or in larger growth cones (*arrow*). This pattern is virtually identical to that seen with conventional childhood neuroblastoma cells; only colony formation was less apparent.

The only noticeable difference between differentiated Ewing's sarcoma cells and neuroblastoma cells was the lack of bundle branch recruitment or large fascicles of parallel neurites interconnecting cell colonies, typically seen in well-differentiated neuroblastoma cultures (Figure 4B versus 4D).

Ultrastructure

The apparent neural differentiation resulting from treatment of the tumors *in vitro* with c-AMP ± NGF or TPA was confirmed by ultrastructural examination and cytochemistry. The processes seen by phase-contrast microscopy were detectable by EM as rather typical neurites, containing neurotubules and neurofilaments (Figure 5A). Often, terminal bulbous swellings of such processes were noted, containing somewhat atypical appearing but apparent true dense core granules (Figure 5A and B). These features were true of all cell lines studied after differentiation by either c-AMP or TPA (Figure 5C). In contrast, they were absent from all original tumors (Figure 2) and every untreated cell line (Figure 3A and C).

Uraniffin Reaction

Recently, an EM immunocytochemical technique, the uraniffin reaction, has been described and demonstrated to be specific for neural crest type dense core granules. Using this technique, we were able to demonstrate the neurosecretory character of the granules found only in differentiated Ewing's sarcoma cells. In the positive control, a differentiated neuroblastoma, only classic dense core granules were stained (Figure 6A). Normal structures such as nuclear chromatin were also stained. In differentiated Ewing's sarcoma cells, the unit membrane bound granules first identified by conventional EM also stained intensely with uranyl acetate (Figure 6B). This result provides nonmorphologic evidence that the granules observed by EM, even though somewhat atypical in appearance, are bona fide neurosecretory granules.

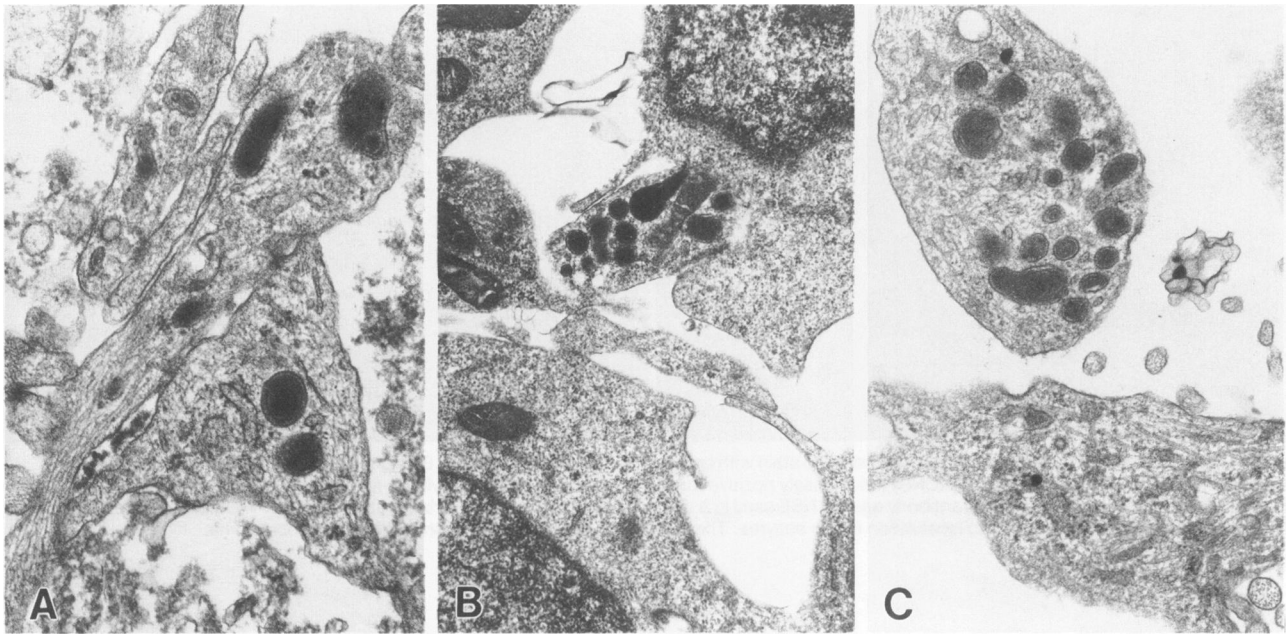

Figure 5—Ultrastructure of differentiation. **A**—The apparent neural character of the cell processes observed in Figure 4C and D is confirmed here. Numerous obliquely sectioned processes containing intermediate filaments and microtubules. Atypical unit membrane-bound dense-core granules are contained within apparent varicosities. Cyclic AMP-induced differentiation. **B**—Detail of AMP-induced dense-core granules. Although great heterogeneity in granule size and shape was observed, typical dense core granules were always found admixed with atypical granules. **C**—TPA-induced neural differentiation. No qualitative or quantitative differences were observed with either agent. Ultrastructural features such as neurotubles and neurofilaments were found (data not shown), and most importantly, dense core granules were readily found, especially in terminal cell processes.

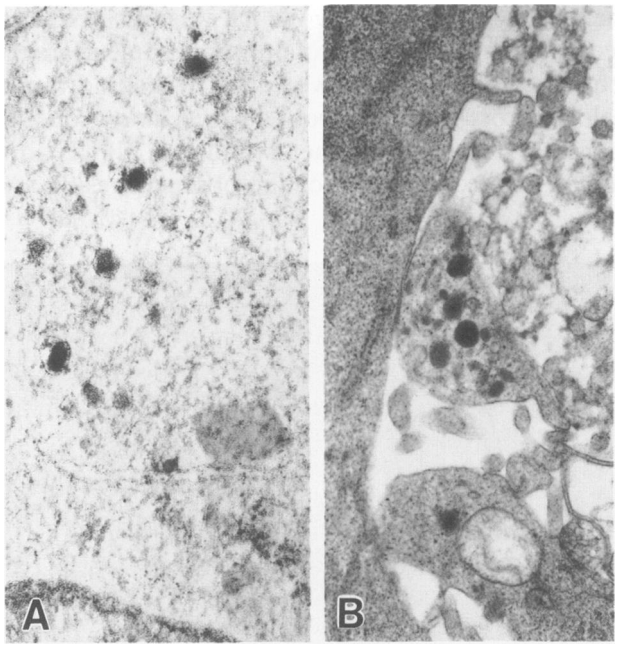

Figure 6—Uraniffin reaction. **A**—Classic childhood neuroblastoma (KCNR) grown in culture in parallel with Ewing's cell lines. Well-developed neural differentiation is normal for this line. Here, four electron-dense neurosecretory granules are obvious in tumor cell cytoplasm. Only the nucleus (*bottom*) is otherwise stained in this unstained section, treated with uranyl salts, as described in Materials and Methods. **B**—Cell from differentiated A4573 Ewing's tumor line after treatment with c-AMP. Note the presence of four or five unit-membrane, dense-core granules with obvious affinity for uranyl salts; these are thereby identified as neurosecretory in nature.

Neuron-Specific Enolase

To further verify the neural differentiation apparently induced in Ewing's sarcoma cells following differentiation with both c-AMP and NGF, or the tumor promotor (TPA), we studied the presence of a known neural-associated enzyme, neuron-specific enolase (NSE), in Ewing's sarcoma cells before and after differentiation. In undifferentiated cells, NSE was not detectable. This was also true of fibrosarcoma cells employed as a negative control (Figure 7A), and unlike untreated positive control cells from a classic childhood neuroblastoma (Figure 7B).

After morphologically defined differentiation, Ewing's sarcoma cells became strikingly positive for NSE, as demonstrated by immunofluorescence localization of the NSE-specific antibody to cell cytoplasm only (Figure 7C). Unlike neuroblastoma cells, differentiated Ewing's sarcoma cells demonstrated variable fluorescence intensity, suggesting unequal induction of enzyme expression. Further, the relative overall fluorescence intensity of the Ewing's cells was generally less than that of neuroblastoma (Figure 7B versus C). Nonetheless, the distribution in perinuclear cytoplasm and that within neurites were comparable (Figure 7D).

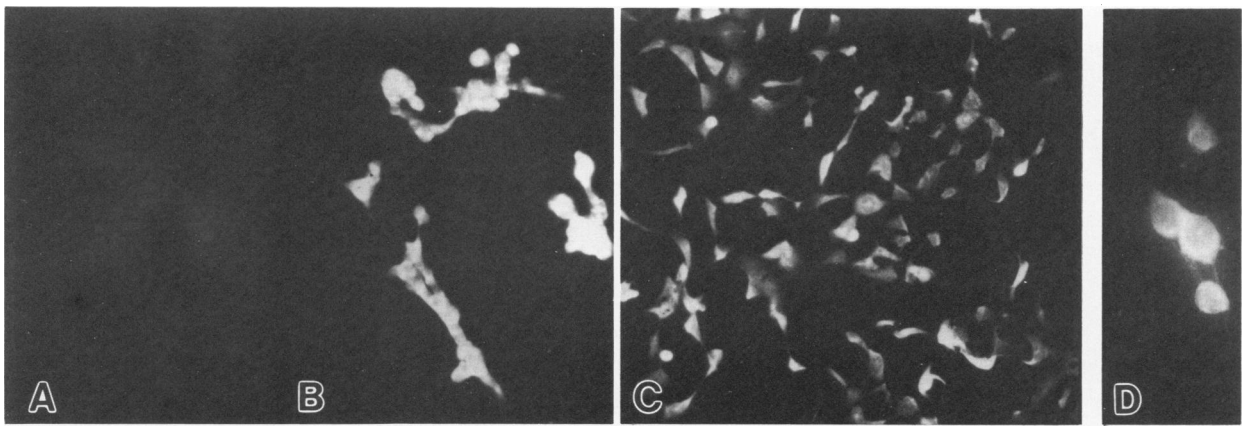

Figure 7—NSE. **A**—Fibrosarcoma cells in culture fail to stain with antibody against NSE. Untreated Ewing's sarcoma cells were, likewise, negative. **B**—Classic childhood neuroblastoma cells (KCNR) are intensely positive for NSE. Processed in parallel with **A**, above. C—Differentiated Ewing's sarcoma cells (c-AMP) after staining with the same antibody against NSE used in **A** and **B**. Note the intense (but variable) staining. **D**—Detail of differentiated Ewing's cells. Note the perinuclear (cytoplasmic) localization of the enzyme. This pattern is virtually indistinguishable from neuroblastoma.

Cholinesterase

Because NSE has been frequently criticized as nonspecific for neural cells, we also assayed the Ewing's sarcoma cells for the presence for an additional neural specific enzyme, cholinesterase. In untreated cells grown in 20% FCS (ie, highly undifferentiated), punctate fluorescence positivity was detectable in the perinuclear Golgi region (Figure 8A). Diffuse cytoplasmic fluorescence was just detectable above background fluorescence, but appeared real. Following differentiation, the cells became overwhelmingly positive for cholinesterase, as demonstrated by intense perinuclear cytoplasmic fluorescence; no Golgi or other localization was discernible (Figure 8B). Even the most delicate neurites, varicosities, and terminal boutons were readily detectable. Negative control cells (fibrosarcoma, lymphoma) were completely nonfluorescent (data not shown).

Cytoskeletal Proteins

An additional piece of evidence to substantiate the putative neural differentiation detected in these tumor cells would be the acquisition of neural tissue-specific cytoskeletal proteins after differentiation. Of the five classes of cytoskeletal proteins, only neural filament triplet protein (NFTP) is specific for neural tissue. Vimentin usually coexists (especially in vitro), but the presence of NFTP protein would be reliable evidence of neural histogenesis. Consequently, we examined HeLa cells, neuroblastoma cells, and Ewing's sarcoma cells, before and after differentiation, for the presence of the 200-kd subunit of NFTP.

As expected, HeLa cells, consonant with their epithelial derivation, were negative for NFTP (Figure 9A). Neuroblastoma cells were intensely positive (data not shown). Untreated Ewing's sarcoma cells showed no evidence of NFTP by immunofluorescence (Figure 9B). After differentiation with c-AMP or TPA, however, these cells became intensely positive for at least the 200-kd subunit of NFTP (Figure 9C).

We also examined the reactivity of all cell types for the presence of vimentin. We felt this was necessary because all cytoskeletal proteins share some degree of sequence homology, and we wished to exclude immunologic cross-reactivity between the ubiquitous vimentin cytoskeletal filaments and our NFTP antibody as an explanation for the observed results. As

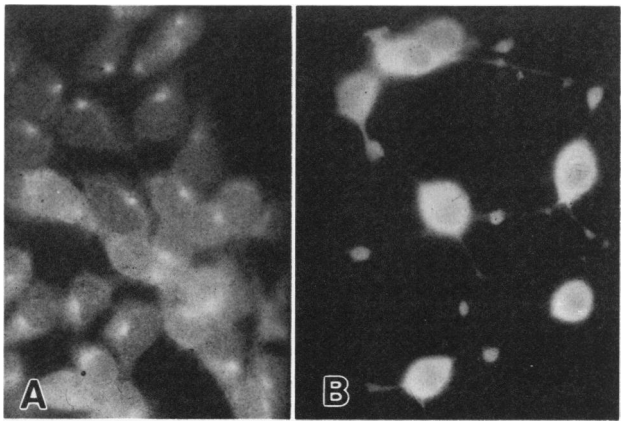

Figure 8—Cholinesterase. **A**—Overexposed micrograph of undifferentiated control cells. An intense spot of perinuclear fluorescence and diffuse, less intense cytoplasmic fluorescence is apparent in each tumor cell. **B**—Cyclic AMP-differentiated cells. Nonfluorescent nuclei are nearly obscured by the diffuse, intense cytoplasmic fluorescence. Even the finest cell processes, and especially the varicosities, are brightly fluorescent.

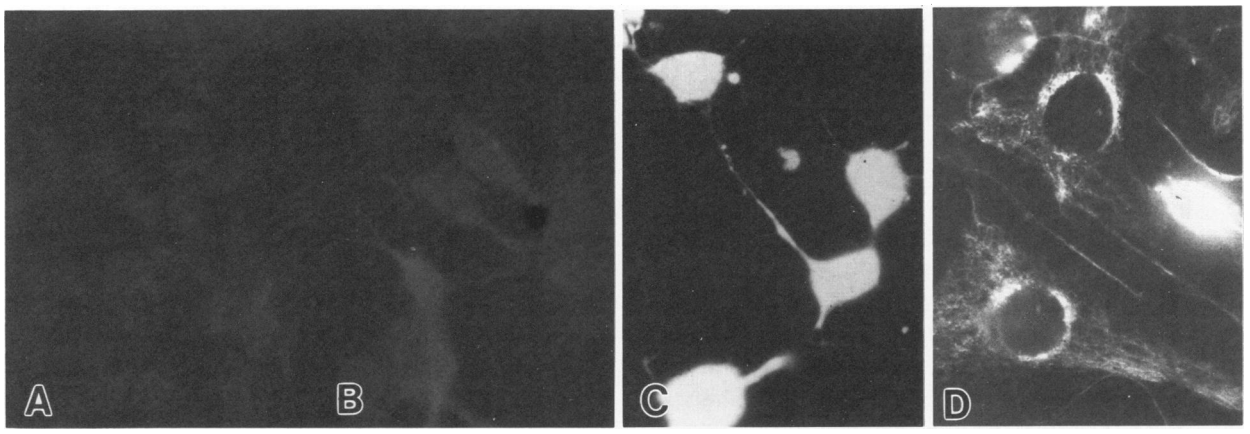

Figure 9—Cytoskeletal proteins. **A**—HeLa cells stained with antibody to NFTP. No fluorescence is detectable, as expected of these nonneural, epithelial cells. **B**—Untreated Ewing's sarcoma cells with antibody to NFTP. Again, no fluorescence is detectable, similar to HeLa cells (8A, above). **C**—Differentiated Ewing's sarcoma cells with antibody to NFTP. In striking contrast to the same cells untreated with cyclic AMP, these treated cells are intensely fluorescent. Even the slender cell processes are positive. The fluorescence is so intense as to obscure the nuclei in this micrograph; negative nuclei were visible, however. **D**—Ewing's sarcoma cells with antibody to vimentin. To confirm the specificity of the result in **C**, above, similar cells from paired, treated flasks were stained with antibody to the ubiquitous cytoskeletal protein vimentin. Somewhat more substrate adherent cells were photographed to illustrate the typical vimentin cytoskeleton to advantage. There is little if any relationship between the vimentin and NFTP cytoskeleton.

expected, all cells under all conditions *in vitro* were positive, but the pattern of fluorescence positivity was strikingly different from that observed with NFTP antibodies. Staining with anti-vimentin antibodies was less intense, distinctly fibrillar, and predominantly perinuclear (Figure 9C), even in highly differentiated Ewing's cells. This contrasted strikingly with the pattern and intensity of NFTP staining in the same cells (Figure 9C versus D). Thus, the NFTP reactivity was deemed specific. The results for all the antibodies and all the cell lines are summarized in Table 3. Remarkably, each of the cell lines showed the same pattern: little (cholinesterase, vimentin) or no (NSE, neurofilaments) reactivity was found in the untreated control cells; but after differentiation, all lines were demonstrably positive for each antibody. There were no exceptions to this general rule, although the degree of positivity that developed with differentiation was variable, as indicated by the semiquantitative scoring noted in the table.

Discussion

Neural Differentiation and Implication for Histogenesis of Ewing's Sarcoma

The results presented here appear to provide compelling evidence for the potential of Ewing's sarcoma cells to undergo marked neural differentiation *in vitro*. The presence of neurites by phase-contrast microscopy and EM, the appearance of large numbers of uraniffin-positive dense core granules by EM, the induction of neural-associated enzymes (NSE, cholinesterase), and the acquisition of a neural-specific cytoskeleton (NFTP) can only be explained by this hypothesis. It is premature at this point, however, to unduly generalize these observations. First, not all Ewing's tumors can be grown successfully in culture (fewer than 30% do so in our hands). We have examined here only five lines (albeit as typical in all respects as any case), but we cannot with certainty state that neural differentiation would be demonstrable with all

Table 3—Immunofluorescence Results

Cell line	NSE	Cholinesterase	Vimentin	Neurofilaments
TC-71	–	+/–	+	–
+AMP	+	+++	++	+
A4573	–	+/–	+	–
+AMP	+	+	++	+
5838	–	+/–	+	–
+AMP	+.	+	++	+
6647	–	+/–	+	–
+AMP	+	++	++	+
TC-106	–	+/–	+	–
+AMP	+	++	++	+

Ewing's sarcoma cell lines, especially because the initial diagnostic criteria are hardly specific. Despite this, the only known positive finding in Ewings sarcoma to date, the reciprocal Chromosome 11 and 22 translocation, was found in all of the present cases, as well as all cases studied to date from fresh patient tumor tissue. This suggests that the findings reported here can be reasonably generalized to at least the majority of Ewing's tumors. The fact that some cases ESB have recently been described[30] which lack the 11:22 translocation in no way diminishes the veracity of this conclusion.

Relationship to Other Childhood Neural Tumors

The findings reported here also provide evidence for a link between Ewing's tumor and at least two other childhood tumors, peripheral neuroepithelioma (PNET, peripheral neuroblastoma) and Askin's tumor, or small-cell tumor of thoracopulmonary region. In the latter case, the authors originally suggested that this tumor might be a form of neural tumor, based on electron-microscopic examination of three cases. Further, they could not determine with certainty whether the tumor arose in bone or soft tissue. The original distinction from Ewing's (ie, PAS negativity) appears to be arbitrary, because PAS-positive variants have been described.[22,23]

It is likely that a spectrum of such bone tumors exists, with the tumor described by Jaffe et al as primitive neuroectodermal tumor (PNET) of bone[14] and Askin's tumor (a tumor whose bone versus soft-tissue origin is unclear) representing obvious neural tumors, and Ewing's tumor the completely undifferentiated version of the same. The finding that both Ewing's and Askin's tumors possess the same *rcp* (11:22) translocation only supports this assertion. This interpretation also allows conceptual integration of recent reports such as that of Schmidt et al of Ewing's sarcoma with neuroblastomatous features[13] and Perez-Atayde et al of neuroectodermal differentiation in bone tumors presenting as Ewing's sarcoma,[15] as well as Jaffe's observation that the PNETs which he described were referred with a diagnosis of Ewing's sarcoma.[14] Apparently, these tumors represent intermediate degrees of differentiation *in vivo* of otherwise typical Ewing's sarcoma. This was one of the possibilities raised by Jaffe et al[14] in their study of neuroectodermal tumors of bone. These tumors appear to be the *in vivo* analogs of the *in vitro* data presented here.

Relationship to Bone Sarcomas

Ewing's sarcoma is only one of several well-defined primary bone tumors. It is unreasonable to expect that all are related to Ewing's sarcoma. Although all 5 Ewing's cases discussed above appear homogeneous in their absent or incipient neural differentiation, additional reports have suggested that at least some Ewing's-like tumors are vascular, pericytic, pluripotential, or otherwise sarcomatous in nature.[3-9] This is especially true of mesenchymal chondrosarcoma and small-cell osteosarcoma. No experimental studies have yet been reported on these entities, and the possibility that they are unrelated or represent an alternative pathway of differentiation cannot therefore be excluded.

The absolute failure of Ewing's sarcoma cells from the five lines used in the present study to undergo any form of nonneural differentiation, even in the presence of nonspecific differentiating agents such as TPA, strongly suggests that a simple mesenchymal origin is not likely for typical Ewing's tumor cells. These tumors show *in vitro* neural differentiation, unlike other bone tumors such as osteosarcoma and MFH of bone, which do not (our unpublished observations). Rather, it is likely that a family of small blue-cell tumors exists that is distinct from bona fide Ewing's sarcoma, as described here. These other, true sarcomas of bone may display vastly different biologic behavior and histogenesis.

Relationship to Soft-Tissue Tumors

Extraosseous Ewing's (EOE) sarcoma has been described by several authors as a tumor that in most respects is indistinguishable from ESB. Although 1 case of estraosseous Ewing's has been reported to possess the same *rcp* (11:22) translocation as ESB,[31] Whang-Peng et al have reported an absence of the *rcp* (11:22) chromosomal abnormality in their cases,[18] and Garvin et al have documented the progression of a case of EOE with a *rcp* (2:13) translocation to a typical case of rhabdomyosarcoma.[32] This at least suggests that EOE sarcoma should not be construed as simply the soft-tissue counterpart of osseous Ewing's sarcoma. Rather, the soft-tissue tumors probably represent a heterogeneous group of completely undifferentiated tumors of potentially disparate histogenesis.

As noted above, neural tumors of childhood other than neuroblastoma occur in bone *and* soft tissue. These soft-tissue tumors, known variously as peripheral neuroblastoma, peripheral neuroepithelioma, and extracranial PNET,[33-38] unlike EOE, appear to be closely related to ESB. Specifically, these tumors also possess the *rcp* (11:22) cytogenetic abnormality and have similar patterns of oncogene expression (ie, N-*myc*-negative, c-*myc*-positive).[39] These observations suggest that peripheral neuroepithelioma of soft

tissue and Ewing's sarcoma are related nonneuroblastomatous neural tumors of childhood.

General Characteristics of Ewing's and Related Neural Tumors

Virtually all of these tumors reported to date lack expression of catecholamines (unlike the overwhelming majority of true neuroblastomas), occur in older patients (adolescents primarily), fail to express the neuroblastoma-associated oncogene N-*myc*, and arise in widely disparate anatomic sites, but never within the sympathetic nervous system, the usual site of origin of most childhood neuroblastomas. This profile describes a family of neural tumors readily distinguished from typical childhood nueroblastoma. The present study documents that Ewing's sarcoma appears to be one, albeit undifferentiated, member of this family of neural tumors.

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