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Immunologic Characterization of Ewing's Sarcoma Using Mesenchymal and Neural Markers

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The two most recent hypotheses about the histogenesis of Ewing's Sarcoma (ES) are that it has a mesenchymal or neuroectodermal origin. Immunologic markers specific to these two tissue origins were tested on cryostat sections from three primary tumors carrying the chromosomal translocation t(11;22)(q24;q12). Cell lines established** in vitro **from two of these three primary tumors were also analyzed. Using antibodies directed against neural components (neurone-specific-enolase [NSE], HNK-1, and neurofilament triplet proteins [NFTP]), positive reactions were observed in cells from two primary tumors and their corresponding cell lines. Results of electron microscopic examination of the primary tumors were compatible with the diagnosis of ES. When using antibodies directed against mesenchymal cell surface antigens (common leucocytes, Leu M1, Leu M2, and Leu M3), the weak positive reactions observed in the three primary tumors were attributed to lymphoid infiltrates within tumor cells. Six additional ES cell lines carrying the translocation t(11;22) were also analyzed by immunocytochemical and flow cytometry methods using antibodies directed against mesenchymal and neural components. Positive reactions were observed in all seven cell lines tested using antibodies directed against NSE, HNK-1, and 200 KD subunit of the NFTP, whereas negative reactions were obtained with Leu M2 antibody. These results are consistent with a neuroectodermal origin of ES cells. (Am J Pathol 1989, 135:847–855)

Ewing's sarcoma (ES) is a malignant proliferation arising primarily in bone. It is composed of small, round, poorly differentiated cells. ES belongs to the group of so-called small-round-cell tumors of childhood.[1] Until now, the only positive diagnostic criterion of ES among the tumors of this group was the chromosomal translocation t(11;22)(q24;q12) associated with ES cells.[2,3] Because of its lack of a differentiation pattern, the histogenesis of ES has been the matter of various hypotheses. That it originates in a primitive mesenchymal cell[1,4,5] or a neuroectodermal cell[6–8] are the most recent hypotheses proposed for the origin of ES cells.

We report here on the immunohistologic characterization of cells from three primary ES tumors using a panel of monoclonal antibodies directed against mesenchymal or neural tissues. Cells from *in vitro*-established cell lines derived from two of the three tumors were also analyzed. An additional six ES *in vitro*-established cell lines were characterized immunocytologically using antibodies directed against mesenchymal and neural components. Antigen expression was also analyzed by flow cytometry in cells from three of these six cell lines.

Material and Methods

Tumors

Surgically removed specimens originated from three patients with primary ES tumors. Clinicopathologic data are given in Table 1.

A part of the specimens was quick-frozen in liquid nitrogen for immunohistologic studies. Another part was used for establishing cell lines. Cytogenetic studies were performed on other parts. The remainder was embedded in paraffin for routine histologic diagnosis and in araldite for electron microscopic examination.

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Table 1. *Clinicopathologic Data of the Primary Tumors and In Vitro-Established ES Cell Lines*

Specimen*	Age (years)/sex	Primary tumor site	Histologic diagnosis	EM	Origin of cell line**
Tumor n°1	35/M	Left rib	ES	0	–
Tumor n°2 DES-1	23/F	Iliac crest	ES	+	–
Tumor n°3† IARC-EW 13	17/M	Right tibia	ES	+	–
IARC-EW 1	19/M	Right rib	ES	0	R
IARC-EW 7	20/F	Left scapula	ES	+	R
IARC-EW 12	16/F	Iliac crest	ES	+	R
IARC-EW 15	20/F	Fibula	ES	0	P
IARC-EW 17	15/M	Left heel	ES	+	R
SKES-1	18/F	Not known	ES	+	–

* The cell lines IARC-EW 1 to EW 17 were established by G. M. Lenoir (International Agency for Research on Cancer, Lyon, France); SKES-1 by J. J. Fogh (Sloan Kettering Institute, New York, New York); DES-1 at School of Medicine, Dijon, France.
† The primary tumors studied at the origin of the cell line were provided by T. Philip, Centre Léon Berard, Lyon, France.
EM, electron microscopy study; +, done; 0, not done; P, primary tumor; R, recurrent tumor.

Cell Lines

Clinicopathologic data of ES cell lines tested in this study are reported in Table 1. Fragments from the primary tumor were dissociated mechanically first, and then with collagenase (type II, Sigma Chemical Co. St. Louis, MO, 200 U/ml final concentration, 2 hours). Disaggregated cells were cultured in RPMI 1640 medium (Flow Laboratories Inc., McLean, VA) supplemented with 20% foetal calf serum, 1% glutamin (Flow Laboratories), and antibiotics. The culture medium was changed twice weekly.

Immunohistology

For immunohistologic studies, 4-μm cryostat sections were prepared from frozen tumor specimens. Using a cytospin centrifuge (5 minutes at 450 rpm), cell smears were prepared from aliquots of cell suspensions from cell lines. Each aliquot contained at least 90% of viable cells. Cryostat sections and cell smears were air-dried and stored at −20 C until use. Just before staining, the slides were fixed in acetone (10 minutes at −20 C).

Immunohistologic studies were performed using indirect immunoperoxidase antiperoxidase methods.[9,10] Briefly, fixed sections were incubated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 minutes to block the endogenous peroxidase activity and then washed in PBS. The nonspecific background staining was blocked with normal swine serum (Dako, Geostrup, Denmark; solution 1: 20) for 30 minutes at room temperature in a humid chamber. The sections were incubated with the appropriate antibody for 1 hour and subsequently incubated for 30 minutes with the second antibody. The peroxidase-anti-peroxidase method was used as described in the Dako kit recommendations. Specimens were treated with diaminobenzidine and H_2O_2 to develop the peroxidase staining and counterstained with hematoxylin.

Antibodies

The primary antibodies used in this study are presented in Table 2. For the indirect immunoperoxidase method,

Table 2. *Panel of Antibodies Used for Immunologic Studies of ES Cells*

Antibodies	Origin	Dilution
Anti-common leucocytes (CL)	Monoclonal, DAKO	1:50
Leu M1	Monoclonal, Becton-Dickinson	1:100
Leu M2	Monoclonal, Becton-Dickinson	1:100
Leu M3	Monoclonal, Becton-Dickinson	1:100
MO 1	Monoclonal, Coultronics	1:200
ILR 2	Monoclonal, Coultronics	1:100
Anti-tranferrin receptor	Monoclonal, Becton-Dickinson	1:50
Anti-vimentin	Monoclonal, Labsystems	1:100
Anti-total keratin	Polyclonal, Biosoft	1:50
Anti-desmin	Monoclonal, Immunotech	1:100
Anti-neurofilament triplet protein (NFT)	Monoclonal, Immunotech	1:100
Anti-68-KD NF subunit	Monoclonal, DAKO	1:200
Anti-160-KD NF subunit	Monoclonal, Boehringer	1:100
Anti-200-KD subunit	Monoclonal, Boehringer	1:100
Anti-GFAP	Monoclonal, Becton-Dickinson	1:100
HNK-1	Monoclonal, Coultronics	1:50
Anti-neuron specific enolase (NSE)	Monoclonal, Dako Kit	—
Anti-S-100	Monoclonal, Dako Kit	—

Table 3. *Reactivity Pattern of ES Cells with a Panel of Monoclonal Antibodies Directed Against Mesenchymal Markers and Neural Markers*

Antibodies	Primary tumors			Cell lines*	
	n°1	n°2	n°3	DES-1	IARC-EW13
CL	(+)	(+)	(+)	0	0
Leu M1	(+)	(+)	(+)	0	0
Leu M2	(+)	(+)	(+)	0	0
Leu M3	(+)	(+)	(+)	0	0
MO1	−			0	0
HLA-DR	+	+	+	0	0
Tranferrin	+	+	+	NT	NT
Total keratin	−	−	−	0	0
Vimentin	++	+	++	10%	69%
Desmine	−	−	−	0	0
NFT	−	+	−	36%	NT
GPAP	−	−	−	0	NT
HNK-1	−	+	+	43%	80%
NSE	−	+	−	37%	0
S-100	−	−	−	NT	NT

* DES-1 and IARC-EW 13 were derived from tumor n°2 and 3, respectively.
NT, not tested; −, no staining in any cell; (+), positive staining in some isolated cells; +, strong staining in an appreciable number of cells; ++, strong positive staining in virtually every cell.

a peroxidase conjugated rabbit-anti-mouse or goat anti-rabbit (Miles Laboratories, Naperville, IL, dilution 1:400) were used.

The primary antibodies were previously tested on various epithelial, fibroblastic, lymphoid, or neural tissues and used as positive controls. Negative controls were performed by omitting the respective primary antibodies.

Flow Cytometry

An indirect immunofluorescence staining was carried out on ES cell lines DES-1, SKES-1, and IARC-EW7 using anti-vimentin and HNK-1 antibodies. Dead cells were eliminated by propidium iodide staining. For each sample 10^4 cells were analyzed.

Neurofilament protein expression was evaluated and compared with DNA content according to the technique described by Bijman et al.[11]

Flow cytometry analyses were performed on an EPICS V cytofluorometer (Coultronics, Margency, France) equipped with an argon ion laser (164 Spectraphysics, Mountain View, CA). The laser was operated at 500 mW with the 488 nm line to excite fluorescein and propidium iodide. A 76-μm nozzle was used. A logarithmic amplifier and scale were used for fluorescein fluorescence signal, whereas propidium iodide fluorescence was measured on a linear scale.

Electron Microscopy

The standard electron microscopy method was used. Briefly, the cells were fixed in phosphate-buffered 3% glutaraldehyde, washed in the same buffer, postfixed in 2% osmiun tetroxide, dehydrated in graded ethanol solutions, and embedded in araldite. The presence of glycogen was demonstrated using the thioemicarbazide-silver proteinate method.[12]

Results

Immunohistology of Primary Tumors and Corresponding Cell Lines

Very few isolated cells from the three primary tumors examined reacted with the monoclonal antibodies recognizing a common leucocytes antigen (CL), Leu M1, Leu M2, and Leu M3. Negative reactions were found with monoclonal antibody MO1 directed against different antigenic determinants of cell surface of monocytes/macrophages. Tumor cells strongly reacted with monoclonal antibody directed against HLA CI II antigen and with a monoclonal antibody directed against transferrin receptor (Table 3).

Using antibodies directed against neural markers, cells from tumors n°2 and n°3 showed the same pattern of reactivity for the detection of HNK-1 antigens and NFT proteins. In addition cells from tumor n°2 also reacted positively to NSE-antibody (Table 3, Figure 1).

Of a wide range of intermediate filament proteins other than NFT proteins, including vimentin, desmin and keratin, only vimentin was found to be present in the three ES tumors tested (Table 3). Negative reactions were obtained using anti-GFAP and anti-S-100 antibodies in the three tumors (Table 3).

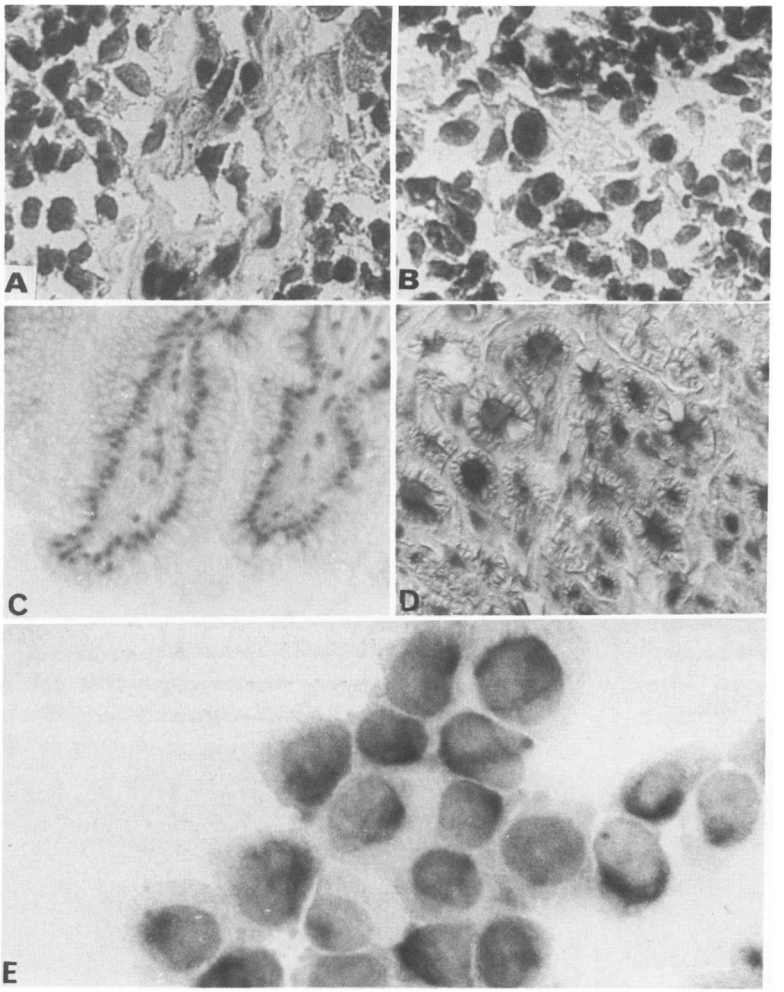

Figure 1. *Immunoperoxidase staining of ES tumor cells (tumor n° 2 and its derived cell line DES-1)* **A** *and* **B**: *Cryostat sections of the primary tumor demonstrated positive reactions with, respectively, HNK-1- and NSE-antibodies (original magnification ×800).* **C** *and* **D**: *Negative (ileal mucosae and submucosae) and positive (nerve fascicules from an axonal neuropathy) controls showing, respectively, negativity and positivity to NFT-antibody (***C**: *original magnification ×400;* **D**: *original magnification ×800).* **E**: *Cell smear preparation of the cell line DES-1 showing NFT positivity. Note the perinuclear cytoplasmic localization of the NF protein staining (original magnification ×1600). Immunoperodidase staining was used for preparations A to E.*

Unlike the primary tumor cells, mesenchymal markers (CL, Leu M1, Leu M2, Leu M3, and MO1) were totally negative in the two cell lines tested (DES-1 and IARC-EW13). As in the primary tumors, vimentin was found to be present in the two cell lines (Table 3). The three neural markers (NSE, HNK-1, and NFTP) reacted positively with cells from DES-1 cell line with a pattern of reactivity similar to the one observed in the original primary tumor (tumor n°2). The pattern of reactivity of the cells from IARC-EW13 cell line was also identical to the one observed in the original n°3 tumor: negative reaction to anti-NSE protein and positive reaction to anti-HNK-1 antigens.

Immunocytology of Six Unrelated ES Cell Lines

Six additional ES cell lines were characterized immunocytologically using Leu M2, HNK-1, S-100, NSE, GFAP, vimentin, and NFT proteins (Table 4). All cell lines expressed vimentin, and none were stained by Leu M2, anti-S-100, and anti-GFAP antibodies.

On the other hand, HNK-1 antigens, NSE, and NFT-proteins were detected in the six ES cell lines. HNK-1 was revealed in 29% to 88%, NSE in 12% to 52%, and NFT in 42% to 75% of cells.

Using antibodies directed against the different NF subunits (68-, 160- and 200-KD), we demonstrated that the detection of NFT proteins in all ES cell lines was due to the expression of the 200-KD subunit, which was the only one which reacted positively.

Flow Cytometry

Three of the cell lines immunocytologically characterized (IARC-EW7, SKES-1, and DES-1) were further studied by flow cytometry for NF-subunits (68-, 160-, and 200-KD), vimentin, and HNK-1 expressions.

Table 4. *Antigen Expression by ES Cell Lines*

Cell lines	Cells expressing the antigen (%)*									
	Leu M2	HNK-1	S-100	NSE	Vimentin	GFAP	NFT	NF-68-KD	NF-160-KD	NF-200-KD
IARC-EW 1	0	88	0	17	89	0	44	0	0	43
IARC-EW 7	0	28	0	52	42	0	75	0	0	68
IARC-EW 12	0	44	0	15	64	0	67	0	0	65
IARC-EW 15	0	29	0	12	42	42	42	0	0	35
IARC-EW 17	0	80	0	21	NT	0	2	0	0	35
SKES-1	0	83	0	18	10	0	72	0	0	55

* The percentage of cells expressing the corresponding antigen was determined on a total of 300 cells.
NT, not tested.

The 200-KD subunit was always strongly expressed regardless of the cell line tested (Figure 2). Only small differences in the amount of expression of this protein were observed according to the line considered. Under these conditions, IARC-EW7 expressed the higher level of 200-KD subunit. The 68-KD and 160-KD subunits were not detected in any cell line.

Differences in the level of vimentin and HNK-1 antigens expressions were also observed from one cell line to another (Figure 3). Vimentin was always expressed at a low level by the three ES lines, which ranged from 50% for IARC-EW7 to 19% for SKES-1 and 17% for DES-1. On the other hand, important differences were observed in the expression of HNK-1 antigens: 90% of the cells expressed this antigen at a high level for SKES-1, whereas only 48% of the cells expressed this antigen slightly for IARC-EW7. For DES-1, HNK-1 antigens were present in 50% of the cells at an intermediate level when compared with the two other ES lines.

These percentages of cell positivity obtained by flow cytometry corresponded to those observed using the indirect immunoperoxidase method on cytospin preparations.

Morphology

All ES samples were stained by hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) for light microscopic examination. Tumors were formed by small polyedral cells with round or ovoid nuclei that had powdery chromatin and often one or two nucleoli. All cells were arranged in compact broad sheets (Figure 4A).

Electron microscopy study was performed on the cells from five of the eight primary tumors and cell lines tested (Table 1). No evidence of neuroectodermal differentiation such as dense core neurosecretory-type granules or microtubules was demonstrated, although intracellular glycogen, very few cytoplasmic organits, and a poorly developed endoplasmic reticulum were observed (Figure 4B and personal communication from E. Tabone, Centre Léon Berard, Lyon, France for tumor n°3 and cell lines IARC-EW12 and SKES-1).

Discussion

The absence of unequivocal cell features characterizing ES cells caused many problems for its diagnosis as well as for an approach to its histogenesis. At the present time, the chromosomal translocation t(11;22)(q24;q12), identified in 92% of ES cases,[13] remains the only marker that can be used with a high degree of confidence to differ-

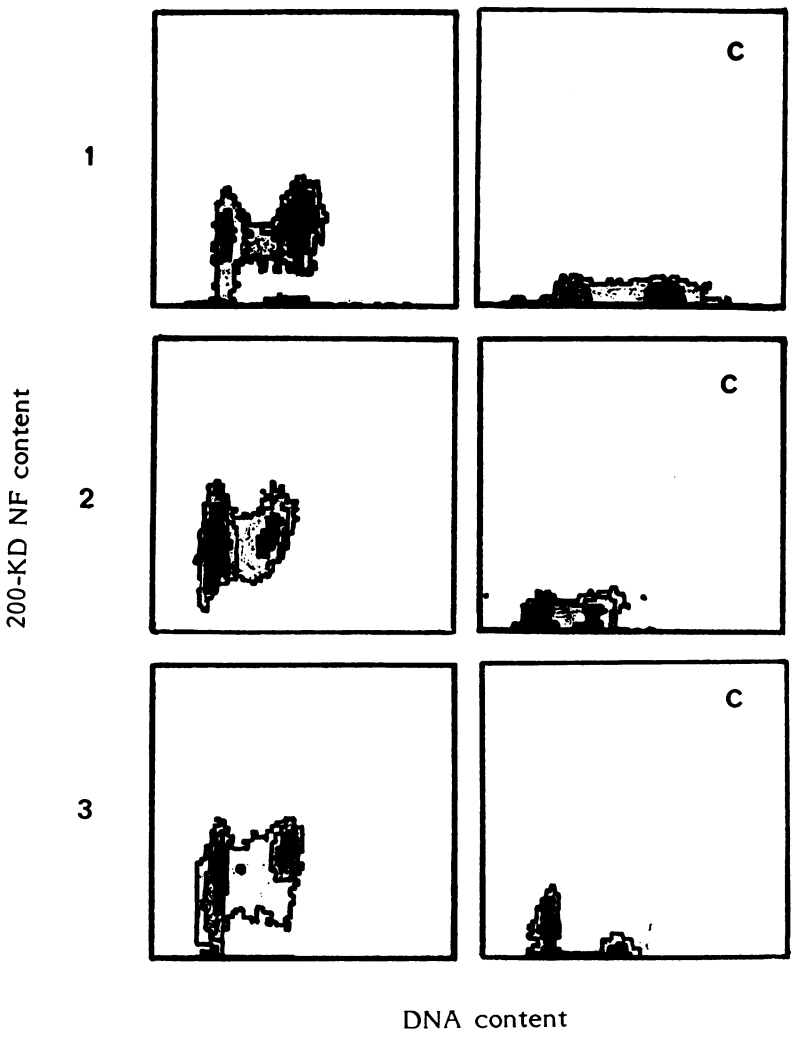

Figure 2. *Flow cytometry analysis of the 200-KD-NF-protein in IARC-EW7 (1), SKES-1 (2) and DES-1 (3) cell lines. Dual analysis (64 × 64 channels) of ES cells stained with propidium iodide (DNA content) and fluorescin indirect immunofluorescence of 200-KD-NF-protein. DNA content was measured on a linear scale and the 200-KD-NF-protein content on a logarithmic scale. C, conjugated control.*

entiate ES from the other tumors among the so-called small-round-blue-cell tumors such as essentially neuroblastomas and rhabdomyosarcomas. However, ES and peripheral neuroectodermal tumors (PNET) cannot be distinguished on the basis of the t(11;22)(q24;q12), which has also been found in some PNETs,[14] tumors that are undoubtedly of neural origin. But PNETs occur at an older age than ES does. For the specimens analyzed in this study, all of which originated from bone, the diagnosis of ES was based on clinical and pathologic data. In addition, in five of eight tumors and cell lines, the final diagnosis of ES was determined on electron microscopic data. Ultrastructural studies showed only the presence of glycogen without any evidence of neural differentiation features such as neurosecretory granules or neurites.

For this report, we made an immunologic study of ES cells carrying the t(11;22) in an attempt to clarify its histogenesis.

The hypothesis of a mesenchymal cell origin for ES was recently proposed by Loning et al[5] on the basis of the presence of antigens recognized by Leu M2 antibody in cells from various fresh Ewing tumors. The data presented in this report do not support this hypothesis.

Although cells from the three primary tumors tested reacted with CL, Leu M1, Leu M2, and Leu M3 antibodies, which are directed against the antigenic determinant of monocytes/macrophages, the number of positive cells was very low, suggesting the presence of lymphoid infiltration within tumor cells. These lymphoid infiltrates had an identical immunologic pattern in all cases that may reflect a cellular immune response. Immunophenotypic evidence of cellular immune reactions is demonstrated by the expression of antigens associated with cell activation, such as HLA CI II,[15] and with proliferation, such as transferrin receptor.[16] Moreover, that cells from the two cell lines established from the tumors did not express any of these mesenchymal markers supports our statement. Additional evidence was provided by the absence of cell surface antigens recognized by Leu M2 antibody on another 6 ES cell lines.

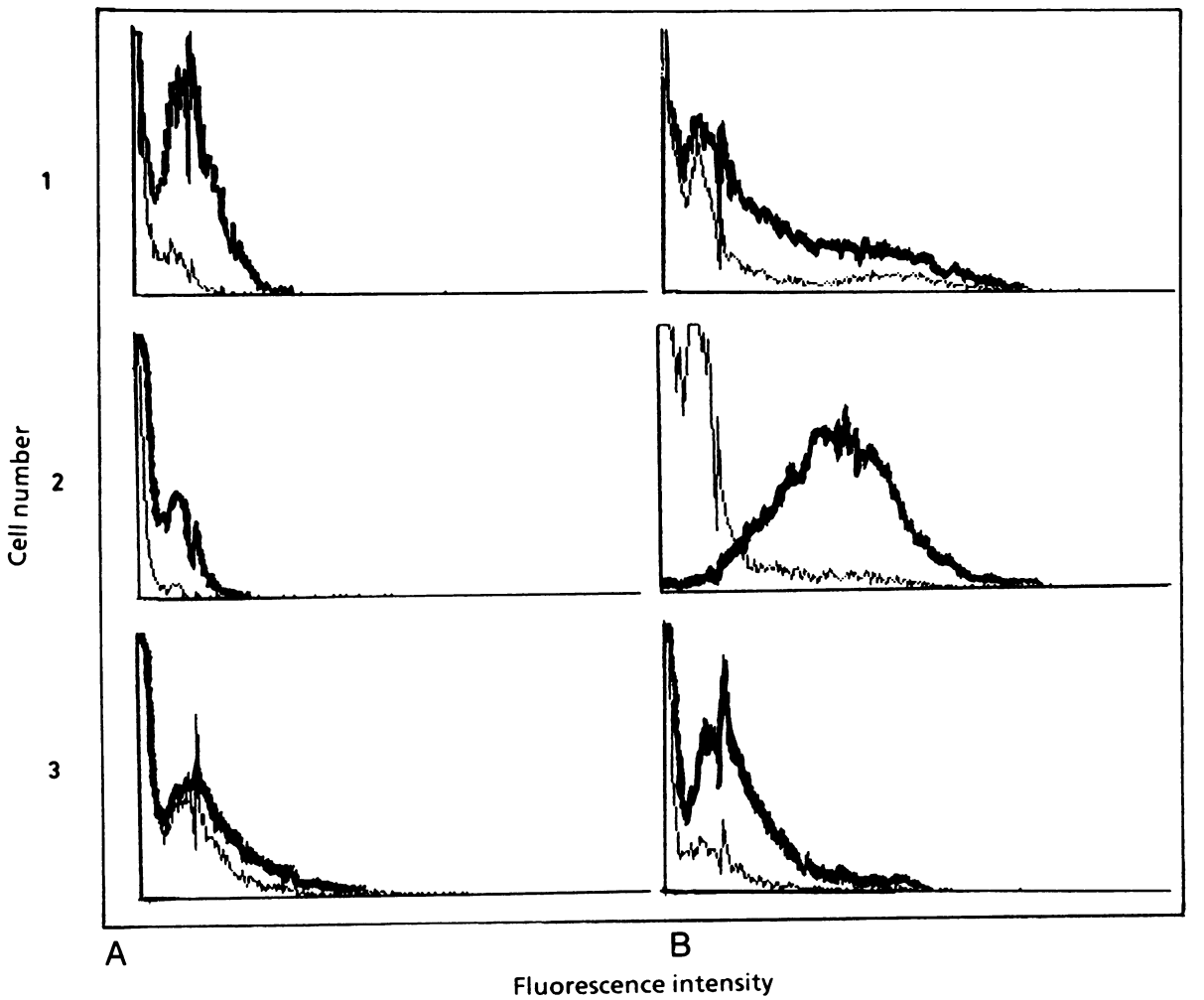

Figure 3. *Profile plots of vimentin* (A) *and HNK-1* (B) *antigens in IARC-EW7 (1), SKES-1 (2), and DES-1 (3) cell lines. Antigen expression was measured on a logarithmic scale (256 channels). Dead cells were eliminated by propidium iodide staining. (—), essay; (■), conjugated control.*

The presence of vimentin in ES cells has been reported previously.[4] This cytoskeletal protein was detected in cells of the ES studied in this report. Vimentin has long been considered an intermediate filament representative of mesenchymal tissues.[17] However, vimentin was recently detected in undifferentiated neural cells where neurofilament proteins were not yet expressed.[18,19] Thus, the presence of vimentin in ES cells cannot be used as a definite argument in favor of their mesenchymal origin.

Antigens commonly associated with neuroectodermal phenotype and recognized by anti-NSE, anti-HNK-1, and anti-NFT antibodies, were previously reported in ES cells.[6-8,20-22]

The NSE-protein, which has long been associated with nerve cells,[20] was detected in all cell lines except one and in two of three tumors tested. However, NSE positivity is not specific to neural cells and is no longer considered definite evidence of a neural phenotype in view of many reports demonstrating its presence in a variety of tumor cells.[23]

Antigens recognized by HNK-1 antibody were among the first neuroectodermal markers detected on ES cells.[6] The present study confirms these previous data, which demonstrated the glycoprotein nature of the epitope.[21]

To characterize neural cells, the detection of NF proteins is of paramount importance. These intermediate filaments are very specific to neural cells and to their precursors, and are remarkably stable during malignant transformation.[24] NFT proteins were detected immunohistochemically in cells from two of the three ES primary tumors, as well as in one of the derived cell lines. The cells of the primary tumor n°1 did not show any reactivity other than with vimentin antibody. The cells from this tumor may be derived from neuroectodermal cells at an early stage

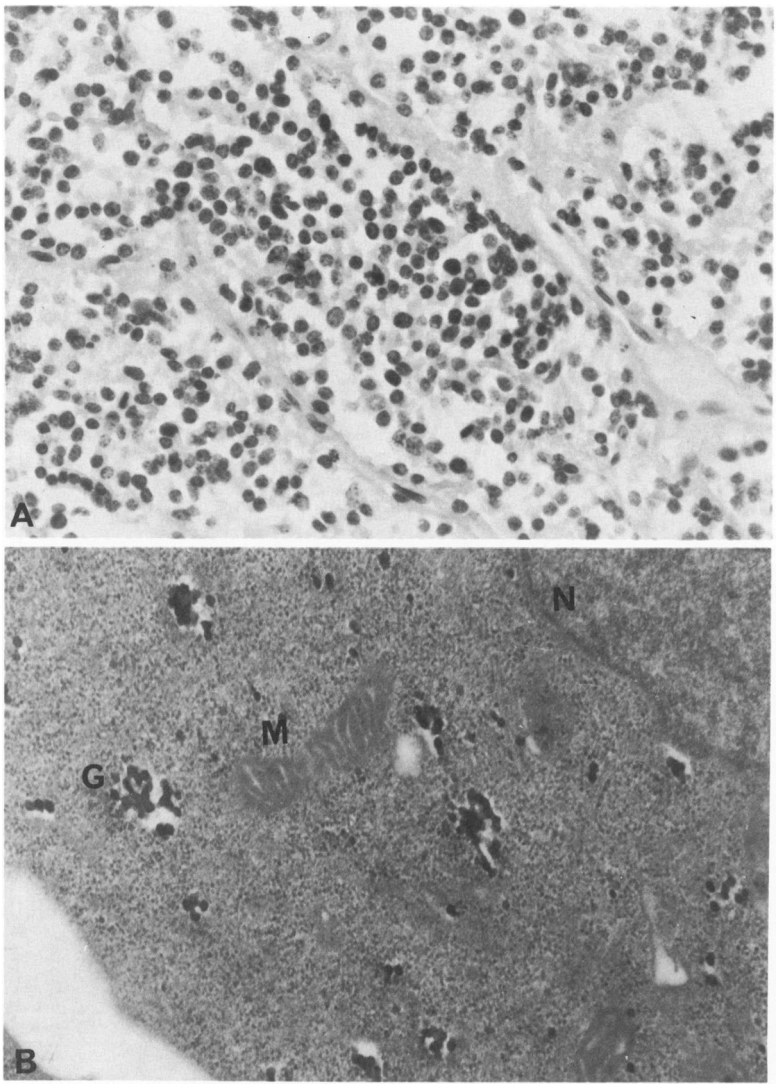

Figure 4. *Morphologic features of ES cells (tumor n° 2).* A: *Light microscopy showing the uniform arrangement of small round blue cells (H&E, original magnification ×400).* B: *Electron microscopy demonstrating cytoplasmic glycogen and few organelles (original magnification × 15000). N, nucleus; M, mitochondria; G, glycogen.*

of differentiation when only vimentin is expressed before the expression of neurofilament proteins.[18,19]

Using the indirect immunoperoxidase method on cytopsin preparations, NFT proteins were also detected in an appreciable percentage of cells from five of the six additional ES cell lines. The variable amount of NFT protein expression may reflect heterogeneous cell differentiation stages within tumor cells. Furthermore, we demonstrated that the positivity to NFT proteins was due to the expression of a single subunit among the three subunits that compose the NFT proteins. The 200-KD subunit was the only one to be consistently expressed in cells from all the cell lines positive to NFT proteins.

The presence of NFT proteins and its 200-KD subunit was confirmed by flow cytometry in cells from three ES cell lines. The flow cytometry method permits the separation of living from dead cells. In addition, the technique, when performed simultaneously for NF protein expression and for cell cycle analysis, allows a quantitative measurement of the intensity of the cell markers in viable cells. Therefore, artefactual positive reactions that might have occurred by immunoperoxidase staining on cytopsin preparations were eliminated.

Until now, NF protein expression in ES cells has not been well documented. Recently, the presence of NFT proteins was reported in three primary ES cells.[24] The 200-KD subunit has been detected in five ES cell lines carrying the t(11;22) but only after induction of cellular differentiation with various inducers.[8] In our cell lines, 200-KD subunit was detected in the absence of any induction of cellular differentiation, indicating that these cells may be at a more committed stage in their differentiation.

The 68- and 160-KD subunits were never detected. It has been noted that tumor progression and promotion in

neural tumors is associated with perturbations in the neural mechanisms that regulate the expression of NF proteins.[25] Such abnormalities of NF expression were observed in neuroectodermal cell malignancies, such as pheochromocytoma and medulloblastoma cell lines.[25-27]

In conclusion, this report demonstrated the absence of mesenchymal markers in ES cell lines, whereas positive cells, probably immunocompetent infiltrating cells, were detected in the primary tumors. On the other hand, the detection by immunohistochemistry and by flow cytometry of significant amounts of a very specific neural marker such as the NFT protein and its 200-KD-subunit constitutes additional convincing evidence that ES cells could have a neural origin.

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