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Osteosarcomas and Ewing's sarcomas

Comparative immunocytochemical investigation of filamentous proteins and cell membrane determinants*

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Summary. Primary malignant bone tumors, osteosarcomas (9 cases), and Ewing's sarcomas (10 cases) were examined for their reactivities with monoclonal and polyclonal antibodies against filamentous proteins and cell membrane determinants of the lymphoid and macrophage marker series. The reactivity of antibodies was studied on snap-frozen tissue probes by using a triple layer immunoperoxidase method.

Osteosarcomas were positive for vimentin and, in part, for HLA-DR. Other types of intermediate-sized filaments were not detected in tumour cells. In a small number of cases (2/9) tumour cells were reactive with antibodies of the macrophage series (Leu M2).

In Ewing's sarcomas, vimentin and HLA-DR was also demonstrated. It was particularly interesting that Leu M2 staining was found in the majority of cases (8/10). The staining pattern supports the assumption that this peculiar tumour is of mesenchymal (monocyte/macrophage) histogenesis.

It was evident from the present study that, in primary osteogenic tumors, none of the examined tumour "markers" were as distinctive as they are for bone metastases. Nevertheless, the reactivity of Ewing's sarcoma cells with monoclonal antibodies of the Leu M2 type throws some highlights on the, as yet, obscure histogenesis of the neoplasm and may be of diagnostic value in conjunction with the known light and electron microscope features of the tumour.

Key words: Osteogenic tumours – Tumour "marker" – Intermediate filaments – HLA-DR – Leu M2

Introduction

In recent years, the production and morphological application of polyclonal and monoclonal antibodies against intracellular and extracellular compo-

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nents (fibrils, cell membranes and ground substance) has helped to increase our knowledge on the histogenesis and differentiation potentials of tumour cells (Osborn and Weber 1983; Hakomori and Kannagi 1983). Those studies have been mainly directed to research on tumours of the lymphoid tissues and, at present, an enormous number of monoclonal antibodies of different commercial sources are available for differentiation of cells of the hematopoetic system and their neoplasms (Reinherz and Schlossmann 1980; Bach and Bach 1981; Haynes 1981; Naiem et al. 1982). Another research field, focussed on tumour differentiation, was born with the development of polyclonal and monoclonal antibodies against filamentous proteins and their subsets (see for review Osborn and Weber 1983).

For primary malignant human bone tumours, electron microscopy and previous immunological studies have shown that these have different lines of cellular differentiation (Ewing 1921; Schulz 1980; Friedman and Gold 1982; Dahlin 1981; Meister 1984; Roessner 1984).

The purpose of this immunocytochemical study of a series of primary bone tumours was:

1. to look for the expression and distribution of known marker molecules of the intermediate-sized filament, Ia-like, T-cell, B-cell, macrophage, accessory cell, endothelial cell series.

2. to scrutinize these reaction patterns with special respect to their value in surgical pathology.

Materials and methods

Tissues. Samples were taken from bone lesions following diagnostic exploration or gross surgery procedures. Immediately after removal the specimens were divided, one part being processed for methacrylate embedding, the other part quick-frozen in OCT-compound in liquid nitrogen and stored at −70° C. Clinical data and pathohistological diagnosis of cases are listed in Table 1.

Immunoperoxidase (Triple layer method). Cryostat-sections (4–6 μm in thickness) were prepared, air dried for two hours and fixed in acetone for 5–10 min at room temperature before and after storage overnight at −20° C.

In the first step, the sections were incubated with the primary monoclonal or polyclonal antibodies (for 45 min, diluted at 1:20 to 1:100).

Consecutively, second and third step antibodies coupled with peroxidase were added to the sections for 45 min, respectively (diluted at 1:10 to 1:20; Löning et al. 1983; Löning 1984; Becker et al. 1985). Primary antibodies were diluted with bovine serum albumin (1%) in TRIS-HCL buffer at pH 7.4. Second and third step antibodies were diluted with complement inactivated human serum in 0.1 M phosphate buffered saline (PBS, dilution: 1:2 of human serum in PBS) at pH 7.4. After each step, sections were extensively washed in PBS buffer at pH 7.4 except for the final 3,3-diaminobenzidine reaction which was performed in TRIS-HCl buffer at pH 7.6. Slides were counterstained with haemalaun, mounted and examined.

Controls. Primary antibodies were previously tested on diverse epithelial, mesenchymal and lymphoid tissues (Löning et al. 1983; Löning 1984; Becker et al. 1985). Polyclonal antisera were replaced by pre-immune sera (Löning 1984), monoclonal antibodies by mouse immunoglobulins of the given Ig type. In these initial control experiments, immune sera were titrated and working dilutions were evaluated. For every case reactivity of primary antibodies was controlled by omitting the respective immune serum.

Table 1. Clinical data

No.	Diagnosis	Age	Sex	Location
1	osteosarcoma	15	m	femur
2	osteosarcoma	44	f	mandible
3	osteosarcoma	40	m	pelvis
4	osteosarcoma	13	m	femur
5	osteosarcoma	9	f	tibia
6	osteosarcoma	20	m	tibia
7	osteosarcoma	16	m	femur
8	osteosarcoma	18	f	femur
9	osteosarcoma	8	f	femur
10	Ewing's sarcoma	19	f	femur, rib, scapula
11	Ewing's sarcoma	14	m	tibia
12	Ewing's sarcoma	16	f	pelvis
13	Ewing's sarcoma	14	m	fibula
14	Ewing's sarcoma	18	m	femur
15	Ewing's sarcoma	18	m	femur
16	Ewing's sarcoma	48	m	pelvis
17	Ewing's sarcoma	12	f	os sacrum
18	Ewing's sarcoma	19	f	pelvis
19	Ewing's sarcoma	14	m	femur

Results

Non-tumorous bone tissue

Among the intermediate-sized filament types, only vimentin was regularly found in all cellular elements: osteoblasts, osteocytes, chondrocytes, osteoclasts, fibrocytes, endothelial cells, lymphoid cells, macrophages and accessory cells (Fig. 1a, 1b, 2a).

Reactivity with HLA-DR antibodies was seen in macrophages and accessory cells as well as endothelial cells (Fig. 2b). Osteoblasts, osteoclasts, osteocytes, were not regularly labeled. Ulex I reacted intensively with all endothelial cells.

Few lymphoid cells of the bone marrow reacted with the T-cell and B-cell markers used.

Some macrophages positive for typical macrophage markers (Table 2) were scattered around within the endosteal spaces.

Malignant osteogenic tumours

Osteosarcomas. Categorization of osteosarcoma cases is shown in Table 1.

All nine cases were strongly positive for vimentin (Fig. 4b, 4c). A close relationship appeared between the size of a tumour cell and the intensity of vimentin staining. Thus, the small cell anaplastic tumour zones were faintly stained in contrast to the large cell osteoblastic, fibroblastic and chondroblastic areas. We never saw tumour cells positive for other types of the intermediate filament system (cytokeratin, desmin, neurofilaments).

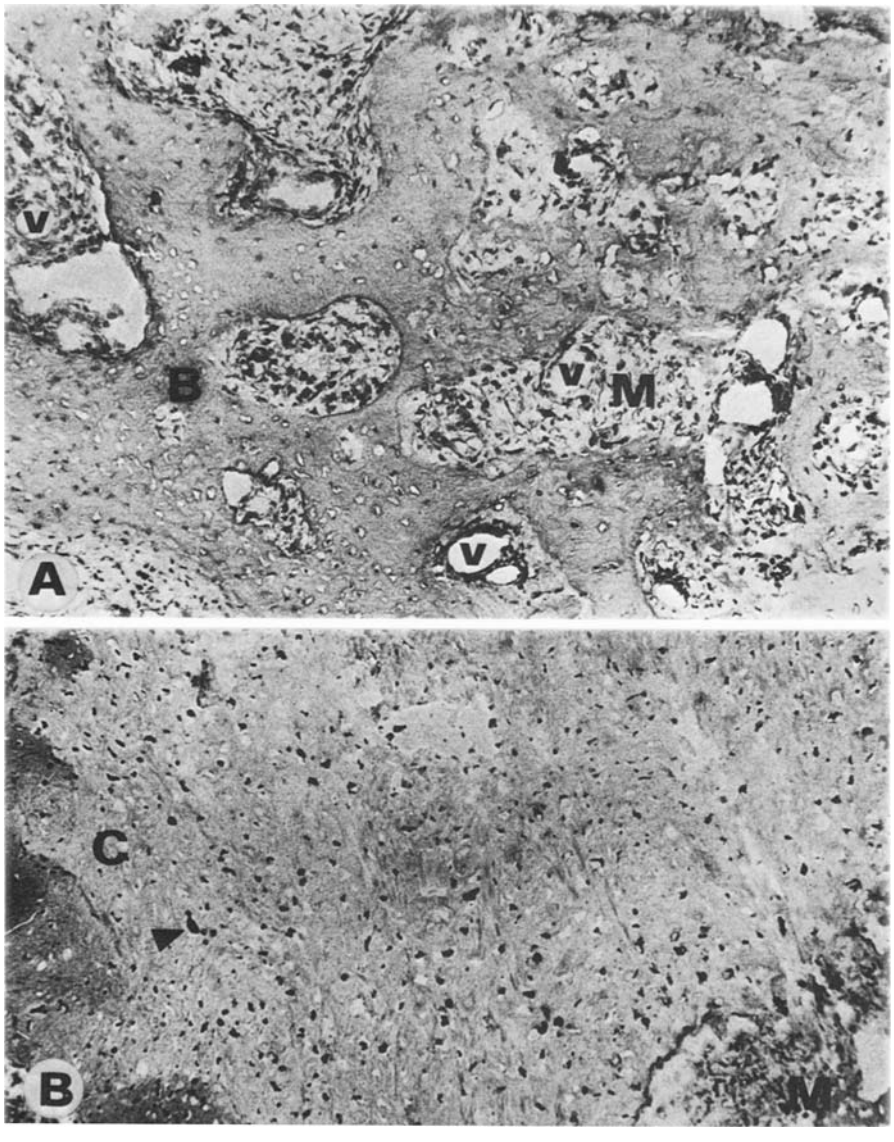

Fig. 1 A. Non-tumorous bone (*B*) stained for vimentin. All mesenchymal cells, especially within the bone marrow (*M*), are strongly labeled (*V* = vessels). Mag. × 63. **B** Fibrous cartilage (*C*) stained for vimentin. Prominent staining of chondrocytes (arrowhead) and cell elements of bone marrow (*M*). Mag. × 63

There were usually some tumour cells which were reactive with HLA-DR-antibodies. In addition, a high number of dendritic cells positive for HLA-DR were seen to be intermingled between tumour cells, (Figs. 3a, 4a). In spindle cell fibroblastic tumour zones, those positive dendritic cells were hard to distinguish from truly malignant HLA-DR-negative cell types. In addition to dendritic cells, vessels also reacted with HLA-DR antibodies.

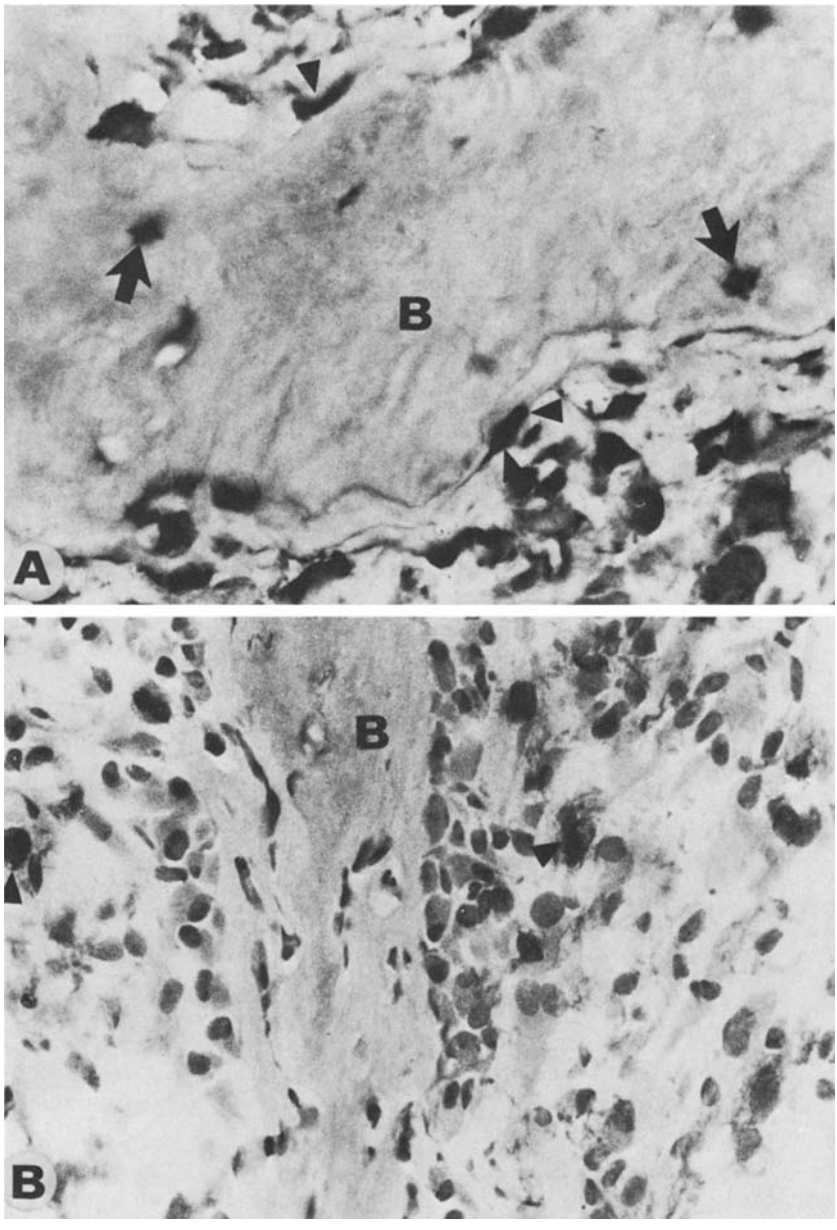

Fig. 2A. Non-tumorous bone (*B*). Staining for vimentin. Arrows point to labeled osteocytes. Note the stained cytoplasm of many lining cells (*arrowheads*). Mag. ×400. **B** Non-tumorous bone (*B*). Staining for HLA-DR. The majority of cells are negative (osteocytes, osteoblasts etc.) Some large cells (macrophages, dendritic cells) within marrow spaces express this antigen (*arrowheads*). Mag. ×250

Table 2. Antibodies/lectins used

Reactivity	Typ	Source
Filamentous proteins	Kl 1	Immunotech
	Vimentin	Labsystems
	419-6	Priv.-Doz. Dr. R. Arndt
	Desmin	Dako
	Neurofilaments	Immunotech
Ia-like molecules	Leu-HLA-DR	Becton-Dickson
	Bl 2	Immunotech
T cell markers	IOT 1a	Immunotech
	Leu 4	Becton-Dickson
	OKT 8	Ortho
B cell markers	OKB 7	Ortho
	IOB 1a	Immunotech
	IOB 1	Immunotech
Macrophages	OKM 1	Ortho
	Leu M 2	Becton-Dickson
Accessory cells	Leu 6	Becton-Dickson
	Bl 6	Immunotech
Endothelial cells	Ulex I	Medac

References:

Priv.-Doz. Dr. R. Arndt	Univers. Krankenhaus Eppendorf, Martinistr. 52, D 2000 Hamburg 20
Becton-Dickson	Tullastr. 8–12, D 6900 Heidelberg 1
Dako	Stormarner Str. 30, D 2000 Hamburg 70
Immunotech	Luminy Case 915, 13288 Marsaille, Cedex 9 France
Labsystems Oy	Pulttitie 9–11, 00810 Helsinki 81, Finland
Medac	Fehlandtstr. 3, D 2000 Hamburg 36
Ortho	Wiesenbacher Str. 65–69, D6903 Neckargemünd

Ulex I labeled endothelial cells exclusively (Fig. 3b). No relationship was seen between labeled endothelial and adjacent negative tumour cells.

T- and B-cell markers were always negative in osteosarcomas.

The majority of tumour cases were negative for macrophage markers. In a few cases (2/9, see Table 1 and 3) labeling of tumour cells was evident. Positive dendritic cells and positive vessels as described for HLA-DR were not found. Accessory cells positive for markers of the T 6 series did not occur in osteosarcomas.

Ewing's sarcomas. In all ten cases of Ewing's sarcomas, a high number of tumour cells were positive for vimentin (Fig. 5a). There was, however, a marked heterogeneity of vimentin staining in different tumour zones. As a rule, larger cells showed a more intense reactivity with vimentin antibodies. In some cases and tumour areas an interesting relationship was seen between vimentin-positive capillaries/venules and tumour cells, where the cells appeared to spread out from those vessels. No staining was seen with antibodies against other intermediate filament types.

Most sarcoma cells were negative for HLA-DR antigens. However, dendritic cells and endothelial cells reacted with HLA-DR antibodies. These

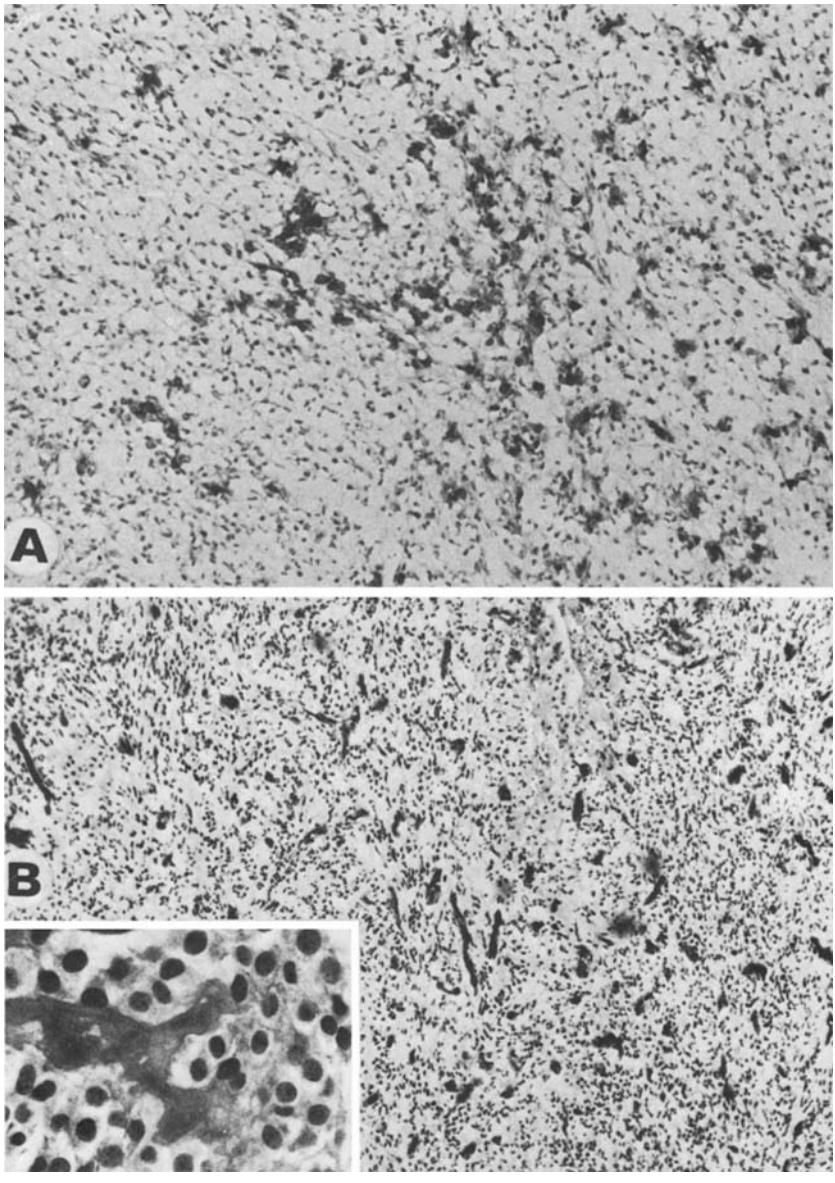

Fig. 3A. Osteosarcoma stained for HLA-DR. The majority of tumour cells are unstained. Some labeled cells may represent innocent bystander cells of the macrophage system. Mag. ×63. **B** Osteosarcoma stained for Ulex I (same case as shown in **A**). Labeled vessels, but negative tumour cells. Mag. ×63. *Inset:* Conventional light microscopy of the same case (methacrylate embedded material) showing tumour cells and osteoid production. Mag. ×400

positive cell types were sometimes hard to distinguish from the negative tumour cells (Fig. 5b).

Ulex I reacted exclusively with endothelial cells.

There was no reactivity of sarcoma cells with antibodies against T- and B-cells. However, T-cell reactions were occasionally seen in the vicinity of

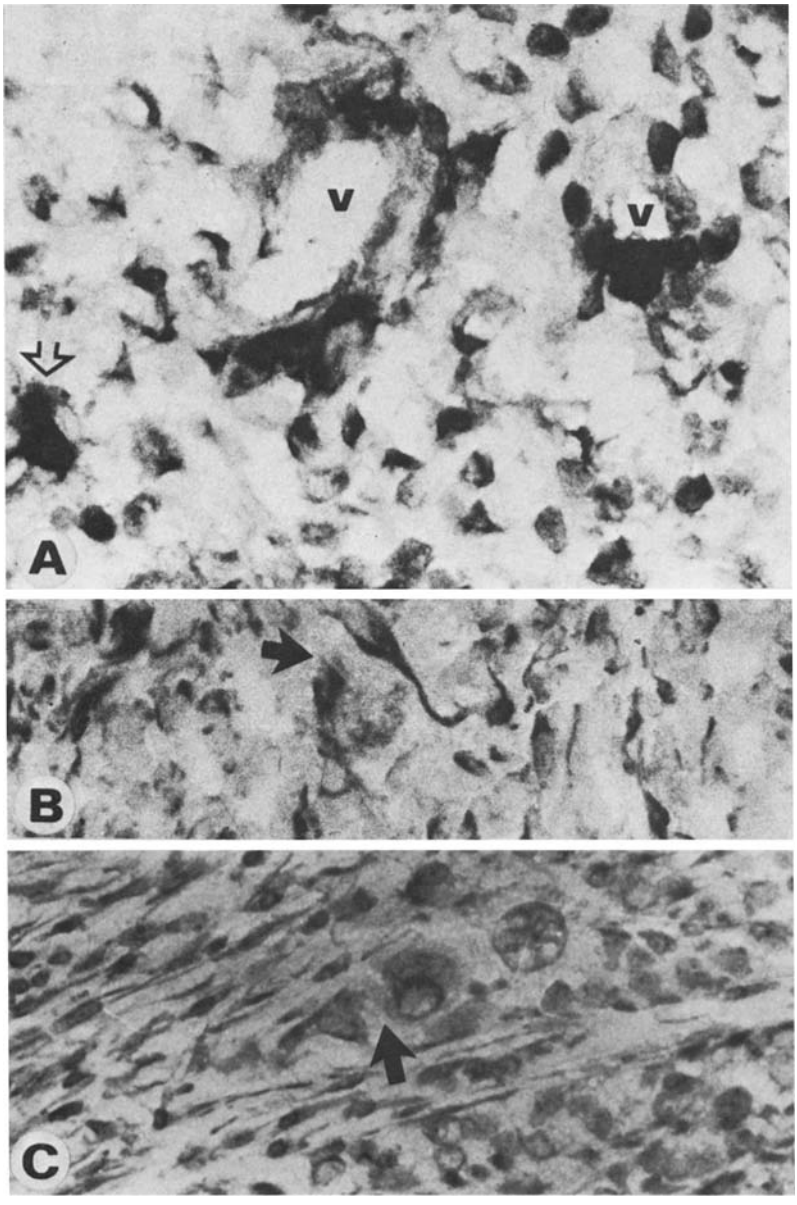

Fig. 4A. Osteosarcoma stained for HLA-DR. Labeling of endothelial cells (*V*-vessels). Few positive tumour cells (*open arrows*). Mag. ×250. **B** Osteosarcoma stained for vimentin. Note the different staining intensity of large cells (*arrow*) and small cell types (*right side of the figure*). Mag. ×250. **C** Osteosarcoma. Vimentin staining. *Arrow* indicate some positive tumour cells containing large irregular nuclei. Mag. ×400

Table 3. Reactivity pattern (first numbers indicate positive cases)

Diagnosis	Keratin	Vimentin	Desmin	HLA-DR	T6	Pant T	Pan B	Leu M2
Osteosarcomas	0/9	9/9	0/9	2/7	0/9	0/8	0/9	2/9
Ewing's sarcomas	0/7	10/10	0/7	4/9	0/10	1/9*	0/9	8/10
Metastatic carcinomas (1)	3/3	1/3	0/3	1/3*	1/3*	2/3*	0/3	2/3*
Rhabdomyosarcomas (1)	0/2	1/2	2/2	0/2	0/2	0/2	0/2	0/2

* = positive stromal cells
1 = for comparison staining pattern of 3 metastatic carcinomas and 2 myosarcomas

tumour areas (Fig. 6b). In many cases, macrophage markers (Leu M 2) were found to be positive in tumour cells (Fig. 6a). Accessory cells of the T6 positive cell type did not occur in Ewing's sarcomas.

Discussion

In this study, cellular elements of non-tumorous bone and bone derived tumours (osteosarcoma, Ewing's sarcoma) were studied for their reactivity with a broad panel of polyclonal and monoclonal antibodies. Initially, we tested a larger panel of antibodies, since recent studies have shown that unexpected or aberrant reactivities can be observed even with well defined monoclonal antibodies. (Naiem et al. 1982; Lane and Koprowski 1982; Schmitt et al. 1982; Wood et al. 1983; Knowles et al. 1984; Delsol et al. 1984).

Thus, in neuroblastomas, it was surprising that tumour cells reacted with monoclonal antibodies characteristic for B-cells (Kennett et al. 1980).

For normal bone tissue, there are antibodies reactive with bone forming and bone resorbing cells (vimentin, see for review Osborn and Weber 1983), dendritic cells (HLA-DR), typical macrophages (OKM 1, Leu M2, lysozyme, alpha-1-antitrypsin), differentiated T- and B-cells (Leu 4, OKB 7 and others), endothelial cells and basement membranes of vessels (Ulex, I-lectin, laminin), collagen fibers and ground substance (Hämmerling et al. 1975; Winchester and Kunkel 1979; Isaacson and Jones 1983; Knowles et al. 1984; Mukai et al 1980; Holthöfer et al. 1982; Roessner 1984).

In osteogenic tumours (osteosarcomas and Ewing's sarcomas), tumour cells were reactive with vimentin antibodies. Vimentin staining has already been reported for a variety of mesenchymal tumours (Ramaekers et al. 1982; Miettinen et al. 1982a; Miettinen et al. 1982b; Schulz 1984). As a histogenetic marker vimentin is only valuable in conjunction with other tissue or cell type specific antibodies, since coexpression of vimentin is known to occur with keratin and desmin proteins in cell lines and tumour samples (Ramaekers et al. 1983). For this reason, we screened all tumours with antibodies against keratin, vimentin, desmin and neurofilaments. We did not find filamentous proteins in osteosarcoma and Ewing's sarcoma cells other than vimentin. The intensity of vimentin staining was very variable depending on the size and the differentiation of tumour cells. Thus, fibro-

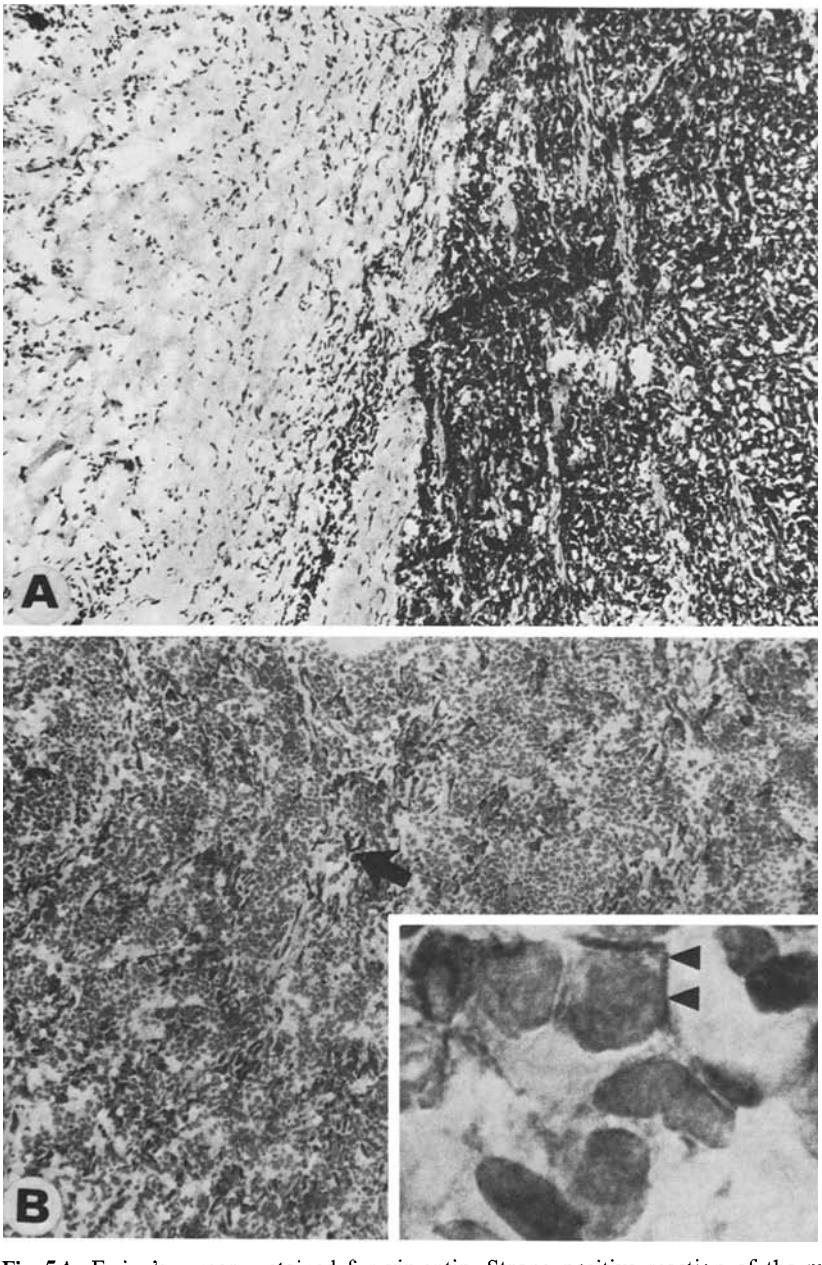

Fig. 5A. Ewing's sarcoma stained for vimentin. Strong positive reaction of the majority of tumour cells (*right side of the picture*). Mag. × 63. **B** Ewing's sarcoma. Staining for HLA-DR. Tumor cell complexes (non-stained) are surrounded by positive dendritic cells and vessels (*arrow*). Mag. × 63. Inset: Some tumour cells react with HLA-DR antibodies. Arrowheads indicate the small positive rim of the cell body. Mag. × 1,000

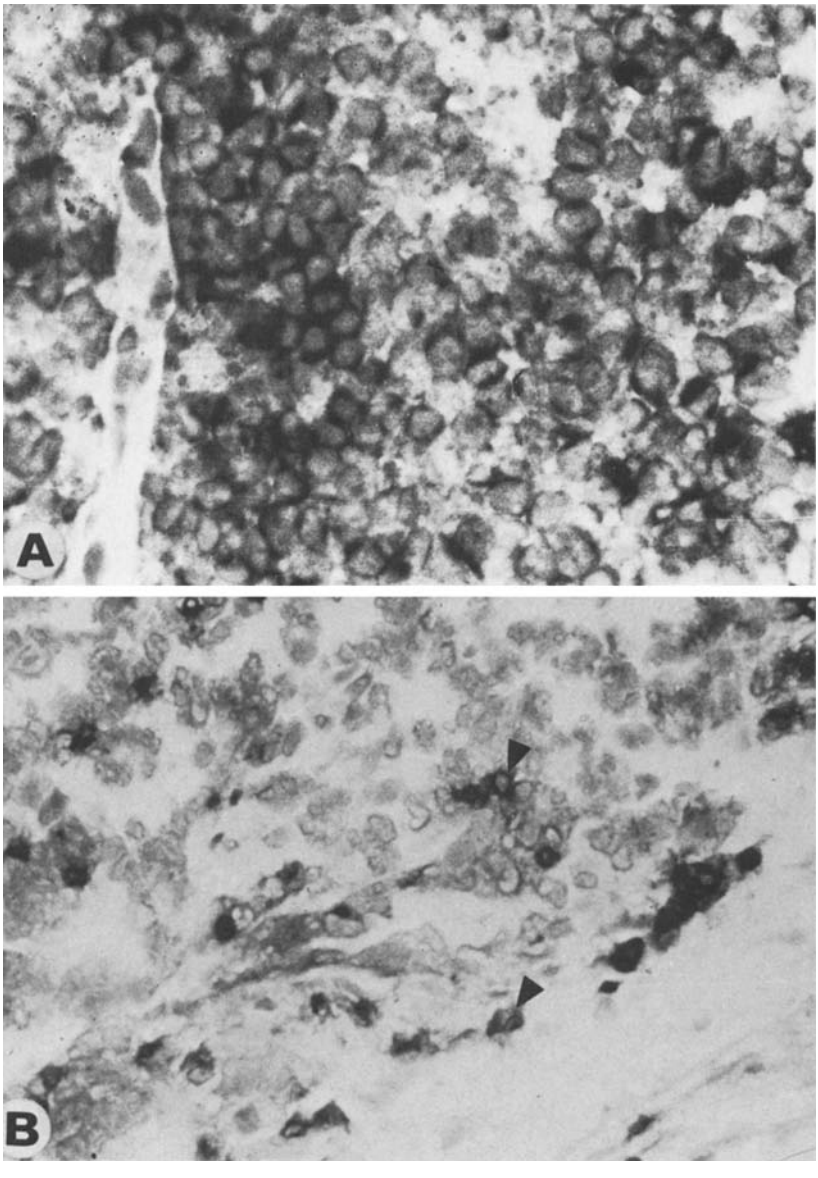

Fig. 6A. Ewing's sarcoma. Staining for Leu M 2. The majority of tumour cells are reactive. Mag. ×400. **B** Ewing's sarcoma. Staining for Leu 4 (reactive with mature T lymphocytes). Negative tumour cells. Arrowheads indicate positive T lymphocytes in close vicinity to the tumour cells. Mag. ×400

blastic and osteoblastic osteosarcomas showed a very strong reactivity with vimentin antibodies in contrast to small cell tumours (Ewing's sarcomas). Occasionally, there was a peculiar relationship of vessels and tumour cells in Ewing's sarcomas which was previously noted (Rössler 1984).

We did not find Ulex affinity to tumour cells neither in osteosarcoma

nor in Ewing's sarcoma. Apparently, markers for vascular tissue (Ulex, Factor VIII) (Sehested and Ho-Jensen 1981; Miettinen et al. 1983b) are absent in tumour cells of osteosarcomas and Ewing's sarcomas.

Among the other cell markers used for osteosarcomas and Ewing's sarcoma the most interesting findings were obtained with HLA-DR and Leu M2 antibodies. Fibroblastic areas of osteosarcoma contained a high number of HLA-DR cells. At present, we cannot unequivocally decide whether all these cells are tumour cells or some reactive cells belong to the tumour stroma. In Ewing's sarcoma, HLA-DR positive cells were found predominantly around the tumour complexes. In view of the presence of HLA-DR in so many cells and tumours (Hämmerling et al. 1975; Winchester and Kunkel 1979; Nadler et al. 1981), it must be pointed out that this marker is of limited value in surgical pathology. Typical macrophage markers (OKM 1) were not seen to be positive in osteosarcomas and Ewing's sarcomas except for Leu M 2 in many Ewing's sarcomas (8/10). This labeling may pinpoint the presumed monocyte/macrophage histogenesis of Ewing's sarcomas (Schulz 1980; Rössler 1984). Further studies on a larger series of tumours and cell culture analysis are clearly needed prior to announce the relevance of this antibody (Leu M2).

There is no doubt that some of the markers used in the series were not of great help for distinction of bone tumors (T- and B-cell marker, Ulex I, laminin, fibronectin). Nevertheless, antibodies against T- and B-lymphocytes have to be included for differential diagnosis of small round cell tumours (Ewing's sarcoma, small cell variant of osteosarcoma, malignant lymphoma, oat cell carcinoma, neuroblastoma). Markers of vascular tissue (Ulex I, factor VIII, laminin) have been reported to be of value in determination of angiosarcomas (Meister 1984; Miettinen et al. 1983b). The large field of collagen and ground substance biochemistry was not investigated and discussed. In this area, further insights may be expected for histogenesis and differential diagnosis of osseous lesions (Roessner et al. 1983; Roessner 1984).

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