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Phenotypic Characterization of Ewing Sarcoma Cell Lines With Monoclonal Antibodies

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The histogenesis of Ewing sarcoma, the second most frequent bone tumor in humans, remains controversial. Four Ewing cell lines were analyzed by immunological methods. A panel of antibodies directed to T, B, and myelomonocytic markers gave negative results. Surface antigens recognized on Ewing cells were found to be related to the neuroectoderm lineage. Ganglioside GD_2, a marker of neuroectodermal tissues and tumors, was present on all lines. These were also stained by the mouse monoclonal antibody HNK-1, which detects a carbohydrate epitope present on several glycoconjugates of the nervous system, including two glycoproteins, the myelin-associated glycoprotein and the neural cell-adhesion molecule (N-CAM), and an acidic glycolipid of the peripheral nervous system. The P61 monoclonal antibody, which reacts with a peptide moiety of N-CAM, and a rabbit antiserum, raised to purified mouse N-CAM and not recognizing the HNK-1-defined epitope, were also reactive. By contrast, all antibodies specific for hematopoietic cell surface antigens were totally negative. Besides these antigenic features, Ewing sarcoma cells are characterized by a specific t(11;22)(q24;q12) translocation also observed in neuroepithelioma, a neuroectodermal tumor, suggesting a possible evolutionary related origin. The recent finding that the human N-CAM gene is located at the vicinity of the breakpoint on chromosome 11 indicates that it might be involved in genetic rearrangements occurring in this region.

Key words: histogenesis, antigenic phenotype, flow cytometry, N-CAM, HNK-1 monoclonal antibody

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Ewing sarcoma, a childhood tumor, was first described in 1921 [1]. It is the second most frequent bone tumor in humans, but also occurs in extraskeletal localizations. Because of its morphological aspect, it belongs to the group of small round cell tumors that, because of absence of unequivocal features of differentiation, poses many problems to the pathologists [2]. For the same reason, the histogenesis of Ewing sarcoma is still debated: The postulated endothelial origin proposed by Ewing has not received confirmation, and most authors consider it to derive from a mesenchymal cell [3,4].

We have used cell lines with the characteristic translocation t(11;22)(q24;q12) [5] also described in fresh Ewing sarcoma [6] to investigate the immunological phenotype of Ewing cells. In the large panel of antibodies used, all those found positive detected antigens whose expression is related to the neuroectoderm lineage, thus supporting our previous hypothesis [7,8] of a developmental relationship between Ewing sarcoma and the neuroectoderm.

MATERIALS AND METHODS

Cell Lines

Four continuous Ewing tumor-derived cell lines were established at the International Agency for Research on Cancer (IARC-EW1, IARC-EW3, and IARC-EW7) and at the Centre Léon Bérard (IARC-EW11), from metastatic cells of different sites of origin in four different patients. The karyotypic analysis of these lines has been performed by Turc-Carel et al and has led to the original description of the t(11;22)(q24;q12) translocation [5].

The HNK-1 hybridoma was purchased from the American Type Culture Collection (Rockville, MD).

All lines were grown as monolayers or in suspension and were carried in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf or horse serum. Adherent cells were usually resuspended using trypsin (1:250, 0.05%)/EDTA (ethylenediamine tetraacetic acid, 0.02%) (Flow Laboratories, UK) with no effect on surface antigen expression, as assessed by comparative staining on cells resuspended with EDTA alone.

Staining Antibodies

Monoclonal antibodies used in this study originated from hybridoma grown in our laboratory or were kindly made available to us. Mouse antibody HNK-1 was initially shown to detect a blood cell population with NK activity [9] but later shown by us and others to identify a neuroectoderm-associated antigen [10–12]. Rat antibody P61 has been raised to purified mouse neural cell-adhesion molecule (N-CAM) [13] and shown to detect a peptide determined epitope of this molecule [14]. This antibody also reacts with N-CAM proteins from all major species tested including man. Mouse antibodies 126 and MB3.6 were donated by David Cheresh (Scripps Clinic, La Jolla, CA). They are directed to gangliosides GD_2* and GD_3, respectively, both highly enriched in neuroectodermal tumors [16]. Antibodies UJ127:11, UJ13A, UJ181, and

*According to the nomenclature of Svennerholm [15].

UJ167, obtained after immunization with human fetal brain and used in a panel of anti-neuroblastoma reagents [17], were provided by John Kemshead (ICRF, London). Human monoclonal IgM antibodies with reactivity to purified myelin-associated glycoprotein (MAG) originated from patients with peripheral neuropathy [18].

A rabbit antiserum was obtained as described [19] after immunization with purified mouse N-CAM. This antiserum does not contain antibodies recognizing the HNK-1-determined epitope [20] and reacts with typical N-CAM proteins in immunoblot from human brain.

Indirect Immunofluorescence Assay

Indirect immunofluorescence assays were performed as described [10] by incubating cells with hybridoma supernatant, ascites fluid, purified antibody, or antiserum at the appropriate dilutions. Reactions were revealed with fluoresceinated goat antisera to mouse Ig or IgM, rat Ig, rabbit Ig, or human IgM, as necessary. Fluorescence of live cells was quantitatively analyzed by flow cytometry (Epics C, Coulter, Margency, France) as described [21], or examined under a fluorescence microscope. Negative controls were included in every experiment and consisted of cells incubated with irrelevant antibodies or in the absence of first antibody.

Neuraminidase Treatment

EW3 cells (10^6) were incubated for 30 min in 50 mM sodium acetate, pH 5.5, 37°C, in the presence or absence of 5 μU of neuraminidase (Calbiochem, Behring Diagnostics, La Jolla, CA), and washed three times in RPMI 1640 supplemented with 2% fetal calf serum prior to immunofluorescence staining.

RESULTS

Immunofluorescence studies using a panel of monoclonal and polyclonal antibodies were carried on cell suspensions from Ewing cell lines EW1, EW3, EW7, and EW11, and analyzed by flow cytometry. All Ewing lines expressed human lymphocyte antigen (HLA) class I but not class II antigens. None was stained by monoclonal antibody 9.4 to the common leukocyte antigen T200. A series of antibodies from the panels of the Second International Workshop on Leukocyte Antigens that are directed to antigens associated with the B, T, and myelomonocytic cell lineages and define clusters of differentiation CD2, CD3, CD4, CD5, CD6, CD8, CD10, CD11, and CDw14 were tested and remained constantly negative (data not shown). By contrast, several molecules associated with the neuroectoderm lineage were demonstrated at the surface of the cells.

Antibody 126, directed to the ganglioside GD_2, stained all Ewing lines (Fig. 1). That the antigenic site resided upon the sialic acid residues of the conjugate was demonstrated by the abrogation of the reactivity with antibody 126 after treatment of the cells with neuraminidase (Fig. 2). Interestingly, antibody MB3.6, directed to GD_3, the metabolic precursor of GD_2, was totally unreactive on these same lines.

Antibody HNK-1 also stained the four Ewing lines to a varying degree (Fig. 3). Two distinct glycoproteins, MAG and N-CAM, both expressed in neuroectodermal tissues, have been reported to carry the HNK-1-defined carbohydrate epitope [20]. Human monoclonal antibodies whose reactivity to purified MAG had been verified by immunoblotting were also tested on Ewing cell lines. They produced similar,

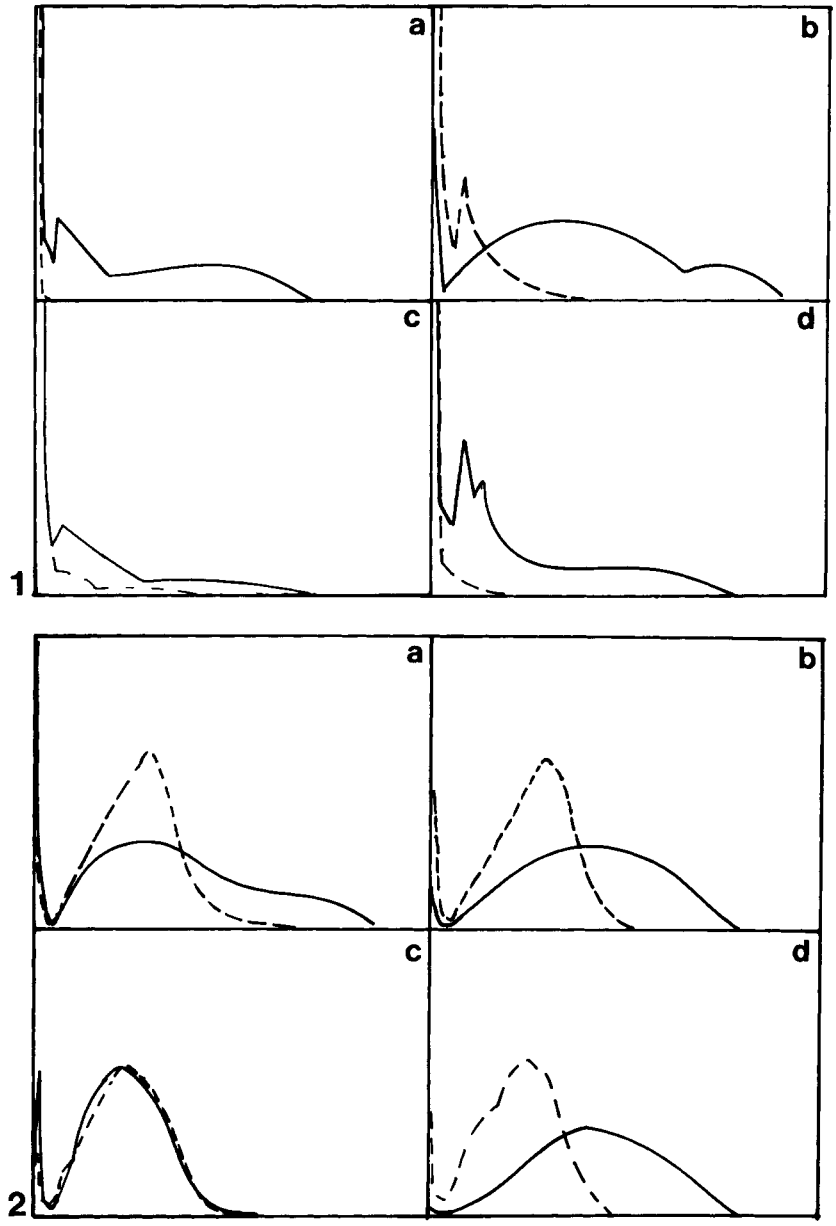

Fig. 1. Immunofluorescence staining of Ewing sarcoma lines EW1 (a), EW3 (b), EW7 (c), and EW11 (d). Cells were incubated for 30 min with 50 μl of hybridoma supernatant containing antibody 126 to GD_2. The reaction was revealed by a fluoresceinated goat antimouse IgM antiserum, analyzed by flow cytometry (—) and compared to controls (-----) obtained with first antibody omitted or replaced by antibody MB3.6 to GD_3. Histograms appear with fluorescence plotted on a logarithmic scale on the x-axis.

Fig. 2. Immunofluorescence staining of neuraminidase and mock-treated cells from Ewing sarcoma line EW3. Cells were incubated for 30 min at pH 5.5, 37°C in the absence (a,b) or presence (c,d) of neuraminidase, then stained with antibody 126 (a,c) or HNK-1 (b,d). Revelation, analysis, and display as in Figure 1.

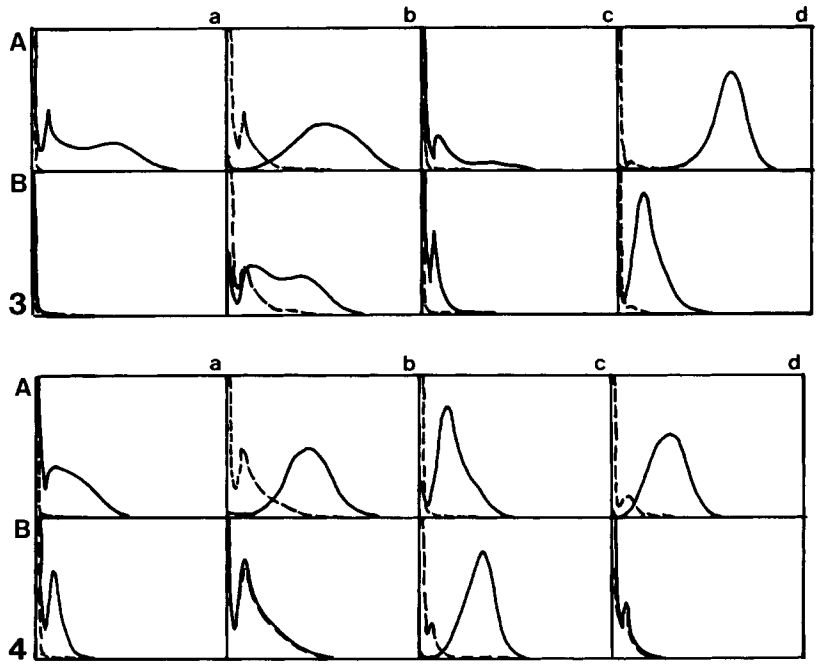

Fig. 3. Immunofluorescence staining of Ewing sarcoma lines EW1 (a), EW3 (b), EW7 (c), and EW11 (d) with mouse monoclonal antibody HNK-1 (A) and with a human monoclonal antibody with MAG reactivity (B). The reaction was as in Figure 1 except that a fluoresceinated antiserum to human IgM was used in B as a developing reagent. Analysis and display as in Figure 1.

Fig. 4. Immunofluorescence staining of Ewing sarcoma lines EW1 (a), EW3 (b), EW7 (c), and EW11 (d) with a rabbit anti-N-CAM antiserum (A) and with the rat monoclonal antibody P61 (B). The reaction was as in Figure 1 except that the reaction was revealed with fluoresceinated antisera to rabbit or rat Ig, respectively. Analysis and display as in Figure 1.

although less intense, stainings. Profiles obtained with one such antibody are shown in Figure 3.

We investigated the reactivity of Ewing lines with two reagents raised to purified mouse N-CAM, a rabbit antiserum and a rat monoclonal antibody that both cross-react with human N-CAM. The rabbit serum bound all Ewing lines (Fig. 4). With this reagent, lines EW3 and EW11 proved more reactive than EW1 and EW7. By contrast, antibody P61 that detects a peptide epitope carried by the 140- and 180-kd species of the N-CAM molecule produced totally different staining profiles, as line EW7 was the most brightly stained, even more so than neuroblastoma cell lines used as controls (data not shown). Line EW1 was more weakly stained while lines EW3 and EW11 seemed to entirely lack the P61 determinant at their cell surface (Fig. 4).

A third glycoconjugate, an acidic glycolipid present in the ganglioside fraction of the peripheral nervous system, also binds HNK-1 antibody [22]. Preliminary results indicate that HNK-1 does detect such a molecule purifying in the trisialo ganglioside fraction extracted from Ewing cell lines EW1 and EW3 (data not shown). That sialic acid residues do not carry the epitope is shown by the persistence of the staining after treatment with neuraminidase in condition that totally abrogated the detection of surface ganglioside GD_2 (Fig. 2).

Finally, we assayed the reactivity of four antibodies to neuroblastoma cells [17]. As shown in Table I, all antibodies but one reacted with one or two Ewing lines.

DISCUSSION

We have used an immunological approach to attempt to clarify the question of the histogenesis of Ewing sarcoma. A preliminary study of Ewing lines had led us to postulate a neuroectodermal origin for Ewing cells [7,8]. We have now obtained additional evidence that the antigens expressed at the surface of Ewing cell lines are indeed related to this lineage.

Ganglioside GD_2 was detected on all Ewing lines. Its expression has previously been analyzed in detail and shown to be restricted to normal tissues and tumors of neuroectodermal origin [23]. The epitope recognized probably contains the sialic acid residues of GD_2 since the reactivity was lost after neuraminidase treatment.

Antibody HNK-1 was also found to bind all four Ewing lines. Staining of a large panel of human solid tumors with this reagent has indicated that its reactivity was restricted to neuroectoderm-derived tumors [12]. HNK-1 has been shown to react with a ganglioside of peripheral nerves [22], but its binding to Ewing cells was not affected by neuraminidase treatment. However, when gangliosides were purified from Ewing cells, HNK-1 was found to detect a moiety present in the trisialoganglioside-containing fraction. This reactivity is likely to be identical with that already described in peripheral nerves [22].

The same carbohydrate epitope seems to be carried by several glycoproteins of the nervous system, including MAG and N-CAM. These two structures are highly glycosylated molecules suspected to play an important role in the nervous system, MAG as a minor constituent of myelin sheaths [24], and N-CAM as a major ligand implicated in the phenomenon of neural-neural cell binding [25]. Also, the antigenic epitope detected by HNK-1 is extremely well conserved during evolution, suggesting that it might exert a major function in the nervous tissues [26]. In this respect, its presence on Ewing cells provides a further indication of the expression on this tumor of neuroectoderm associated structures.

Finally, all Ewing lines tested reacted with an anti-N-CAM antiserum, and furthermore lines EW1 and EW7 were stained by antibody P61 that detects a peptide epitope restricted to the 140- and 180-kd species of the N-CAM molecule [14]. The importance of the finding of neuroectodermal antigens on Ewing cells is strengthened

TABLE I. Binding on Ewing Cell Lines of Monoclonal Antibodies Reacting With Neuroblastoma*

Staining antibody	Ewing cell lines			
	EW1	EW3	EW7	EW11
UJ127:11	+	−	−	+
UJ13A	−	+	−	+
UJ181	−	−	−	+
UJ167	−	−	−	−

*Target cells were incubated for 30 min with staining antibody. The reaction was revealed with a fluoresceinated goat anti-mouse Ig antiserum. Cells were smeared on a slide, and live cells were examined for fluorescence under a fluorescence microscope. Results were scored as positive (+) or negative (−) by comparison with control fluorescence obtained with target cells incubated with second step only.

by the absence of reactivity of these cells with a panel of antibodies directed to differentiation antigens of the B, T, and myelomonocytic lineages. By contrast, monoclonal antibodies raised to human fetal brain that all bind neuroblastoma cells [17] displayed some degree of reactivity with one or two Ewing lines.

To the pathologist, Ewing sarcoma is often difficult to differentiate from other small round cell tumors of neuroectodermal origin [2]. Most often, investigations of specific enzymatic activities and electron microscopic features will nevertheless allow discrimination of a bone metastasis of neuroblastoma from a Ewing sarcoma. Also, neuroblastoma cells usually express very low levels of HLA class I molecules [27], in contrast with Ewing cells. Even more strikingly, the expression of the N-*myc* oncogene is specifically amplified in neuroblastoma [28]. We did not observe such an amplification in DNA from two Ewing lines analyzed here with a specific N-*myc* probe (provided by Frederick Alt, NY) (data not shown). Thus, a variety of biological criteria clearly delineate Ewing sarcoma from neuroblastoma.

The comparison of Ewing sarcoma with peripheral neuroepithelioma raises more problems, as recently underlined [29]. In 1983, the description of a specific translocation t(11;22)(q24;q12) in Ewing sarcoma cells [5,6] provided the first means of positive diagnostic for this malignancy until the same translocation was reported as well in peripheral neuroepithelioma [30] and in another rare neuroectodermal tumor of the thoracopulmonary region [31] described by Askin et al [32], raising questions about the possible implications of this repetitive chromosomal accident.

During normal differentiation of the B cell lineage, genetic rearrangements take place on chromosomes 14, 2, and 22 in the genes coding for heavy and light Ig chains, respectively [33]. Burkitt lymphoma, a B cell malignancy, is characterized by a translocation between chromosome 8 where the c-*myc* oncogene is normally located and one of the Ig gene carrying chromosomes [34]. New DNA configurations are thus obtained that can affect the structure of c-*myc* or the regulation of its expression [35].

Two proto-oncogenes have been localized in the vicinity of the breakpoints observed in Ewing cells: c-*ets* on chromosome 11q23–24, and c-*sis* on chromosome 22 distal to q11. Neither one seems to be involved in the oncogenetic process since, despite being translocated, c-*sis* is neither rearranged nor amplified [36], and c-*ets* has not been found rearranged in five different Ewing tumors [37].

It is nevertheless tempting to imagine that the t(11;22) translocation of Ewing sarcoma and neuroepithelioma could also point to chromosomal breakpoints involved in normal genetic rearrangements occurring during neuroectodermal differentiation. With this hypothesis in mind, it becomes striking that the human N-CAM gene has recently been localized to chromosome 11q23 [38]. Whether this gene is actually translocated in Ewing cells resulting in the transcription of new RNA messengers and expression of abnormal cell surface molecules is presently under investigation at the molecular level.

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