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Ewing's sarcoma lines synthesize laminin and fibronectin*

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Summary. Immunoelectron microscopy was employed to detect laminin and fibronectin cell surface expression on five Ewing's sarcoma lines plus a normal fibroblast line as control. Monospecific antibodies to both glycoproteins were detected on tumour cell and fibroblast layers with colloidal gold – protein A conjugates. All five tumour lines were positive for fibronectin and/or laminin, whereas the fibroblast line expressed fibronectin only, as expected. Fibronectin displayed a dense granular pattern, typically in the cell-cell and cell-matrix adhesion areas; laminin displayed a punctate pattern. ^{3}H-leucine metabolical labelling was also used to demonstrate laminin and fibronectin synthesis. The labelled proteins released in the culture media were separated by molecular weight on SDS-PAGE and identified by immunoprecipitation with the monospecific antibodies. The results substantiated the immunoelectron microscopy data. These findings indicate that Ewing's sarcoma lines produce a complex extracellular matrix including fibronectin and laminin, in addition to the collagens described by other workers. Histogenetic classification of this tumour in terms of extracellular matrix proteins synthesis is thus more difficult than has been supposed. The same complexity must also be borne in mind when using the matrix components as an aid to Ewing's sarcoma differentiation from other childhood tumours.

Key words: Ewing's sarcoma – Laminin – Fibronectin – Extracellular matrix – Tumor histogenesis

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Introduction

The histogenesis of Ewing's sarcoma has been debated since its initial characterization (Ewing 1921). Endothelial cells (Ewing 1921), plasma cells (Ewing 1924), neuroblasts (Willis 1940), myeloblasts (Kadin and Bensch 1971), pericytes (Sato and Takahashi 1975), smooth muscle cells (Stern et al. 1980) and mesenchymal cells (Dickman et al. 1982) have all been put forward as possible origins.

Ewing's sarcoma is a malignant tumour primarily arising in bone. Its typical feature is composition of highly undifferentiated round cells with no specific morphological characteristics other than glycogen deposits. It can easily be mistaken, both clinically and histologically, for other solid tumours of childhood, such as Wilms' tumour and the small-round-cell tumours rhabdomysarcoma, lymphoma and undifferentiated neuroblastoma (Triche 1982). Since no ultrastructural distinction can be drawn between the morphological features of undifferentiated tumours, several immunocytochemical and enzyme histochemical techniques have been devised for the differential diagnosis of Ewing's sarcoma (Triche 1982). Biochemical and immunocytochemical characterization of the extracellular matrix proteins synthesised by the tumour cell has recently been proposed, each protein serving as a histogenetic marker (Alitalo et al. 1980, Alitalo et al. 1982; Krieg et al. 1979; Lanzer et al. 1981; Sariola et al. 1985; Stern et al. 1980).

In support of their view that Ewing's sarcoma is of mesenchymal origin, Dickman et al. (1982) has shown that it produces interstitial collagens (type I–III) as well as type IV collagen. Utilizing antibodies to laminin and fibronectin conjugated to Protein A – colloidal gold spheres as previously

described (Modesti et al. 1984) we demonstrate that Ewing's sarcoma cells express laminin and fibronectin, two typical extracellular matrix components (Engel et al. 1981), together with the listed collagens; by metabolical radiolabelling we further demonstrate that both glycoproteins are synthesized and released in the tumour culture media.

Materials and methods

Cell lines. Five Ewing's sarcoma lines (TC-71, A4573, 6647, TC-106, 5838), were studied plus a normal fibroblast line established from the dermis of a 16-year old female with fibrosarcoma. Lines A4573, 5838, 6647 were derived by Grace Cannon of Litton Bionectics Corporation (Dickman et al. 1982), lines TC-71 and TC-106 were established in the laboratory of Dr. Triche, National Cancer Institute, Bethesda, USA. All lines were derived from the primary or metastatic lesions of five patients with Ewing's sarcoma diagnosed at the National Cancer Institute. Aneuploidy and malignant karyotypes have been demonstrated cytogenetically in all five lines (Biotech Research Lab. Inc., Rokville, MD, USA). Cultures were grown to confluency in RPMI 1640 medium with 10% fetal calf serum and penicillin, streptomycin and gentamycin.

Immunoelectron microscopy. A colloidal gold solution was prepared by reduction of chloro-auric acid with white phosphorus as described by Faulk and Taylor (1971), and Slot and Geuze (1981). Colloidal gold-protein A complexes were obtained by the method of Roth et al. (1978). Ewing's sarcoma and fibroblast cell layers were incubated with monospecific rabbit antibodies to laminin and to human fibronectin (Bethesda Research Laboratories, Bethesda, MD, USA) for 2 h at room temperature. After washing with phosphate buffered saline pH 7.4, the cell layers were incubated with protein A-colloidal gold solution for 1 h at room temperature, washed and fixed in 0.1 M monophosphate buffer with 2.5% glutaraldehyde for 3 h. They were then scraped and pelleted, osmicated, dehydrated through alcohols and embedded in Epon 812 resin.

Protein radiolabelling. Confluent cultures were preincubated for two days in growth arrest medium: RPMI 1640 with decreasing concentrations (5% to 1%) of dialyzed fetal calf serum and 50 μg/ml of ascorbic acid. One hour before labelling, the culture was incubated with Selectamine RPMI 1640 (Gibco) lacking leucine and precursors, without fetal calf serum, with 50 μg/ml of ascorbic acid, 100 μg/ml of beta amino propionitrile fumarate, 3.5 mg/ml of glucose. One hour later 50 μCi/ml of L-(4,5-^{3}H) leucine (120–190 Ci/mmol), (Amersham, Braunschweig, FRG) were added. Twelve hours later the radiolabelled medium was harvested and protease inhibitors were added (5 mM phenyl methyl sulfonyl fluoride, 1 mM ethylenediamine-tetracetic acid).

Protein detection and identification. Incorporation of ^{3}H-leucine was quantified in a Beckman liquid scintillation counter after precipitation of small amounts of radiolabelled media with 10% cold trichloroacetic acid (TCA). A volume equivalent to 100,000 cpm TCA-precipitable counts was removed and was cold ethanol (−20° C) precipitated. The resulting pellet was redissolved in electrophoresis sample buffer (65 mM Tris-HCl pH 6.8, 4 M urea, 20% sodium dodecyl sulfate, 10% glycerol, bromophenol blue), boiled in water for 3 min with or without reduction using 50 mM dithiothreitol (Dtt), and then separated by molecular weight in SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using 3.5% stacking gel and 4% separating gel, as described by Laemmli (1970). Gels were fixed in methanol-acetic acid, treated with fluorographic solution (Autofluor, National Diagnostics, USA), dryed and exposed to photographic emulsion (Kodak XAR-5) at −70° C.

Immunoprecipitation. Radiolabelled media (500,000 cpm TCA-precipitable counts) were alternately incubated with the anti-

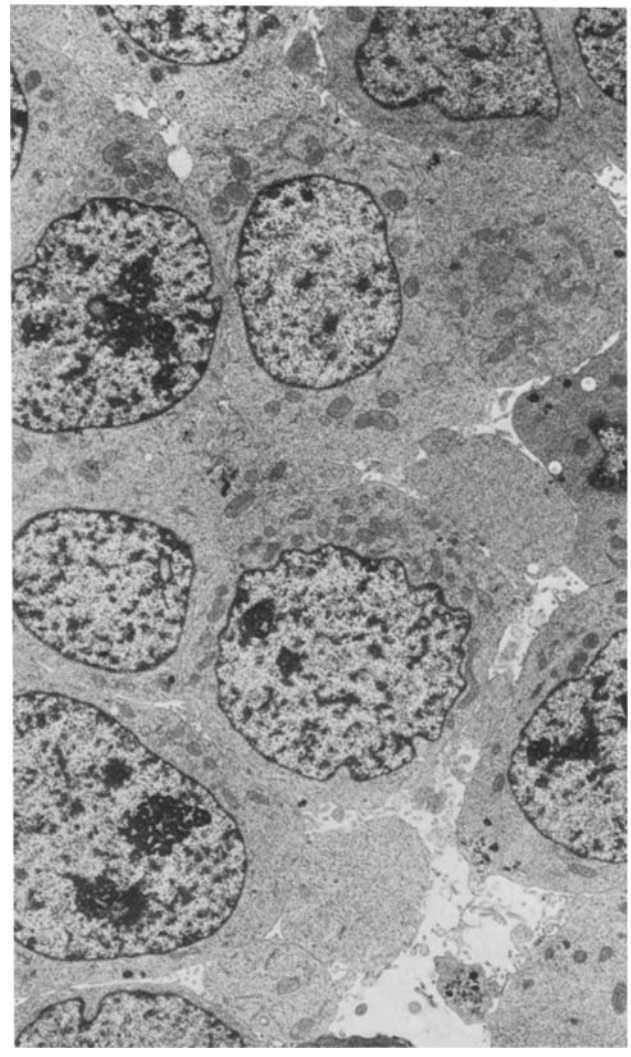

Fig. 1. Ultrastructural appearance of tumour cells from an Ewing's sarcoma line shows cytoplasmic glycogen and simple cytoplasm with few organelles; lysosomes were occasionaly prominent in tissue culture cells

Table 1

Cell line	Cell layer proteins (immunoelectron microscopy)	Media proteins (SDS-PAGE)
A4573	Fn, Lm	Fn, Lm
5838	Fn	Fn
6647	Fn, Lm	Fn, Lm
TC-106	Fn	Fn, Lm
TC-71	Lm	Lm

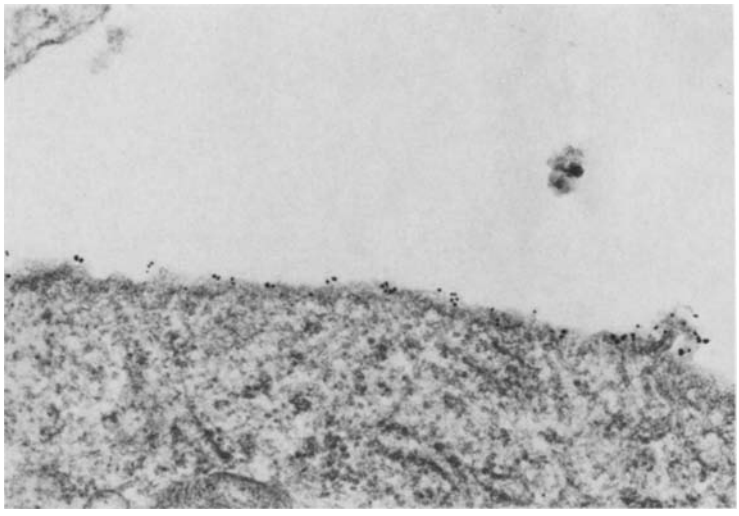

Fig. 2. Ewing's sarcoma cell surface labelling with antibody to laminin-colloidal gold-protein A conjugates. The whole cell is highly stained by the colloidal gold granules with a typically punctate pattern

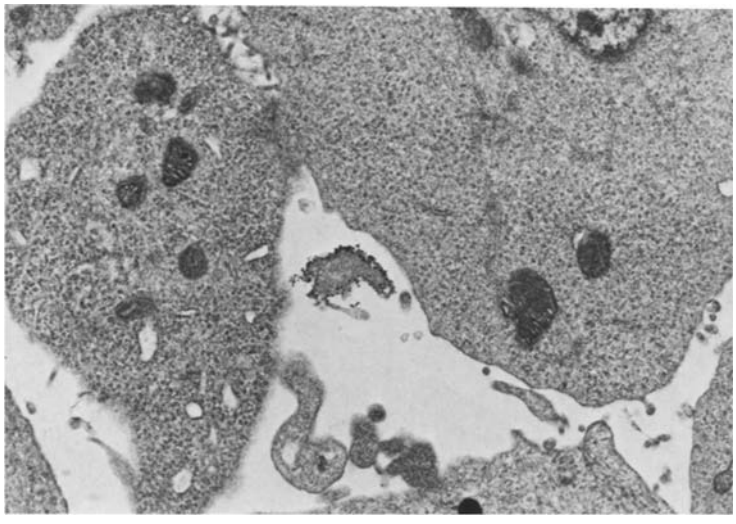

Fig. 3. Ewing's sarcoma cell stained with anti-fibronectin-colloidal gold-protein A conjugates. Granules are visible as electrondense material in the cell-matrix adhesion areas

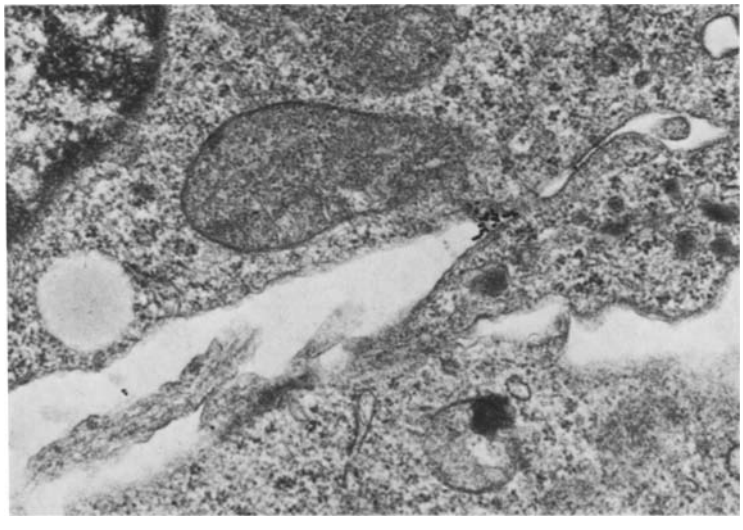

Fig. 4. Immunolabelling for fibronectin of Ewing's sarcoma cells. Fibronectin is concentrated in cell-to-cell contact areas

bodies to laminin and fibronectin for 12 h at 4° C. Next, 50 µl of goat anti rabbit IgG (Cappel, Malvern, PA, USA) were added and incubated for 2 h at 37° C. The antigen-antibody complexes were precipitated by adding 1 ml of cold saline and pelleted in a Beckman microfuge. The pellets were resuspended in electrophoresis sample buffer and analyzed by SDS-PAGE as described before.

Results

The typical ultrastructural appearance of an Ewing's sarcoma cell culture is shown in Fig. 1, all five studied lines expressed essentially the same features.

Laminin and fibronectin were detected as cell layer related proteins on Ewing's sarcoma cultures by immunoelectron microscopy; their synthesis was proved by immunoprecipitation of the radiolabelled culture media. The general agreement between the two sets of results is illustrated in Table 1; the summarized data indicate that all five lines produced fibronectin and/or laminin.

Differences in morphology and distribution on the tumour cell layers were noted: laminin was spread over the whole cell surface in a characteristic punctate pattern (Fig. 2), whereas fibronectin was condensed in the cell-matrix (Fig. 3) and cell-cell adhesion areas (Fig. 4) and had a dense granular pattern.

Controls lacking the primary antisera were generated in parallel with the known positives for comparison (data not shown). As reported elsewhere (Fromme et al. 1982), fibroblasts were intensely stained when treated with gold labelled antibodies to fibronectin. The regular, electrondense layer was particularly concentrated in the cell-matrix (Fig. 5a) and cell-cell (Fig. 5b) adhesion areas and resembled the tumour cell-layer pattern. As expected, all these regions and the whole fibroblast were negative for laminin (Fig. 5c).

Laminin and fibronectin synthesis was confirmed by ^{3}H-leucine metabolic labelling. Fibronectin and interstitial procollagens (type I–III) were the main radiolabelled proteins in the SDS-PAGE separated fibroblast culture media (Figs. 6, 7, lane 1). Extracellular matrix proteins synthesis from the five sarcoma lines, on the other hand, was more varied (Figs. 6, 7, lanes 2–6). Three lines synthesised both proteins (lanes 2–4, Table 1 summary). Four lines produced fibronectin, visible as a sharp band migrating around 240,000 Dalton (240KD) when electrophoresed after reduction (Fig. 6, lanes 2, 3, 4, 6), and at 480K Dalton in unreduced conditions (Fig. 7, lanes 2, 3, 4, 6), (Yamada and Olden 1978).

Four lines synthesized laminin, recognizable as a high molecular weight band migrating in the unreduced SDS-PAGE around 1000K Dalton on the top of the gel (Fig. 7, lanes 2–5); once reduced, 400K Dalton laminin subunit was evident in all four sarcoma lines, while its 200K Dalton band

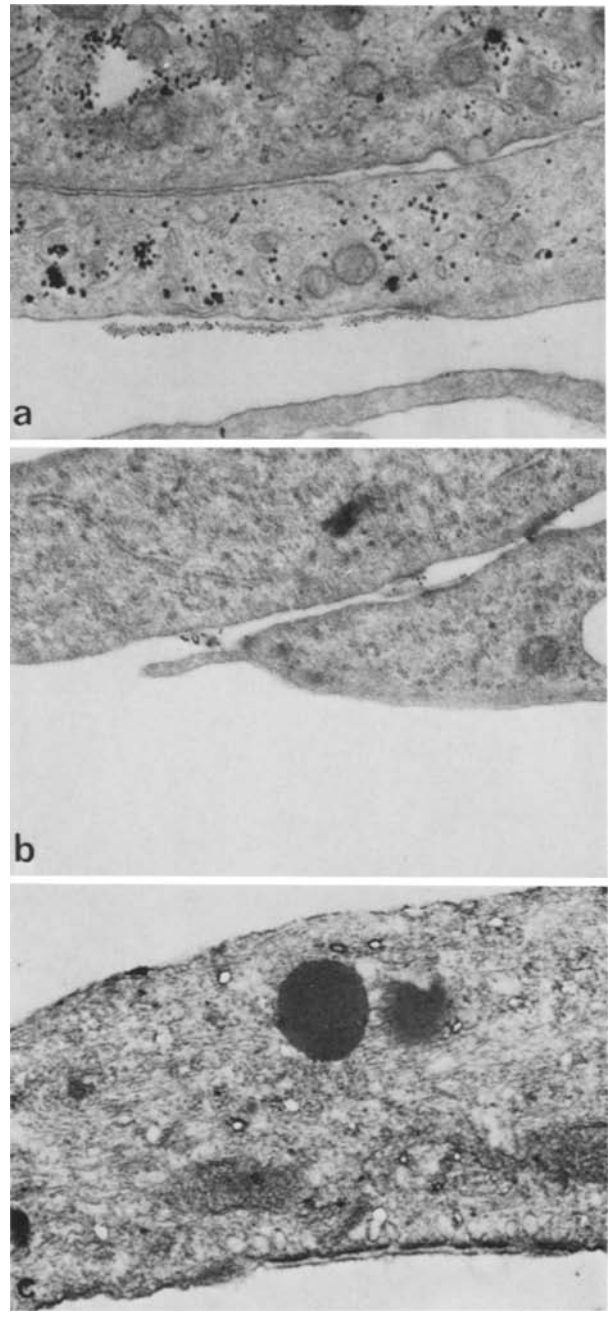

Fig. 5a–c. Fibroblasts labelled with anti-fibronectin **a, b** and anti-laminin **c** antibodies colloidal gold-protein A conjugates. Panels **a, b** show some granules characterized by a dense granular pattern, visible as an electrondense material on the cell-matrix **a** and cell-cell **b** adhesion areas. On panel **c**, in contrast, the electrondense material is unlabelled by anti-laminin-gold conjugates

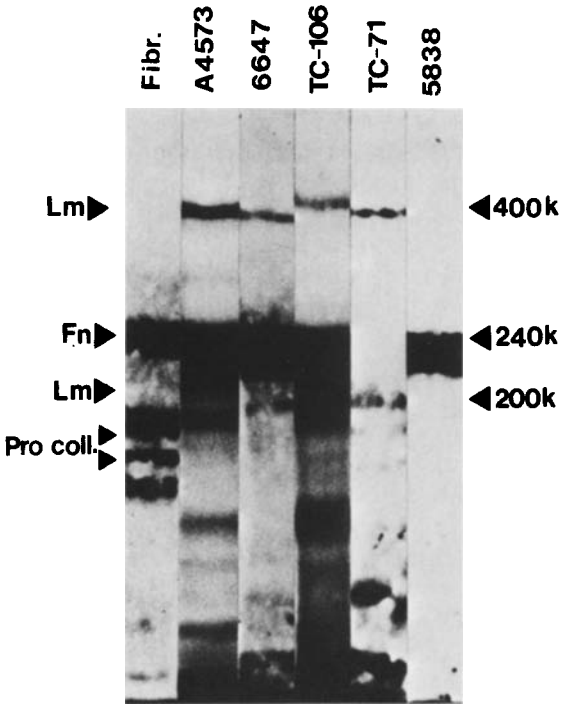

Fig. 6. Autoradiogram of a 4% SDS-PAGE representing the reduced proteins metabolically radiolabelled and released into the culture media by one fibroblast (*lane 1*) and five Ewing's sarcoma (*lanes 2–6*) lines. In the first lane, the radiolabelled products of fibroblasts are fibronectin (*Fn*), visible as a band migrating at *240 KD*, and just below several bands of the interstitial procollagens (*type I–III*). The same fibronectin band migrating at *240 KD* is recognizable in four Ewing's sarcoma lines (*lanes 2, 3, 4, 6*); reduced laminin (*Lm*) *400* and *200 KD* bands are marked in four Ewing's sarcomas (*lanes 2–5*)

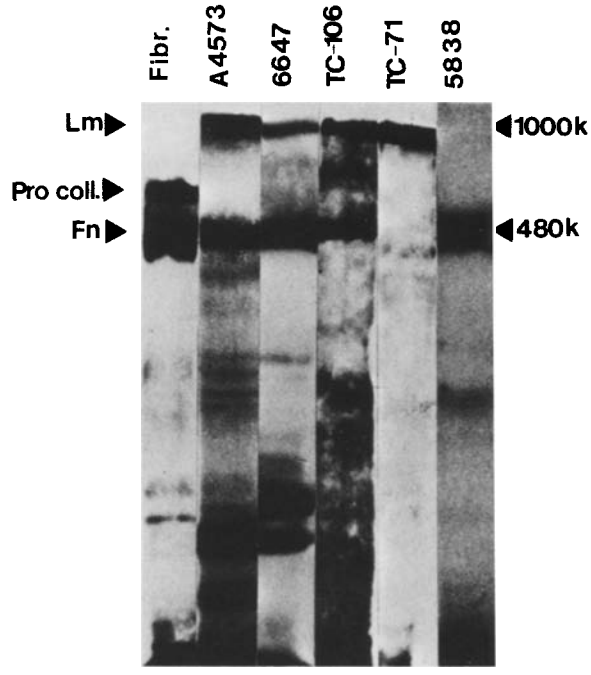

Fig. 7. The same radiolabelled media proteins shown in figure 6 separated on 4% SDS-PAGE without reduction. The first lane represents the proteins synthesized by fibroblasts: a band migrating at *480 KD* corresponds to unreduced fibronectin (*Fn*) and a higher molecular weight band to procollagens. In all the Ewing's sarcoma lines but the fifth (*5838*), a constantly high molecular weight band migrating near the top of the gel at a position corresponding to *1000 KD* unreduced laminin (*Lm*) is evident (*lanes 2–5*). In *lanes 2, 3, 4, 6* the *480 KD* band of fibronectin is visible

was less easily distinguishable (Fig. 6, lanes 2–5), (Engel et al. 1981; Rohde et al. 1979). Band identities were confirmed by immunoprecipitation of each radiolabelled culture media with the monospecific antisera to laminin and fibronectin (Fig. 8).

Antibodies to fibronectin precipitated large amounts from fibroblast radiolabelled medium, whereas antibodies to laminin were always negative (data not shown), in agreement with the immunoelectron microscopy results.

Discussion

Ewing's sarcoma is a highly undifferentiated tumour. Recognition of its characteristic features by conventional electron microscopy is often impossible and its histogenetic classification is equally problematical.

Attempts to determine its origin and permit prompt classification have led to the identification of various "in vitro" and "in vivo" markers (Triche 1982). One of the most recent of these is the extracellular matrix synthesized by the tumour; since many authors have lately described that normal and neoplastic cells, either established cell lines or freshly excised tumour explants, produce extracellular matrix components in characteristic patterns appropiate to their lineage (Alitalo et al. 1980; Alitalo et al. 1981; Bonadio et al. 1984; DeClerck et al. 1985; Stern et al. 1980).

Our results show that the matrix synthesized by five sarcoma lines had a varied pattern and included the glycoproteins laminin and fibronectin, as well a type I-III-IV collagens recently seen as evidence that a primitive mesenchymal cell, undifferentiated and possibly, though not necessarily, from the bone marrow, is the cell of origin (Dickman et al. 1982).

Both radiolabelling and immunoelectron microscopy demonstrated the production of fibronectin and laminin by these Ewing's sarcoma lines. The presence of fibronectin is in line with the suggested mesenchymal origin, since it is the typical

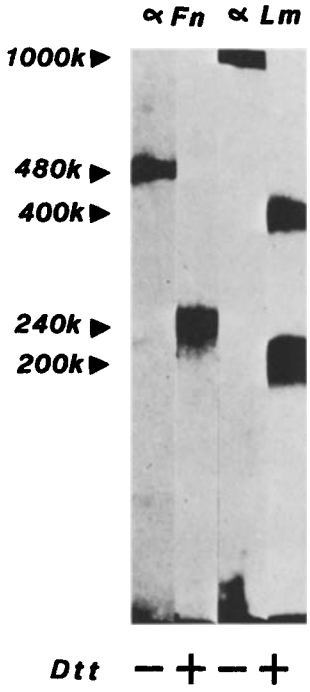

Fig. 8. Ewing's Sarcoma media proteins immunoprecipitated with anti-fibronectin (*Fn*) and anti-laminin (*Lm*) specific antibodies and analyzed on a 4% SDS-PAGE with (*Dtt* +) and without (*Dtt* −) reduction. The first lane represents unreduced *480 KD* fibronectin precipitated by anti-Fn antibody. The same immunoprecipitate run reduced migrates at *240 KD* (*lane 2*). Anti-Lm antibody precipitates the unreduced *1000 KD* band of laminin (*lane 3*). Once reduced, this migrates as two subunits at *200* and *400 KD* (*lane 4*)

product of fibroblastic mesenchymes and their derivatives (Ruoslahti et al. 1981); this was also clear from the results obtained with out fibroblast control line.

Laminin, on the other hand, is, like type IV collagen, a major constitutent of the basement membrane (Timpl et al. 1979) and normally synthesised by endothelial and epithelial cells. Its presence, therefore, while consistent with the production of type IV collagen by Ewing's sarcoma, further complicates the question of its histogenesis.

Inducible neural differentiation has been reported for four Ewing's sarcoma lines (Cavazzana et al. 1985) as well as neuroectodermal differentiation in bone tumours presenting as Ewing's sarcoma (Perez-Atayde et al. 1985). Indeed, many tumours classed as Ewing's sarcoma may be histogenetically related to peripheral neuroepithelioma, another primary neuroectodermal tumour of bone (Donner et al. 1985; Jaffe et al. 1984; Triche et al. 1985). Further evidence of their common origin is their sharing of a unique chromosome abnormality (a reciprocal 11:22 translocation), first described in Ewing's sarcoma (Triche et al. 1985; Turc-Carel et al. 1984). Since laminin is a typical neuroectodermal cell product, therefore, its presence in the extracellular matrix of Ewing's sarcoma can readily be explained. The conclusion to be drawn from this study, in our opinion, is that despite their constant morphology, Ewing's sarcoma cells must be regarded as highly undifferentiated, yet able to produce a large and complex pattern of extracellular matrix proteins. Even so, the synthesis of this extracellular matrix is not specific and characteristic enough to allow a clear tissue origin to be found for Ewing's sarcoma, but it is specific enough to make possible a distinction among the so called small-round-cell tumours of childhood. For example, the production of stromal collagens by Ewing's sarcoma serves to distinguish this tumour from neuroblastoma; also, lymphoma, a tumour commonly confused with Ewing's sarcoma, does not synthesize any extracellular matrix protein (Triche et al. 1980).

Therefore, this study has also served to confirm the utility of using extracellular matrix analysis as an aid in the differential diagnosis of the above mentioned class of undifferentiated paediatric tumours.

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