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Lack of adhesion molecules in testicular diffuse centroblastic and immunoblastic B cell lymphomas as a contributory factor in malignant behaviour

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Abstract Diffuse large B-cell lymphoma of the testis is a rare tumour, often with disseminated disease. According to the Kiel Classification, these lymphomas are of centroblastic or immunoblastic type, corresponding in the Working Formulation to malignant lymphoma, large cell non-cleaved and large cell immunoblastic, respectively. Adhesive cell–cell and cell–matrix interactions are generally assumed to play an important part in the metastatic process, and to find clues to the highly malignant biological behaviour of this tumour we examined expression of integrins and other adhesion molecules on the tumour cells and the presence of matrix proteins. Few adhesion molecules appeared to be expressed. CD44 was expressed in 10/12 lymphomas, CD49f/VLA-6 was positive in 5/12 cases, and CD49d/VLA-4, CD54 and CD62L were detectable in a small number (2–3) of lymphomas. All other adhesion molecules were lacking. This expression pattern is suggestive of a high metastatic potential: the tumour cells seem to be poorly attached to the extracellular matrix, to each other and to other cells (CD54–, CD11a–, CD58–). The adhesion molecules expressed, CD44, CD49f/VLA-6 and CD49d/VLA-4, have been reported to play a part in dissemination, mediating intravasation (CD49f/VLA-6) and extravasation (CD44, CD49d/VLA-4). This profile of adhesion molecules may explain, at least in part, the specific biological behaviour of these lymphomas with early and rapid dissemination.

Key words Testis lymphoma · Non-Hodgkin's lymphoma · Adhesion molecule · Integrin · Extracellular matrix protein

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Introduction

Primary malignant lymphomas of the testis are relatively rare comprising approximately 50% of all testicular malignancies [3] and only 1–3% [3, 4] of all cases of non-Hodgkin's lymphoma. However, it is the most common testicular tumour in men over the age of 60 [3]. The prognosis for patients with testicular lymphoma is poor, with a median survival of 9.5–12 months after diagnosis [24]. Only rarely does the lymphoma remain confined to the testis, and organs with a relatively high incidence of secondary involvement are the skin and subcutaneous tissue, the central nervous system, Waldeyer's ring and adjacent structures, the lung and the contralateral testicle [3]. Most primary malignant lymphomas (ML) of the testicle are diffuse large B cell lymphomas (DLC), of the high-grade polymorphic centroblastic or immunoblastic subtype [5, 24, 35] (Fig. 1) according to the Kiel Classification [2, 36], corresponding to malignant lymphoma, large cell non-cleaved and large cell immunoblastic, in the Working Formulation [22].

Adhesive cell–cell and cell–matrix interactions are important determining factors for the biological behaviour of malignant tumours [18, 28]. Several families of adhesion molecules play an essential part in these interactions, including the integrins. In order to find clues to the highly malignant biological behaviour of testicular diffuse large B cell lymphomas even in comparison to other extranodal large cell lymphomas, we examined expression of integrins and other adhesion molecules on tumour cells and the presence of matrix proteins.

Materials and methods

Frozen tissue was available from 12 cases of diffuse large B cell lymphomas of the testis (8 centroblastic and 4 immunoblastic ML) and was examined in this study. The diagnosis was established on haematoxylin/eosin- and Giemsa-stained paraffin or plastic sections, together with immunohistochemical staining for B cell markers and immunoglobulin expression. The tumours were classified according to the Kiel Classification and the Working Formulation [2, 22, 36].

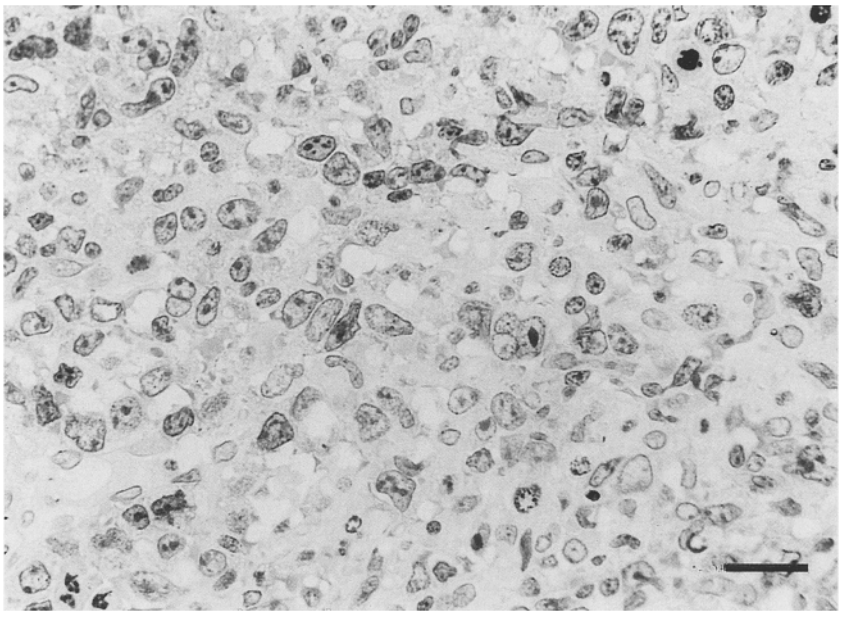

Fig. 1 Diffuse large cell lymphoma of the testis, Kiel-Classification: polymorphic centroblastic malignant lymphoma (ML). HE, ×400, *bar* 25 μm

Tissue blocks were fresh-frozen in liquid isopentane at –80°C and stored at the same temperature. Tissue sections 4 μm thick were cut, air-dried, fixed in acetone and immunostained, or stored at –20°C until use.

A three-step immunostaining procedure was used. The sections were incubated for 60 min with 50 μl of antibody in optimal dilution, as determined previously. The primary antibodies are listed in Tables 1 and 3 [29, 32]. Besides these, antibodies were used against B cells (CD20, CD22/Leu14), T cells (CD2/Leu5, CD3/Leu4), macrophages (CD68/KP1), natural killer cells (CD57/Leu7), immunoglobulin light chains κ and λ, and heavy chains IgM, IgG, IgA, IgD. Leu-antibodies and anti-IgG were obtained from Becton-Dickinson (San Jose, Calif.), and others from Dako (Glostrup, Denmark). Incubation with primary monoclonal mouse and rat, and polyclonal rabbit or goat antibodies was followed by incubation of 30 min with RaM-Biotin (biotinylated rabbit anti-mouse immunoglobulin in a 1:300 dilution), RaR-PO (peroxidase conjugated rabbit anti-rat immunoglobulin in a 1:50 dilution), SaR-Biotin (biotinylated swine anti-rabbit immunoglobulin in a 1:300 dilution) and RaG-PO (peroxidase conjugated rabbit anti-goat immunoglobulin in a 1:50 dilution) respectively. Incubation with RaM-Biotin and SaR-Biotin was followed by incubation with peroxidase conjugated streptavidin. The sections with second-step RaR-PO and RaG-PO were finally incubated for 30 min with SaR-PO (peroxidase-conjugated swine anti-rabbit immunoglobulin in a 1:50 dilution). The second- and third-step reagents were diluted with PBS supplemented with 1% bovine serum albumin (BSA) and 1% human AB serum. After every incubation step, the sections were washed for 5 min in PBS. 3-Amino-9-ethylcarbazole and H_2O_2 were used as substrate for the peroxidase reaction, resulting in a reddish brown precipitate. The sections were counterstained with haematoxylin.

Sections either not incubated with monoclonal antibodies or incubated with isotype-matched, nonrelevant mAb served as negative controls. The expected pattern of staining of endothelial cells, lymphocytes or macrophages, present in all slides, served as an intrinsic positive control.

Results

Tumour cells stained for the pan B-cell antigens CD20/22 and were negative for T cell antigens (CD2 and CD3). On all DLC of the testis, IgM was expressed as the only heavy chain surface immunoglobulin with monoclonal Ig light chain κ in 6 cases and λ in 2 cases. Immunoglobulin light chain expression was lacking in 4 cases.

T lymphocytes, stained with anti-CD2, were present in every tumour, mostly in large or moderate numbers. Macrophages, stained with anti-CD68, were present abundantly in every section. In some lymphomas, small numbers of CD57+ natural killer cells were detected.

The expression pattern of adhesion molecules or subunits on testicular diffuse large B-cell lymphomas is listed in Table 2. Only a few adhesion molecules appeared to be expressed on testicular DLC. CD44 was expressed in 10 of the 12 lymphomas (Fig. 2A). CD49f/VLA-6 was positive in 5 cases. The adhesion molecules/subunits CD49d/VLA-4, CD54 and CD62L were detectable in only a small number of lymphomas (Fig. 2), with a preference of CD54 staining for immunoblastic ML. The subunits CD11a and β2 (CD18) together form the LFA-1 integrin, a specific adhesion molecule of leucocytes. This molecule was absent from all testicular lymphomas (Fig. 3A). Centroblastic and immunoblastic lymphoma differed in CD54 expression (Fig. 3B), being completely negative in all CB-NHL. In the CD49f/VLA-6-positive lymphomas, many tumour cells showed a cytoplasmic dot (Fig. 2D). In cases 6 and 12 there were subsets of tumour cells that expressed CD49d/VLA-4, whereas this adhesion molecule was absent in the rest of the tumour. In case 2, CD62L was expressed in a definite population of tumour cells, while the other cells lacked this molecule.

In most testicular DLC, extracellular matrix proteins were abundant. Data on the presence of specific matrix proteins are listed in Table 3. The fibers of a specific type of matrix protein often formed a tight network (Figs. 4, 5).

Table 1 Antibodies reactive with adhesion molecules (Antigens: *VLA* very late (activation) antigen, *VnR* vitronectin receptor, *int* integrin, *ICAM* intercellular adhesion molecule, *LFA* lymphocyte function associated, *CR* complement receptor, *LECAM* leucocyte–endothelial cell adhesion molecule, *NCAM* neural cell adhesion molecule; ligands: *Coll* collagen, *Epil* epiligrin, *Fb* fibrinogen, *Fn* fibronectin, *HEV* high endothelial venules, *Hy* hyaluronate, *ICAM* intercellular adhesion molecule, *Inv* invasin, *LFA* lymphocyte function-associated antigen, *Ln* laminin, *Ost* osteopontin, *Tsp* thrombospondin, *VCAM* vascular cell adhesion molecule, *Vn* vitronectin, *vWF* von Willebrand's factor; cellular distribution: *BM* membrane associated, *EN* endothelial cells, *Eo* eosinophils, *EP* epithelial cells, *Er* erythrocytes, *F* fibroblasts, *FDC* follicular dendritic cells, *Ly* lymphocytes, *Ly′* activated lymphocytes, *M* monocytes/macrophages, *Mus* muscle, *NC* neural crest cells, melanocytes, *NK* natural killer cells, *PG* proteoglycans, *Pl* platelets, *PMN* polymorphonuclear cell (neutrophil), *Th* thymocytes; *CD* cluster of differentiation)

Antigen	CD	Clone[a]	Source[b]	Ligands	Cellular distribution
VLA-1	CD49a	TS 2/7	Sp	Coll(I,IV)	F,M,BM,T-ly′,B-ly′, Mus
VLA-2	CD49b	CLB-thromb/4	So	Coll(I-III,IV)Ln,Fn	Pl,F,EN,Ep,T-ly′
VLA-3	CD49c	P1B5	T	Coll(I,IV)Ln,Fn, Epil	EP,F,BM,B-ly
VLA-4	CD49d	B-5G10	H	FN,VCAM-1,ICAM-2	M,Eo,Ly,F,NC,NK,Th
VLA-5	CD49e	FNR	Sa	Fn,Inv	Th,T-ly,F,EP,EN,Pl, PMN,M,Mus
VLA-6	CD49f	GoH3	So	Ln,Inv	P1,T-ly,EP,Th,M
VnR	CD51		T	Fn,Vn	NC,F
β1-int	CD29	TS 2/16	Sp	see α-chains	see α-chains
β2-int	CD18	LFA-1/1	L	see CD11a,CD11b, CD11c	see CD11a,CD11b, CD11c
β3-int	CD61	CLB-thromb/1	B	Fb,Fn,vWF,Vn,Tsp,Coll, Ost (αvß3)	B-ly′,M,EN,Pl (αvß3)
β4-int	CD104	3E1	T	Ln (α6ß4)	EP (α6ß4)
H-CAM	CD44	NKI-P1	P	HEV,Hy,	B-ly(not all),N,Th Er,F,PMN,Eo,M,T-ly
ICAM-1	CD54	My 13	C	LFA-1,CR3	M,FDC,EN
LFA-1	CD11a	LFA-1/2	L	ICAM-1,ICAM-2,ICAM-3	All leukocytes
CR3	CD11b	Leu 15	BD	ICAM-1,1C3b,Factor X,Fb	PMN,M,EP,NK
p150,95	CD11c	B-ly6	O	iC3b,Fb,Tsp	B-ly′,T-ly′,PMN,M, Eo,NK
LFA-3	CD58	TS 2/9	Sp	CD2	widespread
LECAM-1	CD62L	Leu 8	BD	PNAd, sialyl Lewis-X	non-activated white blood cells (PMN,M,Ly)
NCAM	CD56	MOC-1	EU	NCAM,PG	NC

[a] All antibodies are monoclonal mouse antibodies, except GoH3 (anti-α6), a monoclonal rat-derived antibody

[b] B, Dr. A.E.G.Kr. von dem Borne, Central Laboratory, Blood Transfusion, Amsterdam, The Netherlands; BD, Becton Dickinson, Erembodegem, Belgium; C, Dr. C. Civin, Johns Hopkins Oncology Center, Baltimore, USA; D, Dako, Glostrup, Denmark; EU, Eurodiagnostics, Apeldoorn, The Netherlands; H, Dr. M.E. Hemler, Dana-Farber Cancer Institute, Boston, USA; L, Dr. R.A.W. van Lier, Central Laboratory, Blood Transfusion, Amsterdam, The Netherlands; O, authors' own laboratory; P, Dr. S.T. Pals, University of Amsterdam, The Netherlands; Sa, Sanbio, Uden, The Netherlands; SBA, Southern Biotechnology Associates, Inc., Birmingham Ala.; So, Dr. A. Sonnenberg, The Netherlands Cancer Institute, Amsterdam, The Netherlands; Sp, Dr. T.A. Springer, Harvard Medical School, Boston, USA; T, Telios, San Diego, Calif., USA

Table 2 Expression[a] of adhesion molecules/subunits in testicular diffuse large B-cell lymphomas (*CB* centroblastic lymphoma, *IB* immunoblastic lymphoma, – no expression, + weak expression (all cells), ++ moderate/strong expression (all cells), p++ moderate/strong expression in part of the tumour)

Patient	Type	CD49d/VLA-4	CD49f/VLA-6	CD29/ß1	CD44	CD54/ICAM-1	CD62L/L-selectin
1	CB	–	–	–	++	–	–
2	CB	–	–	++	++	–	p++
3	CB	–	–	–	++	–	–
4	CB	–	–	+	++	–	–
5	CB	–	++	++	++	–	–
6	CB	+	++	++	++	–	–
7	CB	–	–	–	+	–	–
8	CB	–	–	–	++	–	–
9	IB	–	+	+	++	++	–
10	IB	+	–	+	–	–	–
11	IB	–	+	++	++	++	+
12	IB	p++	++	++	–	++	–

[a] The following adhesion molecules/subunits were lacking: VLA-1, VLA-2, VLA-3, VLA-5, CD51/VnR, CD11a, CD18/ß2, CD61/ß3, CD104/ß4; CD58, CD11b, CD11c; CD56

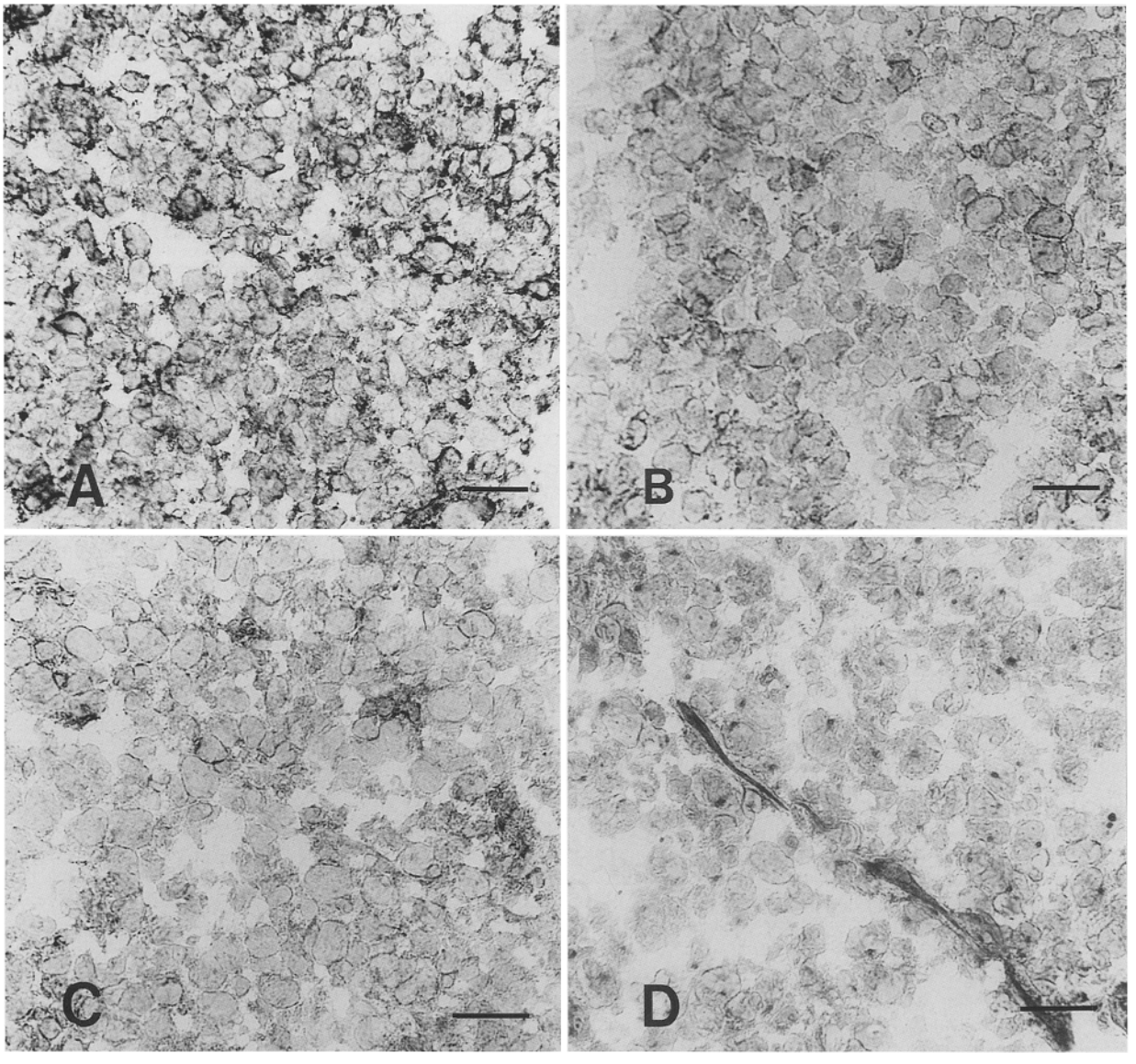

Fig. 2A–D Adhesion molecules in testicular large cell lymphoma. **A** Strong CD44 expression of diffuse centroblastic lymphoma. **B** Variable staining for L-selectin (CD62L) of centroblastic lymphoma. **C** Few lymphomas show any staining for VLA-4/CD49d (immunoblastic lymphoma) **D** VLA-6/CD49f staining of centroblastic lymphoma. Many VLA-6 positive tumour cells show cytoplasmic dots. A VLA-6 positive vessel is seen in longitudinal section. All immunoperoxidase, ×400, *bar* 100 μm

Table 3 Presentation of extracellular matrix proteins in the interstitium of 12 testicular diffuse large B-cell lymphomas (+++ tight network, abundant matrix protein present, ++ tight network, matrix protein present in moderate amounts. ++/+ partly tight, partly loose network, + loose network, sparse matrix protein present, pc polyclonal antiserum, mc monoclonal antibody)

Matrix protein	CB +++	IB +++	CB ++	IB ++	CB ++/+	IB ++/+	CB +	IB +	Origin of antibody
Vitronectin	7	3	1	1	–	–	–	–	pc, rabbit
Laminin	3	2	2	2	–	3	–	–	pc, rabbit
Collagen III	2	3	–	3	–	2	1	1	mc, mouse
Collagen I	1	3	4	1	–	3	–	–	pc, goat
Fibronectin	1	2	5	2	–	2	–	–	mc, mouse
Tenascin	–	1	2	1	5	1	–	2	mc, mouse
Collagen IV	–	–		1	3	2	4	2	pc, goat

[a] Collagen I and IV were obtained from Southern Biotechnology Associates, collagen III from Heyl, and all others from Telios

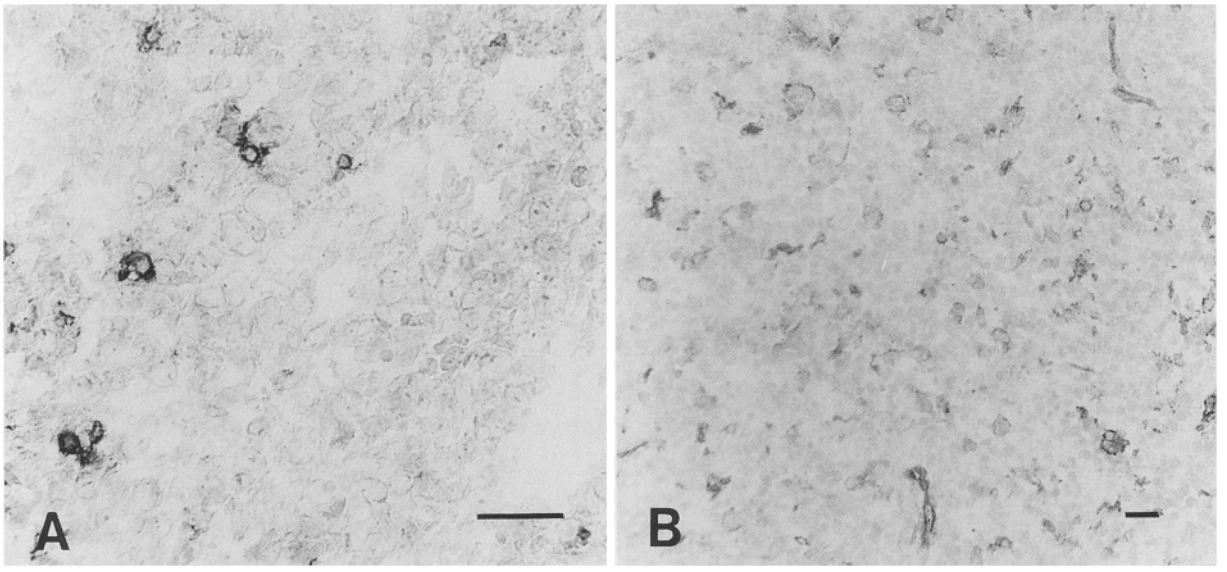

Fig. 3 **A** Immunoblastic lymphoma, stained with anti-CD11a antibodies. The tumour cells do not express CD11a, whereas other cells, probably T-lymphocytes, are strongly positive. Immunoperoxidase, ×400, *bar* 25 μm. **B** Diffuse centroblastic lymphoma stained for CD54. CD54 is not expressed on the neoplastic population, while the endothelial cells stain weakly. Immunoperoxidase, ×160, *bar* 25 μm

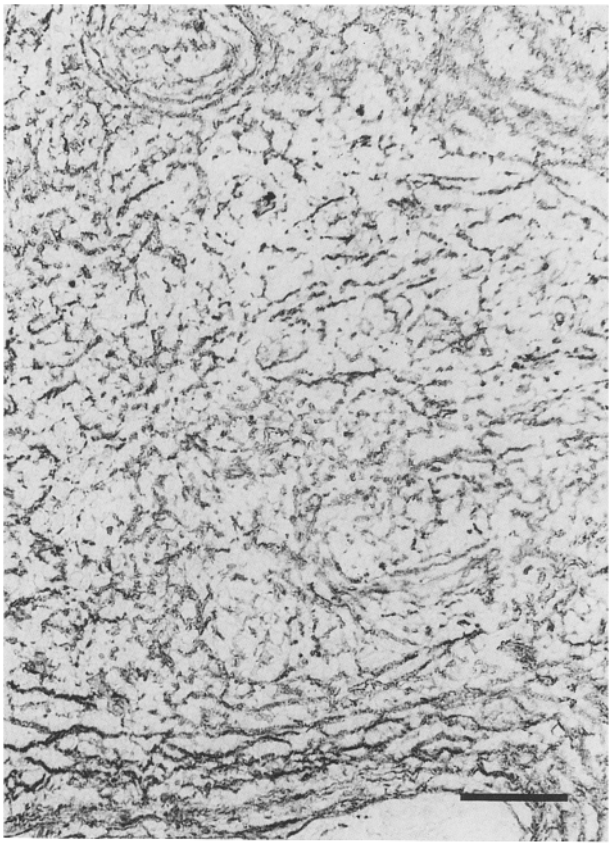

Fig. 4 Staining of vitronectin in testis DLC. Vitronectin is extensively present in the extracellular matrix, forming a tight network. Immunoperoxidase, ×160, *bar* 100 μm

Discussion

Like other lymphohaematopoietic malignancies, diffuse large B cell lymphoma is characterized by the clonal expansion of cells that have been arrested at a specific stage of maturation. Diffuse large B cell lymphomas generally consist of centroblasts (centroblastic lymphoma) or immunoblasts (immunoblastic lymphoma), B cells frozen in a certain stage of differentiation in or after the germinal centre (GC) reaction [16]. Normal centroblasts and immunoblasts are proliferating and differentiating B cells, leading to B memory cells and antibody-producing cells [15].

The expression pattern of adhesion molecules of many leukaemias and lymphomas correlates, to a certain extent, to the expression pattern of their normal counterparts [20, 21, 32]. The GC B cells, which include centroblasts and immunoblasts, express CD11a, CD58 and low amounts of CD49d/VLA-4 and CD44. The adhesion molecules CD11a and CD49d/VLA-4 on GC B cells interact with CD54 and CD106/VCAM-1 on follicular dendritic cells (FDC). CD11a and CD58 on GC B cells adhere to CD54 and CD2 on T lymphocytes in the GC [15]. These adhesive interactions with FDC and T cells are considered to be very important in B cell development in the GC. The adhesion between FDC and GC B cells is also thought to anchor the B cells. Except for CD49d/VLA-4 [15], GC B cells do not express VLA integrins [20]. Testicular DLC appear to have a different profile of adhesion molecules from the profile of normal GC centroblasts and immunoblasts. Centroblasts and immunoblasts show high expression of CD11a and CD58 and low expression of CD49d/VLA-4 and CD44, where-

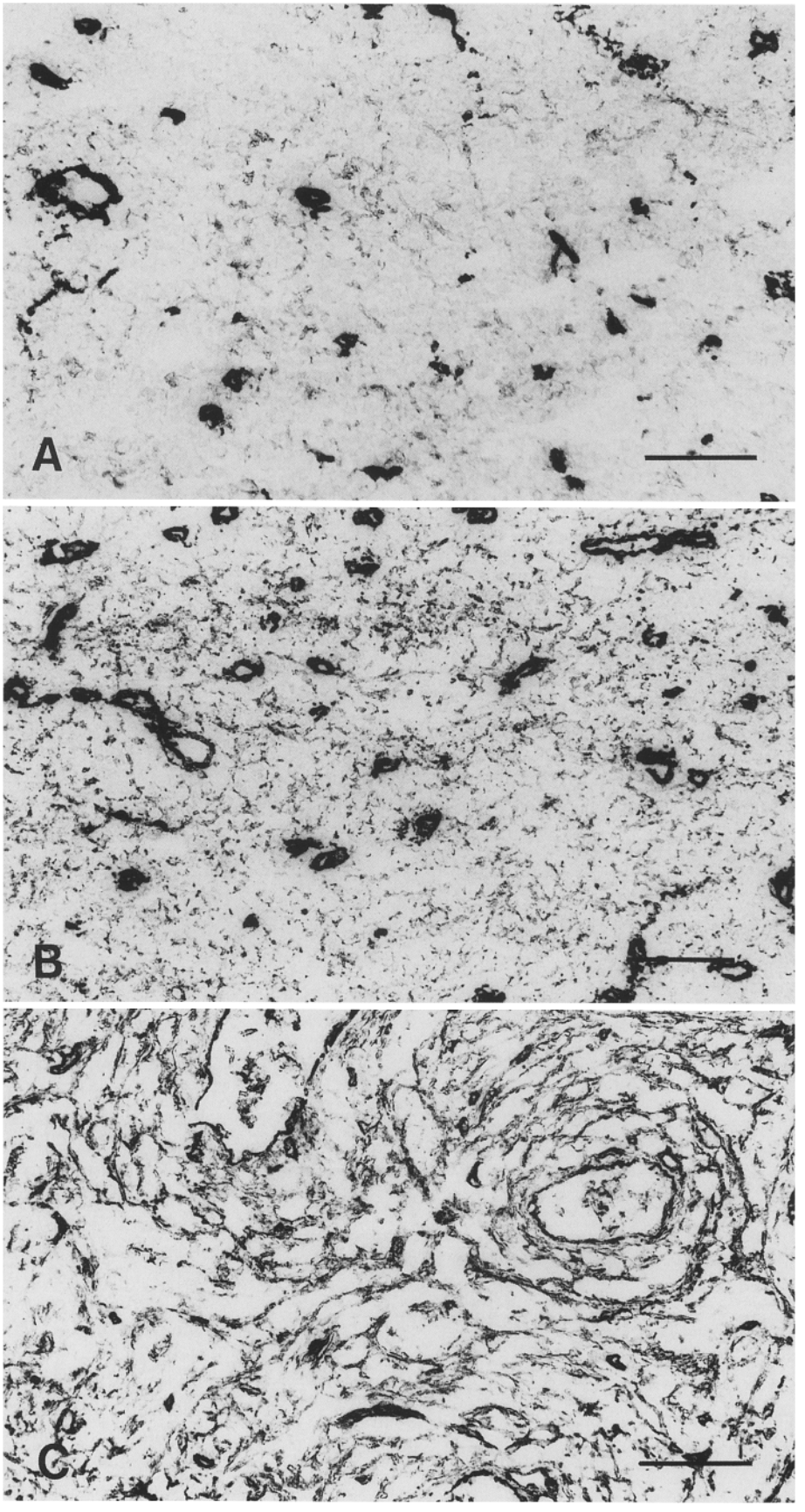

Fig. 5A–C Variable pattern of extracellular matrix as observed for laminin; basal membranes of vessels are clearly positive, but also interstitial matrix can be extensively present. **A** Sparse matrix protein present. **B** Semitight network, moderate matrix protein present. **C** Tight network, coarse presence of matrix. All centroblastic lymphomas. Immunoperoxidase, ×160, *bar* 100 μm

as their testicular malignant counterparts express CD44 (strongly), CD49f/VLA-6 in about half of the cases and CD49d/VLA-4, CD54 or CD62L in a very few cases. This may be an indication of tumour progression, compatible with increased host independence.

On testicular DLC, CD49a/VLA-1, CD49b/VLA-2, CD49c/VLA-3, CD49d/VLA-4 (in most cases), CD49e/VLA-5 and αvß1 of the β1-integrins were absent. Integrins of the β2, β3 and β4 group were also lacking in most cases. Integrins are the most important adhesion molecules involved in cell–matrix interactions [27]. Thus, although ECM proteins are abundantly present in testicular DLC (Figs. 4, 5), the tumour cells do not seem to adhere very tightly to the extracellular matrix, at least not as mediated by β1–integrin interaction. This lack of adhesion may be favourable for dissemination. CD49d/VLA-4 is an integrin that not only binds to extracellular matrix proteins (fibronectin), like the other VLA integrins, but also to CD106/VCAM-1 [8], an inducible counterreceptor found on endothelium, FDC and bone marrow stromal cells. On tumour cells, CD49d/VLA-4 is only expressed in a few cases, and no correlation was found between CD49d/VLA-4 expression and amount of fibronectin. CD49f/VLA-6 is expressed on half of the DLC, and laminin, located in basement membranes and, diffusely, in the interstitium is an important ligand. By binding to laminin, tumour cells are fixed to the basement membrane; they may penetrate it and enter the circulation [13]. Most tumour cells showed a peculiar cytoplasmic dot-staining with anti-VLA-6 antibody for which there is no ready explanation, but we speculate that this represents production of the VLA-6 protein in the Golgi region, preceding surface expression.

CD11a was absent in all, and CD54 in all centroblastic, cases of testicular DLC. The counterreceptors CD11a and CD54 are important mediators of the intercellular adhesion in haematopoietic cell aggregation [34]. The CD11a/CD54 pathway may also be important in the tumour processes, mediating close adhesive interactions between tumour cells. In B-lymphoid tumours, lack of CD54 expression was correlated with metastatic behaviour [1, 23], although this correlation was less clear within a group of large cell lymphomas [10]. Altogether, this suggests that the (relative) lack of CD54 and CD11a in testicular centroblastic ML may facilitate metastatic spread of tumour cells. In combination with the absence of CD49d/VLA-4, it may also be responsible for the diffuse growth pattern of the tumour. We speculate that the metastatic capacity of immunoblastic testicular ML may be less pronounced than that of the centroblastic type, considering that CD54 was expressed in three out of four cases of immunoblastic ML.

T lymphocytes were present in most testicular DLC. CD58, which binds to CD2 on T cells, was not expressed on the tumour cells, and it is likely, that these T cells are reactive and are not involved in paracrine interaction with tumour cells by supplying growth-inducing cytokines. CD62L (L-selectin) is an adhesion molecule that mediates lymphocyte homing to peripheral lymph nodes via binding to HEV [6]. CD62L is reported to be involved in the lymph node localization of NHL and in dissemination to these sites [25]. Nodal lymphomas are significantly correlated with expression of CD62L; gastrointestinal lymphoma (extranodal) is significantly correlated with lack of expression [25]. As in gastrointestinal lymphoma, CD62L is absent on most testicular DLC.

CD44 was expressed on almost all tumour cells. Besides several other adhesive functions, this adhesion molecule is an important lymphoid homing receptor [7] and binds to HEV, mediating invasion of lymphoid cells. Tumour cells may use the same mechanism in invasion of lymphoid and nonlymphoid tissues. In NHL, expression of CD44, among other adhesion molecules, is suggested to be important in dissemination. Several studies have revealed a significant correlation between CD44 expression on NHL and metastatic spread [10, 14, 25], whereas another group found no significant relationships [26]. Thus, the expression of CD44 on testicular DLC may play an important part in metastasis in the case of this tumour.

Testicular DLC disseminate more often than diffuse large B cell lymphomas of other localizations. The general expression pattern of these nontesticular large B cell lymphomas is: CD49a/VLA-1–, CD49b/VLA-2–, CD49c/VLA-3+, CD49d/VLA-4–, CD49e/VLA-5–, CD49f/VLA-6–, CD44+, CD11a+, CD54+ and CD62L± (nodal/extranodal) [9–12, 14, 17, 19, 20, 23, 25, 26, 30, 31, 33]. These tumours seem to adhere weakly to the matrix, by binding of CD49c/VLA-3 to extracellular matrix proteins. In several tumours, the cells bind tightly to each other via CD11a/CD54 interaction. The expression of CD44 may be involved in dissemination, but is also known to adhere to extracellular matrix proteins, mostly proteoglycans. The CD49f/VLA-6 integrin, a possible favourable factor for metastatic spread, is generally not expressed. This expression partly suggests metastatic possibilities (CD44), but less pronounced (CD11a+, CD54+, CD49f/VLA-6–, CD49c/VLA-3+) than indicated by the expression pattern of testicular DLC.

In conclusion, primary testicular diffuse large B-cell lymphomas appear to have an expression pattern of adhesion molecules that is suggestive for metastatic potential. The tumour cells seem to be poorly attached to the extracellular matrix (lack of integrins), to each other and to other cells (CD54–, CD11a–, CD58–). This seems more pronounced for centroblastic (CD54–) than for immunoblastic (CD54+) ML. The expressed adhesion molecules CD44, CD49f/VLA-6 and CD49d/VLA-4 may play a role in dissemination, mediating intravasation (CD49f/VLA-6) and extravasation (CD44, CD49d/VLA-4). This expression pattern may, at least partly, explain the specific biological behaviour of these lymphomas, with early and rapid dissemination.

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