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Dendritic reticulum cell-related immunostaining for laminin in follicular and diffuse B-cell lymphomas*

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Summary. We performed a comparative immunohisto-cytochemical study of the distribution patterns of laminin and follicular dendritic reticulum cells (DRCs) within their follicular microenvironment in both nodular or diffuse B-cell non Hodgkin's lymphomas (NHLs). Twenty nine cases of immunophenotypically diagnosed B-cell NHLs (19 of follicular center cell origin-FCCL- and 10 of the diffuse well differentiated lymphocytic type-WDLL-) and five reactive lymph nodes with follicular hyperplasia were analyzed by immunoperoxidase and immunofluorescence techniques. Serial frozen sections and cytospin preparations were tested either with single antibodies anti laminin and DRC-1, or paired reagents in double labeling immunofluorescence. Our results indicated consistently that within both the reactive germinal centers and the neoplastic nodules of FCCL laminin immunostaining visualized a punctate-granular pattern apart from the linear vascular basement membrane positivity. Double immunofluorescence assay demonstrated that there was a close parallelism between this laminin staining pattern and DRC-1 distribution showing a well developed DRCs meshwork; in the diffuse tumour areas of both FCCL and WDLL, laminin immunoreactivity was found only in those cases in which nests of DRCs were observed. Double immunofluorescence studies performed on cytospin preparations demonstrated that the groups of cells containing DRC-1 positive cells, contained a positivity for laminin, although within the cell the staining for DRC-1 was intense and diffuse, while that for laminin was granular and more sparse. Our results suggested that these laminin and DRC-1 positive reactive sites may be present on the same cells. Since the reduction in number or loss of both DRCs and their related immunostaining for laminin within the microenvironment was consistently associated with a loss of nodularity by lymphoma cells, whereas nodularity in reactive and neoplastic conditions was associated with a rich DRCs meshwork and the related laminin immunostaining, a trapping function of DRCs exercised in the presence of laminin should be considered.

Key words: Laminin – Basement membrane – Follicular dendritic reticulum cell – B-cell lymphoma – Immunohistology – Immunofluorescence

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Introduction

Follicular dendritic reticulum cells (DRCs) are non lymphoid, possibly autochtonous, "stromal" cells that are located in B-cell dependent areas of peripheral lymphoid tissue (Fossum and Ford 1985). They are normally found in lymph node primary follicles as well as in both the germinal center of the secondary follicle and its surrounding mantle zone (Carbone et al. 1985b; Carbone et al. 1988; Naiem et al. 1983; Stein et al. 1982). They have also been consistently identified within both nodular or diffuse B-cell non-Hodgkins' lymphomas (NHLs) of follicular center cell origin (FCCL) (Carbone et al. 1985a; Carbone et al. 1986; Carbone et al. 1987; Garcia et al. 1986; Harris et al. 1984; Mori et al. 1988; Nash 1986; Stein et al. 1982; Stein et al. 1984; Van der Valk et al. 1983), although with different distribution patterns (Carbone et al. 1987; Van der Valk et al. 1983).

Follicular center B-lymphocytes and DRCs appear to be closely interactive. Their antigen-trapping capability, due to binding of immune complexes by specific receptors for the Fc fragment of the immunoglobulins and for C3 (Nossal et al. 1968; Papamichael et al. 1975) suggests that DRCs present antigens to germinal center B cells (accessory function) (Fossum and Ford 1985) and act in the generation of memory B cells (Klaus et al. 1980; Tew et al. 1980). Further, as DRCs are capable of trapping normal or neoplastic lymphoid cells (Gerdes et al. 1983; Heinen et al. 1984; Lilet-Leclercq et al. 1984; Stein et al. 1982), they may play a role in the maintenance of B-cells within the follicles (Naiem et al. 1983; Stein et al. 1982). However, the mechanisms by which this property is exerted are still unknown.

In a previous study (Gloghini et al. 1989), we found a close parallelism between the laminin immunostaining and the dendritic network of DRCs within reactive germinal centers and neoplastic nodules of FCCL, as visualized by DRC-1 monoclonal antibody (Naiem et al. 1983). Considering the basic function of laminin in cell attachment (Abrahamson 1986; Martinez-Hernandez and Amenta 1983; Timpl et al. 1983) we report the results of an analysis of DRCs and their related laminin immunostaining in FCCL showing nodular or diffuse growth patterns, and in a series of B-cell NHLs of non germinal center origin. The aim of the study was to verify possible microenvironmental differences between nodular and diffuse lymphomas. In addition, the relationship between the immunoreactive sites for laminin and DRC-1 antibodies was investigated by double immunofluorescence assay on cytospin preparations from cell suspensions.

Materials and methods

The material for study consisted of paraffin embedded and frozen samples of involved tissues from 29 cases of immunophenotypically diagnosed B-cell NHLs (Table 1). Five reactive lymph nodes showing florid follicular hyperplasia were also analyzed for comparison studies.

Cell suspensions from biopsy specimens involved by nodular lymphoma (three cases) and from reactive lymph nodes with follicular hyperplasia (three cases) were also available for study; lymphoid tissues were minced and passed through a metal mesh (⌀ 400 mcs). The cells were resuspended in RPMI 1640 (Flow Lab. In.) containing fetal calf serum at 5% (Flow Lab. In.) and were washed. The concentration was adjusted to 2×10^6 cells/ml and then cytospin slides were prepared. The preparations were fixed immediately in acetone for 5′ and stored at −20° C until immunostained.

Immunophenotypic analyses were performed by testing the expression of surface immunoglobulin (sIg), common acute lymphoblastic leukemia antigen (CALLA-CD10) and B and T-cell-associated differentiation antigens (Leu12-CD19, Leu14-CD22, OKB7-CD21, BA1-CD24, BA2-CD9, HLA-DR, Leu4-CD3, Leu2a-CD8, Leu3a-CD4, Leu1-CD5, OKT6-CD1, OKT10-CD38, Leu7, Leu8, LeuM1-CD15) and carried out as previously described (Carbone et al. 1986; Carbone et al. 1987). In all cases immunophenotypic profiles supported the B-cell origin of NHLs.

Table 1. Histological Classification of the 29 B-cell non-Hodgkin's lymphomas

Working Formulation Category (1982)	Rappaport Classification (modified) (Nathwani 1979)	Kiel Classification (Stansfeld et al. 1988)	Number of cases
A (small lymphocytic)	WDL	Lymphocytic	10
B (follicular, predominantly small cleaved cell)	NPDL	CB-CC, follicular	3
C (follicular mixed, small cleaved and large cell)	NMLH	CB-CC, follicular	4
D (follicular, predominantly large cell)	NH	CB-CC (large), follicular	3
E (diffuse, small cleaved cell)	DPDL	CC	3
G (diffuse, large cell)	DH	CB	6

WDL: well differentiated lymphocytic; NPDL: nodular poorly differentiated lymphocytic; NMLH: nodular mixed lymphocytic histiocytic, NH: nodular histiocytic; DPDL: diffuse poorly differentiated lymphocytic; DH: diffuse histiocytic; CB: centroblastic; CC: centrocytic

Serial frozen sections were tested with the following antibodies: affinity purified rabbit anti (mouse EHS sarcoma) laminin (Carbone et al. 1986; Timpl et al. 1979) (1:40), monoclonal antibody anti DRC-1 (Dakopatts a/s, Glostrup, Denmark) (1:15). The specificity of laminin was assayed by solid phase radioimmunobinding against several extracellular matrix components, as previously reported (Colombatti et al. 1989).

Immunostaining was performed by using an avidin-biotin-peroxidase complex (ABC) method (Hsu et al. 1981), as previously described (Carbone et al. 1986). In selected cases an indirect labelled ExtrAvidin-biotin immunofluorescence assay was also performed as follows: air dried frozen sections were kept under vacuum for 12 h at 4° C and then fixed in a 1:1 solution of acetone and chloroform for 10 min; sections were hydrated with phosphate buffered saline (PBS) and then incubated with normal goat serum (ABC Kit PK-4001, Vector Lab.) or normal horse serum (Sera Lab.) (1:50 for 20′ at room temperature – RT) depending on the source of the primary antibody; next, antibodies anti laminin or DRC-1, were applied at working dilution (for 1 h at RT); after washings with PBS, sections were incubated with biotinylated horse anti mouse immunoglobulin (Ig) (ABC Kit PK-4002, Vector Lab.) (1:200 for 30′ at RT) or biotinylated goat anti rabbit Ig (ABC Kit PK-4001, Vector Lab.) (1:200 for 30′ at RT); and then with ExtrAvidin-FITC (Sigma Chemical Co.) (1:20 for 1 h at RT).

Double immunofluorescence staining was performed using, as first sequence, laminin and the ExtrAvidin-biotin immunofluorescence assay described above, except for the use of TRITC (instead of FITC) labeled ExtrAvidin (Sigma Chemical CO.). The sections were subsequently incubated with monoclonal antibody anti DRC-1, overnight, at 4° C and then with rabbit anti mouse Ig-FITC conjugated (Dakopatts a/s, Glostrup, Denmark) (1:40, with the addition of 3% normal human serum, for 1 h at RT).

Cytospin preparations were tested with affinity purified rabbit anti (mouse EHS sarcoma) laminin (1:40), monoclonal antibody anti DRC-1 (1:15); immunostaining, including double immunofluorescence assay, was performed as described above, except for a prolonged incubation time of the primary antibody (i.e. overnight at 4° C).

Controls. Negative control experiments were performed by incubating the sections with the immunoglobulin fraction of non-immune rabbit serum (Dakopatts a/s, Glostrup, Denmark) or an antibody directed against a non correlated antigen (i.e. rabbit anti CEA – Dakopatts a/s, Glostrup, Denmark) or by omitting the primary antibody. A further negative control was performed by pre-absorbing primary antibody anti laminin with excess of antigen (for 1 h at RT) and then carrying out immunostaining as described above. Preincubation of anti DRC-1 antibody with purified laminin was also carried out. None of the negative control sections was immunostained: preincubation of anti DRC-1 antibody with purified laminin did not affect staining in follicles.

In selected cases immunoreactivity for laminin was also tested by using an antibody, raised against human laminin, from a commercial source (HEYL Vertriebs GmbH & Co. KG, Berlin, Germany Federal Republic); in these cases immunostaining results for both antibodies were superimposable.

Results

Immunoperoxidase and immunofluorescence methods gave similar results concerning the pattern of distribution of the antigens tested, but in addition we observed a slight increase in sensitivity with the use of the indirect labelled ExtrAvidin-biotin immunofluorescence method.

As expected, reactive lymph nodes immunostained with DRC-1 revealed a fairly well-circumscribed dendritic meshwork of immunostained DRCs that was located more densely in germinal centers than in mantle zones. Laminin immunoreactivity outlined both perifollicular and follicular vascular structures and clearly localized in the basal membranes of the marginal sinus according to its known distribution (Carbone et al. 1986; Karttunen et al. 1986); the most striking feature was its additional presence within germinal centers. This laminin immunoreactivity, which was confined to the follicles, had a punctate-granular pattern. Double immunofluorescence assay demonstrated that there was a close parallelism between the punctate-granular staining pattern of laminin and the dendritic network of DRC-1 stained cells, in agreement with our previous results (Gloghini et al. 1989).

All the ten nodular B-cell lymphomas of follicular center cell origin showed a well developed and sharp outlined spherical meshwork of DRC-1 positive DRCs (Fig. 1); this staining pattern closely resembled that of the reactive follicles. Diffuse areas of neoplastic growth, when present, were accompanied by less dense, faintly outlined, small aggregates of DRCs.

Among the nine diffuse lymphomas DRC-1 immunostained DRCs were found in three cases; they were very few in another case. In one case (G group) they composed small and loosely aggregated nests that were located in histologically defined areas featuring a vaguely nodular tumour pattern. In the other two cases (E group), they were fairly numerous, formed loosely aggregated nests with blurred outlines and featured an irregular pattern.

Immunostaining for laminin, besides the linear vascular basement membrane positivity, visualized the punctate-granular positivity within the neoplastic nodules (Fig. 1) as well as in some diffuse areas of nodular lymphomas. By double immunofluorescence assay, we clearly observed that this immunostaining always occurred in areas of immunoreactivity for DRC-1 (Fig. 1). Punctate-granular immunoreactivity for laminin within the neoplastic nodules appeared sometimes coarser and in larger quantity than that of germinal centers of reactive lymph nodes.

In diffuse lymphomas laminin immunostaining of the punctate-granular type was absent, except for the cases in which DRCs were present; in these cases the positivity had an apparently irregular distribution, but by double immunofluorescence we observed that punctate-granular laminin immunostaining and DRC-1 positivity were located in the same neoplastic areas.

DRCs, revealed by DRC-1 were present in four out ten cases with WDLL; one of these cases had DRCs located within residual hyperplastic germinal centers. In the other three cases DRCs were present inside the neoplastic population (Fig. 2). They had an ill defined network of loosely aggregated DRCs which were associated with histologically defined pseudofollicular proliferation growth centers. In these cases immunostaining for laminin showed a punctate-granular positivity which was restricted to the areas with DRC-1 positivity (Fig. 2). This finding was confirmed by double immunofluorescence studies.

The punctate-granular positivity was lacking in the cases that were devoid of DRCs.

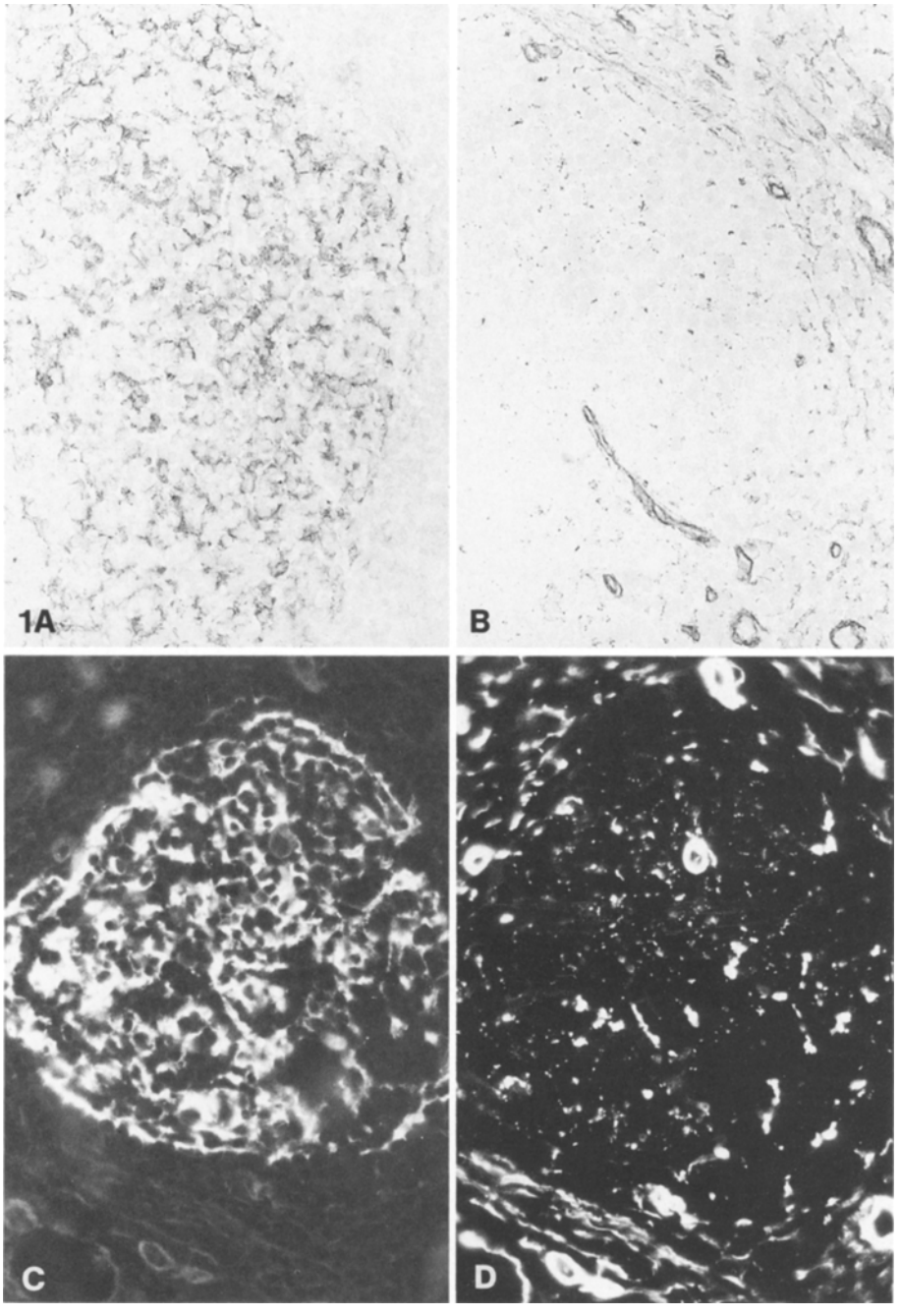

Fig. 1 A–B. Serial frozen sections illustrating the same neoplastic follicle.
A DRC-1 positive DRCs form a dense, well developed meshwork.
B Besides immunostained small vessels, laminin immunostaining pattern of the punctate-granular type can be observed. (Avidin-biotin-peroxidase complex, haematoxylin counterstain, ×250).
C–D Frozen section of nodular lymphoma (FCCL with nodular pattern).
Double immunofluorescence with anti DRC-1 (**C**) and anti laminin (**D**) antibodies. The punctate-granular positivity for laminin within the neoplastic nodule mostly occurs in areas of DRC-1 immunoreactivity. This type of laminin positivity is lacking in the right-lower part of the nodule, that is devoid of DRCs. (×500)

Cell suspensions from biopsy specimens of nodular lymphomas (3 cases) and follicular hyperplasia (3 cases), with immunohistologically identified DRCs on frozen sections, were also studied. DRC-1 immunostaining on cytospin preparations revealed the presence of giant cells with cytoplasmic branching processes within groups of neoplastic as well as normal reactive lymphoid cells (Fig. 3). Immunostaining for laminin appeared to be present on the same cells, although staining was less continuous (Fig. 3). Double immunofluorescence assay demonstrated that the groups of cells containing DRC-1 positive cells showed positivity for laminin; however, within the cell the staining for DRC-1 antibody was intense and diffuse, while that for laminin was granular and more sparse (Fig. 3). Moreover, the possibility that either DRC-1 or laminin immunostaining was present on the lymphocytes, in addition to being on the DRCs, could not be excluded.

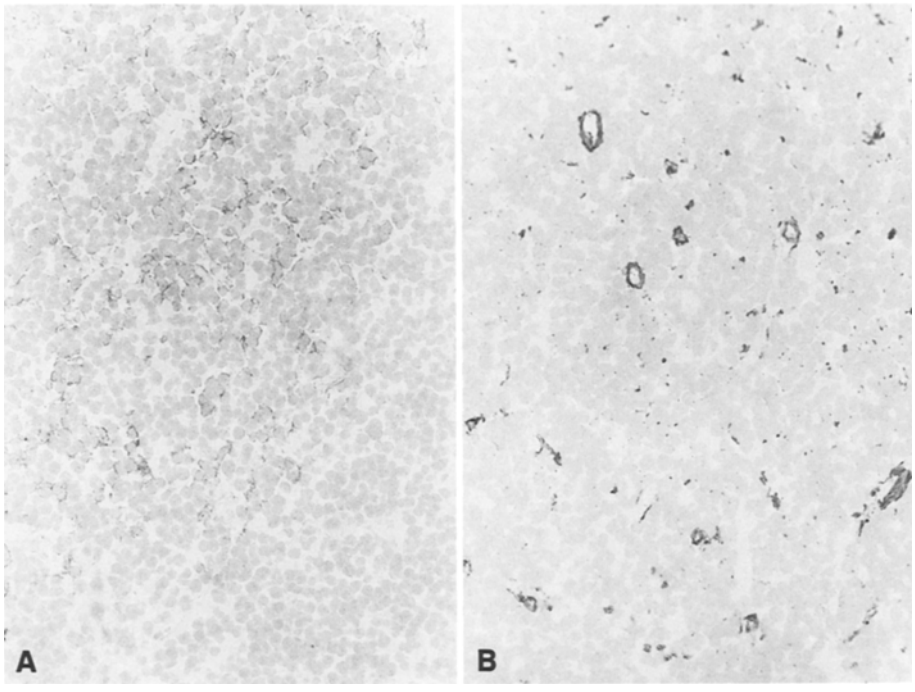

Fig. 2A–B. Serial frozen sections illustrating a diffuse lymphomatous area of WDLL. The irregular pattern of DRC-1 immunostained DRCs (**A**) well correlates with the distribution of laminin immunostaining of the punctate-granular type (**B**) (Avidin-biotin-peroxidase complex, haematoxylin counterstain, ×250)

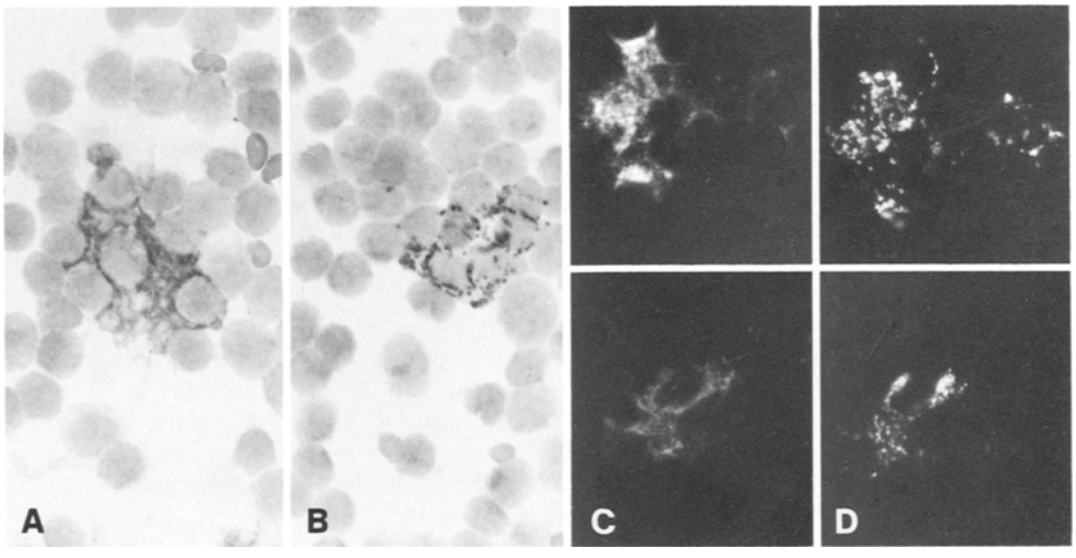

Fig. 3A–D. Cytospin preparations from FCCL with nodular pattern. Groups of neoplastic lymphoid cells contain DRC-1 immunostained cells (**A**) having cytoplasmic branching processes. A similar finding is shown by laminin immunostaining (**B**). This staining is of the punctate-granular type. (Avidin-biotin-peroxidase complex, haematoxylin counterstain, ×400). Double immunofluorescence with anti DRC-1 (**C**) and anti laminin (**D**) antibodies demonstrates that the compared markers are present on the same cells, but the staining for DRC-1 is intense and diffuse, while that for laminin is more sparse and punctate-granular in nature. (×500)

Discussion

The interaction between lymphoid cells and the non lymphoid cell microenvironment in the evolution from nodular to diffuse growth in FCCL has been considered by many researchers in the past (Carbone et al. 1987; Carbone et al. 1988; Collins et al. 1985; Harris et al. 1984; Mori et al. 1988; Nash 1986; Stein et al. 1982; Stein et al. 1984).

It has been assumed that DRCs play a basic role in this microenvironment since a quite sharply defined nodular meshwork of DRCs can be visual-

ized with specific antibodies to DRCs (e.g. DRC-1) in FCCL showing a nodular growth pattern (Carbone et al. 1985a; Carbone et al. 1986; Carbone et al. 1987; Grogan et al. 1988; Harris et al. 1984; Naiem et al. 1983; Stein et al. 1982; Stein et al. 1984). In diffuse NHL's even in cases of small lymphocytic type (WDLL) there are often fewer DRCs, not organized into discrete follicles (Carbone et al. 1987; Chilosi et al. 1985; Harris et al. 1985; Medeiros et al. 1987; Nash 1986; Ratech et al. 1988). DRCs loss has, therefore, been correlated with the inability of the neoplastic follicle to keep its own structure (Grogan et al. 1988), even if it is unclear whether DRCs loss might be the cause or the effect of this condition.

The function of DRCs in follicular immunological reactivity (Klaus et al. 1980; Tew et al. 1980) has been confirmed by immunohistochemical demonstration in tissue sections and cell suspensions, of C3 receptor on their cell surface (Heinen et al. 1984; Stein et al. 1982). In contrast, the mechanism by which the interaction between DRCs and B-cells takes place in the trapping within the follicle is still unclear.

In a previous study (Gloghini et al. 1989), mainly performed on hyperplastic follicles of reactive lymph nodes, we observed that laminin, a basement membrane glycoprotein implicated in a variety of cell matrix and cell-cell adhesive interactions, showed a pattern of distribution within the germinal centers quite similar to that of DRC-1 immunostained DRCs. The laminin staining pattern was of the punctate-granular type, possibly due to the size of the fragment of the laminin molecule against which the antibody has been raised demonstrating only a fraction of the molecule. It may also be the case that the molecule is still in the process of intracellular assembly, or is partly disintegrated. The finding suggests two hypotheses: first, a possible direct interaction between DRCs and laminin, derived from DRCs ability to produce this protein or to bear receptor sites on their cell membrane; second, a possible coexistence of immunoreactive sites (Gloghini et al. 1989).

In this study we found that the close parallelism of patterns between the so called punctate-granular immunostaining for antibody anti laminin and that for DRC-1, namely between a peculiar (non vascular) pattern of distribution of laminin and the pattern of DRCs as visualized by antibody anti DRC-1 was also found in neoplastic conditions. Whenever DRCs were present, independently of the type of lymphomatous pattern of growth, we found positivity for laminin (of the punctate-granular type). Interestingly, in the present study the inability of a lymphoma to maintain nodularity was consistently associated not only with the reduction in number or loss of DRCs, but also with a contemporary reduction or loss of DRCs related laminin immunostaining within the microenvironment.

A close parallelism between the reactions for the markers compared was also found in the WDLL cases. We found DRCs related to pseudofollicular proliferation growth centers in three out ten cases of WDLL. This finding is in accordance with the data of Ratech et al. (1988) who identified DRCs in 8 of 23 cases of WDLL; further as their cases lacked lymphocytosis, they attributed a biological significance to the presence of DRCs in these lymphomas, because the frequency of DRC networks in lymph nodes involved by chronic lymphocytic leukaemia has been reported to be low (Naiem et al. 1983).

The relationship between the immunoreactive sites for laminin and DRC-1 antibodies was verified on cytospin preparations from cell suspensions. Double immunofluorescence assay demonstrated that the immunoreactivity for the antibodies compared was present on the same cells or at least on the same groups of cells, therefore confirming that the parallelism of the patterns displayed by these antigens on frozen sections was not fortuitous and suggesting on microscopic grounds that immunoreactive sites for laminin are present in the DRCs. Moreover, the difference between the two staining patterns seen on cytospin preparations (that for laminin being more restricted and punctate in nature) may also suggest that it may be mainly cytoplasmic. Immuno-electron microscopic studies should be performed to define better the intra- or extracellular localization. The association of DRCs with basement membrane-like material has not been reported in recent studies using immunological and electron microscopy techniques (Fossum 1980; Heinen et al. 1984; Heinen et al. 1986; Imai et al. 1986).

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