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The distribution of fibronectin in lymph nodes infiltrated by Hodgkin's disease

An immunoperoxidase study on paraffin sections

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Summary. The tissue distribution of fibronectin (FN) was examined using a commercial anti-FN serum, the peroxidase-anti-peroxidase (PaP) technique, and paraffin sections of 22 lymph nodes affected by Hodgkin's disease. Vascular basement membranes and reticulin fibres are selectively stained and their structural changes in this pathological condition become readily visible. In contrast to the normal lymph node and to Hodgkin's disease with lymphocytic predominance, cases of mixed cellularity disease contain individual and focally grouped cells displaying intracytoplasmic FN. In nodular sclerosis these cells with fibroblast morphology are consistently numerous in the marginal zones of the cellular nodes. Strongly reacting mastocytes probably absorbed the applied antiserum non-immunologically. All the other cell types giving rise to the varying appearances of Hodgkin's lesions are consistently negative with respect to intracellular FN, including all forms of Hodgkin cells.

We conclude that in Hodgkin's disease the immigration of FN-secreting fibroblasts is an integral part of the early sclerosing reaction, which in itself is a defence/repair mechanism closely related to scar formation.

Key words: Hodgkin's disease – Fibronectin – Fibroblast – Immunoperoxidase study

Fibronectin (FN) is a glycoprotein several types of which can be distinguished immunologically (Atherton and Hynes 1981). Its plasma concentration is of 300 to 400 μm/ml. The molecule possesses distinctive binding domains for fibrin, heparin/actin, gelatine/collagen, cell and bacteria surfaces (Hynes and Yamada 1982). FN is multifunctional and moderates cell adhesion, cell shape, embryonic differentiation and the organization of the cytoskeleton. It was found that cultured human fibroblasts (Vaheri and Rouslahti 1975; Unsworth et al. 1982), endothelial cells (Jaffe and Mosher 1978) and monocytes/macrophages synthetize FN. FN was detected intracytoplasmatically in "fibroblast-rich and epitheloid-differentiated" older tissue cultures (Alitalo et al. 1980). In tissue FN is detectable in argyophilic fibres but not in collagenous

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fibrills (Stenman and Vaheri 1978); healing wounds are rich in FN and seem to contain FN-positive proliferating fibroblasts (Stenman and Vaheri, preliminary data). Nevertheless, little is known about the origin of FN in vivo. From the indirect evidence available Clark et al. (1982a) concluded that an in situ synthesis of FN by endothelial cells takes place as a reaction to tissue injury.

In FN research Hodgkin's disease has only been studied on two occasions. Resnick and Nachman (1981) showed FN-positive vascular basement membranes and adventitial tissue in frozen sections and reported having detected a positive reaction for FN in the cytoplasm of Sternberg-Reed cells, while all other cells were negative. This was so in 5 cases of nodular sclerosis. Harris et al. (1982) used comparable methods and did not find any intracellular FN in their series. Despite great efforts, the normal counterpart of the Hodgkin and Sternberg-Reed cells has not yet been identified and a positive proof of FN secretion by these cells would restrict their potential origin to the cell types mentioned above, which are known to produce FN in culture. In order to study this important aspect as well as the structural changes in FN distribution in Hodgkin's lesions, we examined the traceable binding sites for a commercial polyclonal antiserum directed against human plasma FN in paraffin sections, applying the PaP technique.

Methods

After screening the routine stains (Haematoxilin/eosin, Giemsa, periodic acid Shiff's reaction, Masson's trichrome and Gomöri's silver stain), 25 paraffin-embedded lymph nodes affected by Hodgkin's disease were selected from our files with regard to apparently good preservation and fixation. 2 to 4 µm thick sections were made, using a special knife holder with disposable blades (Feather, Japan) to minimize cutting artifacts. The peroxidase-anti-peroxidase technique was performed as described elsewhere (Möller et al. 1982). In this series, a commercial rabbit-derived antiserum directed against human plasma fibronectin (FN) from Dako, Denmark, Lot. 091A, Exp. 0987, was used as the primary antibody. In our hands a 1:120 dilution of this serum in PBS pH 7.6 gave the best results. Digestion of the sections prior to antibody application (Pronase (Merck, FRG) 1 mg/ml for 10 min, at room temperature) had a beneficial effect on the staining intensity (Høkund et al. 1982). From the 25 cases investigated, 22 yielded satisfactory results; in 3 cases non-specific staining occurred, probably due to tissue degradation. Among the 22 remaining cases there were 5 of the lymphocytic predominance type (comprising 3 nodular paragranulomas), 5 of mixed cellularity, 10 of nodular sclerosis and 2 of the lymphocytic depletion type, one emerging from mixed cellularity and thus presenting itself as the reticular type, the other one being of the diffuse type of fibrosis. In control series an exponential dilution of the primary antibody was carried out for the reasons mentioned in 'Results' and in order to observe the fading of the staining of the different tingible tissue components. The result in staining was entirely negative when one of the antibodies was omitted, thus furnishing evidence that the endogenous peroxidase had always been blocked sufficiently and that the staining was only due to the fixation of the primary antibody.

Results

In paraffin sections FN is detectable immuno-histologically in certain matrix structures and within the cytoplasm of few cells only, creating a reproducible

pattern in lymphogranulomatosis. This is, in some aspects, correlated with the morphological type.

Vascular basement membranes are constantly FN-positive (Fig. 1b); their thickening becomes obvious in areas relatively depleted of lymphocytes which, in nodular sclerosis, sometimes undergo necrosis (Fig. 3b, c). In the vascular lumina the plasma fraction is inconsistently stained. Inserted at the capillary basement membrane, FN-positive reticulin fibres irradiate and form meshes which in an ideal case seem to be identical with the pattern of the corresponding silver stain (Fig. 1a, b). Because of its thickness, the basement membrane of epitheloid venules is always prominent. Larger arteries show FN-positive and reticularly arranged fibres in the inner layer of the media. Variations in the calibre of the reticulin fibres, which have been characterized as reticulosis (Nakanishi et al. 1981), are clearly discernible (Fig. 3a) and prominent at the margin of the sclerotic bars in nodular sclerosis, in areas of mixed cellularity depleted of lymphocytes and in the case of reticular sclerosis examined (Fig. 3b). Hyalin bands only show a superficial positivity and have a negative core. The broad sclerotic ribbons typical of nodular sclerosis and diffuse fibrosis show a varying degree of extracellular FN content negatively correlated to their cellular density: when densely packed, the FN-negative collagenous fibres only have a dust-like positivity between them; in the neighbourhood of the cellular nodules reticular fibres can be seen in an increasing number. Necrotic areas are characterized by a loss of the reticular fibre-associated FN pattern (Fig. 3c). In areas undergoing fibrosis while still having a considerable cellular content the presence of large fibroblasts that are distinctively positive for FN in their cytoplasm is a consistent phenomenon. Such areas are detectable in all forms of Hodgkin's lesions except for the lymphocytic predominance type. In regions still poor in connective tissue, these FN-positive cells have an asteroid shape and long arachnoid cytoplasmic processes by which they contact one another or reticulin fibres. Their nucleus is large, bubble-like and poor in chromatin; it contains predominantly small, sometimes enlarged, and always clearly discernible nucleoli in a paracentral position (Fig. 3a). In regions rich in fibres, FN-positive fibroblasts are mostly fusiform (Fig. 2b). Even the largest fibroblasts could always be definitely distinguished from mononuclear Hodgkin cells on the basis of their cytomorphology, especially by their cytoplasmic processes. All forms of Hodgkin cells are constantly FN-negative (Fig. 1b), and so are lymphocytes, epitheloid cells, macrophages and dendritic reticulum cells in cases in which germinal centres are preserved.

With the cytoplasmic granules of mastocytes, however, our serum reacted even more intensely than with the structures described above. In contrast to all the other tingible structures, this staining did not fade in the same manner upon exponential dilution of the primary antiserum. Even in otherwise non-reactive parts of the section which were completely devoid of background staining, mastocytes still reacted with the anti-FN applied (Fig. 4). The controls with an incomplete antibody sequence, however, showed no reaction at all.

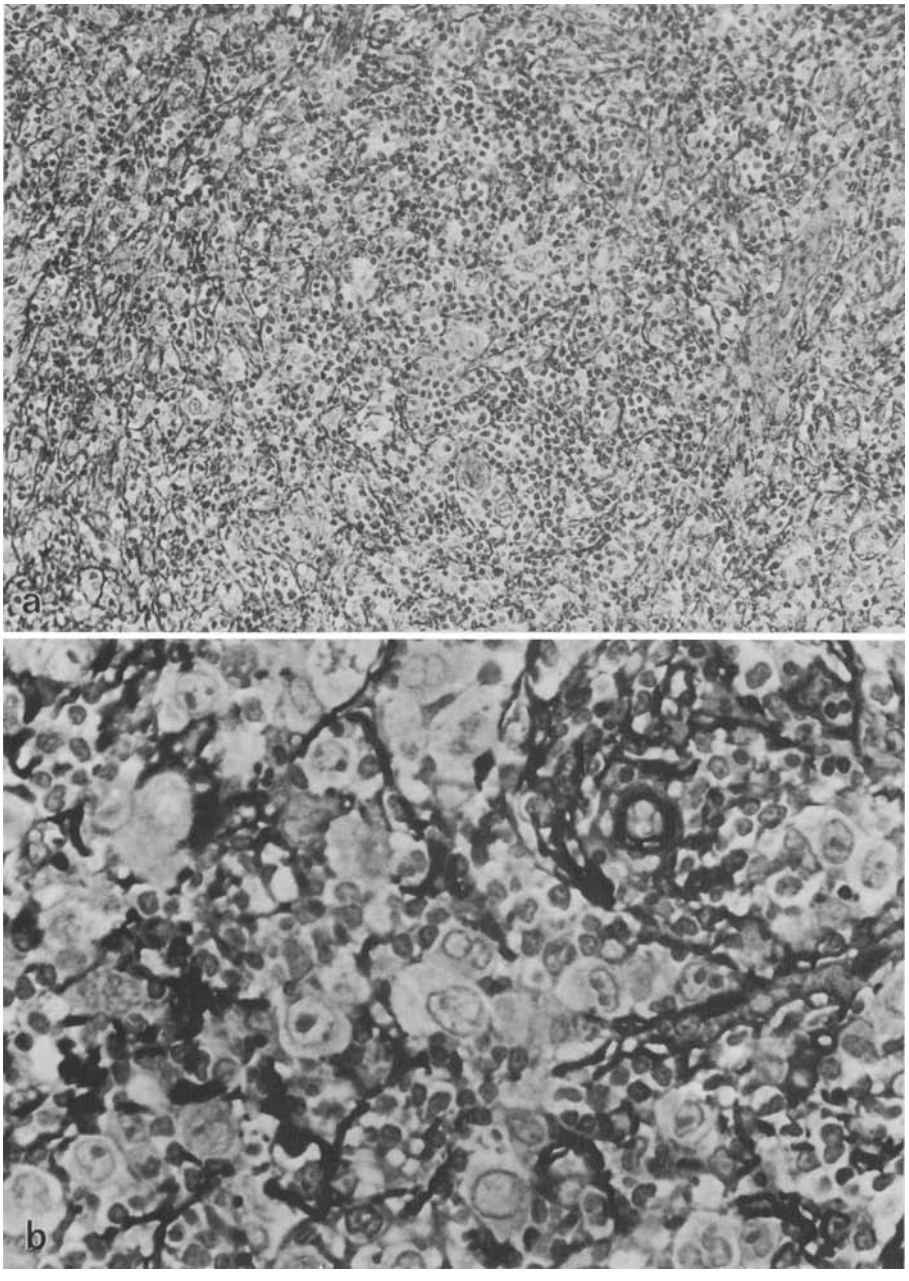

Fig. 1. a Lymph node, mixed cellularity type of Hodgkin's disease: distinct staining of reticulin fibres by the anti-fibronectin (FN) serum. (Peroxidase-anti-peroxidase technique (PaP); diaminobenzidine (DAB)/haemalaun (H); ×190). **b** Lymph node, mixed cellularity type of Hodgkin's disease: FN-positive capillary basement membrane (*top right*) and intensely FN-positive reticulin fibres. Note the non-reactive mononuclear Hodgkin cell and other atypical blast-like cells devoid of FN (PaP; DAB/H; ×474)

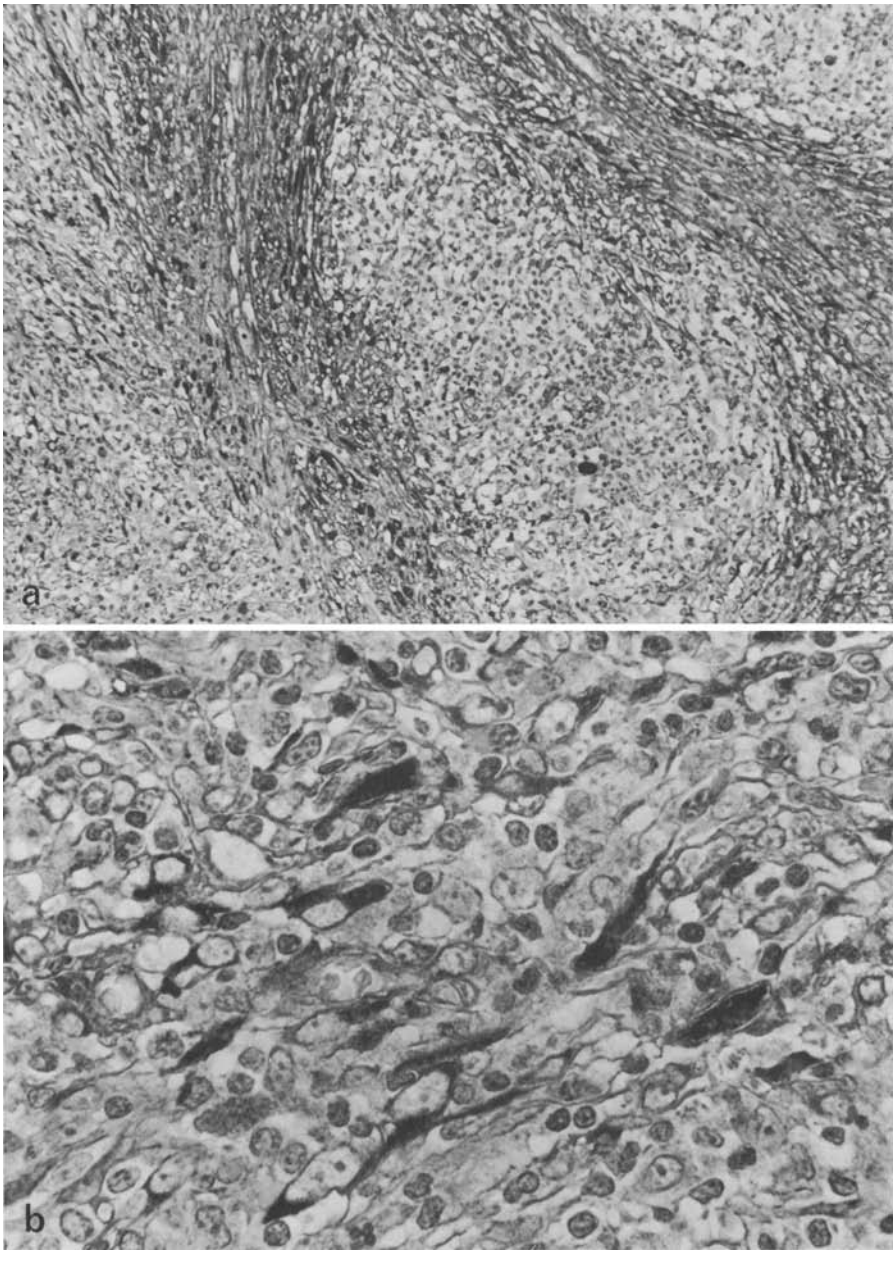

Fig. 2. **a** Lymph node, nodular sclerosis type of Hodgkin's disease: directed arrangement of fusiform cells strongly reactive with anti-FN at the border of the nodules in connection with thickened FN-positive reticulin fibres. (PaP; DAB/H; ×148). **b** Detail of **a**: fusiform large fibroblasts with large, bubble-like nucleus and small globular nucleoli showing a net cytoplasmic reactivity for FN (×592)

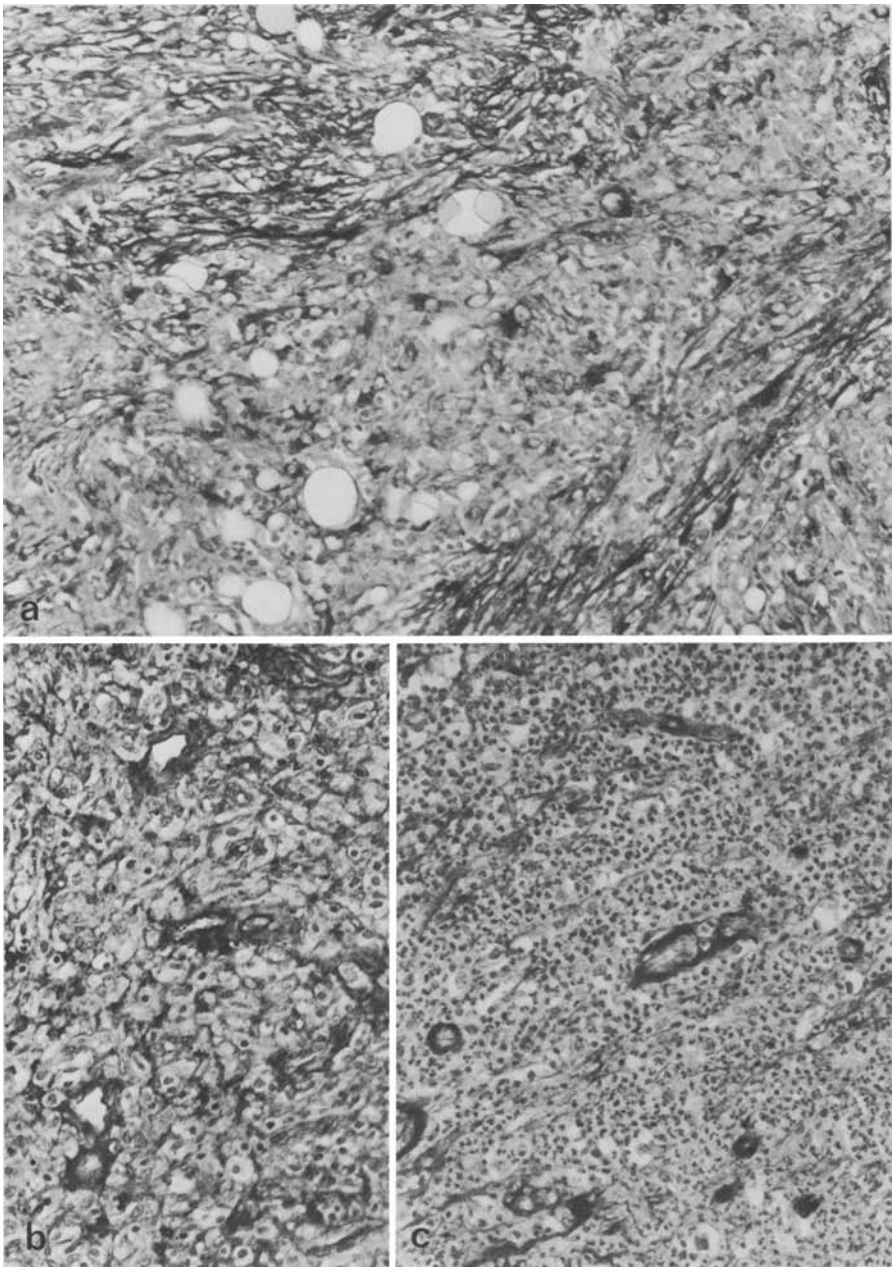

Fig. 3. a Lymph node, nodular sclerosis type of Hodgkin's disease: anti-FN decorates thickened reticulin fibres and the cytoplasm of large blastic cells being exclusively fibroblasts which have an asteroid shape in the pericentre of the nodules. Note some non-reactive collagenous bundles in the upper left corner. (PaP; DAB/H; ×190). **b** Lymph node, lymphocytic depletion type of Hodgkin's disease presenting itself as reticular fibrosis: anti-FN binds to perivascularly concentrated interstitial deposits of amorphous material and bulky reticulin fibres. (PaP; DAB/H; ×190). **c** Lymph node, nodular sclerosis type of Hodgkin's disease; area of necrosis: within necrosis fading of the FN-reactivity of reticulin fibres and thickening of vascular basement membranes strongly reactive with anti-FN (PaP; DAB/H; ×190)

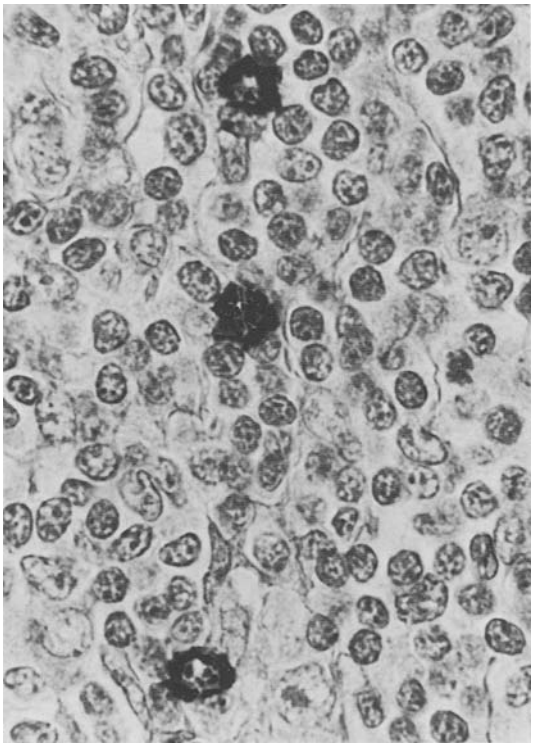

Fig. 4. Lymph node: very strong and resistant affinity of the anti-FN-serum used in this study to the cytoplasmic granules of mastocytes detectable even in otherwise non-reactive parts of the section; most probably an artifact. Note that reticulin fibres are unstained. (PaP; DAB/H; × 562)

Discussion

Our results confirm the data given by Stenman and Vaheri (1978), Dixon and Burns (1982), Unsworth et al. (1982), Laurine et al. (1982) and especially the results of Harris et al. (1982) on Hodgkin's disease obtained by immunofluorescence or electron-microscopic methods, acknowledging the presence of FN in basement membranes and reticulin fibres. The increased amount of FN in the thickened basement membranes of vessels which we found in areas undergoing necrosis and reticular fibrosis seems to corroborate the statements of Clark et al. (1982a, b) about an 'increased vascular FN content' in combination with endothelial proliferation which were observed by means of fluorescence. FN synthesis by human fibroblasts and "fibroblastoid macrophages" has been repeatedly demonstrated in tissue cultures (Vaheri and Rouslahti 1977; Alitalo et al. 1980) and has even been detected in cultured endothelial cells (Jaffe and Mosher 1978); Clark et al. (1982a, b) have also clained that endothelial cells produce fibronectin in vivo. However, it has not yet been possible to demonstrate FN within the producer cells in their natural microenvironment (Clemmensen et al. 1982; Linder et al. 1978; Scott et al. 1981; Stenman and Vaheri 1978, 1981). Though the polyclonal antiserum we applied is stated to be directed against human plasma FN, part of the antibodies apparently recognize cytoplasmic FN, which is less surprising since – in spite of the fact that they can be distinguished by monoclonal antibodies – plasma and cytoplasmic fibronectins

are very similar in structure and function (Atherton and Hynes 1981; Hynes and Yamada 1982).

Thus, using the sensitive PaP technique and extremely thin paraffin sections from our routinely fixed material, we were able to detect FN-positive cells. They have the morphology of large fibroblasts with a marked bubble-like nucleus and clearly-defined but mostly small nucleoli. This nuclear gestalt might be the expression for functional activation. We observed that in normal lymph nodes as well as in the lymphocytic predominance type of Hodgkin's disease these cells do not occur or are very rare. In contrast, they are an integral part of fibrosing areas relatively depleted of lymphocytes in the other types of lymphogranulomatosis, especially at the borders of the cellular nodules in nodular sclerosis. This observation corresponds to findings by Stiller and Katenkamp (1978) who described "activated macrophage-like cells" in nodular sclerosis which occur in areas rich in fibrillar intercellular substance. Electron-microscopically these cells were conspicuous because of their high content of rough endoplasmatic reticulum and their prominent Golgi apparatus. In agreement with Gabbiani et al. (1972), Seemayer et al. (1979) label this cell "myofibroblast". It is said to be non-existent in normal lymph nodes (Levine and Dorfman 1975). Resnick and Nachmann (1981) describe and depict FN-positive Sternberg-Reed cells. If this is the case and considering that FN synthesis by endothelial cells or macrophages in their natural environment has not yet been demonstrated unequivocally, the normal equivalent of the Hodgkin cell would be a fibroblast (or a "fibroblastoid macrophage"). Our findings suggest that the opposite is the case; probably the large FN-positive fibroblasts were confused with Hodgkin cells by these authors. This differentiation may be difficult in frozen sections and in the negative contrast picture of immunofluorescence lacking nuclear morphology. The relation between cell shape and the ability to get intercellular contact on the one hand and the capability to bind FN on the cell surface on the other was recently pointed out by various authors (Singer 1982; Grinnell et al. 1982); a spherical form is correlated with a loss of FN-binding capacity. This phenomenon is regarded as an expression of a functional or malignant transformation (Hynes and Yamada 1982). Likewise, highly metastatic experimental tumour lines lose their ability to metastasize if FN-binding daughter-clones are selected for transplantation (Benedetto et al. 1982; Dennis et al. 1982). Taking into account our results and the well-known fact that in their micro-environment Hodgkin cells are spherical, individually scattered, and surrounded by lymphocytes (Möller 1982), it seems reasonable to conclude that Hodgkin cells neither produce nor bind FN.

We do not yet understand why our anti-FN serum reacts with the vesicles of mastocytes. There are at least four possible interpretations: 1. Mast cell granules contain large amounts of FN. We could not find any support for this assumption in the literature. 2. The serum is contaminated by some undefined substance. However, contamination is not very likely, given the fact that only these granules are affected. 3. There is cross reactivity because of partial identity of epitopes on FN and on an unknown site of the mast

cell granule. 4. The antibody reacts with the granules via a substrate-specific non-immunologic adsorption. This effect was pointed out by Sternberger (1979) for immunohistology and was attributed at least partially (Bergroth et al. 1982) to tissue fixation. Point 3 cannot be excluded but 4. seems most likely to us, since mastocytes tend to absorb antisera (M.R. Parwaresch, personal communication). Therefore we believe that the FN-positivity of mast cells is an intrinsic artifact.

The dense arrangement of FN-positive fibrills in foci of grouped FN-containing fibroblasts might be an argument for their genesis from locally produced and secreted FN (corresponding to Stiller's and Katenkamp's (1978) view on the intercellular substances in Hodgkin's lymphomas). However, since in our material endothelial cells are consistently negative, we assume that the perivascular FN-positive material is plasma-derived and not, as Clark et al. (1982a, b) suspected, produced by the endothelium.

Our findings concerning FN distribution in areas of sclerosing or fibrosing reaction are clearly concordant with the results of several authors who have worked on the dynamics of experimental granuloma formation. Kurkinen et al. (1980) noticed the initial appearance of FN and fibroblasts on day 4, followed some days later by collagen type III, whereas when collagen had matured into bundles (after 5 weeks), FN diminished or disappeared. FN is therefore considered to be a primary matrix for fibroblast adherence (Grinnell et al. 1981) and for the organization of the collagenous connective tissue in scar formation. We think that these principles can also be applied to the fibrotic and sclerotic changes in lymphogranulomatosis, which can be considered as a local (and in the case of nodular sclerosis – centripetal) defence/repair mechanism. In our view the fibroblasts invading the lymph node parenchyma originate from capsular and trabecular sites; however, we still do not know the trigger within the cellular part of the Hodgkin's lesion. Newcom and O'Rourke (1982) have reported on a factor acting as a proliferative stimulus for fibroblasts. They were able to isolate it from cell cultures of nodular sclerosing Hodgkin's disease which were enriched with Hodgkin cells and depleted of fibroblasts.

FN fragments act chemotactically on human blood monocytes in vitro (Norris et al. 1982). FN enhances the monocyte/macrophage-mediated cytotoxic and tumoricidal activity (Perri et al. 1982). It is still a matter of speculation whether the frequent occurrence of non-ischaemic necroses, which is typical of nodular sclerosis, can be attributed to the increased local FN content.

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