Detection of sarcolectin-specific receptors like the cytokine macrophage migration inhibitory factor in rheumatoid nodules

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Summary. The objective of this study was the evaluation of the relation between the N-acetyl-neuraminic acid-binding endogenous lectin sarcolectin and the cytokine macrophage migration inhibitory factor (MIF) during development of rheumatoid nodules (RN) in seropositive rheumatoid arthritis (RA). Sarcolectin was purified and biotinylated. The binding patterns of this probe were analyzed in RN from patients with RA (n=23) and compared with the distribution of antibodies with specificity for MIF, fibrin, fibronectin. In early RN, all areas of the inflammatory tissue displayed presence of receptors for sarcolectin. Macrophages were especially positive. In mature rheumatoid nodules binding of sarcolectin was restricted to the periphery of necrotic areas, to endothelial cells and perivascular connective tissue of marginal zones. Distribution patterns of MIF were similiar but not identical. The histological staining characteristics demonstrate sarcolectin-binding receptors in RN that are altered upon disease progression. The finding suggests that specific interactions between this endogenous lectin and MIF may be involved in the course of RA.

Key words: Macrophage migration inhibitory factor, Rheumatoid arthritis, Rheumatoid nodule, Sarcolectin

Introduction

Rheumatoid nodules (RN) are the result of a necrotising process particularly associated with rheumatoid factor-positive rheumatoid arthritis (RA). They are commonly detected in the subcutaneous tissue overlying bony prominences (Gordon and Hastings,

1998). In its fully developed form, the lesion (Fig. 1) demonstrates a characteristic histopathological morphology (Fassbender, 1994). An intermediate cellular zone of elongated epitheloid cells, composed mainly of activated macrophages surrounds a central necrotic mass. The marginal connective tissue consists of an inner sector that contains inflammatory cell infiltrates, mostly isolated macrophages, which merge with the radially arranged palisading cells. Whereas this area is largely devoid of vasculature, the outer sector shows aggregations of small vessels that are surrounded by lymphocytes and plasma cells. Although the morphology of the RN has been subject of numerous investigations, the pathogenic mechanism of its formation has been open to debate. According to current evidence mechanical trauma and aggregation of rheumatoid factors initiate the granulomatous reaction of connective tissue (Ziff, 1990). It has been suggested that attraction and activation of macrophages is the main mechanism of the necrotising process (Palmer et al., 1987). Staining with proliferation markers such as Ki67 has provided evidence that aggregation of palisaded macrophages is a consequence of vascular influx and not from cell proliferation (Lalor et al., 1987). Enhanced secretion of cytotoxic enzymes and coagulation factors may result in deposition of cell remnants and inflammatory-induced molecules such as fibrin and fibronectin.

Numerous factors, besides rheumatoid factor immune complexes, have been implicated in the pathogenesis of RNs by inducing monocyte chemotaxis and inflammatory stimulation (Arend et al., 1982). Examples are interferon-γ, interleukin 1, tumour necrosis factor α, transforming growth factor ß and complement components (Chantry et al., 1989; Brennan et al., 1997; Langdon et al., 1997). As indicated by its name, the human lymphokine macrophage migration inhibitory factor (MIF) belongs to this group of effectors with impact on monocyte mobility; the pathways for

activation between different elicitors being different (Herriot et al., 1993). Since this factor is assumed to play a critical role in immune regulation and in the systemic response to tissue invasion, its expression in disease-affected tissue can provide relevant information (Calandra et al., 1994). In this respect, it is noteworthy that MIF has been described to be the major binding protein of an α-/ß-interferon antagonist and growth promotor in human placenta, named sarcolectin (Zeng et al., 1993). This protein has lectin-like properties with specificity to N-acetylneuraminic acid and also reactivity with sialoglycoconjugates, apparently exerting regulatory functions in the immune system (Jiang et al., 1988; Zeng et al., 1992). Its binding to the lymphokine, which is mediated by protein-protein recognition (Zeng et al., 1994a,b), can neutralize the factor-dependent inhibition of macrophage migration (Zeng et al., 1993). Histochemically, sarcolectin can be employed to localize MIF, as emphasized in a study that revealed a correlation of extent of binding in lung cancer with survival of patients (Kayser et al., 1994). On this experimental basis and due to the present lack of information on MIF expression in this area we correlated the presence of binding sites for sarcolectin with the development of RN. In the present study we demonstrate characteristic binding patterns that differ from the distribution of fibrin and fibronectin, respectively. Thus, it is tempting to speculate on a participation of MIF in RN-related processes and on the potential for interference of such effects by sarcolectin. However, owing to the complexity of the inflammatory process, care should be exerted in interpreting morphological data.

Material and methods

Rheumatoid nodules

Subcutaneous nodules were removed from 23 patients (16 females/7 males) under local anaesthesia. Each had erosive disease that satisfied ARA criteria for classical RA (MacGregor, 1994). All patients had a positive latex agglutination assay result for rheumatoid factor. The median age was 59 years (27-78 years), and the mean disease duration was 9 years (3-24 years). The nodules were grouped according to the duration of the manifestation. Lesions that had been present between 4-9 months were classified as early RN. Their maximum diameter ranged between 8 and 19 mm. The remaining 18 specimens had been present for more than 21 months. They were referred to as mature RN. Their diameters varied between 12 and 36 mm. All biopsies were immediately fixed in 10% neutral-buffered formol saline, dehydrated and embedded in paraffin according to standard methods. Five normal dermal biopsies, treated identically, served as controls.

Biochemical procedures

Sarcolectin was purified from human placenta by a standard procedure involving pepsin digestion, gel filtration on Sephacryl S-200 columns and fractionation on DEAE cellulose (Jiang et al., 1984; Zeng et al., 1994a,b). Biotinylation without impairment of its MIF-binding capacity was achieved with biotinamidocaproyl hydrazide and ethyldimethylaminopropyl carbodiimide dissolved in dimethylsulfoxide and pyridine-HCl (pH 4.8) and polyclonal antibodies against human recombinant MIF were produced in goats according to standard procedures (Gabius et al., 1991; Kayser et al., 1993).

Histochemical examination of tissue sections

Serial 6 μm sections were mounted on gelatine-coated glass-slides (Menzel, Braunschweig, FRG), deparaffinised and rehydrated. Endogenous peroxidase-activity was blocked with 0.3% H_2O_2 in methanol for 30 min. To minimize non-specific binding reactions, specimens were successively covered for 15 min with 0.1% bovine serum albumin, with a solution of avidin, then with a solution of biotin in phosphate buffer (PBS), followed by rinsing three times in PBS. Specimens were incubated with either biotinylated sarcolectin (45 min) or primary antibodies (Table 1) at room temperature. When necessary, sections were treated for 20 min with 0.1%

outer/marginal zone

outer region/sector
= small vessels

inner region/sector
= isolated phagocytes

intermedate zone
= aggregated cells

inner/central zone/core

fibrillary necrotic area

granular necrotic area

homogenous necrotic area

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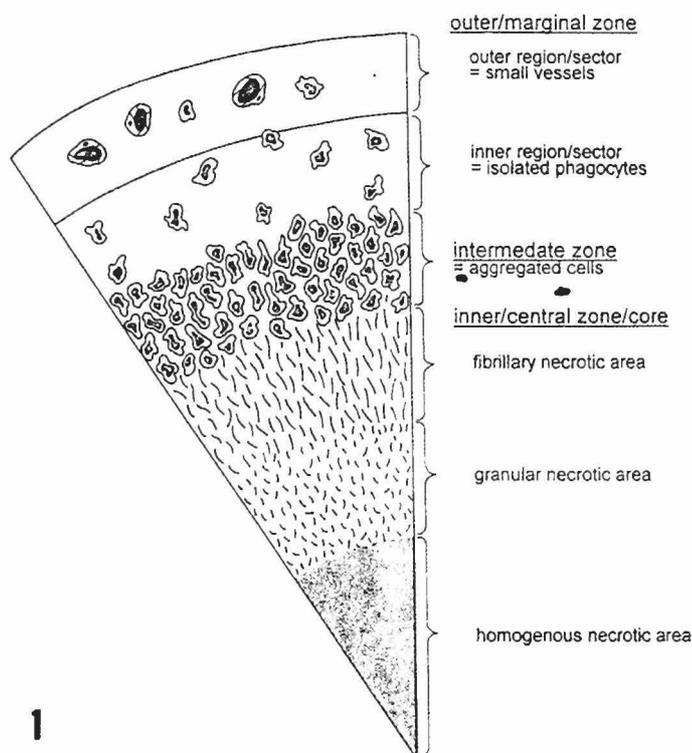
Fig. 1. Schematic representation of the structure of the RN to define the nomenclature used in this investigation.

Table 1. Commercially available monoclonal and polyclonal* primary antibodies applied in the present study.

ANTIBODY	SPECIES	SOURCE	DIGESTION	REFERENCE	CELL/ANTIGEN SPECIFICITY
OKM-I	mouse	Ortho	-	Talle et al., 1983	monocytes/macrophages
PG-M1	mouse	Dako	+	Falini et al., 1993	macrophages/CD 68
AMH 152	mouse	Dianova	+	Stastny and Nunez, 1984	activated macrophages
FMC 4	mouse	Biozol	+	Zola and Kupa, 1990	HLA-DR, non complement fixing
A 245*	rabbit	Dako	+	Christensen, 1990	fibronectin
AHP 061*	sheep	Camon	-	Kudryk et al., 1984	fibrinogen

trypsin (pH 7.8; 0.1% $CaCl_2$) (Kirkpatrick and D'Ardenne, 1984). In the case of primary antibodies, sections were reacted for an additional 30 min with biotinylated species-specific secondary antibodies (Dianova, Hamburg, FRG). Demonstration of the binding reaction was carried out according to Hsu et al. with the avidin-biotin-peroxidase (ABC) technique (ABC-Vectastain, Wiesbaden, FRG) (Hsu et al., 1981). The kit reagent-binding sites were visualised by incubation with a fresh solution of 0.02% H_2O_2 and 0.1% diaminobenzidine hydrochloride for 5 min. Finally, the samples were dehydrated, cleared and mounted. The intensity of binding reactions was graded subjectively and scored as negative: -, faintly visible: (+), weak: +, medium: ++, and strong: +++. Control experiments consisted in the omission of labelled sarcolectin, the primary antibodies or avidin, respectively.

Results

Early rheumatoid nodules

Very often the necrotic centre was not fully surrounded by palisading cells, making it sometimes difficult to delineate the classical structure of the RN. In one specimen multiple independent foci of necrosis were detected in serial sections. In three small (and presumably recent) biopsies a nearly uniform distribution of patchy sarcolectin-binding structures could be demonstrated (Fig. 2). Especially phagocytes and endothelial cells displayed a high binding affinity (Fig. 3), and cytoplasm of epitheloid cells reacted moderately. The staining intensity gradually decreased towards the surrounding unaltered connective tissue, showing no definite border. Immunoperoxidase reactions for MIF and fibrin revealed an analogous distribution, except that endothelial cells were not labelled by anti-MIF, whereas fibrin-positivity was restricted to few vessels that additionally showed distinct inflammatory alterations. Fibronectin staining was especially pronounced in necrotic areas. Most of the palisading cells revealed the macrophage markers OKM 1 or PG-M1, respectively, whereas the percentage of cells binding FMC 4 or AMH 152 was slightly reduced. Cell debris in the outer zone of necrotic areas also stained positive, whereas the central region was completely negative. Plasma cells and T lymphocytes were most prominent in the vascular zone. Only few (< 1%) scattered polymorph leukocytes were observed.

In the remaining two RNs the extracellular structures of the central core revealed no affinity for sarcolectin. Fibronectin or fibrin expression, respectively, was demonstrated in only slight amounts. Few dispersed macrophages were intensely positive for all three markers. In contrast to appearances in the central region, the extracellular matrix at the periphery of the necrotic zone ("fibrillary area") was intensely labelled by sarcolectin. Additionally, vascular cells together with surrounding connective tissue were intensely stained. The remaining fibres in the intermediate as well as in the peripheral zones showed a significantly reduced affinity or were (nearly) negative. Epitheloid-like cells constantly revealed no binding reaction. After incubation with MIF-specific antibodies significant parts of the necrotic core were labelled to a moderate amount. In the intermediate or peripheral zones results were nearly identical compared with sarcolectin, except that vasculature was constantly negative. Fibrin deposition was mainly restricted to perivascular tissue or (rarely) to vessel walls and intravascular thrombi. Fibronectin was seen between the palisading phagocytes in slight amounts, as well as in moderate quantity in the connective tissue of the inner peripheral zone (Fig. 4). Its extent of expression was significantly less in the vascular zone, gradually declining towards the periphery.

Mature rheumatoid nodules

In all specimens from biopsies the characteristic complex structure with its radial arrangement could be detected. Histochemical analysis revealed that most cells of the palisade layer reacted positively with the monocyte/macrophage-specific antibodies OKM I, FMC 4, PG-M1 or AMH 152, respectively. In serial sections it could be documented that multiple necrotic centres with localised small extensions were the rule. The extracellular matrix of necrotic cores showed no or only faint binding reactivity for sarcolectin. Dispersed histiocyte-like cells were invariably positive, although binding affinity varied significantly between different patients. In contrast, fibrillary debris in the outer part of necrotic zones was constantly labelled with high intensity. In the intermediate and peripheral zones morphological findings could be clearly divided into two groups. In two specimens sarcolectin-binding sites were diffusely dispersed within the extracellular matrix, resembling the
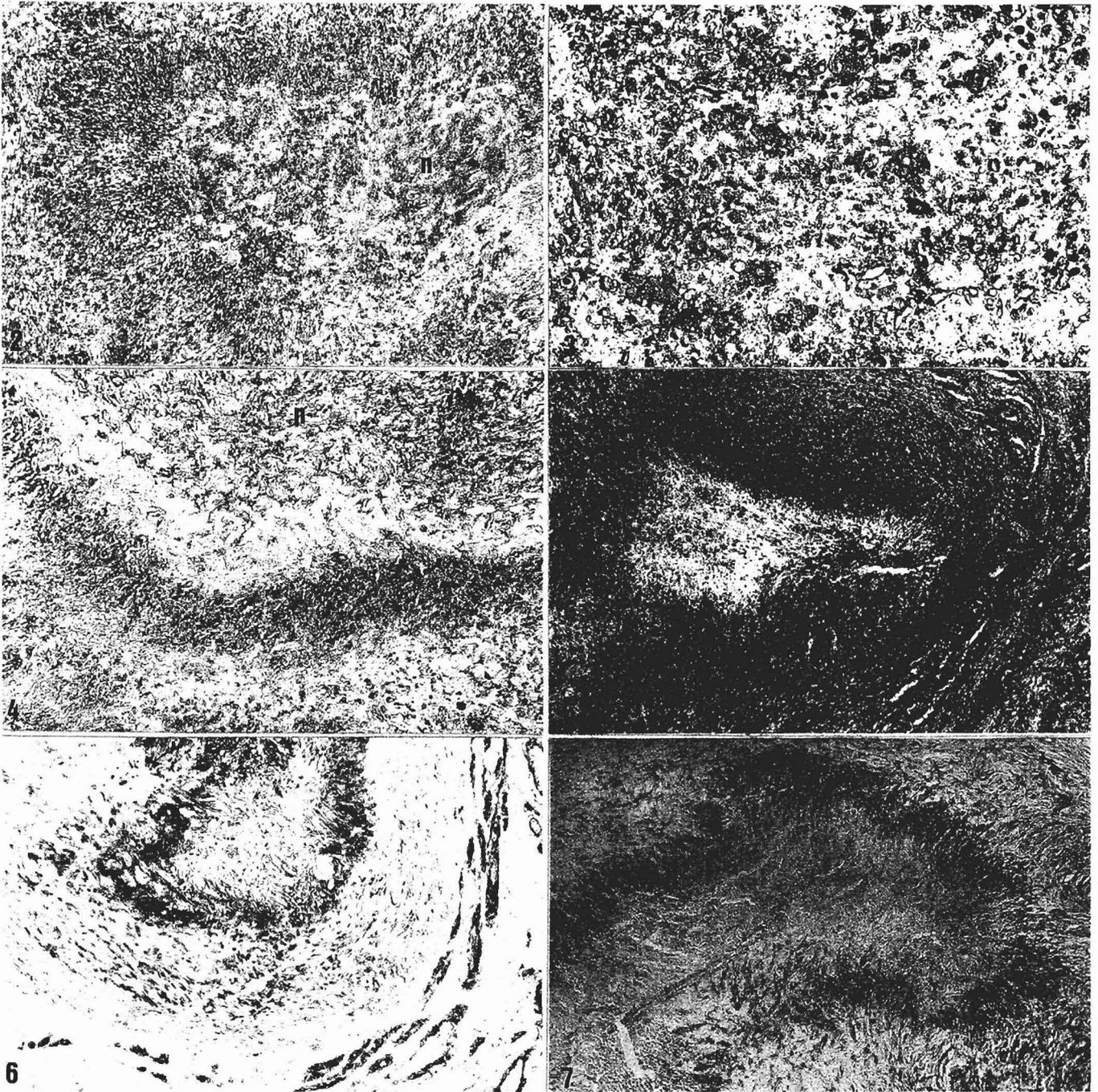
Fig. 2. Immunperoxidase staining of early RN. Diffuse binding affinity for sarcolectin in all 3 segments of the granulomatous tissue. n: necrotic centre. x 100

Fig. 3. Higher magnification of Fig 2. Especially cytoplasm of phagocytes and endothelial cells are strongly labelled. n: necrotic centre. x 120

Fig. 4. Immunperoxidase staining of early RN. with A 245 (anti-fibronectin). Especially the fibrillary area of the necrotic core and the inner sector of the marginal zone are stained. x 100

Fig. 5. Immunperoxidase staining of mature RN with sarcolectin. In the centre of the necrotic zone only phagocytes are positive. x 100

Fig. 6. Immunperoxidase staining of mature RN with sarcolectin. Especially vasculature and marginal areas of the necrotic core are intensely labelled. x 140

Fig. 7. Immunperoxidase staining of mature RN with MIF-antibody. Especially the fibrillar border of the necrotic zone labels positive. Vasculature shows no binding affinity. x 80

results seen in "early RNs" (Fig. 5). Likewise, the binding intensity decreased towards the surrounding mature connective tissue. The cytoplasm of aggregated macrophages stained moderately positive as well. In the remaining 16 specimens palisaded cells revealed no affinity and extracellular fibres were not or only weakly positive. As a consequence an annular corona was formed around the necrotic centre of RNs. Additionally, endothelial cells as well as perivascular fibres were constantly stained with high intensity (Fig. 6). No significant difference was observed between the distribution patterns of sarcolectin-binding molecules and the staining results after incubation with MIF-antibody (Fig. 7), except that endothelial cells and perivascular structures were negative. In all sections investigated, fibronectin and fibrin were co-distributed in the necrotic zone. Whereas the central core showed only slight affinity, binding intensity increased towards the palisaded macrophages infiltrating the clefts between the innermost palisaded cells. Significant presence of fibronectin was additionally detected in the connective tissue outwardly of the palisaded layer, whereas fibrin presence was restricted to occasional perivascular areas and/or intravascular thrombi.

Control specimens

Only in two specimens a faint labelling of endothelial cells by biotinylated sarcolectin was observed, the remaining structures being constantly negative. No binding reaction was detected after incubation with antibodies specific for MIF, fibrin or fibronectin, respectively. Only few dispersed histiocytes reacted for OKM I and PG-M1, whereas FMC 4 and AMH 152 were constantly negative.

Discussion

The objective of the present study was to histo-chemically demonstrate that sarcolectin-binding proteins like the lymphokine macrophage migration inhibitory factor are expressed in the granulomatous tissue of RNs. However, several difficulties are inherent in an approach to integrate our results into the pathogenic framework associated with the necrotising process. The objections partly reside in the slow proceeding over periods of months/years during evolution of RN lesions. Additionally, subtle but distinct differences between different areas of the same biopsy exist in the expression of morphological markers used as inflammatory parameters (i.e. number of inflammatory cells or deposition of fibrin). Especially the choice of mono-clonal antibodies applied to delineate cellular activity can significantly influence judgement (Bernhagen et al., 1998). Analogous conclusions have been drawn explaining rheumatoid joint pathology (Lindblad and Hedfors, 1985). Therefore, the morphological findings have to be interpreted very carefully. However, our immunhistochemical studies concerning cellular

components of the inflammatory reaction correspond with results published in the literature (Palmer et al., 1987; Melbye et al., 1991; Edwards et al., 1993). Most of the palisading cells revealed macrophage markers. Additionally, presence of the glycoproteins fibrin and fibronectin correlates with the distribution patterns reported in previous studies (Holund and Clemmensen, 1984). Therefore, it seems reasonable to assume that the RNs analysed in this investigation are characteristic granulomata in RA.

Until recently it was thought that activated T-cells were the primary, if not exclusive, sites of MIF synthesis (Calandra et al., 1994). According to this opinion non-specific deposition during the course of sustained inflammation has to be discussed, because T-cells are only a minor component of the inflammatory infiltrate in RNs (Firestein, 1998). However, it is now known that activated epithelial as well as mesenchymal cells in many tissues and also macrophages synthesize MIF (Wistow et al., 1993; Paralkar and Wistow, 1994). It was speculated that this protein may have a general involvement in the process of proliferation and differentiation. Significant parallels in the spatial pattern of expression with reference to N-myc were mentioned by the same authors, although not further specified (Wistow et al., 1993). Interestingly, high expression of oncogenes seems to be mainly restricted to aggregated synovial cells in seropositive RA (Asahara et al., 1997).

Recent research has documented that sarcolectin seems to be at least related to a physiological subfraction of serum albumin, its impact on regulation of immune processes currently being unclear (Kayser et al., 1994; Zeng et al., 1994a,b). It is reasonable that not only inflammation-inducing cytokines, but also inhibitory factors seem to be involved in the formation of the RN. With respect to the binding patterns of the MIF-specific antibodies and of sarcolectin, close similarities with few differences were constantly detected. This was especially true for vascular structures that were intensely labelled by sarcolectin. It is presently not possible to decide unequivocally whether another sarcolectin-binding protein is present in these compartments or whether MIF presentation to the immuno- and ligand-histochemical tools yielded the differences. It can be relevant to note that human albumin or subfractions thereof can specifically bind to endothelial receptors (Ghinea et al., 1989). Besides protein-protein recognition, lectin-carbohydrate interactions also have to be considered, because it was shown that certain negatively charged glycoconjugates can inhibit binding reactions between sarcolectin and MIF in a concentration-dependent manner (Zeng and Gabius, 1992). A plethora of different mechanisms modifies carbohydrate composition of connective tissue during sustained inflammation. The patterns of glycoproteins can be significantly altered by inflammation-induced secretion of matrix molecules through local cells and/or by exsudation of serum factors. Additionally, it has been shown that substrate glycosylation can be dramatically modified by cytokines

(van Dijk et al., 1994). Of crucial importance, however, seems to be the release of multiple enzymes partially degrading proteoglycan sequences as well as the oligosaccharide part of glycoproteins. As a result significantly altered glycoconjugate structures are induced that often exhibit atypical carbohydrates in terminal position, leading to notably modified lectin-binding patterns. Recent studies have shown that misdirected glycosylation is of crucial importance in numerous stages of the inflammatory process in connective tissue (Hounsell and Davies, 1993). However, their impact in this context is presently speculative, but not proven. Combined histochemical and cell biological investigations are now required to clarify the importance of MIF-sarcolectin interactions in RN.

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