Detection of Interleukin 1ß (IL-1ß), IL-6, and Tumor Necrosis Factor-α in Skin of Patients with Fibromyalgia

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ABSTRACT. ***Objective.*** To determine if abnormal collagen metabolism is correlated with neurogenic inflammation, a potential activator of collagen metabolism, in patients with fibromyalgia (FM).

Methods. The presence of inflammatory cytokines, interleukin (IL)-1ß, IL-6, and tumor necrosis factor (TNF)-α was investigated in skin tissues by using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. Fifty-three skin biopsies from female patients with FM (30-65 years of age) were examined and compared to skin biopsies of 10 age and sex matched healthy controls. Biopsies were obtained from the left deltoid region. Rheumatoid arthritis synovial fibroblasts and tissues were used as positive controls for the expression of cytokines. Total RNA isolated from the tissue samples were reverse transcribed (RT) by random hexamers as the primer for RT followed by PCR amplification using specific primers for IL-1ß, IL-6 or TNF-α. Expression of IL-1ß, and TNF-α protein was investigated in the skin by immunohistochemistry using specific antibodies (avidin-biotin method).

Results. Positive signals (RT-PCR) were detected in skin tissues of 19/50 (38%) FM patients for IL-1ß, in 14/51 FM patients (27%) for IL-6, and in 17/53 patients (32%) for TNF-α. None of the cytokines could be detected in healthy control skin. Immunoreactivity for IL-1ß and TNF-α was demonstrated in certain skin tissues of our FM patients.

Conclusion. The detection of cytokines in FM skin indicates the presence of inflammatory foci (neurogenic inflammation) in the skin of certain patients (about 30% of FM patients), suggesting an inflammatory component in the induction of pain. This may explain the response to nonsteroidal antiinflammatory therapy in a subset of FM patients. (J Rheumatol 2003;30:146–50)

Key Indexing Terms:
FIBROMYALGIA CYTOKINES INTERLEUKINS
TUMOR NECROSIS FACTOR SKIN

Fibromyalgia syndrome (FM) is a chronic, painful disorder commonly seen in women[1]. The pathogenesis of FM is not known but consideration has been given to genetic predisposition[2], traumatic injury[3,4], affective psychopathology[5,6], viral infections[7], and immunological mechanisms[8]. It is characterized by the presence of widespread musculoskeletal pain, tender points at characteristic sites, fatigue, and poor sleep. Associated disorders are restless leg syndrome, irritable bowel syndrome, irritable bladder syndrome, cognitive dysfunction, cold intolerance, multiple sensitivities and dizziness recognized as an important clinical problem associated with high levels of functional disability, and emotional distress[9].

Much research has been carried out in FM to better understand the neurobiology of chronic pain in these patients. Research suggests that dysregulated pain modulation may play an important role in FM[10]. Other factors such as elevated levels of substance P (SP) in cerebrospinal fluid, a mediator of nociception[11], altered serotonin metabolism in at least a subgroup of patients with FM[12,13], and lower melatonin secretion during the sleep[14,15], have been reported. There are some hypotheses and pilot studies suggesting that cytokines may play a role in FM[16,17]. Although cytokines are suspected to play a role in FM, their presence and functions have not yet been characterized[18].

Our group reported that decreased levels of collagen crosslinking in serum and urine of FM patients may contribute to remodeling of extracellular matrix and collagen deposition around the nerve fibers[19]. Altered collagen metabolism and highly ordered cuffs of collagen were observed around the terminal nerve fibers in the skin of such patients for unknown

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Supported in part by the Zurzach foundation, the American Fibromyalgia Syndrome Association (AFSA), and the Albert Böni Foundation.

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Submitted March 18, 2002; revision accepted May 24, 2002.

reasons. Therefore, the question was raised whether abnormal collagen metabolism is correlated with neurogenic inflammation, a potential activator of collagen metabolism, in FM patients.

The production and the spectrum of cytokines may reflect underlying mechanisms of disease. Interleukin (IL)-1ß, tumor necrosis factor (TNF)-α, and IL-6 are generally thought of as prototypical proinflammatory cytokines[20-22]. The presence of these inflammatory cytokines was investigated by using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC).

MATERIALS AND METHODS

Patient selection and biopsies. Skin biopsies were taken from 53 female patients with FM (mean age 46.6 ± 2.5 years, range 34-61) and a mean time period of 18.7 years (range 2–36) since FM symptom onset, and 10 age-matched healthy women. The patients were diagnosed with FM according to the American College of Rheumatology criteria[23]. The study had the approval of the ethics committee, and subjects were informed orally and in writing about the biopsy procedure and gave written informed consent. Skin tissues were obtained by punch biopsy of the anesthetized left deltoid region. Immediately after the procedure, a section of each tissue was snap-frozen in liquid nitrogen and stored at –70˚C until RNA extraction.

Detection of IL-1β, TNF-α and IL-6 mRNA by RT-PCR. mRNA expression of cytokines was detected by PCR amplification of complementary DNA (cDNA). Total RNA was isolated (Trizol® LS reagent Gibco BRL, Basel, Switzerland) from frozen skin tissue samples. First strand cDNA was synthesized from 0.1–1 µg of total RNA in 20 µl RT reaction using random hexanucleotide primers, 20 mM of each dNTP, 10× first strand buffer solution, M-MulV reverse transcriptase (Boehringer Mannheim, Germany), and DEPC-H_20. PCR amplification of the cDNA from the skin tissue was performed with *Taq*-DNA polymerase (Boehringer Mannheim) in the presence of primers specific for IL-1ß, TNF-α or IL-6 (Table 1). PCR solution containing 10× buffer, $MgCl_2$ (25 mM), dNTP (20 mM), primer reverse (20 µM), primer forward (20 µM), *Taq*-DNA polymerase (5 U/µl GIBCO BRL, Technologies Ltd, Scotland). The samples were placed on a PCR thermal cycler amplified at 95˚C for 5 min, annealing at required temperature for each primer for 30 s and with an extension of 72˚C for 1 min, for 40 cycles. The PCR-amplified samples were run on a 1.5% agarose gel. G3PDH was used as an internal control for loading differences. Negative controls included no template control and RNA control to check for genomic contamination. Synovial tissue and cultured fibroblasts from patients with rheumatoid arthritis (RA) were used as a positive control for the expression of cytokines.

Table 1. IL-1ß, TNF-α, and IL-6 primer sequences used for RT-PCR reaction.

Primer	Primer Sequence (5′–3′)	Annealing Temperature, ˚C	PCR Product
IL1-ß		56	276 bp
forward	AGATGATAAGCCCACTCTACAG		
reverse	ACATTCAGCACAGGACTCTC		
TNF-α		50	271 bp
forward	CCCGAGTGACAAGCCTGTAG		
reverse	GATGGCAGAGAGGAGGTTGAC		
IL-6		55	168 bp
forward	ACAGCCACTCACCTCTTCAG		
reverse	CCATCTTTTTCAGCCATCTTT		
G3PDH		55–60	453 bp
forward	ACCACAGTCCATGCCATCAC		
reverse	TCCACCACCCTGTTGCTGTA		

Immunohistochemical staining (IHC). Pieces of skin samples from each patient were rapidly frozen in isopentane (Fluka, GmbH, Buchs, Switzerland), cooled with liquid nitrogen and covered with OCT-embedding solution (Tissue-Tek®, Sakura, The Netherlands). Six micrometer cryostat sections were fixed in 4% paraformaldehyde (Fluka, Buchs, Switzerland) for detection. IHC was performed by using the avidin-biotin method. Mouse monoclonal IgG1 anti-human IL-1ß antibodies (MAB601, R&D Systems Europe, Abingdon, UK), and rabbit polyclonal anti-human TNF-α antibodies (MAB210, R&D Systems Europe) were used as primary antibodies. Equal concentrations of purified mouse and rabbit IgG were used as negative controls. The slides were stained using chromogen (Super Sensitive ready to use detection kit, No: QP000-5L, Biogenex, CA) or BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 50 µg/ml) substrates. To examine whether cytokines are produced at the site of neurons, monoclonal antibodies against human PGP 9.5 (protein gene product, 31A3, UltraClone Limited, Wellow, Isle of Wight, UK) antibodies, which can stain neuronal tissues in the central and peripheral nervous systems, were used. One synovial tissue sample from a patient with RA was used as a positive control for the staining.

RESULTS

Production of IL-1β, IL-6, and TNF-α in skin of patients with FM. To investigate whether proinflammatory cytokines are produced in FM patients, IL-1β, IL-6, and TNF-α were studied for their expression in the skin of FM patients (Figure 1). Positive signals (RT-PCR) were detected in 19/50 (38%) FM patients for IL-1ß, in 14/51 (27%) FM patients for IL-6, and in 17/53 (32%) patients for TNF-α (Figure 2). Figure 3 shows the production of each cytokine and combinations of 2 and all 3 cytokines together. None of the cytokines could be detected in control samples.

Expression of IL-1ß, IL-6, and TNF-α in skin of patients with FM, detected by IHC. To address whether the expression of IL-1ß, IL-6 and TNF-α mRNA was associated with corresponding protein, we performed IHC on FM tissues (Figure 4). Consistent with the mRNA data, immunoreactivity for IL-1ß, IL-6 and TNF-α was seen on FM skin tissues in fibroblast-like cells and certain mononuclear cells. However, the number of positive cells and the intensity of staining were low. In order to detect neurogenic inflammation at sites of cytokine expression, slides were double labeled with the PGP 9.5 neuronal marker, which showed the expression of cytokines at the site of nerves.

DISCUSSION

Cytokines have long been associated with peripheral inflammatory responses, but there is no clear evidence revealing their presence in FM patients. Certain studies suggest that cytokines may play a role in FM, but their origin and localization has not yet been shown. In our previous work, altered collagen metabolism and highly ordered cuffs of collagen were observed around the terminal nerve fibers in the skin of FM patients[19]. It has been suggested that FM has an immunologic component that includes cutaneous deposition of immunoreactants[24,25]. To know whether FM correlated with neurogenic inflammation, we searched for the presence of the

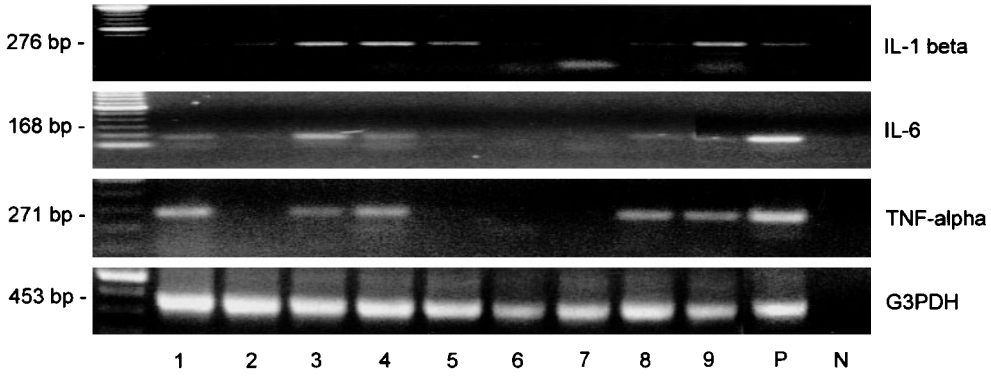

Figure 1. The mRNA expression of IL-1ß, TNF-α, and IL-6 in skin tissues of patients with FM detected by RT-PCR analysis using specific primers. Quality and quantity of RT cDNA was controlled by the G3PDH housekeeping gene. Lanes 1-9: individual patients, P: positive control (rheumatoid arthritis synovial tissue), N: negative control (no template control).

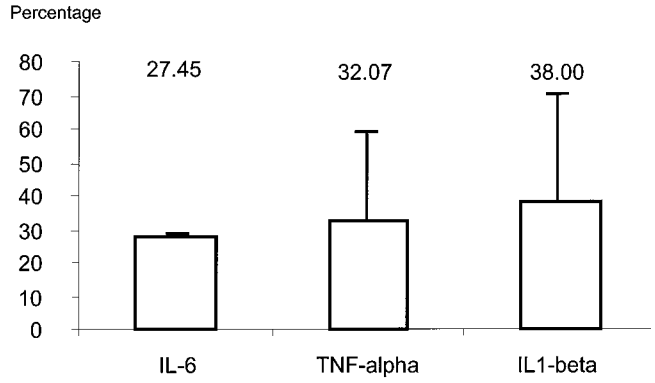

Figure 2. Percentage of patients with FM showing positive expression of cytokines IL-1ß, IL-6, and TNF-α. Means ± SD are given.

inflammatory cytokines such as IL-1ß, TNF-α, and IL-6 in the skin of FM patients[26]. By using RT-PCR, we could detect all 3 cytokines in certain FM patients with the IL-1ß as most prevalent cytokine, suggesting that they may play a role in FM. In previous studies Wallace, *et al* suggested that cytokines such as IL-8 as promotor of sympathetic pain and IL-6 as inducer of hyperalgesia, fatigue, and depression may play a role in modulating FM symptoms[27]. Maes, *et al* reported that pain and stiffness in FM may be accompanied by a suppression of some aspects of the inflammatory response system and that depressive symptoms in FM are associated with some signs of activation of the inflammatory response system[28]. Another group reported altered IL-2 secretion in patients with FM syndrome[17].

In this study, we showed the presence of the cytokines IL-1ß, TNF-α, and IL-6 in the skin of certain FM patients within fibroblast-like cells and mononuclear cells at sites of nerve tissue. These results suggest an inflammatory component in the

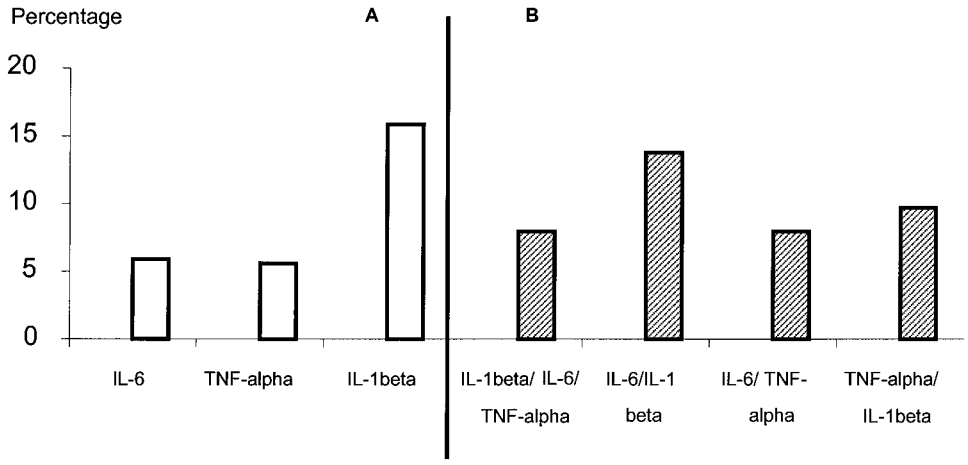

Figure 3. Percentage of expression of cytokines separately and in combination. A. Percentage of each expressed cytokine; B. Combination of the expression of 2 cytokines or all 3 cytokines together.

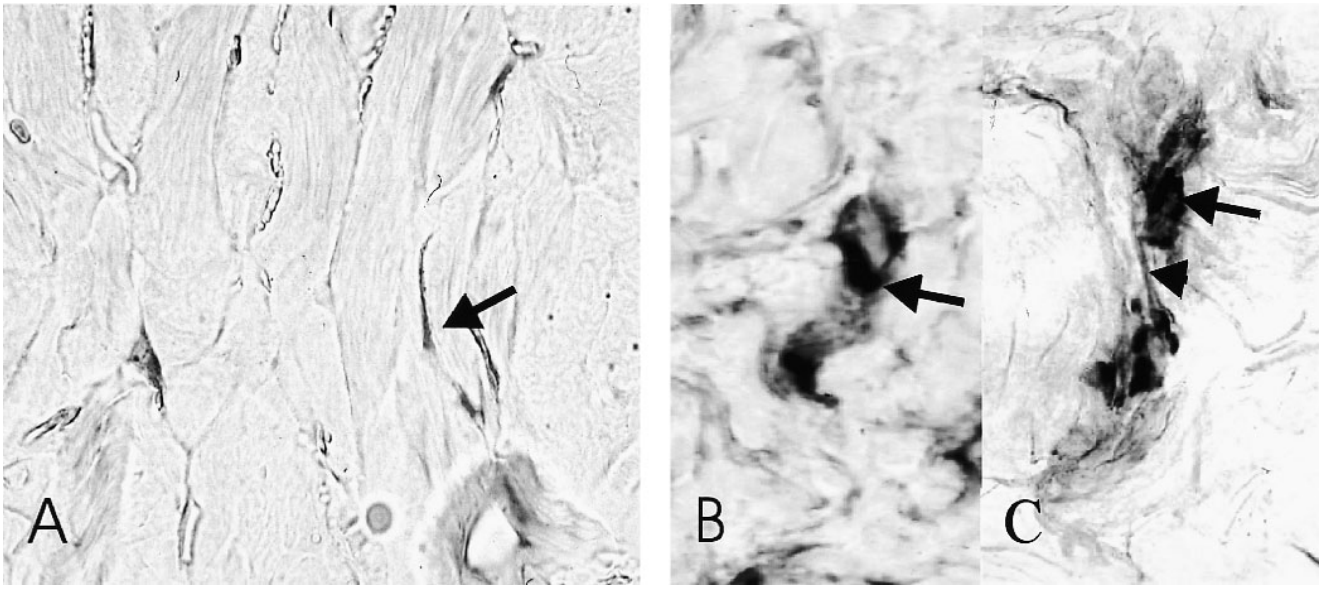

Figure 4. A. Skin sections of FM patients showing positive signals (red) of IL-1ß protein in the subcutaneous region in fibroblast-like cells (× 630), example marked with arrow. B. Area showing positive signals in a skin section of a patient with FM indicating the expression of IL-1ß in a mononuclear cell, stained in blue. C. Same section double labeled with the PGP 9.5 protein, a marker of nerve tissue, stained in brownish red color (arrow head) together with the blue IL-1ß staining (arrow).

skin of certain FM patients. Neurogenic inflammation may occur when substance P and other neuropeptides released from sensory nerve fibers produce an inflammatory response[29]. In this regard it has been reported that FM skin biopsies had higher values of IgG deposits in the dermis and vessel walls[30]. It is not yet clear whether these observations are part of a certain pathway or simply epiphenomena.

However, the detection of cytokines in FM skin biopsies suggests the presence of inflammatory foci in the skin of some FM patients (about 30%) and may explain the response to nonsteroidal antiinflammatory therapy in this subset of patients with FM.

REFERENCES

1. Wolfe F. Fibromyalgia: the clinical syndrome. Rheum Dis Clin North Am 1989;15:1-18.
2. Yunus MB, Khan MA, Rawlings KK, Green JR, Olson JM, Shah S. Genetic linkage analysis of multicase families with fibromyalgia syndrome. J Rheumatol 1999;26:408-12.
3. Culclasure TF, Enzenauer RJ, West SG. Post-traumatic stress disorder presenting as fibromyalgia. Am J Med 1993;94:548-9.
4. Weinberger LM. Traumatic fibromyositis: a critical review of an enigmatic concept. West J Med 1977;127:99-103.
5. Hudson JI, Hudson MS, Pliner LF, Goldenberg DL, Pope HG Jr. Fibromyalgia and major affective disorder: a controlled phenomenology and family history study. Am J Psychiatry 1985;142:441-6.
6. Ahles TA, Khan SA, Yunus MB, Spiegel DA, Masi AT. Psychiatric status of patients with primary fibromyalgia, patients with rheumatoid arthritis, and subjects without pain: a blind comparison of DSM-III diagnoses. Am J Psychiatry 1991;148:1721-6.
7. Goldenberg DL. Fibromyalgia and its relation to chronic fatigue syndrome, viral illness and immune abnormalities. J Rheumatol Suppl 1989;19:91-3.
8. Caro XJ. Is there an immunologic component to the fibrositis syndrome? Rheum Dis Clin North Am 1989;15:169-86.
9. Siegmeth W. Panalgesia and the fibromyalgia concept. Wien Med Wochenschr 1999;149:558-60.
10. Okifuji A, Turk DC, Marcus DA. Comparison of generalized and localized hyperalgesia in patients with recurrent headache and fibromyalgia. Psychosom Med 1999;61:771-80.
11. Schwarz MJ, Spath M, Muller-Bardorff H, Pongratz DE, Bondy B, Ackenheil M. Relationship of substance P, 5-hydroxyindole acetic acid and tryptophan in serum of fibromyalgia patients. Neurosci Lett 1999;259:196-8.
12. Offenbaecher M, Bondy B, de-Jonge S, et al. Possible association of fibromyalgia with a polymorphism in the serotonin transporter gene regulatory region. Arthritis Rheum 1999;42:2482-8.
13. Sprott H, Bradley LA, Oh SJ, et al. Immunohistochemical and molecular studies of serotonin, substance P, galanin, pituitary adenylyl cyclase-activating polypeptide, and secretoneurin in fibromyalgic muscle tissue. Arthritis Rheum 1998;41:1689-94.
14. Wikner J, Hirsch U, Wetterberg L, Rojdmark S. Fibromyalgia: a syndrome associated with decreased nocturnal melatonin secretion. Clin Endocrinol Oxf 1998;49:179-83.
15. Press J, Phillip M, Neumann L, et al. Normal melatonin levels in patients with fibromyalgia syndrome. J Rheumatol 1998;25:551-5.
16. Wallace DJ, Bowman R, Wormsley S, Peter J. Cytokines and immune regulation in the primary fibromyalgia (fibrositis) syndrome. Arthritis Rheum 1989;32:1334-5.
17. Hader N, Rimon D, Kinarty A, Lahat N. Altered interleukin-2 secretion in patients with fibromyalgia syndrome. Arthritis Rheum 1991;34:866-72.
18. Gur A, Karakoç M, Nas K, Cevik R, Denzli A, Saraç J. Cytokines and depression in cases with fibromyalgia. J Rheumatol 2002;29:358-61.
19. Sprott H, Muller A, Heine H. Collagen crosslinking in fibromyalgia. Arthritis Rheum 1997;40:1450-4.
20. Sporri B, Bickel M, Limat A, Waelti ER, Hunziker T, Wiesmann UN. Juxtacrine stimulation of cytokine production in cocultures of human dermal fibroblasts and T cells. Cytokine 1996;8:631-5.

21. Hu J, Cotgreave IA. Differential regulation of gap junctions by proinflammatory mediators in vitro. J Clin Invest 1997;99:2312-6.
22. Buchwald D, Wener MH, Pearlman T, Kith P. Markers of inflammation and immune activation in chronic fatigue and chronic fatigue syndrome. J Rheumatol 1997;24:372-6.
23. Wolfe F, Smythe HA, Yunus MB, et al. The American College of Rheumatology 1990 criteria for the classification of fibromyalgia. Report of the Multicenter Criteria Committee. Arthritis Rheum 1990;33:160-72.
24. Caro XJ, Wolfe F, Johnston WH. A controlled and blinded study of immunoreactant deposition at the dermal-epidermal junction of patients with primary fibrositis syndrome. J Rheumatol 1986;13:1086-92.
25. Caro XJ. Is there an immunologic component to the fibrositis syndrome? Rheum Dis Clin North Am 1989;15:169-86.
26. Salemi S, Rethage J, Kaeser L, et al. Detection of IL-1-beta, IL-6 and TNF-alpha in skin of fibromyalgia patients [abstract]. Arthritis Rheum 2001;44 Suppl:S69.
27. Wallace DJ, Linker-Israeli M, Hallegua D, Silverman S, Silver D, Weisman MH. Cytokines may play an aetiopathogenetic role in fibromyalgia: a hypothesis and pilot study. Rheumatology 2001;40:743-9.
28. Maes M, Libbrecht I, van Hunsel F, et al. The immune-inflammatory pathophysiology of fibromyalgia: increased serum soluble gp 130, the common signal transducer protein of various neurotrophic cytokines. Psychoneuroendocrinology 1999; 24:371-83.
29. Meggs WJ. Neurogenic switching: a hypothesis for a mechanism for shifting the site of inflammation in allergy and chemical sensitivity. Environ Health Perspect 1995:103:54-6.
30. Enestrom S, Bengtsson A, Frodin T. Dermal IgG deposits and increase of mast cells in patients with fibromyalgia- relevant findings or epiphenomena? Scand J Rheumatol 1997;26:308-13.