Neutral endopeptidase (EC 3.4.24.11) in labial salivary glands in healthy controls and in patients with Sjögren’s syndrome

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Abstract

Objective—Neuropeptides from nerve fibres can cause neurogenic inflammation. The potency of these peptides in vitro has led to the hypothesis that enzyme degradative systems are operative in vivo to limit their action. To consider this question neutral endopeptidase (NEP) in labial salivary glands in patients with Sjögren’s syndrome was studied.

Methods—Synthesis of NEP mRNA in situ in labial salivary glands was studied using the reverse transcriptase polymerase chain reaction (RT-PCR). Immunohistochemical staining was used to localise the NEP enzyme protein and its neuropeptide substrates and fluorophotometry to measure the corresponding enzyme activities in saliva.

Results—NEP was found in nerve fibres and in perivascular, periductal, and periacinar axon terminal varicosities. Double labelling of PGP 9-5 and NEP confirmed this neuronal localisation of NEP. Although some fibroblast-like cells and occasional intravascular neutrophils were NEP positive, NEP mRNA was not found in labial salivary glands. Patients with Sjögren’s syndrome and healthy controls did not have nerves containing NEP or neuropeptides (vasoactive intestinal peptide, substance P, or calcitonin gene related peptide (CGRP)) in lymphocyte foci. Salivary NEP activity was not decreased in patients compared with controls.

Conclusion—NEP in labial salivary glands is almost totally of neuronal origin and plays a part in proteolytic modulation of neuropeptides in salivary glands and saliva. These regulatory interactions seem to be altered in focal lymphocyte accumulations in Sjögren’s syndrome.


Sjögren’s syndrome is a systemic autoimmune disease of unknown aetiology. It is characterised by lymphocyte infiltrates, acinar atrophy, and disturbed function of exocrine glands. This results in keratoconjunctivitis sicca and xerostomia.1–4 Secretory function of exocrine glands is controlled by autonomic sympathetic and parasympathetic innervation. More recently, it has become evident that the autonomic nervous system, in addition to its role in an integrated vascular, secretory, and motor control of reflex salivary flow, also delivers trophic and regulatory neuropeptide stimuli to salivary gland parenchymal tissue.5–12 It has been shown that labial salivary gland tissue contains many neuropeptides which are able to modulate salivary gland functions.8–10 12–20 By contrast with classic biogenic amines, which are regulated by reuptake into nerve terminals, neuropeptide actions are rendered time specific and site specific by proteolytic degradation mediated by specific peptidases.

The neutral endopeptidase (NEP, EC 3.4.24.11, enkephalinase, CD10, or CALLA) cDNA sequence predicts a 750 amino acid integral membrane protein with a single hydrophobic 24 amino acid domain near the NH2 terminus that functions as a transmembrane anchor region.21–25 NEP is able to cleave neuropeptides and thus modulate the actions of neuropeptides,26–28 hormones, and immune mediators.26 27 29 30 Despite its potentially important role in salivary gland function and pathology, the eventual presence and tissue distribution of NEP on cell membranes and saliva have not been studied. In this study NEP was studied in labial salivary glands and saliva at the mRNA, protein, and biochemical activity level. Changes associated with eventual disease were looked for by a comparison between healthy controls and patients with Sjögren’s syndrome.

Patients and methods

SAMPLES AND BIOPSIES

All patients and controls participating in the present study received verbal and written information and gave their informed consent. The principles of the Declaration of Helsinki (Hong Kong amendment, September 1989) were followed throughout the work. The local ethics committee approved the study protocol. Collection of the animal material was approved by the local ethics committee and in the care and handling of animals the recommendations of the Strasbourg Agreement (18 March 1986) were followed.

Labial salivary glands were obtained from 12 patients with primary Sjögren’s syndrome and from eight normal controls. From each patient 5–10 labial salivary glands were taken under local anaesthesia as part of routine diagnostic
procedure and fixed in Zambone's fluid for six hours. Preliminary studies had shown that snap frozen sections fixed in cold acetone gave staining artefacts in the form of diffusion of the NEP antigen; due to this diffusion artefact, it was not possible to localise NEP to peripheral nerve fibres. In acetone-fixed tissue, with NEP seemed to be localised in extracellular pericellular and perivascular space and in nearby cells, as, for example, in pericytes around blood vessels. After a wash with 15% sucrose in 0.1 M phosphate buffered saline (PBS), pH 7.4, the glands were embedded in Tissue-Tek OCT compound (Lab-Tek Products, Division Miles Laboratories, Elkhart, IN, USA) and stored at −70°C until analysed.

**IMMUNOHISTOCHEMISTRY**

Antibodies were purchased as follows: monoclonal mouse antihuman NEP of IgG, class from Dako a/s, Glostrup, Denmark; rabbit antihuman PGP 9-5 antiserum from Ultraclone, Cambridge, UK, and rabbit antihuman vasoactive intestinal peptide (VIP), CGRP, and substance P from Cambridge Research Biochemicals, Cambridge, UK.

**IMMUNOPEROXIDASE METHOD**

Six μm thick sections were mounted on poly-L-lysine (Sigma Chemical Co, St Louis, MO, USA) coated slides. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 20 minutes followed by sequential incubations in normal horse serum (1:5) for 20 minutes, mouse antihuman NEP IgG (1:200) for 60 minutes, biotinylated horse antimus NEP IgG (1:100) for 30 minutes, and avidin-biotin-peroxidase complex (ABC) for 30 minutes. Finally, the sections were incubated for five minutes in a chromogen solution of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.006% H2O2 solution in TBS (0.05 M tris[hydroxymethyl]aminomethane, 0.15 M saline, pH 7.4) amplified with the glucose oxidase – nickel ammonium sulphate (Sigma) method. After each step, the slides were rinsed three times in PBS for five minutes each. All incubations were performed at 22°C. Stained sections were dehydrated in ethanol, cleared in Inhibisol (Kalon Chemicals, Cramlington, UK), and mounted in Pertex (Histolab Products Ab, Gothenburg, Sweden). Neuropeptide (VIP, CGRP, and substance P) staining was performed as described in detail elsewhere.16

The staining controls used were (a) omission of the primary monoclonal antibody from the staining sequence, and (b) replacement of specific monoclonal primary antibodies with inappropriate mouse myeloma protein. In addition, histochemical staining of untreated (no inhibition of endogenous peroxidase) tissue sections was carried out to show endogenous peroxidase.

**INDIRECT DOUBLE LABELLING METHOD**

Six μm cryostat sections were cut and mounted on chrome-alum-gelatine coated glass slides and air dried for 60 minutes. Thereafter, sections were serially treated as follows: 5% normal swine serum in phosphate buffered saline (PBS) at 22°C for 30 minutes; rabbit antihuman PGP 9-5 antiserum (1:1000 in PBS) in a humid atmosphere at 4°C for 24 hours; after washing in PBS, murine anti-mouse antihuman NEP antibodies (1:200 in PBS) in a humid atmosphere at 4°C for four hours; after washing in PBS, biotinylated goat antirabbit IgG (RPN 1004; Amersham, UK; 1:100 in PBS with 1% bovine serum albumin or (PBS-BSA)) at 22°C for 60 minutes; after 60 minutes in PBS, fluorescein conjugated streptavidin (RPN 1232; Amersham, UK; 1:100 in PBS-BSA) at 22°C for 15 minutes; after 60 minutes in PBS, rhodamine TRITC conjugated goat antimouse IgG (Jackson Immunoresearch Lab Inc, PA, USA; 1:20 in PBS) at 22°C for 60 minutes. After staining, the sections were thoroughly washed with PBS and mounted in glycerol-PBS (3:1). The specimens were viewed through a Zeiss Aristoplan or Ortophan epi-illumination fluorescence microscope equipped with filters allowing selective demonstration of FITC or TRITC.

**REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)**

The RT-PCR was carried out on five patients with Sjögren's syndrome and five healthy controls. For each mRNA extraction nine 5 μm tissue sections were used. The mRNA was extracted using oligo (dT)25 covalently attached to superparamagnetic polystyrene microbeads via a 5' linker group according to the manufacturer's protocol (Dynal, Oslo, Norway). Briefly, for each sample 30 μl of beads (binding capacity 2 ng of poly(A)+ mRNA/μl beads) in suspension were pipetted into a sterile Eppendorf tube and placed in a magnetic particle collector. As the magnetic field of the collector attracted the beads to the wall, the supernatant was pipetted off from the opposite side. The Eppendorf tube was removed from the collector and 100 μl of lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 5 mM DTT) was pipetted in to re-suspend the beads. The buffer was pipetted off using the magnetic collector just before mixing with the sample. To the sectioned samples 100 μl of lysis/binding buffer was added. After mixing and spinning, genomic DNA was sheared by passing the sample 10–15 times through a 1 ml syringe with a 22 G needle. The sample was incubated with the (dT)25 beads for five minutes at room temperature under continuous gentle stirring, washed twice with 200 μl washing buffer with LiDS (10 mM Tris-HCl pH 8.0, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS), twice with washing buffer without LiDS, and finally with 0.5 × RT buffer (45 mM KCl, 5 mM Tris-HCl, pH 8.3).

The rTth reverse transcription (Perkin Elmer, Branchburg, NJ, USA) reaction was performed using 5 U of the enzyme in a total volume of 20 μl (90 mM KCl, 10 mM
NEP in labial salivary glands

Tris-HCl, pH 8·3, 1 mM MnCl₂, 200 μM of dATP, dCTP, dGTP, and dTTP in thick wall PCR tubes (Plastic Trade, Helsinki, Finland) topped with 50 μl mineral oil (Sigma) using a thermal cycler (Pharmacia, Sollentuna, Sweden). The reaction was run for one minute at room temperature, one minute at 37°C, five minutes at 95°C, and 10 minutes at 70°C with mixing at one minute intervals. After the first strand synthesis the beads were collected with the magnet, resuspended in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8·0), and heated at 95°C for one minute. The beads were immediately collected using the magnet and the supernatant was pipetted off with aerosol resistant pipette tips (Molecular Bio-Products Inc, San Diego, CA, USA).

Target specific primers were designed based on published cDNA sequences corresponding to the bases 1737-1757 for sense (5'GGT CAT AGG ACA CGA AAT CAC3') and 2256-2233 bases for antisense (5'TGA AGA TCA CCA AAC CCG GCA CTT3') oligonucleotide primers. The primers were designed so that they also recognise NEP from the proximal tubules of rabbit kidney, which was used as a positive sample control. The beads were washed once with 100 μl 1 × PCR buffer (10 mM Tris-HCl, pH 8·8, 1·5 mM MgCl₂, 50 mM KCl, 0·1% Triton X-100) and the second cdDNA synthesis (total reaction volume was 30 μl) was carried out in 1 × PCR buffer with 5' primer (0·42 μM final) and 200 μM of dATP, dCTP, dGTP, and dTTP in thin wall PCR tubes topped with 50 μl mineral oil. Hot start at 80°C was used, 2·0 U of the thermostable DNA polymerase (Finnzymes, Espoo, Finland) was added, samples were denatured for two minutes at 95°C, and annealed at 61°C for one minute followed by five minute extension at 72°C with mixing at one minute intervals. After melting the strands for two minutes at 95°C the beads were collected with the magnet and the second strand cdDNA supernatant was transferred to a new PCR tube, from which the 3' primer (0·42 μM final) was pipetted and 45 cycles of one minute at 95°C, one minute at 61°C, and one minute at 72°C were performed. For the last cycle a five minute extension was used (Pharmacia, Gene ATAQ Controller, Sollentuna, Sweden). Amplified DNA was run on a 1% modified agarose gel for size verification.

**FLUOROPHOTOMETRY METHOD**

Salivary NEP was measured from unstimulated saliva in patients with Sjogren’s syndrome (n = 7) and in healthy controls (n = 5) by using a well established activity measurement protocol. All samples were centrifuged immediately after collection and the supernatant was used for analysis. Briefly, 50 μl of 100 μM Suc-Ala-Ala-Phe-AMC in 50 mM Tris-HCl, pH 7·6, was incubated for one hour at 37°C with 150 μl of saliva diluted 1:5 and 1:25 to 50 mM Tris-HCl, pH 7·6, containing 0·1% Triton X-100. Blanks contained 1 μM phosphoramidon (a selective NEP inhibitor) and were run in parallel with the samples. The reaction was stopped by adding 1 μM phosphoramidon and heating at 95°C for 15 minutes. The Phe-AMC formed was analysed completely by incubation for one hour at 56°C after addition of 0·75 μg of aminopeptidase M. This reaction was stopped by heating at 95°C for 15 minutes. Fluorescence was measured using excitation at 367 nm and emission at 440 nm.

**Results**

**IMMUNOLOCALISATION OF NEP**

In thick 20 μm tissue sections NEP immunoreactivity was seen to form dense networks around the acini and ducts of the labial salivary glands. The use of thin 6 μm tissue sections disclosed that NEP immunoreactivity had an appearance typical for small nerve fibres and preterminal varicosities and nerve terminals (fig 1); NEP immunoreactivity was also found in larger peripheral nerve trunks occasionally seen in labial salivary gland biopsies (fig 2). Staining of NEP in labial salivary glands of

![Figure 1](http://ard.bmj.com/)

**Figure 1** Avidin-biotin-peroxidase complex (ABC) staining of neutral endopeptidase (NEP) with monoclonal mouse IgG, antibodies to human CD10 amplified with the glucose oxidase – nickel ammonium sulphate method without counterstaining. Nerve fibres immunoreactive (IR) to NEP in a thin 6 μm tissue section are seen around the labial salivary gland acini and ducts in the form of small diameter nerve fibres, preterminal varicosities, and nerve terminals. (A) Original magnification ×250; (B) original magnification ×1000.
patients with Sjögren’s syndrome was otherwise similar to that of healthy controls, but focal lymphocyte infiltrates did not contain NEP-immunoreactive (NEP-IR) structures except in their peripheral zones (fig 3). Interestingly, a similar phenomenon was noted in the few foci occasionally found in controls with healthy labial salivary glands, which contained less than one focus per 4 mm² labial salivary glands tissue (focus score value < 1). Furthermore, NEP-IR nerve terminals and fibres were seen in labial salivary glands in patients with Sjögren’s syndrome in areas containing diffuse (by contrast with focal) lymphocyte and plasma cell infiltration. Occasionally NEP was also seen in fibroblast-like cells in the tissue interstitium or inflammatory cell infiltrates and in polymorphonuclear neutrophilic leucocytes in blood vessels (not shown). In properly processed tissue (see earlier), vascular endothelial cells, smooth muscle cells, and pericytes of the blood vessels did not show any positive NEP staining.

NEP mRNA IN LABIAL SALIVARY GLANDS
Messenger RNA coding for NEP was regularly found in positive control tissue in accordance with its synthesis in proximal tubules in epithelial cells in rabbit kidney (fig 4). By contrast, labial salivary glands from healthy controls and from patients with Sjögren’s syndrome did not contain NEP mRNA (fig 4).

IMMUNOLocalisation of NEUROPEPTIDES
In normal labial salivary glands PGP 9-5-IR and VIP-IR fibres were seen perivascularly, around acini, and in close proximity to all salivary ducts. In patients with Sjögren’s syndrome, PGP 9-5 and VIP, as NEP (see earlier), were found in the peripheral zones but not in the middle of the inflammatory cell infiltrates. Outside the focal inflammatory cell areas PGP 9-5 and VIP-IR nerves were similarly distributed in Sjögren’s syndrome and in normal labial salivary glands. In double labelled sections NEP was found in PGP 9-5-IR axon terminals, usually located around the salivary acini. However, most PGP 9-5-IR nerve fibres seemed to be NEP negative. The CGRP-IR and substance P-IR nerve fibres were mainly seen in association with blood vessels; CGRP-IR and SP-IR free nerve endings were only occasionally found in the peritubular and periacinar tissue and, in Sjögren’s syndrome, in the periphery of the inflammatory cell infiltrates.

Figure 2 NEP-IR nerve trunk in a 6 μm thick labial salivary gland section showing typical morphological appearance. ABC staining, glucose oxidase – nickel ammonium sulphate amplification without counterstaining. Original magnification × 400.

Figure 3 NEP-IR nerve fibres and varicosities around acini and ducts in a labial salivary gland from a patient with Sjögren’s syndrome. As well as normal looking salivary gland tissue, this field contains a lymphocyte rich focal infiltrate (FI), which is a typical feature in Sjögren’s syndrome. The NEP-IR peripheral nerves are seen in the peripheral zones of this lymphocyte rich infiltrate, but do not penetrate into its centre. In the midst of the lymphocytes, occasional fibroblast-like, NEP-IR mononuclear cells (arrows) are present. ABC staining, glucose oxidase-nickel ammonium sulphate amplification without counterstaining; original magnification × 400.

Figure 4 Representative examples of neutral endopeptidase messenger RNA amplification products after 45 cycles in the RT-PCR. Amplification products were subjected to electrophoresis on 1% modified agarose gel, stained with ethidium bromide, and photographed under UV light. The first lane contains a 100 bp ladder; the second lane the amplification product from the proximal tubule epithelial cells in rabbit kidney. Lanes 3 to 5 contain the amplification products from labial salivary glands of healthy controls and lanes 6 to 8 the amplification products from labial salivary glands of patients with Sjögren’s syndrome.
SALIVARY NEP
Analysis of activity of salivary NEP disclosed slightly higher values in patients with Sjögren’s syndrome than in healthy controls. The difference, however, was not significant. The mean (SEM) Δ absorbance (sample-phosphoramidon inhibited control) value at 1:5 dilution at 42 (8) (absorbance) units in patients with Sjögren’s syndrome compared with 26 (13) units in healthy controls (p > 0.05, Mann-Whitney U test) with the corresponding figures at 1:25 dilution being 21 (7) v 9 (4) (p > 0.05).

Discussion
Neutral endopeptidase has been earlier localised to non-T and non-B acute lymphocytic leukaemia cells, early lymphoid progenitor cells, germinal centre cells, fibroblasts, granulocytes, renal epithelial cells, gut epithelial cells, some lymphomas, melanomas, and glioma cell lines, and some neuronal cells. In the present study specific mouse antihuman NEP antibodies used in immunohistochemical staining showed NEP in an unexpected topographical pattern in human salivary glands – namely, in the peripheral nerves. These immunohistochemical findings on NEP in labial salivary glands were supported by the demonstration of NEP in the corresponding body fluid – saliva – by using a well established activity measurement protocol.

The endopeptidase was found in the nerve terminals and preterminal varicosities and in small diameter nerve fibres in labial salivary glands. This neuronal localisation was not evident – and would have been missed – if routine tissue processing and acetone fixation had been used. Neuronal localisation of NEP was only evident in Zamboni fixed tissues. This unexpected finding was confirmed by double labelling of NEP and PGP 9-5, a generalised neuronal cell marker. Furthermore, NEP was localised in large peripheral nerve trunks, which were unambiguously identified by morphological criteria. These findings were in accordance with those of Pollard and co-workers, who reported NEP in the central nervous system; the present study is the first to show that NEP also occurs in peripheral nerves.

Localisation of NEP in large nerve trunks would be in accordance with axonal transport of NEP, which was first reported by Back and Gorenstein, based on their studies on axotomy of hypoglossal nerve. We hypothesised that if NEP is mainly or exclusively synthesised in nerve cell ganglia (and not in situ in labial salivary glands), the signal for NEP mRNA in labial salivary glands would be weak or negligible, even if a sensitive RT-PCR would be used. This was demonstrated. This hypothesis was verified by using RT-PCR for labial salivary gland samples run in parallel with identically processed positive control samples. Despite occasional fibroblast-like NEP-IR cells, NEP mRNA was totally absent in sections of labial salivary glands. This finding indicates that labial salivary gland NEP is synthesised outside the salivary gland. Taken together, the typical varicotic appearance and tissue localisation structures containing NEP, colocalisation of NEP with a well known neuronal marker, the presence of NEP in large nerve trunks, and the absence of local synthesis, suggest that salivary gland NEP is of extraglandular, neuronal origin.

Saliva also contains NEP. This suggests that NEP, primarily synthesised as an integral cell membrane protein, is in part proteolytically cleaved and solubilised from the cell membrane and secreted into saliva. Interestingly, marker peptides of the autonomic nervous system – namely, VIP and neuropeptide Y – have also been recently described in normal and Sjögren’s syndrome saliva. (Santavirta et al, unpublished observations). This may in part relate to the hypolemmal localisation of these terminal nerve fibres. Saliva also contains various other potential NEP substrates, such as growth factors and glioma cell line-derived neurotrophic factors. Because activity measurements were used, it is not established, despite the relatively specific substrate and inhibitor used, that salivary NEP-like activity was actually exerted by NEP. On the other hand, as NEP in saliva was demonstrated based on activity, it also follows that this NEP-like activity would be able to regulate the action of various bioactive peptides produced by salivary glands for “export”.

Orientation of NEP on the cell surface is such that the active site is located towards the extracellular space. It is attached to the cell membrane by a short 24 amino acid hydrophobic membrane spanning domain, which is connected to a 26 amino acid domain of unknown function on the cytoplasmic side of the plasma membrane. Distribution of NEP in the axon terminals and preterminals would seem to focus its effects on neuropeptides to the immediate perineurial space. Interestingly, NEP cleaves substrates at the amino side of hydrophobic amino acids and its target substrates include VIP (neutral endopeptidase cleaves VIP principally into inactive VIP and VIP fragments, and substance P (neutral endopeptidase can cleave SP at tree bonds, including Gly-Leu, which generates inactive fragments)).

Labial salivary glands are densely innervated by VIP-IR nerves, which are seen around acini, salivary ducts, and blood vessels. Because VIP is a trophic factor for salivary acini, lack of it causes acinar atrophy. Accordingly, surgical parasympathectomy and lack of solid food (leading to lack of mastication induced stimulation or “functional parasympathectomy”) lead to acinar atrophy. Such atrophy cannot be attained by treatment with atropine to block the muscarinic acetylcholine receptors. By contrast, acinar atrophy caused by parasympathectomy could be reversed by local infusion of VIP. Recent studies have shown a close spatial relation between hypolemmal VIP-IR post-ganglionic parasympathetic nerves and acinar endpiece cells. Furthermore, acinar endpiece cells contain high affinity VIP receptors on their basal parts. This trophic effect of VIP represents one of the so-called non-adrenergic, non-cholinergic neurotransmission mechanisms regulating acinar cell function.
non-cholinergic (NANC) effects of the autonomic nervous system. It was therefore of interest to localise a major VIP degrading enzyme in normal and diseased salivary glands. Both VIP and NEP were found in peripheral nerve fibres and terminals. Patients with Sjögren’s syndrome had an altered tissue distribution of such structures, with lack of NEP nerves and VIP-IR nerves in lymphocyte foci. Findings were similar in the few lymphocyte foci found in normal glands. By contrast, diseased salibary glands from patients with Sjögren’s syndrome contained NEP-IR terminals and fibres in areas diffusely infiltrated by lymphocytes and plasma cells. These findings suggest that lack of VIP-IR nerves in foci may play a part in the formation of foci rather than in the genesis of Sjögren’s syndrome itself.

Focal lymphocyte infiltrates in the parenchyma of canine glands form a hallmark of Sjögren’s syndrome. In the present study VIP-IR, substance P-IR, and CGRP-IR nerves were seen in the marginal zones but not in the centres of these accumulations of focal lymphocytes. Interestingly, NEP showed a similar distribution. Under these conditions, NEP could balance the proinflammatory effects of neurotransmitters. Neuropeptides released in the marginal zone of the focal lymphocyte accumulations may diffuse and act on the local inflammatory cells without being degraded by NEP. This might prolong the local half-life of neuropeptides and thus augment the neurogenic component of inflammation. Substance P has in fact been shown to induce the release of interleukin-1, tumour necrosis factor α, and interleukin-6 from monocytes, whereas VIP modulates lymphocyte migration and recirculation and increases B cell mediated immunoglobulin production. Apart from this difference between the normal and Sjögren’s syndrome glands, there was no difference in topographical distribution of NEP between the two study groups. However, NEP activity was slightly but not significantly higher in saliva of patients with Sjögren’s syndrome than in healthy controls, which may be due to either upregulation of neuronal NEP synthesis or, perhaps more likely, its enhanced solubilisation and secretion as a result of local inflammation.

We are grateful to Mrs Eija Kaila, Mrs Paula Hasenstrom, and Mrs Maria Astramaki for their skilful technical assistance and to Mr Reijo Karppinen for producing the photographs. This study was supported by the Finnish Academy of Science, Finnish Medical Society, the Samu and Anna Gyllenhielm Foundation, and the Finnish Rheumatism Research Foundation.


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doi: 10.1136/ard.55.8.513

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