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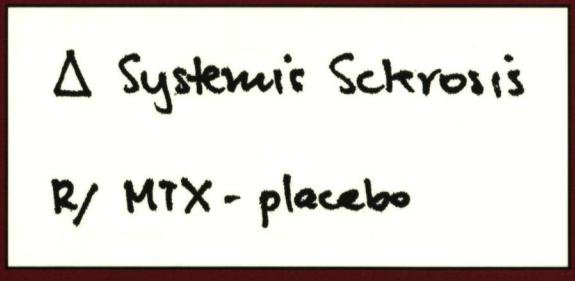
Systemic sclerosis: Effects of treatment with methotrexate

Frank H.J. van den Hoogen



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Contents

Chapter 1

General introduction

SYSTEMIC SCLEROSIS

Definition

Systemic sclerosis (scleroderma, SSc) is a generalized disorder of connective tissue characterized by induration and thickening of the skin, by microvascular and larger vessel lesions, and by fibrotic degenerative changes in muscles, joints and viscera, mainly the esophagus, intestinal tract, heart, lungs and kidneys. These changes are caused by variable degrees of extracellular matrix accumulation and associated with specific autoantibodies, most notably anticentromere and antitopoisomerase I antibodies[1,2].

History

In the writings of both Hippocrates and Galen allusions are made to diseases of the skin. The first detailed report of SSc was made by the Italian physician Curzio, who described hardness of the skin of a 17-year-old girl in Naples in 1753[3]. The disease was given the name sclérodermie by Gintrac in 1847[4]. From then, patients with scleroderma and widespread skin thickening were gradually known to have progressive internal organ disturbances. Only in 1945 did Goetz[5] propose the name progressive systemic sclerosis. Because not all SSc is progressive, the designation progressive should be abandoned[6].

Classification

In 1980, a subcommittee of the American Rheumatism Association (ARA) set up preliminary criteria for the classification of SSc[7](Table).

ARA scleroderma criteria cooperative study: Preliminary criteria for the classification of systemic sclerosis (scleroderma)

1. Major criterion:	- Proximal scleroderma
2. Minor criteria :	- Sclerodactyly
	- Digital pitting scars or loss of substance of the digital finger pad
	- Bibasilar pulmonary fibrosis

Proximal scleroderma was defined as tightness, thickening, and non-pitting induration proximal to the metacarpophalangeal or metatarsophalangeal joints; sclerodactyly as skin changes limited to fingers and toes. Digital pitting scars or loss of substance of the digital finger pad was defined as depressed areas at tips of digits or loss of digital pad as a result of digital ischemia rather than trauma or exogenous causes. Bibasilar pulmonary fibrosis was considered to be present when a bilateral reticular pattern of linear or lineonodular densities, not attributable to primary lung disease, were present on standard chest X-rays. One major or

two or more minor criteria were found in 97% of patients with definite systemic sclerosis, but in only 2% of the comparison patients with systemic lupus erythematosus, polymyositis/dermatomyositis, or Raynaud's phenomenon. Patients with localized scleroderma and pseudosclerodermatous disorders were excluded[7]. These criteria were derived from a population of definite SSc patients and developed to allow comparability of patient groups in clinical trials, not to diagnose SSc in the early stages. A further subclassification was proposed by LeRoy[8] in limited (cutaneous) SSc (lSSc) and diffuse (cutaneous) SSc (dSSc). In lSSc the skin involvement is limited to hands, feet, face and/or forearms; in dSSc skin thickening is present also on upper arms and trunk. It is currently the most widely used subclassification of SSc. Limited SSc is associated with a high incidence of anticentromere autoantibodies (70-80%), the existence for years of Raynaud's phenomenon and a significant late incidence of pulmonary hypertension[9]. The acronym CREST (Calcinosis, Raynaud's phenomenon, oEsophageal dysmotility, Sclerodactyly and Telangiectasia) fits into this subclassification. Diffuse SSc is associated with an early incidence of interstitial lung disease, hypertensive crisis and renal failure, diffuse gastrointestinal disease and myocardial involvement[9]. In approximately 30% of dSSc patients antitopoisomerase I autoantibodies can be detected[10-12].

Epidemiology

The rarity of SSc makes it a difficult disorder to study epidemiologically. It has been described in all races and is global in its distribution. Most of the available data concerning incidence are derived from records of patients attending hospitals draining a defined denominator population. Its incidence is suggested to be in between 4 and 12 individuals per million population per year[13,14]. The disease seldom occurs in childhood[15,16], is 3 - 4 times more common in women than men and its onset is highest in the fourth and fifth decade of life[17]. The incidence of SSc seems to be increasing[14], probably because of better ascertainment.

Prognosis

The natural course of the disease is variable: a few patients experience spontaneous remission; the majority undergoes progression of skin and internal organ involvement, resulting in considerable morbidity and mortality[18,19]. Several studies report 5-year cumulative survival rates ranging from 34-73%[20]. Older age, male gender, internal organ involvement, presence of antitopoisomerase I autoantibodies, and diffuse skin involvement adversely affect outcome[21-23].

Etiology

Although the etiology of SSc is still unknown, genetic and environmental factors appear to play a part[24]. The familial and genetic predisposition to SSc is indicated by its occurrence predominantly in the female sex[17,25], the reported familial clusterings of SSc[26,27] and related diseases[28], and by the fact that autoantibodies

associated with SSc are found frequently in blood from relatives of patients with SSc[29]. Studies concerning the pattern of the major histocompatibility complex (MHC) antigens in scleroderma patients have led to conflicting results from the different geographic areas in which they have been performed[30]. In the Dutch population an increased incidence of the haplotype HLA A1, B8 and DR3 was found[31]. The finding of an abnormal fibronectin gene in Japanese patients, suggesting a gene defect as cause of SSc, could not be confirmed in Dutch patients[32]. Some chemicals, such as toluene and benzene[33], trichloroethylene[33], and organic solvents[34] are associated with systemic-like conditions. Likewise, exposure to vinylchloride[35], epoxy resins[36], silica dust[37] and aniline-adulterated rape seed oil[38], and treatment with drugs such as pentazocine[39], L-tryptophan and carbidopa[40] and bleomycin[41] may cause scleroderma-like disease. Microvascular lesions associated with the development of fibrosis[42] or direct stimulation of collagen production[40,43] have been mechanisms proposed for the sclerodermatous changes.

SSc has also been reported to occur among recipients of silicone breast implants[44]. However, the cause-effect relationship is unknown and there are no concrete data as to whether the removal of silicone implants will halt the progression of the disease.

Finally, the chronic graft-versus-host reaction, developing after allogenic bone marrow transplantation, can cause sclerosis of the skin, muscles and bones[45].

At present, there is no adequate experimental model that reproduces all the characteristics of SSc. Skin changes resembling scleroderma occur in a mutant line of white leghorn chickens, the so called UCD L-200 line[46], and in the tight-skin mouse[47]. Both animal models lack internal organ involvement.

Pathogenesis

Although the pathogenesis of SSc remains unknown, the altered connective tissue metabolism, vascular abnormalities and changes in the immune system all seem to be involved.

a. Altered connective tissue metabolism

The deposition of increased amounts of extracellular matrix components (collagen, fibronectin, and glycosaminoglycans) is a hallmark of systemic sclerosis. Tissue cultures of dermal fibroblasts have been found to synthesize collagen at an increased rate[48-50] and to accumulate up to 5 times more glycosaminoglycans than normal skin fibroblasts[51-54]. Likewise, an increase in fibronectin in the deep dermis of involved skin in progressive systemic sclerosis was found[55].

The increase of collagen production is likely to be the result of an increased rate of biosynthesis since both collagenase activity and collagen degradation is normal in cultured scleroderma fibroblasts. The mechanisms through which scleroderma fibroblasts are stimulated to produce more extracellular matrix are unknown; it is suggested that some factors from sera or cells, such as platelet derived growth factor, interleukins 1 and 4, and fibroblast growth factor, promote a high collagen producing fibroblast population[56].

b. Vascular abnormalities

Vascular lesions are widespread in SSc and often considered to be the primary event[57]. Evidence for vascular dysfunction is demonstrated by the presence of Raynaud's phenomenon as indicator of vasomotor instability in up to 90% of scleroderma patients and the microvascular abnormalities characterized by arterial intimal proliferation leading to irregular luminal narrowing and zones of total obstruction. The presence of abnormal nailfold capillaries can be demonstrated in more than 80% of scleroderma patients[58]. Endothelial cell damage seems to be the central event and is reflected by changes in circulating levels of endothelial cell products such as von Willebrand factor, plasminogen activating factor, and prostacyclin/thromboxane metabolites. This damage may lead to altered permeability of the vessel wall and increased passage of mononuclear cells. Possible explanations of endothelial cell damage in SSc are exposure to endothelial cell cytotoxins produced by activated T-cells[59-61] or complement activation due to reduced expression of the cytoprotective proteins CD 59 and decay accelerating factor on the endothelial cell surface[62].

c. Autoimmune alterations

- Aberrant humoral immune responses

About 95% of SSc-patients have detectable antinuclear antibodies when HEp-2 cell lines are used as the detection substrate[63]. Anticentromere autoantibodies are directed against the centromere of chromosomes and are detected in sera of approximately 30% of unselected scleroderma patients. Anticentromere autoantibodies have a predilection for patients with limited cutaneous involvement. Patients with anticentromere autoantibodies and only Raynaud's phenomenon have an increased risk of eventually developing limited SSc eventually[64]. Antitopoisomerase I autoantibodies are directed against the enzyme topoisomerase I which modulates the topological states of DNA. They occur in about 20% to 50% of SSc patients and are associated with diffuse cutaneous involvement. Antitopoisomerase I autoantibodies were originally designated anti-Scl 70 or anti-Scl 86[10], and are associated with more frequent occurrence of internal organ involvement[65].

Other nucleolar autoantibodies detected in less than 10% of patients with SSc are anti-RNA polymerase I and III antibodies, anti-Th-antibodies, anti-U1 RNP- and anti-U3 RNP antibodies and anti-PM-Scl antibodies.

Other circulating antibodies to self proteins frequently detected in SSc and mainly in the early stages of the disease are antibodies against type I, II, IV and VI collagen; these antibodies are associated with the severity of interstitial lung disease[66].

Whether the production of autoantibodies is important in the pathogenesis of SSc or whether they are mainly 'innocent bystanders' is still a subject of debate.

- Aberrant cell-mediated immune responses

T lymphocytes and plasma cells are found in excess in the involved skin during the early course of the disease[67]. In the peripheral blood, the number of T cells is decreased with a normal ratio of T to B cells but the ratio of helper T cells to

suppressor T cells is increased in approximately 30% of the patients[68-70], mainly because of a reduction in suppressor T cells[67,69]. The number of natural killer cells in peripheral blood of SSc patients appears to be normal[71] but the activity of these cells is reduced, predominantly in patients with active disease and widespread visceral involvement[72,73]. Scleroderma mononuclear cells spontaneously release interleukin 1 (IL-1) and T-cell response to IL-1 is defective[74]; IL-1 production by mononuclear cells is reduced[75]. Serum levels of interleukin-2 (IL-2) and IL-2 receptors are increased[76-80] and correlate positively with skin thickness[81,82].

The changes known to occur in connective tissue metabolism, vascular endothelium and immune system have led to several hypothetical schemes of the pathogenesis of systemic sclerosis, in which an unknown inciting event breaks down endothelial cells and activates the immune system. The activated immune system enhances endothelial cell damage which results in release of cytotoxic products causing fibroblast proliferation and enhanced extracellular matrix deposition with, ultimately, fibrosis[83].

Treatment

Many drugs have been advocated in the treatment of SSc, but none has been proven to be effective in properly controlled trials. In assessing efficacy of a drug in SSc, the variable disease course with sometimes spontaneous improvement after several years, and variable rate of progression of SSc make it necessary to perform double-blind, preferably placebo controlled trials. Such double-blind therapy trials are limited in systemic sclerosis and have not provided evidence of benefit in systemic manifestations.
Based upon a better understanding of the pathogenesis of SSc, therapeutic trials have focussed on drugs affecting vascular changes, collagen and other extracellular matrix components produced by fibroblasts, and immunologic changes.
Drugs aimed at improvement of the vascular pathology include aspirin, dipyridamole, ketanserin and iloprost[84-86]. These treatments can improve Raynaud's phenomenon and digital ulcers but were not found to improve skin thickening or stop organ damage. They are less likely to be effective once vessels are totally occluded.
Drugs aimed at inhibition of formation of collagen and other extracellular matrix components by fibroblasts are D-penicillamine, colchicine, cyclofenil, N-Acetylcysteine and interferon-γ. D-penicillamine is able to cleave the labile cross-links of newly formed collagen and is currently the most widely used treatment of SSc although its presumed efficacy has never been tested in a double-blind trial. Retrospective studies showed improvement in skin thickening and improved 5-year survival compared to similar historical controls[87,88]. A multicenter double-blind trial is under way comparing 750 mg D-penicillamine with 62.5 mg D-penicillamine per day[89]. Cyclofenil inhibits production of proteoglycans and was reported to produce slight changes in skin softening in two controlled trials but allergic and hepatic reactions limit its use[90,91]. N-Acetylcysteine, which is

presumed to promote collagen breakdown, was shown to be ineffective in a double-blind trial[92] and the evaluation of colchicine, which affects collagen deposition, gave conflicting results[93]. Interferon-γ and interferon-α inhibit fibroblast proliferation and collagen production. Interferon-γ gave promising results in open uncontrolled trials[94-96] while interferon-α seemed less effective[97].
Drugs aimed at affecting immunologic changes, such as azathioprine, cyclophosphamide and cyclosporine A have been studied in several uncontrolled trials[98]. Although cyclosporine A gave promising results, the high frequency of renal toxicity limits its use in SSc[99-100].
Chlorambucil[101] and 5-fluorouracil[102] have been shown not to be superior to placebo in double-blind trials. Photopheresis was compared with D-penicillamine in a single-blind multicenter controlled trial[103]. Although photopheresis was reported to be superior to D-penicillamine, the conclusions of this study have been doubted by others[104,105].

Outcome measures in systemic sclerosis

Various outcome measures have been used in clinical trials of SSc. These measures include skin scores (determining the area and degree of the skin involved on a three- or four point scale)[106,87], physical function using the HAQ disability scale or the functional index, global assessments by patient or physician and physical performance assessments such as maximal oral opening, flexion and extension index (distance between third finger and the distal palmar crease in full flexion respectively extension), grip strength[107]. Outcome measures concerning visceral involvement comprise pulmonary function tests and chest X-rays, barium swallow of the oesophagus and oesophageal manometry or scintigraphy, creatinine clearance, electrocardiograms, echocardiograms and Holter monitoring[107]. Many of these outcome measures did not detect significant differences between a presumed active drug and placebo in double-blind trials, although this could be explained by the inefficacy of the treatments used.
Although mortality is an important endpoint in SSc, significant differences are unlikely to be found between treatment groups in trials of less than three years duration. In a recent review dealing with outcome measurements for treatments of scleroderma patients in clinical trials Pope and Bellamy concluded that skin score measurements and global assessment are the best primary outcome measures for clinical trials; good secondary measures include variables of internal organ involvement, mortality, functional assessment, and physical parameters such as grip strength and extension index[107].

METHOTREXATE

Methotrexate (4-amino-4 deoxy-N10-methylpteroylglutamic acid, MTX) resembles folic acid and is an inhibitor of folate dependent enzymes such as dihydrofolate reductase (DHFR), 5 aminoimidazole-4-carboxamide ribonucleotide, thymidine synthetase and glycinamide ribonucleotide transformylase[108]. These enzymes are involved in the pyrimidine (DNA) synthesis and the de-novo purine synthesis of DNA and RNA[109,110]. DHFR is the major of these enzymes and its inhibition by MTX leads to depletion of tetrahydrofolates, that are essential for DNA, RNA and protein synthesis.

MTX is absorbed rapidly and completely after dosages not exceeding 30 mg/m^2 [109,110] and, after absorption, fifty to seventy percent is bound to plasma proteins, mainly albumin. MTX is transported via an active carrier-mediated system into the hepatic cells[109,110], where it is converted to MTX polyglutamates. At low doses, such as used in rheumatic diseases, MTX is not subjected to much metabolism and 80 to 90% is excreted by the kidneys. MTX is also secreted in the bile, but most of it is reabsorbed. When renal function is impaired, biliary excretion is increased[111].

Low-dose MTX is a widely accepted treatment of rheumatic diseases such as rheumatoid arthritis[112-115], poly- and dermatomyositis[116,117]. Dosages administered in these diseases vary from 7.5 mg/week to 50 mg/week in one single dose or divided into three fractional doses at 12-hour intervals. Low-dose MTX has also been reported to be effective in SLE[118-120], periarteritis nodosa[121,122] and Wegener's granulomatosis[123]. The mechanisms by which low dose MTX affects the inflammatory process in rheumatic disease remain poorly understood; most studies of immune function in RA patients treated with low dose MTX show only marginal effects on humoral or cellular immune responses and the rapid clinical response to treatment as well as the equally rapid flare of disease subsequent to its discontinuation suggests an anti-inflammatory effect[124]. A recent study suggested inhibition of synovial fibroblast proliferation to be a possible action of MTX in rheumatoid arthritis[125].

Adverse reactions of MTX mainly comprise gastrointestinal complaints, gastrointestinal ulceration and hemorrhage and elevation of liver enzymes [126,127]. There is no evidence of increased liver toxicity after 2 grams MTX in rheumatoid arthritis patients[128]. MTX pneumonitis is a seldom occurring, though life threatening, complication of MTX therapy and has not been related to the cumulative dose, age or route of administration[129,130]. Hematological toxicity (leucopenia, thrombocytopenia, megaloblastic anaemia, pancytopenia) occurs in approximately 3% of patients treated with low-dose MTX[131] and seldom occurring side effects include dermal toxicity[132] and increased susceptibility to infections[133].

METHOTREXATE AND SYSTEMIC SCLEROSIS

Low-dose MTX as a treatment for patients with SSc has been described only in one report[134]. It concerned two patients, one suffering from limited SSc and the other from diffuse SSc complicated by pulmonary fibrosis. The dosage used was 25 mg MTX biweekly over a period of 6 months. In both patients improvement was obtained as assessed by amelioration of the general condition, Raynaud's phenomenon and skin thickening. For unknown reasons, this report has never been quoted in the literature concerning treatment of SSc.

AIM AND CONTENTS OF THIS THESIS

The major objective of this thesis is to determine the efficacy and toxicity of low-dose MTX in the treatment of patients suffering from systemic sclerosis.

In chapter II the literature on immunomodulating therapy is reviewed.

In chapter IIIa the first two scleroderma patients that received MTX in our department are described; in chapter IIIb we report on the data of an open study in which eight patients with SSc were treated with MTX.

The results of a randomized, double-blind, placebo controlled trial comparing low-dose MTX with placebo are described in chapter IV.

Chapter V comprises the effects of low-dose MTX on glycosaminoglycan synthesis by scleroderma fibroblast in culture.

The development of a recombinant topoisomerase for application in an ELISA is described in chapter VI.

Chapter VIIa offers changes in antitopoisomerase I antibody titers during plasmapheresis in a patient with SSc, and chapter VIIb deals with effects of MTX on antitopoisomerase I antibody titers, measured in patients involved in the double-blind trial.

In chapter VIII the evolution from MCTD to SSc, SLE, RA or a combination of these diseases in patients with anti-(U1)snRNP antibodies is described.

Finally, a summary of the results and the conclusions is offered in chapter IX.

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Immunomodulatory treatment of systemic sclerosis

Van den Hoogen FHJ, Boerbooms AMTh, Van de Putte LBA. Effects of immunomodulatory treatment in systemic sclerosis. Clin Rheum 1990;9;319-324.

Summary

We reviewed studies concerning immunomodulating therapy in systemic sclerosis. These comprise mostly uncontrolled trials and case reports. Some of these studies hint at a possible beneficial effect of antimetabolites (azathioprine, 5-fluoro-uracil and methotrexate), cyclosporine and interferon-γ. However, to give a clearcut answer on the efficacy of these drugs in systemic sclerosis, controlled studies are urgently needed.

Introduction

Systemic sclerosis (SSc) is a multisystemic disease with characteristic vascular lesions and fibrosis of the skin, synovium, muscles and internal organs, notably the gastrointestinal tract, lung, heart and kidney[1]. The etiopathogenesis of the disease is unknown, but a role for (auto)immune mechanisms is suggested by the wide range of autoantibodies found in at least 90% of SSc patients[2,3], the accumulation of T cells and an elevated T helper/T suppressor ratio in skin biopsy specimens due to a decrease in the number of suppressor cells[4,5].
Evaluation of treatment of this disorder has been difficult because of the variable course in the individual patient with occasional spontaneous remissions, and the unavailability of objective parameters on which to assess clinical improvement. No single drug or combination of drugs has proved to be of value in adequately controlled studies[6]; the literature on this subject comprises mostly retrospective or uncontrolled studies and case reports.
In view of the presumed immune-mediated pathogenesis of systemic sclerosis, drugs with immunoregulatory activities have also been used in the treatment of patients with SSc. If one excludes corticosteroid therapy, these drugs consist of alkylating agents, purine antimetabolites, cyclosporine and interferon-γ. Experience with these drugs in the treatment of SSc will be briefly reviewed.

Alkylating agents

Chlorambucil

Mackenzie[7] treated 11 patients with chlorambucil (0.1 mg/kg a day) for 18 months and noted absence of further progression in every organ system studied, with healing of all fingertip ulcers and increased finger movements. There was also improvement in pulmonary function studies in 6 patients. Ansell et al.[8], using chlorambucil in doses between 4 and 7 mg daily in the treatment of a 15-year-old girl, noticed prolonged considerable loosening of the skin and improvement of the flexion contractures, even after discontinuation of the drug. These encouraging results could not be substantiated by Steigerwald[9], who treated 27 patients with the same dosage of chlorambucil: seven patients died after 2 to 37

months of therapy (two from renal disease and five from progressive cardiorespiratory involvement); the remaining patients showed no evidence of any regression or even stabilization of the disease except for improvement in myositis and arthritis. Saporta et al.[10], using 0.2 mg/kg a day during 2 years in two patients, found no changes in sclerotic skin or systemic manifestations, and an extensive double blind placebo-controlled study with 0.05-0.1 mg/kg a day by Furst et al.[11], conducted over a 3-year period in 65 patients, failed to show significant differences between treatment groups.

Cyclophosphamide

Cyclophosphamide has been reported incidentally in the treatment of a day for 6 months without any effect, and Chaouat et al.[12] treated two patients with cyclophosphamide: one showed some clinical improvement while the other did not (data on cyclophosphamide dosage and duration of therapy were not given).
Hurd and Giuliano[13] studied the effect of therapeutic cyclophosphamide 2 mg/kg a day. They found a reduction of both B and T lymphocytes as early as 2 weeks after onset of treatment in each case, with eventual depletion of both cell types, but no data are reported of any effect on the disease itself.
Tolchin et al.[14] observed a significant increase in the number of cytogenic aberrations in five patients during therapy with cyclophosphamide, 2 mg/kg a day, ranging from 2-25 months; again no mention was made of any effect on the disease itself.
In an uncontrolled trial Dau et al.[15] found a combination of plasmapheresis with prednisone and cyclophosphamide therapy to produce clinical improvement in 14 of 15 patients with varying degrees of skin and internal organ involvement. The extent to which cyclophosphamide might have contributed to this improvement remains unclear.
Kaplan and Ward[16] found a combination of plasmapheresis, steroids and cyclophosphamide effective in reversing previously irreversible serious gastrointestinal and pulmonary complications of SSc in five patients.
To separate effects of combined plasmapheresis and immunosuppression from immunosuppression alone, Wollheim and Åkesson[17] started immunosuppression (cyclophosphamide 2 - 2,5 mg/kg during the first year and azathioprine during the second year) in seven patients, and combined therapy in eight; all patients received prednisolone at the same time. Although far from conclusive, their results showed a tendency in favor of the combined group.

Anti-metabolites

6-Thioguanine

Demis et al.[18] were the first to report in 1964 the use of an anti-metabolite in the treatment of SSc; they used a daily dose of 1 mg/kg 6-thioguanine for two weeks

each month in two patients. After 6 resp. 8 months of treatment they found no clear, objective evidence of significant prolonged improvement, although both patients did report softening of the skin; two years after discontinuation of the drug no progression of the disease was noted.

Azathioprine

Jansen et al.[19] treated 21 patients with azathioprine, 150 mg a day for 5 to 23 months; eight patients were judged to have improved subjectively and by clinical evaluation during the course of the treatment, seven were unchanged and two showed progression of their disease. One patient was lost to follow-up; three had to discontinue the drug because of side effects. These uncontrolled results were said to be slightly better than those observed in other groups of untreated patients.
Kurwa and Denman[20] reported on one patient using a maintenance dose of 150 mg azathioprine on weekdays in combination with 10 mg prednisone daily. They found improvement in the range of joint motion and some softening of the skin on arms and legs.
Saporta et al.[10] treated two patients with the acrosclerotic form of SSc with 2.5 to 3 mg azathioprine/kg a day during 5 and 17 months respectively; no effect on the sclerodermatous skin or visceral manifestations could be observed.
Thivolet and Perrot[21] noted no effect of 6-mercaptopurine, a metabolite of azathioprine (total dose 5250 mg in 34 days), and minor improvements in skin hardening but deterioration of pulmonary function tests after treatment with azathioprine (total dose 2165 mg in 3 months) in two patients.
Maas et al.[22] reported on 19 patients who received 2-2.5 mg azathioprine/kg daily. In 16 cases no further progression of the disease was noted, in particular no further deterioration in lung and kidney manifestations. One patient showed minor deteriorations and two died: one from osteomyelofibrosis 3½ years after azathioprine had been discontinued, the other from right heart failure after recurrent pulmonary emboli. The authors stated that, on the whole, therapy with azathioprine over a long period of time seems in most cases to prevent further progression of the disease.

5-Fluoro-uracil (5-FU)

Casas et al.[23] treated 12 patients with 12.5 mg 5-FU/kg a day intravenously (IV) in 4 - 5 doses, followed by 4 additional doses (8-10 mg/kg/day) given IV every two days, followed by a weekly dose of 10-20 mg/kg IV. Significant and objective improvement occurred initially in the skin and subsequently in the involved viscera and vasculature. The authors suggested that 5-FU may be effective in the treatment of SSc and started a double-blind study in patients with newly diagnosed SSc of which the results are not yet published. Malaviya et al.[24], using the same treatment with slightly reduced doses in 11 patients, had to discontinue this treatment because of major toxicity in three patients, while six other patients showed minor side effects. The duration of their study was too short to assess any beneficial effect of 5-FU.

Methotrexate

Welin et al.[25] treated two patients with SSc complicated by Sjögren's syndrome, with 25 mg methotrexate IV every two weeks up to a total dose of 275 and 350 mg respectively. Both patients improved in range of motions of the fingers and one in softening of the skin of the dorsum of the hands.
The same results were recently obtained by Van den Hoogen et al.[26], who treated one patient with weekly doses of 15 mg methotrexate intramusculary and one patient with 5 mg methotrexate orally (lower dose because of reduced renal function) and noted softening of the skin after 3 months of therapy; during the six months of their study no deterioration of renal function or other organ systems tested occurred.

Cyclosporine

Zachariae and Zachariae[27] noted impressive improvement of the sclerotic skin of the upper extremities and to a certain extent also of the skin of the trunk within four months of initiating cycloclosporin A (CyA) 7 mg/kg a day in a patient with diffuse SSc; they also found increased grip strength and lip-lip distance and decreased pulpar-volar distance in a patient with acrosclerosis, treated with the same dose.
Yocum and Wilder[28] also noticed improvement of sclerotic skin six weeks after starting CyA therapy, 10 mg/kg daily in one patient with diffuse SSc and a marked increase in skin disease after discontinuation of the drug. After reinstitution of CyA therapy gradual improvement reappeared.
The most striking result of CyA therapy was reported by Appelboom and Itzkowitch[29], who treated one patient with pulmonary, cardiac and gastrointestinal manifestations with CyA (drug serum concentration was adjusted to 200 ng/ml during the entire treatment). Not only was dramatic diminution of skin abnormalities seen, but improvement of pulmonary function tests, echocardiography and barium swallow study also occurred. Anti-Scl-70 antibodies, demonstrable before the start of CyA therapy, were no longer detectable at the end. Progressive renal insufficiency developed, but this completely resolved within 4 weeks of discontinuation of CyA.
Russell and Schachter[30] reported on treatment of 5 patients with SSc and one with morphea profundus with CyA. They found improvement in skin score and discomfort in 3 out of 4 patients who were treated for at least 6 months; two patients had permanent healing of severe skin ulcerations. After 8 months of treatment one patient developed renal failure typical of SSc, the other patients showed no deterioration of internal organ involvement during the study.

Interferon-γ

Recently Kahan et al.[31] published the results of a study in which they treated ten patients with recombinant interferon-γ (IFN-γ) once daily for seven days per week by intramuscular injections. The dosage of IFN-γ was gradually increased from 10 μg/day to 100 μg/day in one month and continued for another five months. One patient dropped out of the study after six weeks because of rapid worsening of the disease. The other nine patients showed a significant improvement in total skin score, maximal oral opening, range of motions of wrists and elbows, grip strength, functional index, dysphagia and creatinine clearance. No serious side effects occurred.

No drug with immunoregulatory activities has ever been shown to be effective in the treatment of SSc in an adequately controlled study. There are, however, prospective uncontrolled studies and case reports that hint at a possible beneficial effect of some of these drugs, chiefly anti-metabolites (azathioprine, MTX and 5-FU), cyclosporine and IFN-γ. In the absence of any drug available for the treatment of patients with SSc, these findings, even if uncontrolled, do justify clinical trials with these agents. Because of the extremely variable course of SSc these clinical trials ought to be placebo controlled in order to give a clearcut answer on the efficacy of the drug in the treatment of patients with SSc. The best SSc-patients for study are those whose disease is of recent onset[32]; furthermore, the SSc-patients should be studied over a prolonged period of time, preferably several years. In view of the positive results we obtained in a prospective study in which patients with SSc were treated with MTX[26], we recently started a double-blind placebo-controlled study in which the efficacy of MTX in the treatment of SSc will be assessed.

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Methotrexate treatment in systemic sclerosis: open studies

a. Van den Hoogen FHJ, Boerbooms AMTh, Van de Putte LBA. Methotrexate treatment in scleroderma. Am J Med 1989;87:116-117.

b. Van den Hoogen FHJ, Boerbooms AMTh, Van de Putte LBA, Rasker JJ, Van Venrooij WJ. Low dose methotrexate treatment in systemic sclerosis. J Rheumatol 1991;18:1763-1764.

A. Methotrexate treatment in scleroderma

Many drugs have been used for the treatment of scleroderma (systemic sclerosis, SSc), all without convincing evidence of efficacy[1]. We report the beneficial effect of methotrexate (MTX) therapy in two patients with SSc.

Patient 1.

A 67-year-old man had a two-year history of Raynaud's phenomenon, arthralgias and stiffness in both hands, and symmetric proximal muscle weakness. A diagnosis of overlap syndrome, consisting of SSc and polymyositis, was made based on sclerodactyly and scleroderma skin changes in both forearms and in the face, and typical electromyogram and muscle biopsy alterations with elevated serum creatine kinase values (maximum, 1900 U/L). Other symptoms included a diminution of peristaltic activity in the distal part of the oesophagus on cinefluoroscopic examination and complete left bundle branch block on the electrocardiogram. Autoantibodies to Scl-86[2] or Jo-1 antigen were not detectable. Treatment was started with weekly intramuscular injections of 15 mg of MTX. A gradual improvement of skin hardening was noted about three months after the beginning of therapy and continued for six months, when MTX had to be discontinued because of an aspergillosis lung infection. At that time, the scleroderma skin changes were only detectable in the fingers, the total skin score[3] was 8 compared with 22 at the starting point, and serum creatine kinase values had returned to normal. The lung infection was successfully treated with antibiotics.

Patient 2.

A 55-year-old woman had a two-year history of Raynaud's phenomenon and progressive scleroderma skin changes eventually covering the arms, legs, face and upper part of the thorax. Treatment with D-penicillamine had to be stopped because of severe side effects, and no improvement occurred with corticosteroid therapy (maximum, 15 mg/day). Instead, the scleroderma skin changes increased and an impairment of renal function developed (endogenous creatinine clearance of 45 mL/minute), accompanied by hypertension. Autoantibodies to Scl-86 antigen were not detectable. Treatment with MTX was commenced at a dosage of 5 mg/day (dosage was lower than that in patient 1 because of impaired renal function) given orally; corticosteroid therapy was continued at a dosage of 7,5 milligrams/day and hypertension was controlled with nifedipine and hydrochlorothiazide. After three months of therapy, an improvement occurred in scleroderma skin changes and Raynaud's phenomenon; renal function showed no further deterioration. After six months of therapy, the total skin score was 22 as compared to 41 at the beginning of therapy. No side effects of MTX were noted.

These observations suggest not only an effect of MTX on the progression of skin fibrosis, but also an improvement of already established skin fibrosis in patients with SSc. Our findings are in agreement with those previously reported by J. Welin et al[4], who also showed a softening of fibrotic skin in two patients with SSc receiving MTX therapy. However, the course of SSc is extremely variable and spontaneous improvement can occur; these observations therefore need to be confirmed in a controlled study. At this time, we are performing a double-blind trial of MTX and placebo in patients with SSc to assess the presumptive efficacy of MTX in SSc.

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B. Low Dose Methotrexate Treatment In Systemic Sclerosis

Many drugs have been used in the treatment of patients with systemic sclerosis; however, no convincing evidence of efficacy of any of these drugs could be established[1]. Methotrexate (MTX) has been successfully applied in polymyositis/-dermatomyositis[2,3] and rheumatoid arthritis[4-6], diseases that display overlapping features with systemic sclerosis.
We describe the results of a pilot study in which eight patients with systemic sclerosis according to the preliminary ARA criteria[7] were treated with MTX. The study lasted one year, and prior to the start of it all patients had active disease with either progression of skin fibrosis or persistent digital ulcerations. Four patients had previously been treated with D-penicillamine, but in all these patients this drug was stopped at least four months before the study because of lack of efficacy or side effects. Concurrent low dose prednisone therapy was continued in three patients.

Treatment consisted of 15 mg MTX/week, given intramuscularly for the first six months and orally thereafter. One patient received a reduced dose of 5 mg MTX/week orally throughout the study because of impaired renal function. The demographic and clinical characteristics of the patients are listed in Table 1.
Assessment at entry and after 6 and 12 months consisted of pulmonary function tests; chest, hand and feet radiographs, barium swallow of the oesophagus and electrocardiogram. During the first 6 months, patients' cases were evaluated every month, thereafter every two months. These evaluations included a detailed clinical examination performed by the same investigator and assessments of the degree and extension of skin fibrosis using the total skin score according to Steen[8]. Also measured were maximal oral opening and distance of the third fingertip to the most distal crease of the wrist with the fingers maximally extended (fingertip-wrist distance). During each visit, blood samples were drawn for analysis of blood cell count, liver function tests, creatinine, creatine phosphokinase, immunoglobulins, complement levels and antitopoisomerase-I antibodies, using a cloned topoisomerase protein as substrate in an ELISA[9]. Creatinine clearance was calculated using the Cockroft formula[10]. Student's t-test for continuous variables was used to calculate differences between baseline values and values recorded after 6 and 12 months.

Seven patients completed the study. In one patient MTX was stopped after 6 months because of pulmonary aspergillosis; the one-year evaluation of this patient is included in the overall results. Total skin score diminished from average 28.5 ± 18.3 at baseline to 14 ± 10.5 ($p<0.01$) after one year (fig. 1). Skin softening was usually noted after 2 to 3 months of MTX therapy. Every patient had a reduction in total skin score of at least 25%, except for one patient, who experienced a rapid progression of skin fibrosis with cardiac, pulmonary and gastrointestinal involvement just prior to the start of the study. Maximal oral opening increased from average 39.1 ± 18.3 mm to 46.0 ± 9.4 mm ($p<0.05$). No significant change

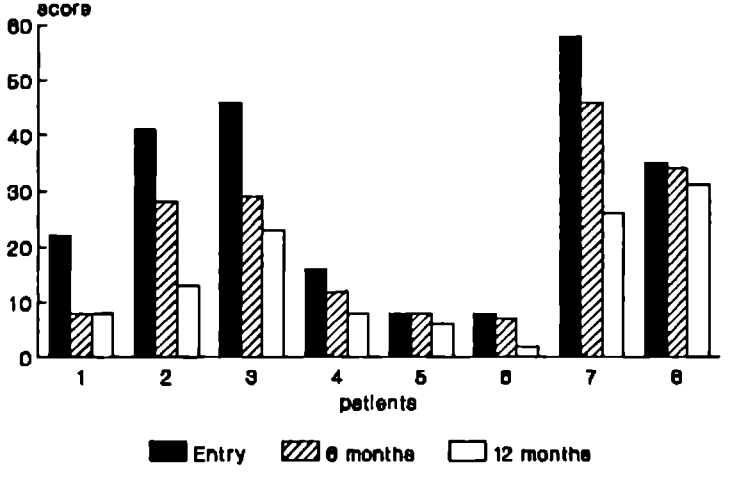
Table 1: Clinical characteristics of trial patients

Pat	Sex	Age	Disease Duration (months)	Ltd Dif	Dig Ulc	Anti Topo	Previous Treatment	ECC	Bibas Fibr	Cardiac Conduct Disturb	Cardio-megaly	Restr Lungf	Hypoth Oesoph	Calc
1	M	67	24	ltd	-	-	-	76	-	+	+	-	+	-
2	F	54	24	dif	-	-	Pred,D-pen	33	-	-	-	-	-	
3	M	40	22	dif	-	-	D pen	131	-	-			-	-
4	F	48	84	ltd	+	+	Pred,D pen	95	+	-	-	+	+	+
5	M	58	19	ltd	+	+	-	90	-	+	-	-	-	
6	F	40	22	ltd	+	-	-	95	-	-	-	+	-	-
7	F	44	26	dif	-	-	Pred,D-pen	98	-	-	-	+	+	-
8	M	54	8	dif	+	+	-	120	+	+	+	+	+	+

ltd = scleroderma limited to lower arms and/or face, dif = scleroderma also of upper arms and/or trunk, dig ulc = digital ulceration, anti topo = antitopoisomerase I antibodies, pred = prednisone therapy, D pen = D penicillamine therapy, ECC = endogenous creatinine clearance (ml/min), bibas fibr = bibasilar fibrosis on chest radiogram, cardiac conduct disturb = cardiac conduction disturbance, restr lungf = restricted lung function, hypot oesoph = reduced oesophageal peristalsis, calc = calcinosis, pat = patient

occurred in fingertip-wrist distance, though a slight improvement was seen in five patients. Likewise, no significant changes occurred in the results of pulmonary function tests and chest radiographs nor were changes observed in cardiac conduction on electrocardiograms. In one patient, barium swallow revealed a diminished peristalsis of the oesophagus after 6 months that was not present at the beginning. Digital ulceration disappeared in three patients and markedly improved in one. From the start of the study there was a gradual though after 12 months significant, decrease of creatinine clearance; mean creatinine clearance fell from 92.2 ml/min ± 29.5 to 85.4 ± 45.3 ml/min ($p<0.05$). The absence of other internal organ involvement suggests that this mild decrease in creatinine clearance may be caused primarily by MTX itself rather than by progression of renal sclerosis. Of all other serological variables tested, a reduction was found in circulating immune complexes from average 7.37 ± 3.54% to 2.25 ± 0.70% ($p<0.01$) after 12 months; IgM levels decreased from 2.06 ± 0.89 g/L to 1.69 ± 0.75 g/L ($p<0.01$) during the first 6 months of the study to remain steady thereafter, and C3-levels increased from 1033 ± 227 g/L to 1303 ± 254 g/L ($p<0.05$) during the last 6 months of the study. The anti-topoisomerase titers declined in all three positive patients. Whether these changes reflect a direct effect of MTX on the immune system or must be looked upon as secondary changes due to inactivation of the disease process cannot be elucidated from our data.

Figure 1 Total skin score at study entry and after 6 and 12 months of low dose MTX treatment



In two patients, MTX therapy was temporarily withdrawn: one patient had a transient rise in transaminase values and the other patient had a herpes zoster infection of the 6th cervical nerve. After restarting MTX no further complications were encountered.

Our data suggest a beneficial effect of MTX in the treatment of patients with systemic sclerosis with good tolerance of the drug. However, the short duration and the open design of the study preclude definite conclusions about any beneficial effect of MTX in systemic sclerosis and a double-blind placebo controlled trial is urgently needed to confirm the results of this pilot study. Such a trial is currently being conducted in our department.

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Methotrexate versus placebo in the treatment of systemic sclerosis

Van den Hoogen FHJ, Boerbooms AMTh, Swaak AJG, Rasker JJ, Van Lier HJJ, Van de Putte LBA. Comparison of methotrexate with placebo in the treatment of systemic sclerosis. A forty-eight week randomized double-blind trial. Submitted.

Summary

Objective - To compare methotrexate with placebo in the treatment of systemic sclerosis in a 48 week, randomized, double-blind, placebo-controlled trial.

Methods. Twenty-nine scleroderma patients were allocated to receive weekly injections of either 15 mg methotrexate or placebo. Patients who responded favorably after 24 weeks continued with the same regimen for a further 24 weeks; those who showed a poor response on placebo were allocated to further treatment with 15 mg methotrexate weekly, and those who responded poorly to treatment with 15 mg methotrexate weekly had their doses increased to 25 mg. Favorable response was defined as improvement of total skin score (TSS) by ≥30%, of single breath diffusion capacity (DL_{CO}) by ≥15%, or of the score on a visual analogue scale of general well being (VAS) by ≥30%, provided that such improvements were not accompanied by persistent digital ulcerations or worsening of DL_{CO} ≥15%.

Results - Seventeen patients were allocated to methotrexate treatment; 12 to treatment with placebo. Comparison between the two treatment groups at week 24 showed improvement in the methotrexate group of TSS (p=0.06), VAS (p=0.17) and creatinine clearance (p=0.12). The number of withdrawals was two in both groups and due to renal crisis (n=2), progressive cardiopulmonary involvement (n=1) and severe headache (n=1). Breaking the code after 48 weeks of double-blind treatment, it appeared that after 24 weeks a significant larger number of patients receiving methotrexate (n=8, 53%) that completed the first 24 weeks of the study had responded favorably compared to patients receiving placebo (n=1, 10%, p=0.03). At week 48, 13 patients received methotrexate from the start of the study and nine during 24 weeks. From these 22 patients, 15 (68%) responded favorably and compared with the start of the study they showed significant improvement of TSS (p=0.04), VAS (p=0.02), grip strength of right hand (p=0.02) and ESR (p=0.01). Two patients, both receiving an enhanced dose of 25 mg methotrexate died during the second 24-week period: one because of progressive disease and one presumably due to acute myocardial infarction.

Conclusion - Low-dose methotrexate appears to be an effective treatment of systemic sclerosis.

Systemic sclerosis (SSc) or scleroderma is a multisystemic disease characterized by excessive deposition of collagen and other extracellular matrix components by fibroblasts, damage to the endothelium of small vessels, resulting in intimal hyperplasia and tissue ischaemia, and activation of the immune system. These phenomena may lead to progressive fibrosis of the skin, muscles, joints, and internal organs, accounting for many of the clinical manifestations[1,2]. The natural course of the disease may vary: a few patients experience spontaneous remission; the majority undergoes progression of skin and internal organ involvement, resulting in considerable morbidity and ultimately in death. Several studies have given estimates of five-year cumulative survival rates ranging from 34-73%[3]. Male gender, older age, involvement of heart, lung or kidney, presence of antitopoisomerase I autoantibodies, and diffuse skin involvement adversely affect outcome[4-6]. SSc is a rare disease, with an estimated annual incidence rate of 20 new cases per million[7]. Its pathogenesis is unclear, but evidence suggests an autoimmune or vascular aetiology[8]. No treatment has proven convincingly to be effective. Several studies have reported favorable effects of certain drugs, but most of them consist of case reports, uncontrolled trials, or studies with historical controls [9-15]. Placebo-controlled, double-blind trials in SSc are scarce, and have given negative or inconclusive results[16-23].

Methotrexate (MTX) is an antifolate drug. In low dosages it has shown favorable effects in the treatment of autoimmune diseases such as rheumatoid arthritis (RA) [24-28] and dermato- and polymyositis[29,30]. We recently reported the results of a one-year pilot study in SSc patients treated with low-dose MTX. In the majority of the patients cutaneous symptoms improved within six months and no further internal organ deterioration was detected[31]. Similar encouraging observations have been reported in the meantime[32]. To obtain more data about the role of MTX in the treatment of SSc we conducted a double-blind trial comparing MTX and placebo, focussing on the efficacy and toxicity of these treatments and on the differences between responders and nonresponders on MTX treatment.

Patients and methods

Patients

The inclusion criteria for the trial consisted of the preliminary criteria for the classification of SSc of the American Rheumatism Association[33] and the requirement that disease duration from the first signs of skin thickening was less than three years. Patients with longer disease duration were also included if they had experienced a progression of skin thickening, persistent digital ulcerations, or a deterioration in pulmonary function, during the last six months. All patients voluntarily signed an informed consent form; the study protocol was approved by the institution's ethical committee.
We applied the following exclusion criteria: age less than 16 years; the presence

of another connective tissue disease or SSc-like illness related to exposure or ingestion; the presence of acute or chronic infection; pregnancy or childbearing potential without an acceptable means of contraception; the presence of liver disease, defined as a value exceeding twice the upper limit of normal for a hepatic function test or the presence of a known liver disease; serum creatinine level >130 μmol/l or a creatinine clearance rate <50 ml/minute as estimated by the method of Cockcroft and Gault[34]; total lung capacity (TLC), vital lung capacity (VC), or single breath diffusion capacity for carbon monoxide (DL_{CO}) <50% of its predicted value; a leucocyte count of <3.5 x 10^9/liter or platelet count <150 x 10^9/liter; the presence of a concurrent neoplastic disease; the presence of insulin-dependent diabetes mellitus; alcohol abuse (more than 4 oz/day); the use of an antifolate drug other than MTX such as sulfonamide derivates, allopurinol or probenecid; and the presence of active peptic ulcer disease.

Study design

The study was set up as a multicenter single observer trial. Twenty-nine patients were enrolled between November 1989 and November 1991. Potentially disease-modifying drugs, such as d-penicillamine and colchicine, were discontinued at least three months prior to study entry. Patients were randomly allocated to treatment with either MTX (Ledertrexate, Lederle Nederland bv, The Netherlands) or placebo, both of which were administered weekly by intramuscular injection. The weekly dose of MTX was initially 15 mg. The two groups were balanced for disease duration (time between onset of skin thickening and entry to the trial) and extent of skin involvement, known prognostic factors for SSc.

Since there is no consensus concerning the outcome measures to be used in assessing disease activity and, hence, response to treatment in SSc, we set up our own criteria by which response can be evaluated. We based them arbitrary on data from previous studies and on our own experience. They employ the following variables: total skin score (TSS), which is the sum of scores of 0-4 obtained manually at 26 anatomic locations, described by Steen et al.[9]; visual analogue scale (VAS) of general well-being, as determined by the patient on a 100-mm scale on which 100 mm represents optimal general well-being; lung diffusion capacity as reflected by DL_{CO}; and presence or absence of digital ulcerations. Response to treatment was defined as favorable if TSS or VAS improved by ≥30% or if DL_{CO} improved by ≥15%. However, when digital ulcerations developed or persisted or when DL_{CO} decreased by ≥15%, despite improvement of VAS or TSS, the response was defined as unfavorable. Evaluation of response criteria was performed double-blind at week 24 and 48. Patients with favorable response after 24 weeks of treatment continued the same regimen for another 24 weeks. Nonresponders on placebo after 24 weeks were started on MTX 15 mg weekly; nonresponders on 15 mg MTX weekly received an increased dose of 25 mg weekly for the remaining 24 weeks of the trial. The clinical examiner and the patients were blind regarding the changes in treatment; the treatment code was broken only after all patients had completed the 48 week study. All subjects were outpatients at the time of enrolment and were admitted to hospital during the

course of the study only in the event that severe complications occurred.

Clinical assessment

At study entry a detailed medical history was taken and the patient was given a thorough physical examination. Each patient was evaluated monthly during the trial year by the same investigator (FHJvdH). The evaluations consisted of: a detailed clinical examination and the determination of TSS, VAS, extension indices for both hands, grip strength in both hands, and maximal oral opening. An extension index is the distance between the tip of the third finger and the distal palmar crease with the hand fully extended. Grip strength was measured by a sphygmanometer (Tonometer, von Recklinghausen, Germany) with a range of 0-300 mmHg; the value recorded was the maximum of three consecutive measurements. At study entry and after 24 and 48 weeks of treatment, pulmonary function tests, including those for TLC, VC and DL_{CO} were performed, barium swallow of the oesophagus was measured, an electrocardiogram (ECG) was made, and a radiologic examination of the chest was performed.

Laboratory assessment

Haemoglobin level, white blood cell (WBC) and platelet counts, and hepatic and renal functions were evaluated weekly during the first month of treatment and then every four weeks for the remainder of the study. Hepatic function tests consisted of the assessment of alkaline phosphatase, serum aspartate aminotransferase (AST), and serum alkaline aminotransferase (ALT) levels; renal function tests, of the assessment of serum creatinine and creatinine clearance rate according to the method of Cockcroft and Gault[34]. Other laboratory variables measured once every four weeks from the beginning of therapy were: erythrocyte sedimentation rate (ESR) according to Westergren, serum levels of IgA, IgM, IgG, C3, C4 and circulating immune complexes and urinalysis. At study entry patients were tested for antinuclear antibodies, by immunoblotting and counter immunoelectrophoresis, and for rheumatoid factor, by the latex fixation test. All laboratory tests except the hepatic and renal function tests and those for ESR and blood cell counts, were performed at the University Hospital Nijmegen, to ensure that uniform methods were used.

Organ involvement

Pulmonary dysfunction was defined as a VC and TLC $\leq$80% or DL_{CO} $\leq$70% of predicted normal values or chronic interstitial changes on chest roentgenograms; oesophageal dysfunction, as diminished motility as determined by barium swallow; cardiac dysfunction, as conduction disturbances on an ECG or cardiomegaly on a chest X-ray; renal dysfunction, as serum creatinine level >130 µmol/l or a creatinine clearance rate of <50 ml/minute as estimated according to the method of Cockcroft and Gault[34]; and muscular involvement was defined as serum creatine phosphokinase (CPK) level more than twice the upper limit of normal,

without proximal muscle weakness and without specific changes on an electromyogram. Muscle biopsies were not performed. SSc was considered diffuse if sclerotic skin changes were present proximal to the elbows or knees; SSc was considered limited if skin changes were present distal to the elbows or periorally with other areas being unaffected.

Concurrent medication

Concurrent treatment with corticosteroids at dosages not exceeding 10 mg/day, nonsteroidal anti-inflammatory drugs, analgesics, nifedipene, ketanserine, cimetidine or omeprazol was permitted. No changes in dosages were permitted from eight weeks before study entry until the end of the trial.

Adverse reactions and withdrawals from the study

At each visit patients were routinely questioned about symptoms related to MTX toxicity. The trial medication was temporarily withheld if the WBC count was less than 3.0 x 10^9/l, if the platelet count was less than 150 x 10^9/l, if liver enzyme levels exceeded three times the upper limit of normal, or if serum creatinine levels exceeded 160 µmol/l, for two consecutive measurements. Treatment was resumed once the values had normalized. If the same abnormality reoccurred a second time, after recovery the patients received a dose reduced by 50%. A third occurrence resulted in the patient's being permanently withdrawn from the study. Patients who failed to keep scheduled appointments at the clinic or whose clinical conditions deteriorated to such an extent as to necessitate other therapy were also permanently withdrawn.

Statistical analysis

Categorical variables were compared with Fisher's exact test. The Wilcoxon test was used to compare the changes in the variables over time in the MTX group with those in the placebo group. Ninety-five percent confidence intervals were calculated. For changes within the MTX group the sign rank test was used. P values of 0.05 or less were considered significant.

Results

Patient characteristics

Twenty-nine patients were enrolled in the study. Seventeen were allocated to MTX treatment; 12 to placebo treatment. This difference in numbers resulted from the fact that by mistake two patients in the MTX group were initially recorded as belonging to the placebo group. The error was discovered after breaking the code at the end of the study. Due to this error the number of patients

with diffuse and limited cutaneous involvement is also different, though not statistically significant, in both groups. Table 1 and the first column of Table 2 give the initial demographic, clinical, and laboratory data of the two groups. The duration of cutaneous involvement, and the prevalence of Raynaud's phenomenon in the two groups were similar. Disease duration was less than one year for seven patients (41%) in the MTX group and four (33%) in the placebo group. Five patients receiving MTX (29%) and six patients receiving placebo (50%) were classified as having diffuse SSc. Digital ulcerations or pitting scars were present in 12 patients (70%) in the MTX group and in five (42%) in the placebo group. No autoantibodies other than antitopoisomerase I-, anti-centromere-, and anti-RNP-antibodies and rheumatoid factor could be detected. CPK values were significantly higher ($p=0.04$) in the MTX group. Muscular involvement was somewhat more prevalent in the MTX group ($p=0.10$). There were no significant differences between the two groups with regard to internal organ involvement or previous treatment.

Course of treatment

The course of the trial is outlined in fig. 1. During the first 24 weeks of treatment two patients in each treatment group had to be withdrawn from the study (see further). After breaking the code at week 48, it appeared that eight (53%) of the 15 patients in the MTX group who had completed the first 24 weeks had responded favorably after 24 weeks, whereas nine (90%) of the patients receiving placebo and completed the first 24 weeks did not respond favorably. This difference is significant: $p=0.03$. The favorable response among the patients receiving MTX was due to improvement in TSS in three cases, to improvement in VAS in four, and to improvement in both in one case. One patient whose VAS improved ≥30% was classified as nonresponder, owing to a ≥15% decrease in DL_{CO}. The patient who responded while undergoing placebo treatment had ≥30% reduction in TSS.
Two further patients, both of whom had been started on an increased dose of 25 mg MTX weekly, had to be withdrawn between weeks 24 and 48 (see further), leaving 23 patients to complete the trial. At week 48, of the nine patients who had been transferred from placebo to MTX treatment after the first 24 weeks, five had responded favorably with ≥30% improvement in TSS. Two patients who had not responded to 15 mg MTX weekly during the first 24 weeks responded favorably to the increased dose of 25 mg with an improvement of ≥30% in VAS. Thus at week 48, of the 22 patients who had completed the trial and had been treated with MTX for at least 24 weeks, 15 (68%) responded favorably according to the predetermined criteria.

Comparison of groups after 24 weeks

The results of only those patients who completed the first 24 weeks of treatment were included in the analysis. Ten patients from the placebo group and 15 from the MTX group (fig. 1) were eligible. The differences between the initial and

Table 1: Patient characteristics at study entry*

Variable		Methotrexate group (n=17)	Placebo group (n=12)
Age, mean ± SD years (range)		52 ± 12 (32-75)	56 ± 11 (39-72)
Duration of cutaneous SSc, mean ± SD years (range)		3 2 ± 6 3 (0 1-27)	3 2 ± 3 4 (0 6-12)
Sclerodermatous skin distribution			
	diffuse	5 (29)	6 (50)
	limited	12 (71)	6 (50)
Male		7 (41)	2 (17)[#]
Raynaud's phenomenon		17 (100)	11 (92)
Duration of Raynaud's phenomenon mean ± SD years (range)		3 5 ± 7 0 (0 4-30)	5 6 ± 9 5 (0 9-34)
Digital ulcers/scars		12 (70)	5 (42)
Autoantibodies			
	ANA	15 (88)	11 (91)
	Anti-TOPO	9 (53)	7 (58)
	Anticentromere	0	1 (9)
	Anti-RNP	1 (6)	0
	Rheumatoid factor	5 (29)	3 (25)
Prevalence of organ involvement			
	Pulmonary	9 (53)	6 (50)
	Cardiac	3 (18)	4 (33)
	Renal	0	0
	Oesophageal	15 (88)	10 (83)
	Muscular	5 (29)	0[@]
Previous treatments			
	Penicillamine	6 (35)	4 (33)
	Prednisone	3 (18)	4 (33)
	NSAID's	5 (29)	1 (8)
	Colchicine	1 (6)	0

* Values given are the number of patients and values in parentheses are percentages, unless otherwise indicated

SSc=systemic sclerosis, ANA=antinuclear autoantibodies, RF=rheumatoid factor; anti-TOPO=antitopoisomerase I antibodies, anticentromere=anticentromere antibodies, anti-RNP=anti-ribonucleoprotease antibodies, [#]p=0 32 and [@]p=0 10 for differences between groups

Table 2: Clinical and laboratory variables: mean ± SD values at study entry and differences after 24 weeks of treatment*

Variable		Baseline		week 24		p-value MTX vs placebo
		MTX group (n=17)	Placebo group (n=12)	MTX group (n=15)	Placebo group (n=10)	
Total Skin Score		20 2 ± 11 0	20 7 ± 11 4	-0 7 (3 9, 2 5)	1 4 (-1 5, 4 3)	0 06
Extension index right (mm)		95 0 ± 12 2	88 4 ± 20 2	-1 7 (-5 1, 1 7)	-1 6 (-3 5, 0 3)	0 78
Extension index left (mm)		97 1 ± 15 0	95 1 ± 13 8	-0 6 (3 4, 2 2)	-0 4 (-3 3, 2 5)	0 77
Grip strength right (mm Hg)		88 7 ± 70 4	116 2 ± 56 0	21 4 (3 4, 46 2)	20 1 (-9 1, 49 3)	0 48
Grip strength left (mm Hg)		99 5 ± 77 1	121 8 ± 68 7	0 5 (18 5, 19 5)	-1 0 (-16 0, 14 0)	0 39
Oral opening (mm)		41 9 ± 9 3	38 7 ± 6 5	-2 7 (3 7, -1 7)	0 2 (-2 0, 2 4)	0 68
General health (0-100mm VAS)		54 1 ± 18 0	56 1 ± 17 3	7 8 (3 7, 19 3)	0 8 (-6 4, 8 0)	0 17
Organ involvement						
Lung	Fibrosis (N,%)	5 (29)	5 (42)	5 (33)	4 (40)	1 0
	TLC (% predicted)	87 2 ± 12 7	82 3 ± 16 9	-1 3 (-4 0, 1 4)	-0 6 (-2 7, 1 5)	0 58
	VC (% predicted)	92 7 ± 18 0	86 1 ± 20 3	-3 1 (-7 8, 1 6)	-2 0 (-6 0, 2 0)	0 60
	DL_{CO}	1 42 ± 0 25	1 45 ± 0 37	-0 04 (-0 08, -0 00)	-0 01 (-0 12, 0 10)	0 94
Cardiac (N,%)		3 (18)	4 (33)	4 (27)	3 (30)	1 0
Renal (N)		0	0	0	0	
Creatinine clearance rate (ml/min)		77 9 ± 18 3	75 4 ± 13 5	4 3 (-0 6, 9 2)	-0 7 (-3 5, 2 1)	0.12
Esophageal (N,%)		15 (88)	10 (83)	13 (87)	8 (80)	1.0
Muscular (N,%)		5 (29)	0	4 (27)	0	0 13

Table 2 (continued)

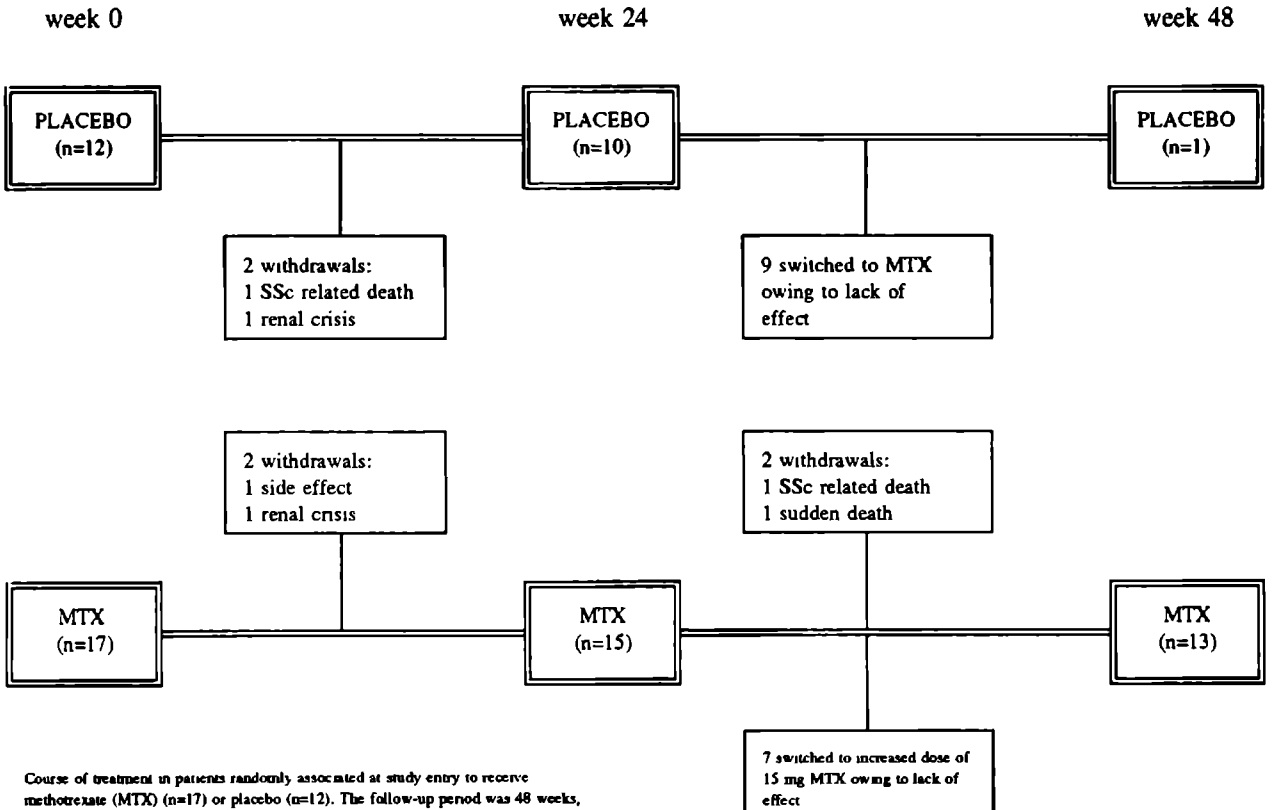
Variable	Baseline		week 24		p-value
	MTX group (n=17)	Placebo group (n=12)	MTX group (n=15)	Placebo group (n=10)	MTX vs placebo
Creatine phosphokinase (U/ml)	132.7 ± 135.4	53 5 ± 37 6[#]	-10 8 (-67 5, 45 9)	4.0 (-4.8, 12 8)	0 14
ESR (Westergren, mm/h)	29.0 ± 20.1	22.4 ± 12.5	-0 66 (-10.6, 9.3)	-1.5 (-5.8, 2.8)	0.89
Haemoglobin (mmol/l)	8.1 ± 0.9	8 1 ± 0.6	-0.2 (-0 5, 0 1)	-0.5 (-0.9, -0.1)	0.04
WBC ($x10^9$/l)	8.4 ± 2.6	7 6 ± 1 0	-1 2 (-2 3, -0 1)	0 5 (-2.2, 1 2)	0 003
Thrombocytes ($x10^9$/l)	349 ± 135	335 ± 66	-60 (-131, 11)	-3 (-37, 31)	0 14
IgA (g/l)	3.57 ± 1 81	2.74 ± 1.31	-0.03 (-0.5, 0 4)	0.15 (-0 18, 0.48)	0.20
IgM (g/l)	1.46 ± 0 46	1 75 ± 0.97	-0.11 (-0 45, 0.23)	0 (-0 17, 0 17)	0.06
IgG (g/l)	16.44 ± 4 35	14.66 ± 3.95	-1.37 (-2.52, -0.22)	-0 22 (-1.24, 0.80)	0.08
C3 (mg/l)	1227 ± 308	1328 ± 300	-126 (-258, 6)	-91 (-306, 124)	0.49
C4 (mg/l)	264 ± 91	263 ± 74	-2 (-48, 44)	15 (-24, 54)	0.50
Circ immune complexes (%)	3 1 ± 2 4	3.4 ± 2 8	0.07 (-1.36, 1.50)	0 (-1.87, 1.87)	0 76

* Values in parentheses are 95% confidence intervals unless otherwise indicated MTX = methotrexate; VAS = visual analogue scale; TLC = total lung capacity, VC = vital capacity, DL_{CO} = diffusion capacity for carbon monoxide

See text for definitions of organ involvement

[#]p=0 04 between MTX and placebo treated patients

Figure 1: Course of the trial



Course of treatment in patients randomly associated at study entry to receive methotrexate (MTX) (n=17) or placebo (n=12). The follow-up period was 48 weeks, assessments of treatment course are shown for week 24 and week 48.
Ssc-related death, death caused by SSc-related cardiopulmonary and/or renal involvement. Sudden death: death due to acute myocardial infarction.

week-24 values of the clinical and laboratory variables are shown in Table 2 for both groups. After 24 weeks, the following differences in improvement of clinical variables between the MTX and placebo treated patients were found:

a. TSS decreased by 0.7 in the MTX group and increased by 1.4 in the placebo group (p=0.06);
b. VAS improved by 7.8 mm in the MTX group and remained approximately the same in the placebo group (p=0.17);
c. the creatinine clearance rate increased by 4.3 ml/min in the MTX group and decreased by 0.7 ml/min in the placebo group (p=0.12).

The CPK values of two MTX patients reflecting pre-existing muscular involvement decreased ≥50% and remained unchanged in two others. A fifth was withdrawn from the study at week 10 due to renal crisis. One patient developed muscular involvement while undergoing MTX therapy. Differences between the groups with respect to laboratory variables consisted of a significantly greater decrease of haemoglobin level in the placebo group (p=0.04) and WBC count in the MTX group (p=0.003). IgM and IgG concentrations also decreased more in the MTX group, but not significantly (p=0.06 and p=0.08 respectively).

Results after 48 weeks

At the end of week 48, the treatment code was broken and it was found that only one patient was still being treated with placebo. Therefore, no comparison could be made between the placebo group and the MTX group. Instead we compared the week-48 values with the initial values of the patients treated with MTX throughout the trial. The data of 13 patients were eligible for analysis, as two more had been withdrawn. Significant improvements were found in VAS (p=0.01) and grip strength of the right hand (p=0.04, Table 3), which was dominant in all patients. There was a tendency of improvement in TSS (p=0.12) and creatinine clearance rate (p=0.11). Other parameters concerning internal organs showed no change. Laboratory analysis demonstrated significant reductions in ESR (p=0.002), WBC count (p=0.04), thrombocyte count (p=0.04), and concentrations of IgM (p=0.0002) and IgG (p=0.0002). There were also minor decreases in the concentrations of C3 and CPK (p=0.08 and p=0.13 respectively) (Table 4).

Responders versus nonresponders

Of the 15 patients who could be classified as responders at the end of the trial, eight achieved a ≥30% improvement in TSS; six, a ≥30% improvement in VAS; and one, a ≥30% improvement in both. The initial values and the differences between the week 48 values and the initial values of clinical and laboratory variables of responders compared with those of nonresponders are shown in Tables 5a and 5b. The difference between the two groups regarding disease duration was due to the presence of two patients with longstanding disease among the responders, but was not significant. Significantly more women than men

Table 3: Clinical variables: differences with entry values after 48 weeks of treatment*

Variable		week 48 MTX group (n=13)	p-value 48 weeks baseline
Total Skin Score		-2 0 (-5 6, 1 6)	0 12
Extension index right (mm)		0 3 (-3 9, 4 5)	0 26
Extension index left (mm)		0 6 (-3 2, 4 4)	0 43
Grip strength right (mm Hg)		27 5 (-2 6, 57 7)	0 04
Grip strength left (mm Hg)		7 5 (-19 4, 34 4)	0 22
Oral opening (mm)		0 4 (-1 2, 2 0)	0 30
General health (0-100mm VAS)		17 5 (1 9, 39 4)	0 01
Organ involvement			
Lung	Fibrosis (N,%)	3 (23)	1 0
	TLC (% predicted)	-1 09 (-3 4, 1 2)	0 78
	VC (% predicted)	-0 9 (-4 8, 3 0)	0 66
	DL_{CO}	-0 04 (-0 14, 0 06)	0 74
Cardiac (N,%)		2 (15)	1 0
Renal (N)		0	
Creatinine clearance rate (ml/min)		3 5 (-1 3, 8 3)	0 11
Esophageal (N,%)		11 (85)	1 0
Muscular (N,%)		2 (15)	0 40

* Values in parentheses are 95% confidence intervals unless otherwise indicated MTX = methotrexate, VAS = visual analogue scale, TLC = total lung capacity, VC = vital capacity, DL_{CO} = diffusion capacity for carbon monoxide See text for definitions of organ involvement

Table 4: Laboratory variables: differences with entry values after 48 weeks of treatment*

Variable	week 48 MTX group (n=13)	p-value 48 weeks-baseline
Creatine phosphokinase (U/ml)	-51.0 (-134.5, 32.5)	0.13
ESR (Westergren, mm/h)	-8.2 (-14.0, -2.4)	0.001
Haemoglobin (mmol/l)	-0.1 (-0.3, 0.1)	0.75
WBC ($x10^9$/l)	-0.6 (-1.9, 0.7)	0.87
Thrombocytes ($x10^9$/l)	-46 (-95, 2.9)	0.04
IgA (g/l)	0.09 (-0.5, 0.7)	0.79
IgM (g/l)	-0.32 (-0.45, -0.19)	0.0002
IgG (g/l)	-1.94 (-3.0, -0.9)	0.0002
C3 (mg/l)	-82 (-207, 43.1)	0.08
C4 (mg/l)	-18 (-62, 26)	0.27
Circ immune complexes (%)	1.67 (-0.08, 3.42)	0.98

* Values in parentheses are 95% confidence intervals.

Table 5a: Clinical variables at study entry and differences after 48 weeks in responders (N=15) and nonresponders (N=7) to methotrexate therapy[a]

	Baseline values		Changes at 48 weeks			
	Responders	Non responders	Responders	p-value	Non responders	p-value
Age (years)	48.6 ± 8.2	53.5 ± 12.3				
Disease duration (years)	2.4 ± 5.7	0.5 ± 0.5				
Males (N,%)	2 (15)	5 (71)[#]				
Diffuse (N,%)	6 (40)	2 (29)				
Limited (N,%)	9 (60)	5 (71)				
Antitopoisomerase I antibodies	5 (33)	6 (86)[@]				
Total Skin Score	18.6 ± 6.5	18.4 ± 9.0	-2.9 (-6.2, 0.4)	0.04	-0.4 (-3.2, 2.4)	0.36
Extension index right (mm)	89.8 ± 14.4	98.0 ± 22.8	0.8 (-1.7, 3.3)	0.26	-5.4 (-12.3, 1.3)	0.05
Extension index left (mm)	90.9 ± 15.8	105.4 ± 8.7	0.9 (-1.8, 3.7)	0.40	-5.3 (-10.2, -0.4)	0.02
Grip strenght right (mm Hg)	106.7 ± 65.0	109.7 ± 72.8	29.5 (2.4, 56.6)	0.02	4.3 (-38.2, 46.8)	0.41
Grip strenght left (mm Hg)	121.1 ± 81.7	115.4 ± 60.7	11.6 (-6.1, 29.3)	0.09	-27.1 (-79.4, 25.2)	0.13
Oral opening (mm)	41.9 ± 7.4	41.1 ± 10.3	0.1 (-1.4, 1.6)	0.43	-0.4 (-4.1, 3.3)	0.39
General health (0-100 mm VAS)	54.4 ± 18.6	59.3 ± 20.9	14.3 (0.1, 28.5)	0.02	2.6 (-8.0, 13.2)	0.39

Table 5a (continued)

	Baseline values		Changes at 48 weeks			
	Responders	Non responders	Responders	p-value	Non responders	p-value
Organ involvement:						
Lung Fibrosis (N,%)	5 (33)	1 (14)	4 (27)	1.0	1 (14)	1.0
TLC % predicted	86.1 ± 16.8	90.8 ± 8.0	0 (-1.8, 1.8)	0.50	-5.2 (-9.4, -1.0)	0.02
VC % predicted	91.6 ± 23.2	94.6 ± 7.2	0.13 (-3.1, 3.3)	0.46	-9.4 (-18.7, -0.1)	0.01
DL_{CO}	1.46 ± 0.27	1.33 ± 0.42	-0.05 (-0.13, 0.03)	0.12	0.1 (0.0, 0.19)	0.45
Cardiac (N,%)	1 (7)	3 (43)	2 (13)	1.0	3 (43)	1.0
Renal	0	0	0		0	
Esophageal (N,%)	12 (80)	6 (87)	14 (93)	0.50	6 (87)	1.0
Muscular (N,%)	2 (13)	1 (7)	1 (14)	1.0	1 (7)	1.0
Creatinine clearance (ml/min)	76.9 ± 12.3	89.0 ± 18.7	2.5 (-1.8, 6.8)	0.21	-1.71 (-8.5, 5.0)	0.28

For definition of responders and nonresponders: see text

*Unless otherwise indicated, values in parentheses are 95% confidence intervals. MTX = methotrexate; VAS = visual analogue scale; TLC = total lung capacity; VC = vital capacity; DL_{CO} = diffusion capacity for carbon monoxide. For definitions of organ involvement: see text.

[a]$p<0.05$; [@]$p=0.06$

Table 5b: Laboratory variables at study entry and differences after 48 weeks in responders (N=15) and nonresponders (N=7) to methotrexate therapy*

	Baseline values		Changes at 48 weeks			
	Responders	Non responders	Responders	p-value	Non responders	p-value
Creatine phosphokinase (U/ml)	102 7 ± 134 4	84 3 ± 103 9	39 4 (-111 5, 32 7)	0 38	5 9 (-27 8, 16 0)	0 27
ESR (Westergren, mm/h)	22 9 ± 12 3	17 7 ± 14 0	-7 5 (13 1, 1 9)	0 01	3 0 (-5 4, 11 4)	0 21
Haemoglobin (mmol/l)	8 2 ± 0 5	8 6 ± 0 8	-0 3 (-0 5, -0 1)	0 003	0 2 (-0 8, 0 4)	0 23
WBC ($x10^9$/l)	7 5 ± 2 3	8 3 ± 1 5	0 3 (1 4, 0 8)	0 25	0 4 (1 4, 0 6)	0 20
Thrombocytes ($x10^9$/l)	320 ± 105	320 ± 68	35 (79, 9)	0 05	-1 (65, 63)	0 48
IgA (g/l)	2 79 ± 1 46	3 59 ± 2 13	0 09 (0 56, 0 38)	0 65	0 64 (-0 05, 1 33)	0 04
IgM (g/l)	1 52 ± 0 68	1 71 ± 0 92	-0 15 (0 48, 0 17)	0 08	-0 06 (-0 53, 0 42)	0 65
IgG (g/l)	15 82 ± 5 41	15 06 ± 2 03	1 64 (2 75, -0 53)	0 005	0 14 (1 48, 1 76)	0 80
C3 (mg/l)	1175 ± 238	1228 ± 224	160 (-295, -24)	0 01	4 (-194, 203)	0 96
C4 (mg/l)	286 ± 82	201 ± 31[#]	26 (-62, 9 7)	0 13	30 (-23, 82)	0 01
Circ Immune complexes	2 9 ± 2 4	4 2 ± 2 5	0 5 (1 3, 2 3)	0 96	2 4 (-0 06, 4 8)	0 08

For definition of responders and nonresponders see text

*Unless otherwise indicated, values in parentheses are 95% confidence intervals

[#]p=0 007 between responders and nonresponders

responded favorably (p=0.05). Antitopoisomerase I antibodies seemed to be identified more frequently in the nonresponders (p=0.06) and mean C4 levels were significantly higher at study entry in the responders. There were no further differences between responders and nonresponders with respect to any of the other variables that were tested at study entry.

By week 48 the responders showed significant improvement in TSS (p=0.04), grip strength of the (dominant) right hand (p=0.02), and VAS (p=0.02). There was also some improvement in the grip strength of the left hand (p=0.09). DL_{CO} decreased slightly (p=0.12), and VC and TLC remained unchanged. Mean ESR, haemoglobin concentration, and thrombocyte count dropped significantly (p=0.01, p=0.003 and p=0.05 respectively). Likewise, mean IgG and C3 levels dropped significantly (p=0.003 and p=0.005 respectively). Among nonresponders, the extension indices of the right and left hand worsened significantly (p=0.05 and p=0.02 respectively), as did TLC (p=0.02) and VC (p=0.01), and mean IgA increased significantly (p=0.04).

Diffuse and limited skin involvement, short and long disease duration

There were no significant differences between the changes that occurred among patients with diffuse skin involvement and those that occurred among patients with limited skin involvement or between the changes that occurred among patients with disease duration of more than one year and those that occurred among patients with disease duration of less than one year (data not shown).

Withdrawals and adverse reactions

Withdrawals and adverse reactions are summarized in Table 6. One patient from the placebo group experienced a severe progression of cardiopulmonary and gastrointestinal manifestations of SSc, eventually accompanied by a renal crisis of which she died in week 10. Another patient receiving placebo had to be withdrawn in week 12, owing to renal failure due to scleroderma renal crisis. Two patients from the MTX group were also withdrawn: one in week 10 because of renal failure due to scleroderma renal crisis, which was successfully treated in the acute phase, but which left the patient with chronic renal failure; another because she suffered from persistent, severe headache after each injection. Two patients died between weeks 24 and 48: one patient died because of cardiorespiratory insufficiency caused by progressive pulmonary fibrosis; the other died suddenly presumably because of acute myocardial infarction. Both patients had been receiving an increased dose of 25 mg MTX weekly. The patient who died of cardiorespiratory insufficiency had suffered shortly before from a pancytopenia. This pancytopenia had completely recovered after temporary withholding MTX. Six patients receiving MTX experienced liver enzyme abnormalities characterized by elevated levels of AST and ALT. These normalized in two to four weeks after the trial medication had been withheld. None of these patients experienced recurrences of hepatic function abnormalities after resuming MTX. None of the

Table 6: Adverse reactions, withdrawals, and deaths during study period

Pat	Sex	Age	Disease Duration	Week	Methotrexate 15 mg	Methotrexate 25 mg	Placebo	Withdrawal due to side effects: temporary	Withdrawal due to side effects: permanent	Death
HH	F	48	2.9	12	x			liver		
NH	F	50	0.1	16	x			liver		
BM	F	55	5.1	36	x			liver		
LG	F	32	2.4	20	x			liver		
DA	M	68	0.6	36	x			liver		
RA	F	41	30.0	44	x			liver		
WE	M	63	0.4	26		x		pancytopenia		
TA	F	75	2.3	5	x				headache	
SC	M	52	0.8	10	x				renal crisis	
MO	F	71	12.4	16			x		renal crisis	
HP	F	67	0.9	36		x				sudden death
WE	M	63	0.4	28		x				SSc-related*
EL	F	68	0 8	6			x			Ssc-related*

*SSc-related death: death caused by SSc-related cardiopulmonary and/or renal involvement.

patients in the placebo group experienced any adverse reaction that could be ascribed to the injections.

Discussion

The design of this study was largely determined by the findings of our pilot study [31], in which skin thickening was reversed within six months of the beginning of MTX therapy. It was therefore felt that, because of the seriousness of SSc, a potentially beneficial treatment could not be withheld from patients in a placebo group for more than six months. In a recent editorial it was recommended that therapeutic trials among scleroderma patients should last at least two years, because of the variability of the course of the disease[35]. However, the same authors concluded later that the best period for evaluating therapies that are intended to affect skin thickening is probably the first year of treatment[36].

The rarity of SSc limits the number of patients available for trials, unless multicenter trials are undertaken. Multiobserver examinations, on the other hand, are liable to diminish the reliability of TSS, which is the most important marker of disease progress. We chose for one observer in this multicentre trial as TSS is at least as reliable in SSc as joint count in rheumatoid arthritis, provided that it is evaluated serially by a single investigator[37].

The paucity of suitable disease variables makes it difficult to evaluate therapies for SSc. It was necessary to develop criteria for separating responders and nonresponders. These employed a combination of TSS, VAS, DL_{CO}, and the presence or absence of digital ulcers. The choice was based on findings reported in the literature and our own experience. Pope and Bellamy recently reviewed the literature dealing with outcome measurements for treatments of scleroderma patients in clinical trials[38]. They too concluded that skin score measurements and global assessment are the best primary outcome measures for clinical trials; good secondary measures include variables of internal organ involvement, mortality, functional assessment, and physical parameters such as grip strength and extension index. After 24 weeks of treatment there was a trend for improvement in the MTX group for the disease activity variables TSS and VAS. Analysis of results at week 48 between the two groups could not be made since at week 24 all placebo treated patients except one were switched to MTX treatment according to the study protocol. Analysis of MTX treated patients at week 48 showed that 68% of the patients in the present study benefitted from MTX treatment: skin scores and global assessment improved in a significantly larger proportion of the MTX group than of the placebo group.

Low-dose MTX is suspected of impairing renal function, principally glomerular and tubular function, especially when it is used in combination with other nephrotoxic drugs[39,40]. We found no reduction in renal function as measured by creatinine clearance rate as we observed in the pilot study. This may have been because only a few patients in the present study used potentially nephrotoxic drugs (nonsteroidal anti-inflammatory drugs or cimetidine in a constant dose).

Only one patient had to be permanently withdrawn from the study because of side effects (severe headache) attributable to MTX. MTX would therefore appear to be reasonably well tolerated and safe in low, weekly, intramuscular dosages. The number of adverse reactions was relatively high, but in general minor and manageable. The occurrence of transient hepatic function disturbances in six patients and pancytopenia in one emphasizes the necessity of closely monitoring hepatic function tests and blood cell counts.

Elevated ESR and thrombocyte count are associated with inflammation. The significant decrease in both variables after 48 weeks of treatment with MTX may reflect a reduction in the inflammatory process. The same may be true of the decrease in IgM and IgG concentrations, although this may also be due to direct inhibition of B-cell activity by MTX. Such an anti-inflammatory effect has been hypothesized as the mechanism through which MTX acts in rheumatoid arthritis, because of rapid clinical response to treatment and equally rapid flare-up upon discontinuation[41]. Somewhat similarly we observed severe and rapid progression of cutaneous and internal organ involvement in two patients six weeks after MTX had been withheld. One of these patients is described in detail elsewhere[42].

The mechanism through which MTX acts in SSc has yet to be established. Direct inhibition of extracellular matrix production by fibroblasts is unlikely: we recently reported that MTX, unexpectedly, did not reduce but even enhance glycosaminoglycan production by scleroderma fibroblasts in culture[43].

More patients whose serum was positive for antitopoisomerase I antibodies and significantly more patients of male gender failed to respond to MTX therapy. Both variables are known to be associated with an unfavorable prognosis in SSc.

The dosage of 15 mg MTX weekly seemed to be sufficient for the majority of patients who responded favorably. A higher dose of 25 mg may be necessary in those cases in which it is not.

This study demonstrates that 68% of SSc patients respond favorably to MTX therapy with reductions in skin thickness or improvements in general well-being and without further damage to internal organs after 48 weeks. Long-term prospective trials examining efficacy and toxicity and randomized trials comparing MTX with, for example, d-penicillamine or cyclosporine will determine the position of MTX therapy in the treatment of scleroderma.

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Methotrexate and glycosaminoglycanproduction by scleroderma fibroblasts

Van den Hoogen, Van der Kraan PM, Boerbooms AMTh, Van den Berg WB, Van Lier HJJ, Van de Putte LBA. Effects of methotrexate on glycosaminoglycan-production by scleroderma fibroblast in culture. Ann Rheum Dis 1993;52;758-761.

Summary

Objective - To determine the effects of increasing concentrations of methotrexate on the proliferation and glycosaminoglycan (GAG) synthesis of cultured dermal fibroblasts from patients with scleroderma.
Methods - Cultured dermal fibroblasts from nine patients with scleroderma and nine normal volunteers were grown for 72 hours in media containing various concentrations of methotrexate. The GAG synthesis in each cell was measured after incubating the fibroblasts with [^{3}H]glucosamine and [^{35}S]sulfate.
Results - A negative correlation was found between the concentration of methotrexate and numbers of fibroblasts from patients with scleroderma and normal controls. A positive correlation was found between GAG synthesis in each cell, as measured by [^{3}H]glucosamine and [^{35}S]sulfate incorporation, and increasing methotrexate concentrations in fibroblasts from patients with scleroderma and normal controls.
Conclusions - These data indicate increased GAG synthesis in scleroderma and normal fibroblasts with increasing concentrations of methotrexate. Therefore the reported beneficial effect of methotrexate on skin fibrosis in scleroderma is most probably not the result of direct inhibition of GAG synthesis by fibroblasts.

Introduction

Systemic sclerosis (SSc) is characterized by proliferative vascular lesions, chronic inflammatory infiltrations, and an excessive accumulation of connective tissue in many organs. Abnormalities in the functioning of the fibroblasts are considered to be responsible for progressive fibrosis in SSc. Tissue cultures of dermal fibroblasts from SSc patients have been found to synthesize collagen at an increased rate[1,2] and to accumulate up to five times more glycosaminoglycans (GAGs) than normal skin fibroblasts[3,4]. There is evidence to suggest that most of the increase in GAG synthesis is in the hyaluronic acid fraction[3,4].
Methotrexate (MTX) in low doses is efficacious in treating several connective tissue diseases, such as rheumatoid arthritis (RA)[5,6], dermatomyositis and polymyositis[7]. Low doses MTX have also been reported to reduce skin thickening in patients with SSc[8,9]. The mechanisms through which low doses MTX affects the inflammatory process in rheumatic disease are as yet unknown. Most studies of immune system in RA patients who have been treated with low-dose MTX show only marginal effects on humoral and cellular immune responses. The rapid clinical responses to treatment and equally rapid flare up upon discontinuation suggest an anti-inflammatory effect[10]. One study suggests that one of the roles of MTX in RA may be to inhibit interleukin-1 mediated proliferation of synovial fibroblasts[11].
This study was undertaken to determine the effects of increasing concentrations of MTX on the proliferation of cultured scleroderma skin fibroblasts and their production of GAGs.

Patients and methods

Patients with scleroderma and normal volunteers

The nine patients fulfilled the American Rheumatism Association's preliminary criteria for the diagnosis of SSc[12]. The mean (SD) age of the patients was 49.4 (8.4) years, with a range of 38 to 63 years. Seven patients were women and two were men. Five had diffuse SSc - that is, with the skin proximal to the elbows affected - and four had limited SSc - that is, with the skin distal to the elbows affected. Disease duration, estimated from the first signs of skin thickening, varied from six months to seven years, the median being 48 months. In six patients antibodies to topoisomerase I could be detected; in two others anticentromere antibodies were found. None of the patients had previously received any drug treatment known to influence connective tissue metabolism. The control group consisted of nine normal volunteers, five men and four women, with a mean (SD) age of 46.4 (11.7) years (range 29-67 years).

Fibroblast explant cultures

Full thickness skin biopsy samples were taken with a 4 mm punch from each patient at a site of dermal thickening on the dorsum of the left forearm; from each donor a biopsy sample was taken from either the dorsal forearm or the upper arm. Each biopsy specimen was minced and placed in a 25 cm^2 plastic tissue culture flask (Costar, Cambridge, MA, USA), to which 4 ml nutrient medium was added. The latter consisted of Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Irvine, Strathclyde, UK) that contained 0.002 M glutamine and 0.02 M HEPES buffer and had been supplemented with 15% fetal calf serum and gentamicin (40 µg/ml, Schering, Kenilworth, NJ, USA). In addition to Hepes buffer the medium pH was controlled by sodium hydrogencarbonate. Each culture was maintained at 37°C in a humidified atmosphere of a CO_2 incubator containing 5% CO_2 in air. The medium was completely replaced one to three times a week, depending on the change in color. Once fibroblasts had grown from an explant culture, the old medium was discarded and fibroblasts were dispersed with 1 ml 0.05% trypsin (Sigma Chemical Co., St. Louis, MO, USA) for 15 minutes at 37°C. The cells were left in the same flask and 4 ml of nutrient medium was added. When they reached confluence, the fibroblasts were trypsinized again and divided equally between two 25 cm^2 tissue culture flasks to each of which 4 ml of nutrient medium was added. When these cell layers had become confluent, they were trypsinized once more and the dispersed fibroblasts of each subculture were divided equally among 20 wells of one of two identical 24-well tissue culture plates (Costar). A 500 µl volume of nutrient medium was added to each of these 40 wells. After 24 hours this was completely replaced with 500 µl fresh medium containing MTX in a concentration of 0, 10^{-8}, 10^{-6}, 10^{-4} or 10^{-2} mol/l. Each of the five concentrations was assigned to four wells on each plate. These cultures were then further incubated under the conditions described. All subsequent determina-

tions were performed on the contents of the quadruplicate wells.

Measurement of GAG synthesis

The fibroblast cultures of one tissue culture plate were labeled with 185 kBq [^{3}H]glucosamine (NEN Products, Dupont, Boston, MA, USA) and 185 kBq [^{35}S]sulfate (NEN Products) in each well, three days after the MTX had been added. The next day, all medium was decanted from each well and supplemented with 200 µl papain (1 mg/ml; Sigma Chemical). The fibroblasts were trypsinized (200 µl/well) and mixed with 100 µl papain (5 mg/ml). The cells and decanted media were incubated at 60°C for 24 hours. A 0.2% solution of cetylpyridinium chloride (CPC) was then added, 400 µl to fibroblasts and 600 µl to medium, to obtain a final concentration of 0.1% in all samples. These were incubated at 37°C for one hour to allow the GAG-CPC complex to precipitate. The resulting pellets were centrifuged and washed, the cell fraction twice and medium fraction three times, with CPC 0.05% to remove any remaining glycopeptides and unincorporated precursors. Each pellet was then supplemented with 0.5 ml Luma solve (Lumac.LSC BV, Olen, Belgium) and, after an incubation of 10 minutes at 60°C, 10 ml Lipoluma (Lumac.LSC) was added. The [^{3}H]glucosamine and [^{35}S]sulfate content of the pellets was determined in a liquid scintillation counter (LKB, Sweden), and appropriate corrections for the [^{3}H]-[^{35}S] overlap were made. As these experiments were not performed simultaneously, the specific activities of the isotopes had been determined before labeling. The results were corrected for the decrease in radioactivity that occurred during the course of the study to obtain figures for the levels of incorporation that could be compared.

Fibroblast count

The second tissue culture plate was used for counting cells. Four days after MTX had been added to the cultures, the media were decanted. The cells were washed twice with 400 µl phosphate buffered saline per well. Following trypsinization (200 µl/well), the cells of each well were counted with a Bürker hemocytometer. The [^{3}H] and [^{35}S] counts/minute (cpm) of each of the quadruplicate cultures were divided by the corresponding number of cells to obtain the GAG synthesis for each fibroblast, to eliminate the effect of MTX on cell proliferation.

Statistical analysis

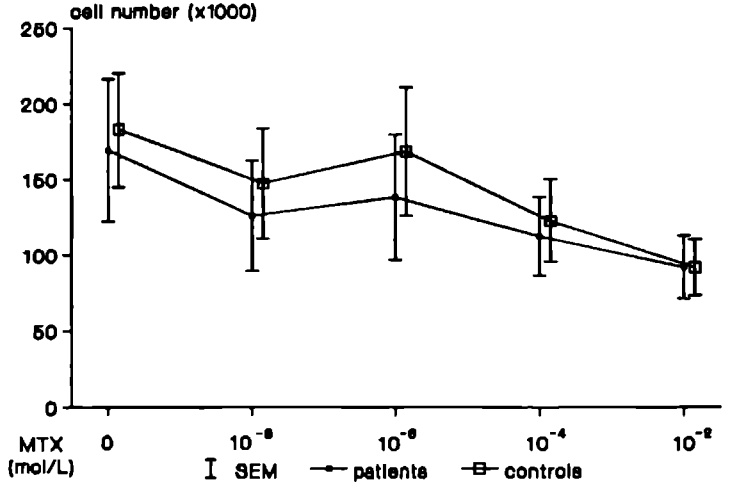
For all of the variables the means were calculated for quadruplicate cultures. Comparisons between groups were performed with the two tailed Wilcoxon test. Spearman rank correlation coefficients were calculated, and Fisher's Z method applied. p Values of 0.01 or less were considered significant.

Results

Fibroblast proliferation

As shown in fig. 1, the mean number of cells was significantly inversely related to the concentration of MTX to which they had been exposed. The mean number of scleroderma fibroblasts in each well was lower than that of control fibroblasts for all concentrations of MTX except 1 x 10^{-2} mol/l, but the differences were not significant.

Figure 1 Mean cell number per well of patients and controls at different methotrexate (MTX) concentrations



Effect of methotrexate on fibroblast proliferation. Methotrexate causes a dose-dependent decrease of scleroderma ($P<0.001$) and normal ($P<0.001$) fibroblast numbers. No differences were observed between the numbers of scleroderma and normal fibroblasts.

GAG synthesis

There was a positive correlation between the total amount of [^{3}H] (mean [^{3}H] count rate in cells plus mean [^{3}H] count rate in media of quadruplicate cultures) and the corresponding concentration of MTX for scleroderma and normal fibroblasts (fig. 2a). Likewise, total [^{35}S] count rate was positively correlated with the corresponding concentration of MTX (fig. 2b).

Figure 2a Total ^{3}H counts of scleroderma and control fibroblasts

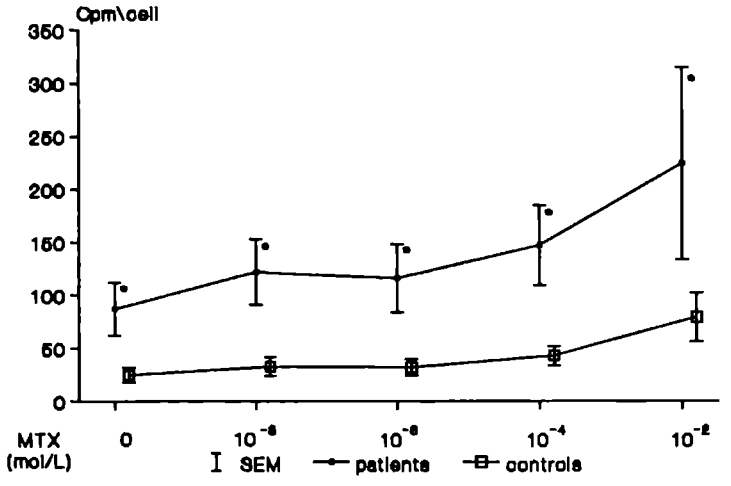
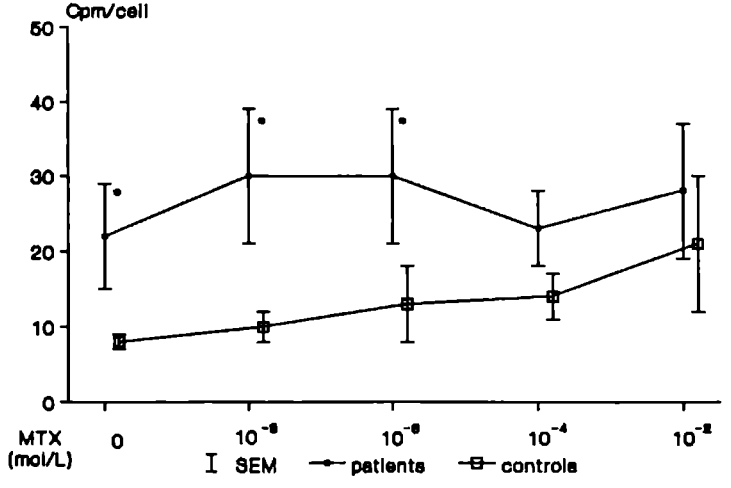


Figure 2b Total ^{35}S counts of scleroderma and control fibroblasts



Mean total [^{3}H] (fig. 2a) and [^{35}S] (fig. 2b) counts per minute (cpm) per cell of scleroderma and normal fibroblasts. Each value represents the mean and SEM of quadruplicate cultures. A positive correlation was found between the concentration of methotrexate and both total [^{3}H] cpm of scleroderma ($P<0.001$) and normal ($P<0.001$) fibroblasts and total [^{35}S] cpm of scleroderma ($P<0.001$) and normal ($P<0.001$) fibroblasts. An asterisk indicates a significant difference ($P<0.01$).

As the extent of skin disease and disease duration in SSc may influence GAG synthesis, we compared the results for fibroblast cultures derived from specimens from five patients with diffuse skin disease with those for cultures derived from specimens from four patients with limited skin disease. For each concentration of MTX the total [^{3}H] and [^{35}S] count rates were higher for diffuse disease fibroblasts than for limited disease fibroblasts ($p<0.01$). There was no difference between the total [^{3}H] or [^{35}S] cpm of limited disease fibroblasts and those of normal fibroblasts.
The results for fibroblast cultures derived from specimens from five patients with a disease duration of more than three years were compared with those derived from specimens from four patients with a disease duration of less than three years. The total [^{3}H] and [^{35}S] count rates were higher for patient with long term disease than for those with short term disease, but the differences were significant for a MTX concentration 1 x 10^{-4} mol/l only. Values of total [^{3}H] and [^{35}S] count rates of normal fibroblasts were between the values of fibroblasts of patients with long term and short term disease and were not significantly different from either of these.
More than half the newly synthesized GAGs were secreted into the media (Table 1). Scleroderma and normal fibroblasts secreted a higher percentage of [^{3}H]-glucosamine labeled GAGs into the media than [^{35}S]sulfate labeled GAGs ($p<0.001$).

Discussion

MTX has been reported to be efficacious in the treatment of SSc and particularly in treating the affected skin[8,9]. If the beneficial effect of MTX is a result of it acting directly on the fibroblasts, this might be either by inhibiting their proliferation or by decreasing the production of extracellular matrix. MTX, an antagonist of folate dependent enzymes, interferes with de novo pyrimidine and purine synthesis of RNA and DNA and, hence, blocks cell proliferation. The inverse relation between the number of cells and MTX concentrations in scleroderma and normal fibroblasts was therefore to be expected. The proliferation rate was approximately the same in scleroderma and normal fibroblasts.
Total [^{3}H] and total [^{35}S] count rates were positively correlated with the concentration of MTX for scleroderma and normal fibroblasts. As total [^{3}H] and [^{35}S] count rates reflect the incorporation of [^{3}H]glucosamine and [^{35}S]sulfate into GAGs, these results show that, in scleroderma and normal fibroblast cultures, GAG synthesis increases with increasing concentrations of MTX.
Production of GAGs by scleroderma fibroblasts as measured by the total [^{3}H] count rate was significantly greater than that by normal fibroblasts for all concentrations of MTX. The production of GAGs as measured by total [^{35}S] count rate was significantly greater in scleroderma fibroblasts in the absence of MTX and at concentrations of 1 x 10^{-8} and 1 x 10^{-6} mol/l. As [^{3}H]glucosamine is incorporated into all GAGs, and [^{35}S]sulfate into sulfate containing GAGs only, thus leaving

Table 1: Mean (± SD) percentages of [^{3}H]-glucosamine and [^{35}S]-sulfate counts per minute (cpm) secreted into the medium of cultured scleroderma and normal fibroblasts

MTX concentration (mol/l)	0	10^{-8}	10^{-6}	10^{-4}	10^{-2}
percentage [^{3}H] cpm medium					
Scleroderma fibroblasts	80 ± 8	76 ± 8	75 ± 7	81 ± 7	83 ± 5
Normal fibroblasts	75 ± 6	77 ± 8	74 ± 7	77 ± 9	80 ± 7
percentage [^{35}S] cpm medium					
Scleroderma fibroblasts	65 ± 11	62 ± 7	59 ± 8	60 ± 11	62 ± 11
Normal fibroblasts	57 ± 9	58 ± 12	56 ± 12	57 ± 13	62 ± 15

Table 1: Percentages of [^{3}H]-glucosamine and [^{35}S]-sulfate counts per minute (cpm) secreted into the medium by dermal fibroblasts of scleroderma patients and control subjects. Differences between scleroderma and normal fibroblasts were not significant for any MTX concentration.

hyaluronic acid unlabeled by [^{35}S]sulfate, this indicates an enhanced accumulation of hyaluronic acid in scleroderma fibroblasts at higher MTX concentrations. The possibility that undersulphated GAGs are produced cannot be totally excluded, however.

As diffuse skin disease was associated with a greater production of GAGs than limited skin disease which was associated with a level of production similar to that of normal fibroblasts, the increased production of GAGs by scleroderma fibroblasts must be attributed primarily to those derived from the biopsy specimens from patients with diffuse skin disease.

SSc is believed to be at its most active during the first three years. We had, therefore, expected a greater production of GAG to be associated with short disease duration than with long disease duration. We found, however, a greater production of GAGs in fibroblast cultures of patients with long term disease. The difference was not significant, perhaps because the number of patients was too small.

The concentration of MTX had no effect on the percentages of newly synthesized [^{35}S]sulfate or [^{3}H]glucosamine labeled GAGs that were secreted into the media by the scleroderma fibroblasts, which were comparable with those found by Bashey *et al.*[4]. This shows that MTX does not cause a shift in the distribution of GAGs between the cells and medium.

Considering the mechanisms through which MTX is known to act, we had expected GAG synthesis to decrease as the concentration of MTX increased. The results show, however, that GAG production by cultured fibroblasts, whether scleroderma or normal, actually increases with increasing MTX concentrations. There are several possible explanations for this. First, the synthesis of extracellular matrix may be suppressed through contact inhibition [13]. As shown in fig 1, MTX reduces the number, and therefore the density, of fibroblasts as its concentration increases, thus creating a population in which less contact inhibition can occur. This could lead to more GAG synthesis for each fibroblast.

Second, MTX may have been responsible for selecting those fibroblasts with high rates of GAG synthesis. It has been established that normal and scleroderma fibroblasts are heterogeneous with respect to their synthetic and proliferative capabilities and that SSc fibroblasts are somehow selected so that high producers of connective tissue are favored at the expense of low producers, which they replace[14]. Third, the increase in GAG synthesis may be due primarily to decreased degradation, as a result of the interference by MTX with the synthesis of GAG degrading enzymes, rather than to increased production. Finally, by blocking DNA synthesis and thus cell proliferation, MTX may promote a fibroblast differentiation that is accompanied by protein synthesis. MTX has been reported to induce differentiation of cultured human keratinocytes, resulting in an increase in protein synthesis by a factor of 2 - 2.3[15].

The results obtained in this study are inconsistent with the reported beneficial effect of low doses of MTX on sclerosis of the skin in SSc. Therefore any beneficial effect of MTX on skin thickening in SSc is most probably not the result of direct inhibition of GAG production by fibroblasts, but is more likely to be based on modulation of the (immuno)inflammatory system.

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A recombinant topoisomerase I used for autoantibody detection in sera from patients with systemic sclerosis

Verheijen R, Van den Hoogen FHJ, Beijer R, Richter A, Penner E, Habets WJ, Van Wanrooij WJ. A recombinant topoisomerase I used for autoantybody detection in sera from patients with systemic sclerosis. Clin Exp Immunol 1990;80:38-43.

Summary

We report the expression of a cDNA clone encoding 695 carboxyl-terminal amino acids of human DNA topoisomerase I (topoI) in Escherichia coli. More than 96% of the anti-HeLa topoI-positive sera from patients with a connective tissue disease displayed also an immunoreactivity with this recombinant protein (the HTopoA protein). Sera from patients with a definite diagnosis systemic sclerosis and reacting with HeLa topoI, all reacted with the HTopoA protein as well. Sera from patients with systemic sclerosis that did not contain anti-topoI antibodies (about 30% of the systemic sclerosis sera), as concluded from HeLa immunoblot, displayed also no immunoreactivity with our recombinant antigen. By expressing different fragments of HTopoA, we were able to assign at least three different autoimmune epitope regions on the HTopoA protein and we show that over a period of 5 years the amount of anti-topoI antibodies against these regions may fluctuate.

Introduction

In the eukaryotic cell, the topological states of DNA are modulated by two enzymes known as type I and type II DNA topoisomerase. The type I enzyme (topoisomerase I; topoI) interconverts different topological forms of DNA by creating a transient single-stranded nick in the DNA backbone, passing the unbroken strand of the DNA through the nick, and resealing the original scission[1,2]. Although topoI is not necessary for the viability of eukaryotic cells[3,4], it does appear to play important roles in chromatic organization[4], mitosis[5], DNA replication[6,7], recombination[8,9] and transcription[10,11].

Autoantibodies directed against DNA topoI in sera of patients with systemic sclerosis have been described by several investigators[12-18]. These autoantibodies are usually detected by the immunodiffusion or by the more sensitive immunoblotting technique[18]. Thymus extracts[18] and HeLa cell extracts[5] are the most widely used antigen sources in these tests. In the present study we report the expression of a cDNA clone encoding 695 carboxyl-terminal amino acids (=91%) of human topoI. The DNA sequence of this clone is identical with nucleotides 418-3409 of the earlier described topoI clone T1B[19]. Our recombinant topoI protein (HTopoA protein) displayed an immunoreactivity with 82 of 85 anti-topoI-positive sera (=96%) indicating the usefulness of the HTopoA protein in routine detection of anti-topoI antibodies in sera from patients with connective tissue diseases.

Furthermore, we were able to assign at least three different autoimmune epitope regions on the HTopoA protein and show that over a period of 5 years the amount of anti-topoI antibodies against these regions may fluctuate.

Materials and methods

Sera

Most patient sera were obtained from the Department of Rheumatology of the University Hospital St Radboud at Nijmegen, The Netherlands. Some additional sera were received from hospitals in Enschede, Deventer and Groningen, the Netherlands.

Cells

Culturing of HeLa S3 suspension cells as well as preparation of HeLa total nuclear protein fraction has been described[20].

Bacteria and growth media

Escherichia coli strains Y1089 and RR1 were purchased from Promega Biotec (Madison, WI). The strains HMS174 and BL21 (DE3) were provided by Dr F.W. Studier (Brookhaven National Laboratory, NY) and grown as described[21,22]. When growing phage- or plasmid-containing cells, ampicillin (Sigma) was added to the medium at a final concentration of 100 µg/ml.

Screening of a λgt11 expression library with antibody probes

A systemic sclerosis serum (diluted 1/500) with a high titre of anti-topoI antibodies was used to screen a human placental cDNA library (Clontech) constructed with the λgt11 vector as described previously[23]. To detect specifically bound antibody, ^{125}I-labelled sheep anti-human immunoglobulin $F(ab)_2$ fragment (Amersham) was used.

Expression of topoI as fusion proteins

The clone λHTopoB (see Results) was transferred to the lysogenic host E. coli Y1089. The lysogen was induced by temperature shift and addition of 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) allowing maximal fusion protein synthesis[24].
The cDNA HTopoA (see Results) was inserted into the *Bam*Hi site of the pET-3c expression vector[21,22] to yield pEHTopoA. E. coli HMS174 was used as host strain for initial cloning of the target DNA into the pET vector and for maintaining the plasmids. E. coli BL21(DE3) was used as host for expression of pEHTopoA. BL21(DE3) contains a single copy of the gene for T7 RNA polymerase in the chromosome under control of the inducible *lac*UV5 promotor. Transcription of pET-3c is controlled by the strong ϕ 10 promotor for T7 RNA polymerase. Addition of 0-4 mM IPTG to a growing culture of BL21(DE3)/pEHTopoA induces T7 RNa polymerase, which in turn transcribes HTopoA in the

pET-3c plasmid.

In this study we also used a topoI cDNA clone which was isolated from a HeLa cell DNA library[25]. The isolated clone, referred to as the A-sequence, contains an insert of 2177 bp, corresponding to nucleotides 1241-3425 of the sequence published by D'Arpa et al.[19]. The cDNA of this clone contains an open reading frame encoding amino acids 344-589 of topoI (see Results) and was cloned into the pEV-vrf1 expression vector[26]. Plasmids carrying the A-sequence were introduced into E. coli RR1(pRK248clts) expressing a temperature-sensitive λcI repressor. Upon heat induction from 30°C to 42°C for 3 h, large amounts of a 33-kD polypeptide (the A-fragment) were synthesized. In addition to the 246 amino acids homologous to topoI, the polypeptide contains four amino-terminal and 21 carboxyl-terminal amino acids not related to the topoI sequence.

Gel electrophoresis, protein blotting and detection of antigens

SDS-PAGE and transfer of proteins from 13% polyacrylamide gels onto nitrocellulose sheets was performed as described by Habets et al.[20]. For the detection of antigen, the protein blots were treated and processed as described[27].

DNA sequence analysis

cDNA fragments were digested with a variety of restriction enzymes. DNA fragments were ligated into the polylinker region of M13 mp18[28]. Sequence analysis of the DNA fragments was performed by the dideoxy chain termination method[29].

Results

Isolation and expression of cDNA clones

Serum from a patient with systemic sclerosis was used to screen a λgt11 cDNA expression library of human placenta for clones encoding topoI using standard methods previously described by Habets et al.[23]. One putative topoI clone with an insert of 1.2 kbp, referred to as λHTopoB, was identified.
A number of longer cDNAs were obtained by rescreening the cDNA library with λHTopoB as hybridization probe. One of these clones, referred to as λHTopoA, contained an insert of 3.0 kbp. The isolated insert of λHTopoA was recloned into the *Bam*HI-site of the pET-3c plasmid vector (see Materials and Methods) to yield pEHTopoA.
Lysogens containing phage λHTopoB produced a β-galactosidase fusion protein (HTopoB protein) with an apparent mol.wt of about 125 kD.
Induced BL21(DE3) harboring pEHTopoA produced a polypeptide (HTopoA protein) with an apparent mol.wt of 74kD (fig. 1). Next to a large amount of this

protein the induced pEHTopoA lysates contained three additional protein products of 56, 42, and 34 kD, respectively, that could not be detected in lysates of the induced wild type. We assume that these proteins are proteolytic degradation fragments of the 74-kD HTopoA protein.

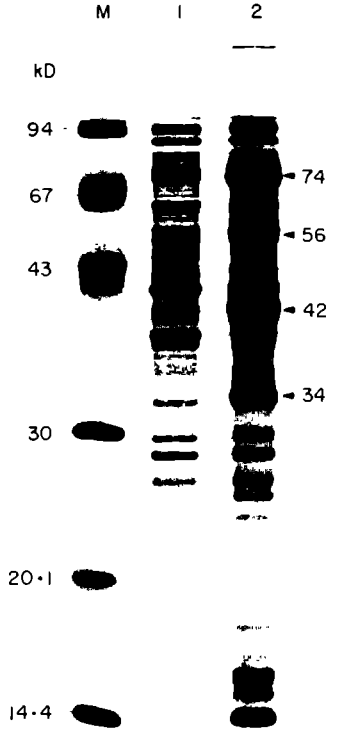

Fig. 1. Expression of pEHTopoA in BL21(DE3). Coomassie brilliant blue stained 13% SDS/polyacrylamide gel of a crude BL21(DE3) lysate containing pET-3c as a control (lane 1) or pEHTopoA (lane 2). In both cases the bacteria were harvested after induction for 3 hours at 37 C with 0·4 mM IPTG. M, mol. wt markers (in kD).

Identification of the cDNA clones

DNA sequencing of λHTopoA and λHTopoB established that we had isolated clones encoding part of topoI. The DNA sequences of HTopoA and HTopoB were completely identical with the corresponding parts in the full-length clone T1B published by D'Arpa et al.[19]. The relative position of the HTopoI sequences to T1B are shown in fig. 2. The clone HTopoB codes for 109 amino acids located at the carboxyterminal end of topoI. HTopoA contains in its 5'-section an open

reading frame of 2091 bp flanked by 900 non-coding nucleotides at the 3'-end. The HTopoA protein contains amino acids 70-765 of the topoI protein fused to only 18 amino acids encoded by the vector and linker sequences (fig. 3). The calculated mol.wt of this protein is 84.2 kD, comprised of a 1.9 kD peptide encoded by the vector linked to 82.3 kD of the topoI protein.

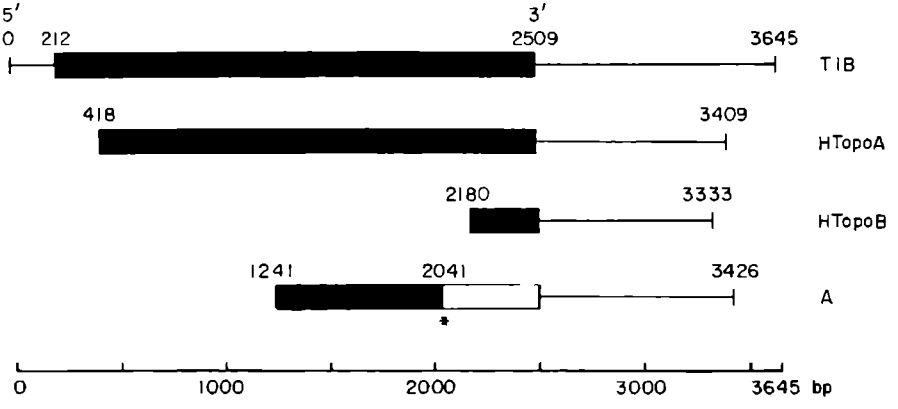

Fig. 2. Schematic representation of the cDNA clones HTopoA and HTopoB and comparison with the full-length cDNA clone T1B published by D'Arpa et al. (1988). The relative position of the cDNA A-sequence is indicated as well. Regions coding for topoI sequences are indicated by solid bars. The open box in the A-sequence represents the out of frame part of the coding sequence of topoI. The premature stop in the open reading frame in the A-sequence is indicated by an asterisk. The 5' and 3' noncoding regions are indicated by thin lines.

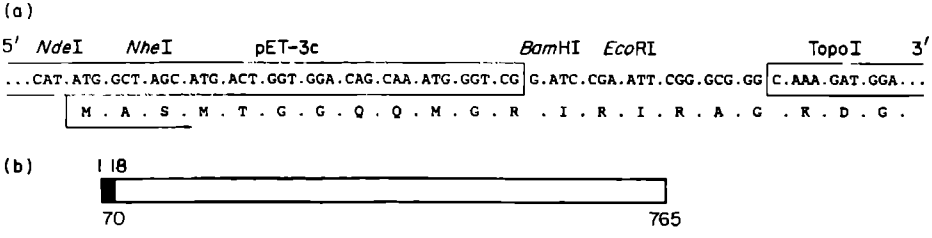

Fig. 3. (a) Nucleotide and amino acid sequences at the protein start point of cloned HTopoA in the pET-3c translation vector (pEHTopoA). The boxed nucleotide sequences at the 5'- and 3'- sites indicate the sequences belonging to the pET-3c vector and the topoI cDNA clone region, respectively. The first 11 amino acids of the fusion protein are derived from the gene 10 protein. The intervening sequence originated from linkers. Locations of the *NdeI*, *NheI*, *Bam*HI and *Eco*RI sites are shown. (b) Schematic representation of the HTopoA protein. The fusion part consist of 18 amino acids followed by amino acids 70-765 of the native topoI protein.

Reactivity of autoimmune sera with the HTopoA protein

Using the immunoblotting technique with a HeLa S3 total nuclear protein extract as antigen source[20], sera from patients with several connective tissue diseases were screened for the presence of anti-topoI antibodies[30]. Fig. 4a shows an example of such an analysis. In this way we selected 85 anti-topoI sera which were subsequently probed on immunoblots containing the HTopoA protein. A typical example of the staining pattern of these sera on such immunoblots is shown in fig. 4b, lane 2. A positive immunoreaction with the 74-kD protein was obtained with 82 of the 85 sera (=96%). All these sera not only recognized the 74 kD protein but also the putative degradation products of 56, 42, and 34kD (see fig. 1, lane 2).

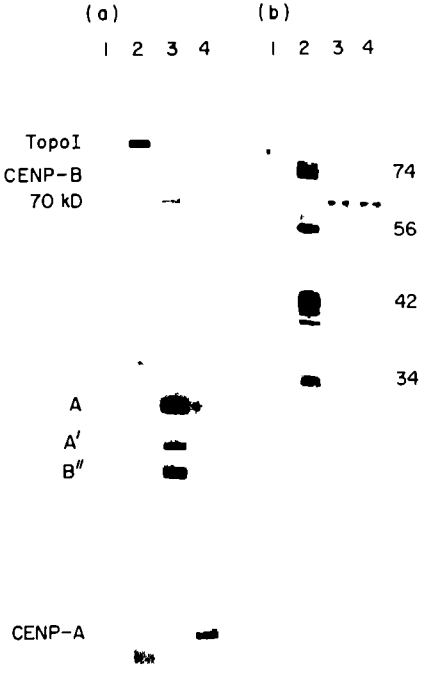

Fig. 4. (a) Characterization of human autoimmune sera Western blots containing HeLa total nuclear proteins were probed with a normal human serum (lane 1), systemic sclerosis serum Z28 showing the topoI band at around 100 kD (lane 2), anti-U1, U2 RNP serum V26 showing the RNP proteins 70K, A, A' and B'' (lane 3), or CREST serum B39 decorating the CENP-A band (19 kD) and the CENP-B ban (80 kD) (lane 4).
(b) Analysis of human autoimmune sera on immunoblots containing total proteins of induced BL21 (DE3)/pEHTopoA Lane 1, normal human serum, lane 2, systemic sclerosis serum Z28, lane 3, anti-U1, U2 RNP serum V26, lane 4, CREST serum B39. The antibody-antigen complexes were detected with horseradish peroxidase-conjugated second antibodies

By comparison, only 85% of the sera that were positive on the immunoblot reacted positively in the Scl-70 immunodiffusion test[31]. Also the three sera that were anti-topoI-positive on a HeLa immunoblot but did not react with the recombinant antigen were negative in this immunodiffusion test.

Reactivity of systemic sclerosis sera with the HTopoA protein

From 40 patients in the group of 85 studied we could obtain a definite diagnosis systemic sclerosis according to the ARA criteria[32]. All these sera showed a strong immunoreactivity with the HTopoA protein. An analogous control experiment was performed with 23 sera from patients with a definite diagnosis of systemic sclerosis that were negative for anti-topol antibodies on a HeLa immunoblot. None of these 23 sera did react with HTopoA protein or its degradation products. These results indicate that both types of immunoblots display a comparable sensitivity for detecting anti-topoI autoantibodies in patient sera.

Autoimmune epitope distribution

To obtain more information about the autoimmune epitope distribution on topoI, immunoblots containing either the HTopoA protein, the HTopoB protein or the A-fragment were probed with anti-topoI positive sera. All proteins were expressed as described in Materials and Methods. As shown in Table 1 three reaction patterns were found, indicating that the various sera recognized different epitopes.

Table 1: Immunoreactivity of anti-topoI-positive sera with the HTopoA protein, the HTopoB protein of the A fragment

Immunoreactivity of anti-topoI sera with:			
HTopoA protein	HTopoB protein	A-fragment	*n* (sera)
+	+	+	38
+	-	+	7
+	-	-	8
+	+	-	0

Serum F14 is placed in the group of sera that gave a positive immunoreaction with all three proteins.

Seventy-two percent of the sera were found to be immunoreactive with all three proteins, 15% reacted with both the HTopoA protein and the A-fragment but contained no detectable level of antibody directed against the protein encoded by HTopoB, whereas another 13% of the sera reacted only with the HTopoA protein. This finding indicates that topoI contains at least three different autoimmune epitope regions, which are distributed over the entire protein (fig. 5).

Fig. 5. Map of the different epitope regions (ER) on the HTopoA protein as recognized by sera from patients with anti-topoI autoantibodies. The numbering of amino acids (70-765) is derived from the full-length clone T1B (D'Arpa et al., 1988). □ ER-1; ■ ER-3; ▨ ER-2.

One epitope region (ER) has to be situated on the A-fragment (ER-2), formed by amino acids 344-589, whereas a second region has to be located on the HTopoB protein which contains amino acids 657-765 (ER-3). As the HTopoA protein bears also one or more epitopes which are not present on the HTopoB protein or the A-fragment, amino acids 70-344 and/or amino acids 589-657 are necessary to form a third ER (ER-1). In none of the sera used in this study could antibodies be detected merely directed against ER-3 without the simultaneous presence of autoantibodies directed against ER-1 and/or ER-2.

We also performed a preliminary study on the epitope distribution of anti-topoI positive sera from systemic sclerosis patients which had been followed longitudinally for about 4 to 8 years. In most of these sera the antibody pattern appeared not to have changed in that period. In some patients, however, the titre of antibody against the various epitopes was found to have been changed significantly during the course of the disease. This was observed, for example, in the serum samples of patient F14 (fig.6) who came to the hospital in 1984 as a severe case of Raynaud's disease. The serum of 1984 of this patient showed a very weak immunoreaction with both the HTopoA protein and the A-fragment. From 1985 up to 1988 this reaction became stronger and clearly positive. In contrast, a significant level of antibody directed against the HTopoB protein (ER-3) was not seen until 1987. A three-fold increase of the HTopoB protein concentration on the immunoblots to enhance the sensitivity of the reaction gave identical results.

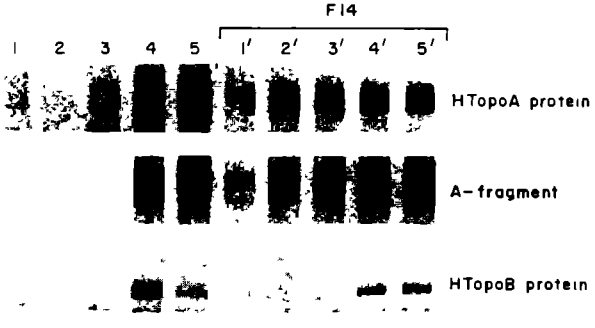

Fig. 6. Characterization of several samples of the SSc serum of patient F14 on immunoblots containing the HTopoA protein (74 kD), the HTopoB protein (125 kD) or the A-fragment (33 kD). Samples were collected in 1984 (lanes 1'), 1985 (lanes 2'), 1986 (lanes 3'), 1987 (lanes 4') and 1988 (lanes 5'). Control sera used were a normal human serum (lanes 1), anti-Sm serum C45 (lanes 2), CREST serum B39 (lanes 3) and SSc sera Z28 (lanes 4) and R25 (lanes 5). The antibody-antigen complexes were detected with horseradish peroxidase-conjugated second antibodies.

Discussion

Using autoantibodies from a patient with systemic sclerosis we isolated a human cDNA clone (HTopoB) encoding DNA topoI from a λgt11 expression library. Screening of the same library with this clone revealed additional recombinants with a longer cDNA insert, the longest being HTopoA. The cDNA HTopoA covered 2091 nucleotides (695 amino acids) of the coding sequence of topoI which consists of 2297 nucleotides (765 amino acids)[19]. This cDNA was expressed in the pET-3c expression vector giving rise to a protein (HTopoA protein) of 84.2 kD. On SDS-PAGE the HTopoA protein migrated as a polypeptide of approximately 74 kD. A similar aberrant behavior of a topoI recombinant protein on an SDS polyacrylamide gel has been described by D'Arpa et al.[19]. Next to the 74-kD protein, lysates of bacteria expressing HTopoA contained several smaller prominent polypeptides (fig. 1). Their presence can be explained either by assuming partial proteolytic cleavage of the 74-kD protein or by the use of alternative ATG initiation codons.

The HTopoA protein was recognized by 96% of our anti-topoI-positive sera. Three sera, scored as weakly positive with topoI on a HeLa immunoblot, did not react with the HTopoA protein. This discrepancy can be explained in several ways. One explanation is that these sera had been scored as false-positive for topoI because of an immunoreaction with another protein migrating in the same mol. wt region as HeLa topoI. As these sera were also negative in the Scl-70 immunodiffusion test, this possibility seems the most likely one. Another possible explanation is that the sera do react with topoI but with an epitope present on amino acids 1-70, i.e. that part of topoI that is missing in the HTopoA protein. Nevertheless, the finding that the HTopoA protein is recognized by at least 96% of our anti-topoI sera makes this recombinant protein a good substitute for native eukaryotic topoI in the screening for anti-topoI antibodies of sera from patients with connective tissue diseases. Since large amounts of purified HTopoA protein can be obtained in a relative simple and easy way this antigen might be very useful in future diagnostic tests. Furthermore, our results confirm an earlier report that about 30% of systemic sclerosis patients do not seem to contain detectable levels of anti-topoI antibodies[14]. Even the use of a very sensitive ELISA test with the recombinant topoI as antigen failed to show the presence of anti-topoI antibodies in these systemic sclerosis patients (data not shown).

In analyzing the immunoreaction patterns of anti-topoI sera with either the HTopoA protein, the HTopoB protein or the A-fragment we found that 72% of the sera contained antibodies directed against all epitope regions on the HTopoA protein. The fact that in the other sera antibodies could be detected against ER-1 and/or ER-2 without the presence of antibodies against ER-3 suggests that the antibody reaction against the various ERs is not developed simultaneously.

A follow-up study of sera obtained between 1984 and 1988 from a systemic sclerosis patient (F14) demonstrated that in the serum sample taken in 1984 no antibodies could be detected against ER-3. Only 3 years after the first detection of autoantibodies against ER-1 and ER-2, could autoantibodies against ER-3 be

detected. As the HTopoB protein is a β-galactosidase fusion protein we considered the possibility that these results were due to a development of antibodies against β-galactosidase. However, all serum samples were negative for the presence of such antibodies (data not shown). One of the possible explanations for our finding is that the autoimmune epitope on ER-3 was developed in a later phase of the disease as compared with the other autoimmune epitopes on topoI. However, this conclusion should be interpreted with great care, as in these experiments one is comparing the immunoreactivity of different types of antibodies on different antigens.
Recently, Eng, Pandit & Sternglanz[33] and Lynn et al.[34] have mapped the tyrosine residue in yeast topoI that is responsible for the formation of the covalent enzyme-DNA intermediate. On basis of alignment of topoI sequences of human and yeast, these investigators proposed Tyr-723 in human topoI to be the equivalent active site tyrosine. This means that only autoantibodies against the HTopoB protein (ER-3) may interact with or near the active center and possibly inhibit topoI activity. Further studies are now in progress to map the epitope regions in more detail in order to select those autoimmune sera that recognize the active site of topoI. Such knowledge may contribute to a better insight into the progression of systemic sclerosis.

Note

Shortly after this paper was accepted for publication, another description B cell epitope on DNA topoisomerase I appeared (Maul GG, Jimenez SA, Riggs E, Ziemnicka-Kotula D. Proc. Natl. Acad. Sci. USA, 1989;86:8492). This autoepitope is contained in the region defined by us as ER-3 (see fig. 5).

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Antitopoisomerase I antibodies and treatment of systemic sclerosis

A. Van den Hoogen FHJ, Verheijen R, Boerbooms AMTh, Croockewit AJ, Van Venrooij WJ, Van de Putte LBA. Rebound of antitopoisomerase I antibody titers after plasma exchange. Ann Rheum Dis 1993;52:246-247.

B. Van den Hoogen FHJ, Boerbooms AMTh, De Jong BAW, Van Venrooij WJ, Van de Putte LBA. Effects of low dose methotrexate treatment on antitopoisomerase I antibody titers in systemic sclerosis. Submitted.

A. Rebound of antitopoisomerase I antibodies titers after plasma exchange therapy in a patient with systemic sclerosis.

Serum levels of a specific antibody induce a negative feedback on the synthesis of antibody of the same specificity[1,2]. If this feedback inhibition of antibody synthesis is reduced by diminishing the concentration of antibody in the circulation by plasma exchange (PE) an enhancement of antibody synthesis, also termed "antibody rebound" occurs. This rebound phenomenon has been shown to occur amongst others in autoimmune mediated diseases such as SLE[3].
Plasma exchange, single or combined with immunosuppressive therapy, has been used in the treatment of systemic sclerosis (SSc)[4-6], although its effectiveness in this disorder remains questionable[7]. Changes in autoantibody concentrations during PE-therapy of scleroderma patients have been used to monitor the efficacy of PE[4,5].

We would like to present the case of a 50 year old woman who had Raynaud's phenomenon since the age of 30 and a 4-year history of sclerodactyly. In a few months time skin thickening progressed to upper arms, face, neck and chest. A diagnosis of rapidly progressing SSc was made. Treatment with azathioprine, 100 mg (2 mg/kg) daily, was initiated. Four weeks thereafter, as no improvement occurred, PE therapy was started. At that time laboratory results included hemoglobin 6.4 mmol/L, white blood cell count 7,300/mm^3, platelets 428 x 10^9/L, Westergren 48 mm/hr. and creatinine clearance 70 ml/min. Total hemolytic complement, circulating immune complex levels, C3/C4 complement components and concentrations of immunoglobulins (Ig) A, M and G were within normal range. Both IgG and IgA isotypes of antitopoisomerase I antibodies (ATA) could be detected, the IgM isotype was below detection level. Radiologic examination disclosed reduced peristalsis of the oesophagus; chest X-ray and pulmonary function tests were within normal limits. During a 29 day period 11 PE sessions were performed. At each session 2000 ml of plasma was exchanged using cryoglobulin free plasma for replacement. The interval between PE sessions varied from 1 to 5 days. Before the start of azathioprine treatment and immediately before and after each PE, total IgA and IgG concentrations were established, as well as IgG and IgA ATA titers, using an enzyme-linked immunosorbent assay (ELISA) with a recombinant topoisomerase I as antigen source[8]. After the 11th session PE was stopped on patient's request. Azathioprine medication was given during the whole period of PE-treatment and continued after the cessation of this treatment.

No change in titers of total IgA and IgG or ATA could be detected before and after 4 weeks of azathioprine treatment, when PE-therapy was started. After each PE however, a reduction of IgG and IgA ATA titers was found (figures 1a and 1b). IgG ATA titers decreased on average 40% ± 16% (range 18% - 72%) and IgA ATA titers 51% ± 14% (range 29% - 77%). Total IgA and IgG levels decreased in the same amount as IgA and IgG ATA concentrations. Within 1 to 5

days after each PE, ATA titers increased to 80% - 125% of pre-PE levels; although an increase in total IgA and IgG was also observed, this increase never exceeded pre-PE values. Five days after the last PE, IgG and IgA ATA levels were respectively 77% and 83% of the initial values. Five weeks after the last PE, the patient still being on azathioprine, both IgG and IgA ATA levels equalled pretreatment values. No changes in the physical condition of the patient could be observed during the PE-treatment.

To our knowledge this rebound of ATA after PE in a patient with systemic sclerosis has not been reported before. The mechanisms of antibody rebound, sometimes leading to post-PE concentrations exceeding pre-treatment values, remain unsolved. A conjectural explanation might be clonal expansion of antibody producing B-cells, caused by re-exposure of antigenic determinants after a decrease in antibody levels or by removing blocking anti-idiotypic antibody[9]. Therefore, PE applied in the treatment of autoimmune diseases is usually combined with cytotoxic drugs to inhibit B-cell proliferation. In our patient azathioprine 100 mg daily could not accomplish a decline of ATA titers and could not prevent the rapid increase of ATA after PE as well. However, 5 days after the last PE, the combination of azathioprine and PE had caused a reduction of about 20% of both IgG and IgA ATA titers. In the study performed by Ferri et al.[5], who treated 6 scleroderma patients with PE without concomitant cytotoxic treatment, autoantibody levels were largely unaffected after a prolonged period of PE-treatment despite a clinical benefit observed in their patients. It was suggested that the unchanged antibody concentration could be attributed to a rapid resynthesis of autoantibodies rather than to insufficient PE. The rebound of ATA titers observed in our patient confirms this presumption. Therefore, in monitoring autoantibody levels during PE-treatment in SSc, rebound of ATA and presumably all other auto-antibodies, should be considered.

Figure 1a IgG anti-topoisomerase antibody titers

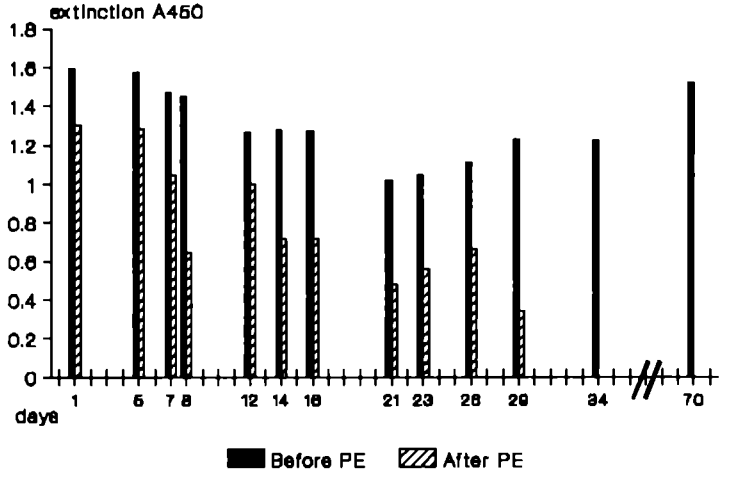
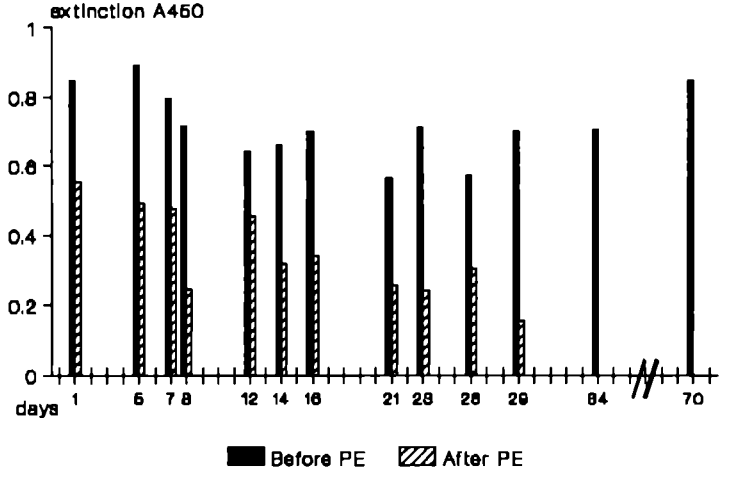


Figure 1b IgA anti-topoisomerase antibody titers



Antitoposisomerase antibody titers were determined by enzyme linked immunosorbent assay (ELISA) in the serum before and immediately after plasma exchange (PE) treatment. The results are given in optical density units (OD), read at 450 nm. The value of pooled normal human serum in this experiment was about 0.1. After day 29 plasma exchange treatment was discontinued.

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B. Effects of low dose methotrexate treatment on antitopoisomerase I antibody titers in systemic sclerosis

Summary

Aims - To study the effects of methotrexate (MTX) treatment on antitopoisomerase I antibody (ATA) titers and to investigate the relation between ATA titers and response to treatment in scleroderma patients.

Patients and methods - Out of 29 scleroderma patients who participated in a double blind study comparing MTX with placebo, 16 patients were positive for antitopoisomerase I antibodies and were involved in this study. The patients received either 15 mg MTX or placebo weekly via intramuscular (im) injection. After 24 and 48 weeks of treatment response was evaluated according to predetermined criteria, consisting of ≥30% improvement of skin score or general well-being, or improvement of ≥15% of single breath diffusion capacity for carbon monoxide. Patients who were considered to respond after 24 weeks on initial treatment continued with the same treatment to the end of the trial at week 48; patients who did not respond on placebo after 24 weeks continued with 15 mg MTX weekly from then on; patients who did not respond on 15 mg MTX continued with an increased dose of 25 mg MTX until week 48. At study entry and after 24 and 48 weeks ATA titers were measured using an ELISA with a recombinant topoisomerase I as antigen source.

Results - No significant differences were observed in pretreatment ATA titers between scleroderma patients receiving MTX or placebo. ATA titers decreased equally during MTX and placebo treatment. Likewise, a similar reduction of ATA titers was found in patients responding on MTX treatment and in patients not responding on MTX treatment.

Conclusion - ATA titers are not useful for monitoring MTX treatment in SSc.

Introduction

Systemic sclerosis (scleroderma, SSc) is a connective tissue disease characterized by fibrosis and vascular obliteration in the skin and internal organs. SSc is a rare disease with an estimated annual incidence rate of 20 new cases per million[1]. The pathogenesis of the disease remains unknown but mechanisms of enhanced extracellular matrix accumulation, vascular endothelial injury and immune cell activation seem to be involved[2]. Evidence for immune cell activation is obtained amongst others by the presence of antinuclear antibodies in over 90% of sera of patients with SSc[3]. Antitopoisomerase I antibodies (ATA) are a specific serological maker for SSc[4] and can be detected in 28 - 59% of scleroderma patients with the diffuse cutaneous form with skin thickening proximal of elbows and/or knees[5-7]. ATA also occur in approximately 14% in patients with limited disease, that is with skin involvement of the distal extremities only[8]. Diffuse SSc is associated with more frequent occurrence of internal organ involvement and hence adversely affects disease outcome[9-11]. Therefore, the presence of antitopo in scleroderma patients is thought to be indicative of a worse prognosis.
Although a wide variety of drugs have been advocated in the treatment of SSc, no single drug has been shown to be effective in this disease. Recently, we reported on the results of a randomized double blind trial in which low dose methotrexate (MTX) was compared with placebo in the treatment of patients with SSc. It was shown that MTX was superior to placebo[12].
The aims of this study were to determine the effects of methotrexate (MTX) treatment on antitopoisomerase I antibody (ATA) titers and to investigate the relation between ATA titers and response to treatment in scleroderma patients.

Patients and methods

The details of the clinical study have been described elsewhere[12]. In short, 29 patients meeting the preliminary criteria for the classification of SSc[13] were enrolled in a 48-week, randomized, double-blind trial comparing methotrexate with placebo. Drugs with potential disease-modifying effects were discontinued at least three months prior to study entry. Dosages of allowed concurrent treatment (including prednisone ≤10 mg/day and non-steroidal anti-inflammatory drugs) was kept stable during the trial. Patients were randomly allocated to receive either 15 mg MTX (n=17) or placebo (n=12) administered weekly as intramuscular (im) injection. Clinical and laboratory data were gathered by one observer (FvdH) every 4 weeks. After 24 and 48 weeks, treatment was evaluated according to predetermined criteria. Good response was defined as ≥30% improvement occurring in either skin score (using the skin score method as developed by Steen et al.[14] or patients' global assessment expressed in a visual analogue scale (0 mm = low general well-being and 100 mm = optimal general well-being) or improvement of ≥15% of diffusion capacity for carbon monoxide (DL_{CO}), whenever not accompanied by persistence of digital ulcerations or worsening of DL_{CO} by ≥15%.

Patients who were considered to respond on initial treatment continued with the same treatment to the end of the trial at week 48; patients who did not respond on placebo continued with 15 mg MTX im weekly from then on, patients who did not respond on 15 mg MTX continued with an increased dose of 25 mg MTX until week 48, at which time a final evaluation took place. At study entry, in sera of 16 of the 29 patients antitopo could be detected on immunoblotting and quantified by ELISA[15].

Antitopoisomerase I antibody measurements

Serum samples obtained at study entry and after 24 and 48 weeks of treatment were used for measuring antitopo-titers. Serum samples were stored at -70°C, and after the study was completed, all sera were thawed and processed at the same time. For detection of ATA activity an ELISA was established using a recombinant topo protein as antigen source[15]. Optimal concentrations of antigen, patient sera and conjugates were established after appropriate chess board titrations. The procedure was performed according to the method previously described[16]. Plates were read on a Bio-Rad model 2550 ELISA reader at 450 nm. OD_{450} was arbitrarily defined as 1 unit. All sera were tested in triplicate in different dilutions and the results were averaged. Interassay and intra-assay variabilities never exceeded 15%.

Statistical analysis

Differences in demographic characteristics between the two treatment groups were judged using the X^2 test for nominal variations. Differences in ATA-titers between groups at baseline were calculated with the Mann-Whitney test. Comparison of changes in ATA-titers between the groups at baseline and during follow-up were calculated with the two sample T test. P values ≤ 0.05 were considered significant.

Results

As none of the 13 patients, in which at study entry ATA activity was not detectable either via immunoblotting had antitopotiters detectable on ELISA at any measuring point during trial, this study focusses only on the 16 antitopo positive patients. The demographic characteristics of the patients are shown in table 1. Nine patients received methotrexate and 7 received placebo treatment initially. No significant changes were observed in demographic characteristics between the two treatment groups. The disease course of the patients is depicted in figure 1.

Seven out of nine patients who started treatment with 15 mg MTX weekly at study entry completed the first 24 weeks of the trial. Two patient were withdrawn, one because of renal crisis and one because of severe headache. At week 24, three patients were considered to be responders and 4 nonresponders, according to the

above mentioned criteria. The four not responding patients were treated with 25 mg MTX weekly from then on; one of these patients died because of respiratory insufficiency due to progressive fibrosis, the remaining three did not respond on the enhanced dose of MTX at week 48.

Table 1: Demographic characteristics of patients positive for antitopoisomerase I antibodies at study entry

	Methotrexate	Placebo
number	9	7
mean age (SD), years	53 8 (8 2)	56.3 (9 2)
male/female	5/4	2/5
limited/diffuse*	6/3	5/2
dis duration <1 year/≥1 year**	4/5	2/5

* limited: skin thickening distal to elbows and/or knees or face only;
diffuse: skin thickening proximal to elbows and/or knees

** dis. duration. disease duration as assessed from first signs of skin thickening

Six out of the seven patients initially treated with placebo, completed the first 24 weeks of the trial; one patient was withdrawn due to renal crisis. After 24 weeks, one of these patients was considered to respond; the five remaining patients were treated with 15 mg MTX from then on and two of these were considered responder as yet on week 48. ATA titers at weeks 24 and 48 of the latter patients were included in the analysis of responders and nonresponders on 15 mg MTX treatment. At the end of the trial, six patients could be classified as responders (5 on MTX treatment, 1 on placebo treatment).

Twelve patients received 15 mg MTX for at least 24 weeks; this group included seven patients who where treated with MTX from week 0 to 24 and five patients treated with MTX from week 24 to 48.

ATA titers from patients that received placebo or MTX, and from patients that responded or did not respond during the trial are presented in table 2. No significant difference was observed in pretreatment ATA titers between the placebo and MTX treated group; likewise, no significant differences were found between responders and nonresponders. Compared with baseline values, after 24 weeks of treatment ATA-titers decreased in the placebo and in the MTX group. The difference of changes between the two groups was 9.7 ± 17.4 (mean ± standard error of the mean, p=0.59). ATA-titers also decreased in patients that responded

Table 2: Antitopoisomerase I antibody (ATA) titers and percent change after 24 weeks in patients receiving either methotrexate or placebo treatment, and in responders and nonresponders.

	ATA titers (U)	% changes at 24 weeks	mean difference of changes between groups
Placebo (n=6)	1520 (220-17493)*	-14 (-43,38)	
Methotrexate (n=12)	3392 (954-4138)	-28 (-60,74)	9 7 ± 17 4 (p=0 59)
Responders (n=6)	1179 (220-3872)**	-40 (-60,8)	
Nonresponders (n=6)	1357 (552-17494)	-24 (-38,74)	26 0 ± 19 7 (p=0 22)

Values of ATA titers and % changes are given as median with between parentheses minimum and maximum values Differences of changes are given as mean ± standard error of the mean ATA titers are presented as units, OD_{450} was arbitrarily defined as 1 unit

* p=0 18 between placebo and MTX treated group, ** p=0 82 between responders and non responders.

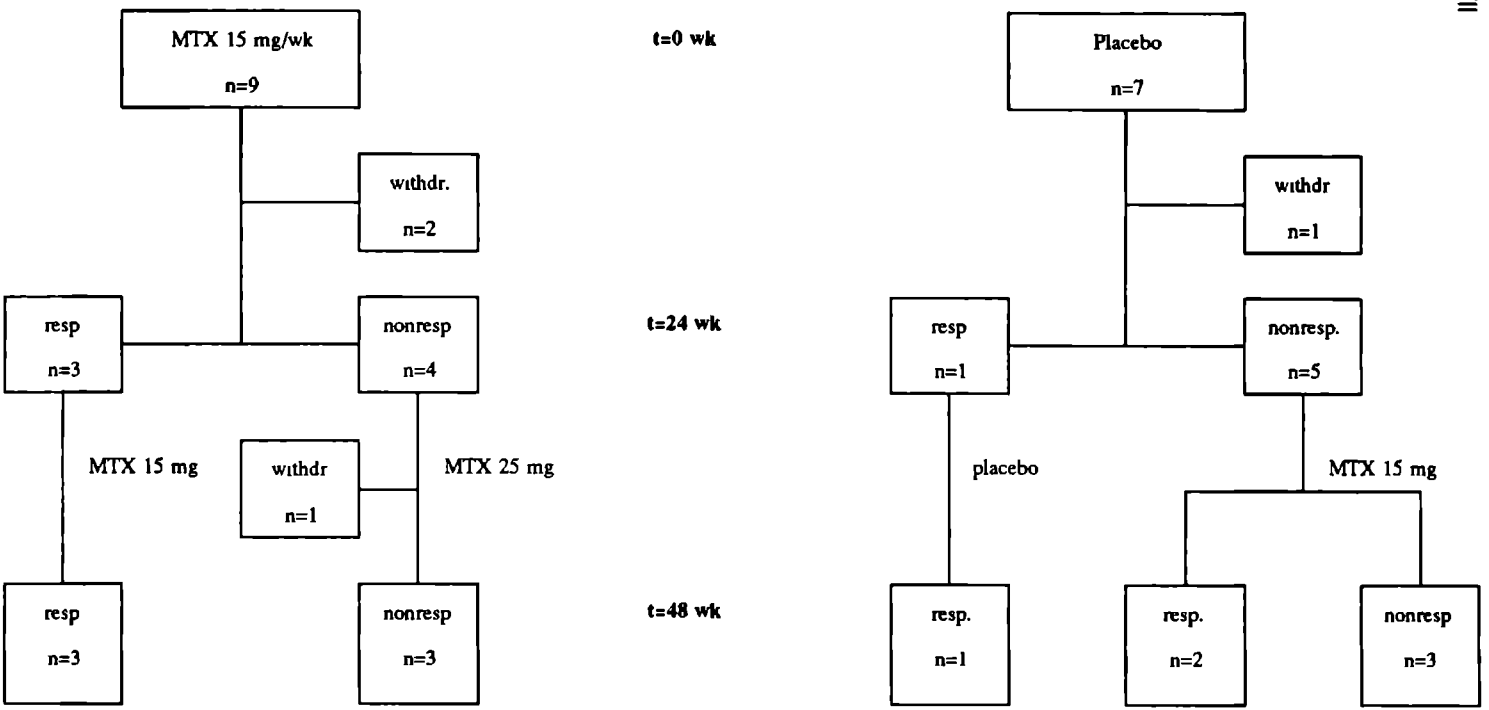
Table 3: Antitopoisomerase I antibody (ATA) titers at study entry in patients with disease duration <1 year and ≥1 year, and in patients with limited and diffuse disease.

	ATA titers (U)	P-value between groups
Disease duration <1 year (n=6)	3440 (768-17493)	0 09
Disease duration ≥1 year (n=10)	1309 (220-3872)	
Limited disease (n=11)	1098 (220-17493)	0 31
Diffuse disease (n=5)	3296 (1520-3584)	

Values of ATA titers are given as median with between parentheses minimum and maximum values

* limited disease skin thickening distal to elbows and/or knees or face only
diffuse disease skin thickening proximal to elbows and/or knees

Figure 1: Disease course of patients with antitopoisomerase I antibodies



withdr.= withdrawal; resp = responders; nonresp.= nonresponders

to either 15 mg MTX (n=5) or placebo (n=1) and in the patients that not responded to 15 mg MTX treatment. The mean difference of changes between the two groups was 26.0 ± 19.7 (p=0.22). No difference in results was obtained when ATA-titers of the only patient responding on placebo were omitted in the calculations (data not shown).

We also compared ATA-titers at study entry of patients with disease duration <1 year and ≥1 year, and of patients with limited and diffuse disease (Table 3). There was a tendency of higher median ATA-titers in the patients with disease duration <1 year as compared to patients with longer disease duration (p=0.09); patients with diffuse disease had no significant different ATA-titers as compared with patients with limited disease (p=0.31).

Discussion

Serial assessments of antibody levels in autoimmune rheumatic diseases such as anti-dsDNA in systemic lupus erythematosus[17-19] and anticytoplasmic antibodies in Wegener's disease[20] can be helpful in monitoring disease activity. An increase of the titer of one of these autoantibodies may precede a disease exacerbation; response to treatment may be accompanied by a decline of autoantibody titer. Both autoantibodies have a high disease specificity, which is also the case for the ATA in scleroderma[4]. Antitopoisomerase I antibodies are reported to occur predominantly in patients with diffuse scleroderma. However, in this study only 5 of the 16 patients with ATA could be classified as diffuse SSc. ATA are associated with a bad disease outcome[9-11,21]. As far as we know this is the first study that examined the relation between the ATA titers and efficacy of treatment in patients with systemic sclerosis.

We found a reduction of ATA-titers both in patients receiving MTX and patients receiving placebo for 24 weeks. The difference of the changes between the two groups after 24 weeks was not significant indicating that low-dose MTX cannot be the cause for the decline in ATA-titers.

There was no significant difference in ATA-titers between responders and nonresponders at the start of either MTX or placebo treatment; therefore response to treatment is not predicted by pretreatment ATA-titers.
After 24 weeks of treatment, ATA-titers decreased equally in the responders and nonresponders. This indicates that ATA-titers do not reflect clinical response as defined by criteria consisting of ≥30% improvement of skin score or general well-being, or ≥15% improvement of DL_{CO}, that we applied in this study.

Only in three patients an increase of ATA-titers was observed. This increase could not be related to MTX or placebo treatment, diffuse or limited disease, or disease duration <1 year or ≥1 year. ATA-titers decreased in all the other patients studied

during follow-up, irrespective of MTX or placebo treatment, and irrespective of response to treatment. We have no good explanation for this decrease but it could be explained by an inverse relation between ATA-titers and disease duration. Some support for this explanation is derived from the fact that there was a tendency of higher ATA-titers at study entry in patients with disease duration <1 year as compared to patients with longer disease duration. However, ATA-titer measurements in larger groups are required to substantiate this assumption.
On the basis of the results of this study, we conclude that ATA titers are not useful in monitoring MTX treatment in SSc.

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Long-term follow-up of 46 patients with MCTD

Evolution towards systemic sclerosis, systemic lupus erythematosus and rheumatoid arthritis

Van den Hoogen FHJ, Spronk PE, Boerbooms AMTh, Bootsma H, De Rooij DJRAM, Kallenberg CM, Van de Putte LBA. Long-term follow-up of 46 patients with anti-(U1)snRNP antibodies. Br J Rheumatol, in press.

Summary

The records of 46 patients with anti-(U1)snRNP antibodies and a minimal period of follow-up after first clinical presentation of at least five years were examined with emphasis on symptoms contributing to established criteria of systemic lupus erythematosus (SLE), systemic sclerosis (SSc), rheumatoid arthritis (RA) or dermato- or polymyositis (DM/PM). At first clinical presentation 13 (28%) of the 46 patients studied fulfilled ARA-criteria for SLE (n=10), RA (n=2) and SSc (n=1), and 33 (72%) were classified as mixed connective tissue disease (MCTD). During follow-up 18 patients initially classified as MCTD were now classified as SLE (n=5), SSc (n=7), RA (n=3), or a combination of these disorders (n=3). A transformation of MCTD towards these connective tissue diseases occurred 2.6 ± 3 years (mean ± SD) after first clinical presentation. At the end of the follow-up period 67% of the patients fulfilled ARA criteria for SLE, SSc, RA or a combination of these diseases.

The majority of patients with anti-(U1)snRNP antibodies have or will develop a classified connective tissue disease within 5 years after clinical presentation. This undermines the concept of MCTD being a distinct clinical entity.

In 1972 Sharp et al. described a syndrome, which they called mixed connective tissue disease (MCTD), that was clinically denoted by features of two or more defined autoimmune connective tissue diseases, namely systemic lupus erythematosus (SLE), systemic sclerosis (scleroderma, SSc) and dermato- or polymyositis (DM/PM), and the presence of a high titer of circulating antibodies to an RNA-se sensitive extractable nuclear antigen[1]. This antigen proved to be a ribonucleoprotein (RNP) that plays an important role on pre-messenger ribonucleic acid (RNA)-splicing[2] and is composed of a small nuclear (sn) RNA designated U1, and three or more distinct U1-RNA binding proteins[3]. Immunoblot studies have shown that the immunoreactive sites are located on the protein moiety. Antibodies directed against the (U1)snRNP (anti-(U1)snRNP antibodies) are considered to be the hallmark of MCTD. Additional clinical features of MCTD appeared to be infrequent renal disease, good response to low doses of corticosteroids and a favorable prognosis[1]. However, since then the concept of MCTD being a truly distinct disease entity has been questioned in many reports. Some hold that MCTD should be viewed as a part of the spectrum of SLE rather than a different disease subset[4], while others have not found a close association between the mixed clinical characteristics of the different autoimmune connective tissue diseases and antibodies to RNP[5-7]. Anti-RNP antibodies are not restricted to MCTD, but occur in other connective tissue diseases as well[4,8-11]. Occasionally anti-(U1)snRNP antibodies have been reported in other autoimmune diseases[12]. Furthermore, many of the cases initially diagnosed as MCTD have evolved to SSc or SLE[6,7,13,14], and some have developed well defined overlap syndromes[15,16].
Since the concept of MCTD being a distinct clinical entity is still controversial and the diagnostic and prognostic value of anti-(U1)snRNP antibodies are unresolved, the aim of this study was to evaluate the disease course of patients with anti-(U1)snRNP antibodies during a minimal follow-up period of at least five years.

Patients and methods

Patients

Patients with signs and symptoms indicating inflammatory connective tissue disease treated at or referred to the Departments of Rheumatology of the University Hospital or St. Maartenskliniek Nijmegen, or the Department of Clinical Immunology of the University Hospital Groningen between 1975 and 1986 were analyzed for the presence of antinuclear antibodies (ANA). All sera with ANA activity were routinely analyzed for antibodyspecificity by counterimmunoelectropheresis (CIE) and from 1981 onwards also by immunoblotting. Patients positive for anti-(U1)snRNP were selected for further analysis.

Methods

Charts of all patients were examined according to a predetermined protocol with particular attention paid to the presenting symptoms and symptoms contributing to the well-defined classification criteria of SLE, RA, SSc and DM/PM[17-20], as well as to the presence of clinical and serologic manifestations characteristic of connective tissue diseases. During follow-up patients were seen at regular intervals; serologic parameters, that from 1980 onwards were measured yearly, consisted of rheumatoid factor (RF), ANA, anti-dsDNA antibodies, and anti-(U1)snRNP antibodies as assessed by CIE and immunoblotting. For each patient, clinical and serologic data were stored on a cumulative way.
The presence of ANA was determined by indirect immunofluorescence using human fetal fibroblasts[21] or Hep2 cells as a substrate. Serum was considered ANA positive when a serum dilution of ≥1:40 on human fibroblasts and ≥1:10 on Hep2 cells appeared positive. Anti-dsDNA antibodies were considered positive when a positive immunofluorescence was obtained with a serum dilution of ≥1:10 in the Crithidia luciliae assay[22]. Antibodies to extractable nuclear antigens (anti-ENA) were detected by CIE according to Kurata and Tan[23] using a crude extract from rabbit thymus acetone powder as substrate and reference sera showing identity with the corresponding Center for Disease Control (CDC, Atlanta, GA) references. Immunoblotting was performed with extracts from HeLa cell nuclei as previously described[24]. Anti-(U1)RNP antibodies were considered when the 70kDa and/or the A and/or the C band were present, anti-Sm antibodies in the presence of the D band with or without the BB' band, antitopoisomerase I antibodies in the presence of the 86kDa band and anticentromere antibodies in the presence of the 17kDa band. RF was considered positive when IgM-RF, measured by enzyme linked immunosorbent assay (ELISA), was >10 U/ml.

Results

Demographic characteristics

In 49 patients anti-(U1)snRNP antibodies could be detected on two consecutive occasions on both immunoblot and CIE. Three patients had moved and were lost for follow-up, leaving 46 patients with anti-(U1)snRNP antibodies who at the time of the study had a minimal follow-up period of five years. There were 33 females and 13 males. Mean age ± SD of the patients was 49 ± 15 years (range 24 - 81 years) with a mean disease duration of 17 ± 6 years and an average period of follow-up of 15 ± 6 years.

Diagnosis at clinical presentation

At clinical presentation ten patients could be classified as having SLE, two patients as having RA and one as having SSc; the remaining 33 did not fulfil

classification criteria of any of these connective tissue diseases but could be designated MCTD according to Sharp's criteria[1]. The presenting symptoms of the patients with a diagnosis of the various autoimmune diseases are depicted in Table 1. This shows that none of the presenting symptoms was specific for any of the autoimmune diseases studied.

Table 1: Presenting symptoms in 46 patients with anti-(U1)snRNP-antibodies, classified according to initial diagnosis

	MCTD (n=33)	SLE (n=10)	RA (n=2)	SSc (n=1)
Raynaud's (N,%)	17 (52)	3 (30)	1 (50)	1 (100)
Arthralgia (N,%)	14 (42)	5 (50)	0	0
Arthritis (N,%)	2 (6)	2 (20)	2 (100)	0
Nephropathy (N,%)	1 (3)	0	0	0
Discoid lesions (N,%)	1 (3)	1 (10)	0	0
Puffy hands (N,%)	2 (6)	1 (10)	0	0

MCTD mixed connective tissue disease, SLE systemic lupus erythematosus,

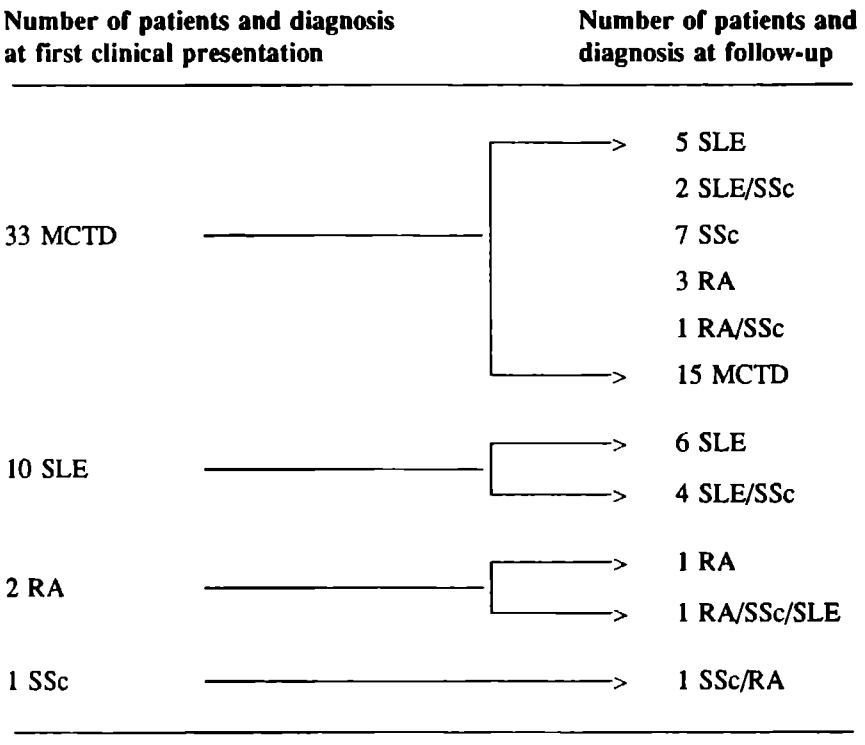
RA rheumatoid arthritis, SSc systemic sclerosis

Diagnosis at follow-up

At the end of the follow-up period, 18 (55%) of the 33 patients initially classified as MCTD could be diagnosed as having one or a combination of two connective tissue diseases (Table 2). The majority of these patients developed SSc, SLE or a SSc-SLE overlap syndrome, while in none of the patients a diagnosis of DM or PM could be made. In the group of patients initially designated MCTD the time lapse between first clinical presentation and diagnosis of a classified connective tissue disease was on average 2.6 ± 3 years (median 2 years). The patients who remained classified as MCTD had a mean follow-up of 12.7 ± 4.2 years (median 12 years).

The initial characteristics of these latter patients were not different from patients who evolved into a well-defined connective tissue disease (data not shown). Follow-up data of those patients, who fulfilled diagnostic criteria of SLE, SSc or RA at first clinical presentation, are also shown in Table 2. It appeared that during follow-up four patients with SLE and one with RA also fulfilled classification criteria for SSc and one patient with RA fulfilled classification criteria of SLE, the patient with SSc developed signs and symptoms of RA.

Table 2: Diagnoses at first clinical presentation and at follow-up of 46 patients with Anti-(U1)snRNP-antibodies



Finally, 31 (67%) of all anti-(U1)snRNP antibody positive patients entered in this study fulfilled ARA diagnostic criteria for SLE, SSc, RA or a combination of these diseases.

Autoantibody profiles

In addition to anti-(U1)snRNP antibodies other autoantibodies appeared to be present at first clinical presentation as well (Table 3). RF was the most prevalent antibody; it could be detected in sera of 26 (79%) patients with MCTD, of five (50%) patients with SLE and of both patients with RA, and of the one patient with SSc. Anti-dsDNA antibodies were the second most frequent autoantibodies found and occurred in three (9%) of the patients with MCTD and eight (80%) of patients with SLE. Other autoantibodies that could be detected each in one patient only were anti-Sm antibodies, anti-SSA and -SSB-antibodies and anticentromere antibodies.

Table 3: Concurrent autoantibodies in anti-(U1)snRNP positive patients at first clinical presentation

	MCTD (n=33)	SLE (n=10)	RA (n=2)	SSc (n=1)
Rheumatoid factor	26	5	2	1
Anti-dsDNA	3	8	1	--
Anti-Sm	--	--	1	--
Anti-SSA	--	1	--	--
Anti-SSB	1	--	--	--
Anticentromere	--	--	1	--
Antitopoisomerase I	--	--	--	--

For abbreviations see Table 1.

During the follow-up period anti-(U1)snRNP antibodies remained detectable in the serum of every patient. The three patients with MCTD and anti-dsDNA antibodies at presentation all developed SLE during follow-up. Patients with MCTD throughout the follow-up period did not develop other autoantibodies than those present at clinical presentation. However, in four other patients out of the 18 patients with an initial diagnosis of MCTD who developed a classified connective tissue disease, autoantibodies appeared during follow-up (RF in two patients, one of whom developed SLE and one SSc; anti-dsDNA antibodies and anti-Sm/anti-dsDNA antibodies both in one patient who developed SLE).
In seven patients with an initial diagnosis of SLE other autoantibodies than those present at first clinical presentation appeared during follow-up; these autoantibodies consisted of RF in three cases, anti-dsDNA in two and anti-SSA and anti-Sm both in one patient. Patients with an initial diagnosis of SSc or RA did not develop other autoantibodies than those initially present.

Treatment

In most of the patients with MCTD (n=15) throughout the follow-up period, symptoms could be managed with a combination of NSAIDs and hydroxychloroquine (n=14). Five (34%) patients received an additional treatment of 5 mg prednisone daily. In only two (13%) patients prednisone dosage was temporarily enhanced to 15 mg a day because of mild thrombocytopenia. Patients with an initial diagnosis of MCTD but who developed SLE or SSc, required treatment with high doses prednisone (>15 mg a day) and/or azathioprine, methotrexate or

cyclophosphamide more frequently than those with persistent MCTD. Four of the patients who developed SLE and two of those who developed SSc received those treatments because of evolving glomerulonephritis, serositis or vasculitis.

Discussion

In this study, 13 of 46 patients with anti-(U1)snRNP antibodies could be diagnosed as SLE, SSc or RA at first clinical presentation. This confirms other reports which state that anti-(U1)snRNP antibodies are not specific to any of the connective tissue diseases[4,8-11]. Moreover, in 18 (55%) of 33 patients with anti-(U1)snRNP antibodies with a diagnosis of MCTD according to Sharp's criteria at presentation, a diagnosis of SLE, SSc, RA or a combination of these disorders could be made during a minimal follow-up period of five years. This is in agreement with reports in the literature[6,7,13-16] and supports the concept that MCTD is a transient phase of a connective tissue disease, not yet having reached its final expression.

None of the presenting symptoms in the MCTD patients appeared to be predicative for the evolution towards a classified connective tissue disease. However, the de novo appearance of specific autoantibodies during the course of the disease must alert the physician of an ongoing development towards SLE or RA. In some patients initially designated MCTD, the appearance of anti-dsDNA antibodies made a diagnosis of SLE possible.

Patients initially diagnosed as MCTD who evolved into SLE, SSc, RA or a combination of these diseases, developed complications that required more aggressive treatment than was required for those with persistent MCTD. Anti-(U1)RNP-antibodies are therefore not associated with a favorable disease course as stated in the original report of MCTD by Sharp[1] but refuted later[13,25-27].

Anti-(U1)RNP-antibodies have been reported to appear or disappear during the course of disease[28]. In this study, however, anti-(U1)RNP-antibodies persisted in all patients throughout the follow-up period, irrespective of treatment. Neither was a switch observed in antibody production from anti-(U1)RNP to Sm or vice versa, as mentioned in several reports[28,29]. During the follow-up other autoantibodies than those present at clinical presentation were produced by a number of patients initially diagnosed as MCTD who developed a classified connective tissue disease, as well as in those patients with a classified disease at presentation.

We conclude that the majority of patients with anti-(U1)snRNP antibodies have or will get a classified connective tissue disease such as SLE, SSc, RA or a combination of these diseases within five years after presentation.

We agree with previously published proposals[30,31] to abandon the concept of MCTD as a distinct clinical entity and to reserve the demotion *undifferentiated connective tissue disease* for patients with signs and symptoms of a connective tissue disease not satisfying the established classification criteria for any of the connective tissue diseases, irrespective the presence of anti-(U1)RNP antibodies.

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Chapter IX

Summary and Conclusions

The main objective of this thesis is to assess the efficacy and toxicity of low-dose MTX in the treatment of patients with systemic sclerosis (SSc). Further objectives are to assess the effects of MTX on extracellular matrix production of scleroderma fibroblasts, to investigate whether antitopoisomerase I antibody titers can be used in assessing response to treatment in SSc, and to confirm assumptions that patients with MCTD develop SSc in due course.

Chapter I is a general introduction including definition, classification, epidemiology and prognosis, etiopathogenesis and treatment of SSc, and outcome measures used in SSc. Moreover, MTX and its use in rheumatic diseases is briefly discussed.

A review of the literature regarding immunomodulatory treatment of SSc is presented in chapter II. In uncontrolled trials applying numerous immunomodulating drugs, such as chlorambucil, azathioprine, cyclophosphamide, 5-fluoro-uracil and cyclosporine, a beneficial effect was claimed on the progression of SSc. However, on the rare occasion that a double-blind placebo controlled trial with any of these drugs was performed, these effects could not be substantiated. Low-dose MTX was reported once in the treatment of two patients with SSc; improvement was obtained in softening of the skin of the dorsum of the hands and in range of motions of the fingers.

In chapter III we report on our first experiences with MTX treatment of scleroderma patients. In chapter IIIa the first two scleroderma patients receiving MTX since Welin's case report in 1973, are described and in chapter IIIb the results of an open 12 months study in eight scleroderma patients are reported. Patients received 15 mg MTX weekly except for one patient, who received 5 mg MTX weekly because of impaired renal function. It appeared that after 12 months a significant improvement occurred in skin thickening and maximal oral opening while no significant deterioration was detected in internal organ tests performed. First signs of improvement were usually noted after 3 months of treatment. We concluded that these data, obtained in an open study, suggested beneficial effects of MTX in the treatment of patients with SSc but that a double-blind placebo controlled study was necessary before definite conclusions could be drawn.

Chapter IV describes the results of a double-blind randomized 48 week trial. In this trial MTX was compared to placebo in 29 patients with SSc. Patients were randomly allocated to treatment with either MTX or placebo, both of which were administered weekly by intramuscular injection. The weekly dose of MTX was initially 15 mg. The two groups were balanced for disease duration and extent of skin involvement.
Clinical and laboratory evaluations were made monthly by the same investigator. After 24 and 48 weeks of treatment response to treatment was evaluated. Response to treatment was defined as favorable if the total skin score (TSS), reflecting the area and degree of skin involvement, or general well-being, as determined

by the patient on a 100-mm visual analogue scale (VAS) improved by ≥30%, or if the diffusion capacity for carbon monoxide (DL_{CO}) improved ≥15%. However, when digital ulcerations developed or persisted or when DL_{CO} decreased by ≥15%, despite improvement of TSS or VAS, the response was defined as unfavorable. Patients with favorable response after 24 weeks of treatment continued with the same drug for another 24 weeks. Nonresponders on placebo after 24 weeks were started on 15 mg MTX weekly; nonresponders on 15 mg MTX weekly received an increased dose of 25 mg weekly for the remaining 24 weeks of the trial. Seventeen patients were allocated to receive MTX, 12 to receive placebo. The difference in number of patients in both groups was caused by a misrecording of two patients in the MTX group, discovered after breaking the trial code after the final patient had completed the trial.
During the first 24 weeks of the trial 2 patients in each treatment group had to be withdrawn from the study because of side effects (n=1), renal crisis (n=2) and death due to progressive disease (n=1). After breaking the code at week 48, it appeared that a significantly higher percentage of patients receiving MTX had responded according to the predefined criteria, as compared to the patients receiving placebo (53% versus 10%, P=0.03). Only one patient was left in the placebo group making comparison between placebo and MTX treatment groups at week 48 impossible. Between week 24 and 48, two patients receiving an enhanced dose of 25 mg MTX died, one presumably because of acute myocardial infarction, the other due to progression of the disease. At week 48, out of the 22 patients who had completed the trial and had been treated with MTX for at least 24 weeks, 15 (68%) could be classified as responders. With respect to any of the variables tested, it appeared that significantly more women than men responded favorably (p=0.05) and that mean C4 levels were significantly higher at study entry in the responders. Moreover, antitopoisomerase I antibodies seemed to be identified more frequently in the nonresponders (p=0.06).
Adverse reactions occurred only in patients receiving MTX and included: severe headache after each injection (n=1), necessitating discontinuance of MTX; transient elevations of liver enzymes (n=6) and pancytopenia (n=1). The latter two side effects normalized within 2 to 4 weeks after the trial medication was withheld and did not recur after resuming the same dose of MTX.
We concluded that this double-blind trial demonstrated efficacy of MTX in the treatment of patients with SSc and that the number of adverse reactions was relatively high but, in general, minor and manageable.

In chapter V we determined the effects of increasing concentrations of MTX on the proliferation and GAG synthesis of cultured dermal fibroblasts from patients with scleroderma. Fibroblasts in SSc are known to produce excessive amounts of extracellular matrix components, mainly collagen, glycosaminoglycans (GAG) and fibronectin. Cultured fibroblasts from nine patients with scleroderma and nine normal volunteers were grown for 72 hours in media containing various concentrations of MTX. The GAG synthesis in each cell was measured after incubating the fibroblasts with [^{3}H]-glucosamine and [^{35}S]-sulfate. We found a negative correlation between the concentration of MTX and numbers of fibro-

blasts from patients with scleroderma and normal controls. A positive correlation was found between GAG synthesis in each cell and increasing MTX concentrations in fibroblasts from SSc patients and normal controls. It was concluded that the beneficial effect of MTX on skin fibrosis in scleroderma is most probably not the result of direct inhibition of GAG synthesis of fibroblasts.

Chapter VI presents the expression of a cDNA clone encoding 695 carboxyl-terminal amino acids of human DNA topoisomerase I (topoI) in Escherichia coli. Immunoreactivity with this recombinant protein (the HTopoA protein) was obtained in more than 96% of the anti-HeLa topoI-positive sera from patients with a connective tissue disease. All sera from patients with SSc and reacting with HeLa topoI reacted with the HTopoA protein. Sera from patients with SSc not containing antitopoisomerase I antibodies did not display immunoreactivity with the HTopoA protein. At least three different autoimmune epitope regions on the HTopoA protein could be assigned and it was shown that over a period of 5 years the amount of antitopoisomerase I antibodies against these regions may fluctuate.

Chapter VIIa reports on the effects of plasma exchange (PE) on the synthesis of antitopoisomerase I antibody production. Levels of autoantibody concentrations during PE-therapy of scleroderma patients have been used to monitor the efficacy of PE. The case of a 50 year old woman is presented with systemic sclerosis and progression of skin thickening. After four weeks of azathioprine treatment, PE was started and during a 29 day period 11 PE sessions were performed. Immediately before and after each PE, antitopoisomerase I antibody concentrations were measured using an ELISA with HTopoA as antigen source. After each PE, IgG and IgA antitopoisomerase I antibody levels decreased on average 40% and 51% respectively. However, within 1 to 5 days after each PE, antitopoisomerase I antibody titers increased to 80% - 125% of pre-PE levels. It was concluded that rebound of antitopoisomerase I and presumably all other autoantibodies should be considered in PE treatment of patients with SSc.

In chapter VIIb the effects of low dose MTX treatment on antitopoisomerase I antibody titers are reported. In sera from 16 scleroderma patients who participated in the double-blind trial comparing MTX with placebo, antitopoisomerase I antibodies could be detected. At study entry and after 24 and 48 weeks, antitopoisomerase I antibody titers were measured using an ELISA with HTopoA as antigen source. We observed an equal decrease of antitopoisomerase I antibody titers in sera from patients treated with MTX or placebo. Likewise, a similar reduction of antitopoisomerase I antibody titers was found in sera from patients responding on MTX treatment and in patients not responding. The conclusion drawn from these findings was that antitopoisomerase I antibody titers are not useful for monitoring MTX treatment in SSc.

In chapter VIII the long term follow-up of patients with anti-(U1)snRNP antibodies is described. Anti-(U1)snRNP antibodies are considered to be the hallmark of mixed connective tissue disease (MCTD). However, since the original description of MCTD by Sharp and co-workers in 1972, the concept of MCTD being a distinct disease entity has been questioned. We examined charts of forty-six patients with anti-(U1)snRNP antibodies and a follow-up period of at least 5 years. It was found that at first clinical presentation 28% of patients with anti-(U1)snRNP antibodies fulfilled ARA classification criteria for systemic lupus erythematosus (SLE), SSc or rheumatoid arthritis (RA). At the end of the follow-up period, 67% met ARA classification criteria for SLE, SSc, RA or a combination of these diseases. This transformation from MCTD towards a defined connective tissue disease occurred on average 2.6 years after first clinical presentation. We concluded that MCTD is a transient phase of a connective tissue disease and agreed with previous proposals to abandon the concept of MCTD.

General conclusions of this thesis are:
- Methotrexate is effective in the treatment of patients with SSc;
- Methotrexate is well tolerated but the occurrence of hematological side effects and hepatic function disturbances necessitate close monitoring;
- The reduction of skin fibrosis by MTX is unlikely to be caused by direct inhibition of glycosaminoglycan production of fibroblasts;
- A recombinant DNA topoisomerase I (the HTopoA protein) reacts with all sera from patients with SSc that react with HeLa topoI;
- Antitopoisomerase I antibody titers are not helpful in assessing clinical response to either plasma exchange therapy or MTX treatment;
- Mixed connective tissue disease is a transient phase of a connective tissue disease.

Aims for future research

One of the objectives for future research is to compare MTX treatment with presumed beneficial treatments, such as D-penicillamine, cyclosporine or interferon-γ. Such trials require large numbers of patients. Given the rarity of the disease it is difficult to recruit patients for clinical trials in one single center and multicentre studies are necessary. Multicentre studies urge standardization of outcome measures. It will be the major goal of centers involved in clinical trials in scleroderma, to reach consensus on which outcome measures will be used in future trials and to standardize these outcome measures.

Samenvatting

Sclerodermie of systemische sclerose is een gegeneraliseerde bindweefselaandoening gekenmerkt door fibrosering van de huid, spieren, gewrichten en inwendige organen, met name slokdarm, longen, hart en nieren, hetgeen leidt tot een stoornis in de functie van de betreffende organen. Bij ongeveer 90% van de patiënten met sclerodermie treedt het fenomeen van Raynaud op. De fibrosering wordt veroorzaakt door een overmatige ophoping van extracellulaire matrixbestanddelen zoals collageen, fibronectine en glycosaminoglycanen. De oorzaak van deze aandoening is onbekend; echter de bij sclerodermie gevonden afwijkingen in de extracellulaire matrix, in de microcirculatie en het immuunapparaat hebben tot de volgende hypothese geleid: endotheelcellen worden door onbekende factoren beschadigd waardoor het immuunsysteem geactiveerd wordt met een toename van de endotheelschade als gevolg. Hierdoor komen cytotoxische producten vrij die fibroblasten stimuleren tot zowel proliferatie als overmatige depositie van extracellulaire matrixbe-standdelen, hetgeen uiteindelijk leidt tot fibrose.
Op grond van de uitgebreidheid van de huidafwijkingen wordt sclerodermie onderverdeeld in een diffuse vorm, waarbij de huidfibrose zich tot proximaal van de ellebogen en knieën uitbreidt, en een gelimiteerde vorm, waarbij de huidfibrose zich beperkt tot de onderarmen. Antitopoisomerase I antistoffen zijn aantoonbaar in ongeveer 50% van de sera van patiënten met een diffuse vorm, anticentromeer in ongeveer 50% van de patiënten met een gelimiteerde vorm. Andere autoantistoffen, zoals anti-Th, anti-(U3)RNP en anti-PMScl, worden slechts bij 5% van de sclerodermiepatiënten aangetoond.
Sclerodermie is een zeldzame aandoening; in verschillende studies wordt een incidentie opgegeven variërend van 4 tot 12 per miljoen per jaar. Sclerodermie komt in alle rassen voor, 3 tot 4 keer vaker bij vrouwen dan bij mannen en begint veelal tussen het veertigste en vijftigste levensjaar.
Het natuurlijk ziektebeloop is wisselend en kan variëren van een spontane remissie, hetgeen zelden voorkomt, tot geleidelijke of snelle progressie van huid- en inwendige orgaan afwijkingen. De mortaliteit is aanzienlijk; in verscheidene epidemiologische studies wordt een cumulatieve 5-jaars overleving vermeld van 34-73%. Aantasting van inwendige organen, hoge leeftijd, mannelijk geslacht, uitgebreidheid van de huidafwijking en aanwezigheid van antitopoisomerase I antilichamen zijn prognostisch ongunstige factoren.
Verscheidene medicamenten worden toegepast in de behandeling van sclerodermie, veelal op grond van positieve bevindingen in case reports of ongecontroleerd onderzoek. Echter nimmer werd in dubbelblind, placebo gecontroleerd onderzoek effectiviteit van deze medicamenten aangetoond. Onderzoek naar de effectiviteit van een geneesmiddel bij sclerodermie wordt bemoeilijkt door het wisselende ziektebeloop met soms spontane verbetering, en de wisselende mate van progressie van sclerodermie; een dergelijk onderzoek dient derhalve bij voorkeur dubbelblind en placebo gecontroleerd te zijn.
Methotrexate is een foliumzuurantagonist en interfereert in de synthese van DNA en RNA. Methotrexaat is een effectieve behandeling van reumatische aandoeningen zoals reumatoïde artritis, polymyositis en dermatomyositis. De toegepaste doseringen hierbij variëren van 7.5 mg/week tot 50 mg/week. Het werkingsmechanisme van methotrexaat bij deze aandoeningen is vooralsnog onbekend; de relatief

snelle klinische respons na start van de behandeling en de eveneens snel optredende exacerbatie van ziekteactiviteit na staken van methotrexaat suggereren een anti-inflammatoir effect.
Slechts eenmaal is methotrexaat in de literatuur beschreven als mogelijke behandeling van sclerodermie. Het betreft een onderzoek uit 1973 waarbij twee patiënten, één met een gelimiteerde vorm van sclerodermie en één met een diffuse vorm gecompliceerd door longfibrose, werden behandeld met 25 mg methotrexaat om de 14 dagen gedurende een periode van 6 maanden. Bij beide patiënten verbeterde de algehele conditie, verminderde de huidfibrose en nam de intensiteit van het fenomeen van Raynaud af.

Het voornaamste doel van dit proefschrift is onderzoek naar de effectiviteit en de toxiciteit van methotrexaat in de behandeling van patiënten met sclerodermie. Andere doeleinden zijn onderzoek naar effecten van methotrexaat op de produktie van extracellulaire matrix door fibroblasten, onderzoek naar de waarde van antitopoisomerase I antistoftiters om respons op behandeling te meten en onderzoek naar het ziektebeloop van patiënten met mixed connective tissue disease, een aandoening klinisch gekenmerkt door verschijnselen van sclerodermie, systemische lupus erythematosus en polymyositis.

In Hoofdstuk I wordt een definitie gegeven van het ziektebeeld sclerodermie en wordt nader ingegaan op de door de American Rheumatism Association voorgestelde voorlopige classificatiecriteria voor sclerodermie. Tevens wordt een overzicht van de literatuur gegeven betreffende etiopathogenese, epidemiologie, outcome variabelen, behandeling en prognose van sclerodermie. Methotrexaat en de toepassing ervan in reumatische aandoeningen worden in het kort besproken.

Hoofdstuk II is een overzicht van de literatuur over de behandeling van sclerodermie met immunomodulerende geneesmiddelen. Beschreven wordt dat in ongecontroleerd onderzoek positieve effecten op de progressie van sclerodermie worden gemeld van immunomodulerende medicamenten zoals azathioprine, chlorambucil, cyclophosphamide, 5-fluoro-uracil en cyclosporine. Dubbelblind, placebo gecontroleerd onderzoek werd slechts uitgevoerd met chlorambucil en 5-fluoro-uracil, waarbij geen verschillen werden gevonden tussen deze medicamenten en placebo.

In Hoofdstuk IIIa beschrijven we de eerste twee patiënten met sclerodermie die sedert de publikatie van Welin in 1973 methotrexaat kregen toegediend. Aangezien tijdens behandeling met methotrexaat de huidafwijkingen bij deze twee patiënten afnamen en er geen progressie van aantasting van inwendige organen optrad, werd een pilot studie gestart, waarvan de resultaten worden beschreven in Hoofdstuk IIIb. Acht patiënten werden behandeld met 15 mg methotrexaat per week, met uitzondering van één patiënt, die een gereduceerde dosis kreeg van 5 mg per week in verband met een gestoorde nierfunctie. Na 12 maanden behandeling bleek er een significante verbetering te zijn opgetreden in de uitgebreidheid

en mate van huidfibrosering, en van de maximale lip-lip afstand. Significante veranderingen in de onderzochte inwendige orgaanfuncties werden niet waargenomen. We trokken de conclusie dat methotrexaat mogelijk een effectieve behandeling van sclerodermie is, doch dat dubbelblind, placebo-gecontroleerd onderzoek noodzakelijk is om dit vermoeden te bevestigen.

De resultaten van een 48 weken durend, dubbelblind, placebo gecontroleerd en gerandomizeerd onderzoek worden weergegeven in Hoofdstuk IV. Negenentwintig patiënten met sclerodermie werden behandeld met wekelijkse intramusculaire injecties met 15 mg methotrexaat of placebo. Beide groepen werden gerandomizeerd naar ziekteduur en uitgebreidheid van de huidafwijkingen. Bij patiënten, bij wie een gunstig effect werd waargenomen na 24 weken behandeling, werd dezelfde medicatie gedurende 24 weken gecontinueerd. Bij patiënten zonder gunstig effect op placebo werd de behandeling voortgezet met 15 mg methotrexaat, bij patiënten zonder gunstig effect op 15 mg methotrexaat werd de dosering methotrexaat verhoogd tot 25 mg. Een gunstig effect op de toegediende medicatie was gedefinieerd als een verbetering van de total skin score (TSS) of van de visual analogue scale (VAS) betreffende algeheel welbevinden van tenminste 30%, of een verbetering van de CO-diffusie capaciteit van de longen (DL_{CO}) van tenminste 50%, mits er geen digitale ulceraties ontstonden of persisteerden en mits de DL_{CO} niet verslechterde met 15% of meer. Twaalf patiënten kregen placebo toegediend en 17 patiënten methotrexaat tijdens de eerste 24 weken van het onderzoek. Dit verschil in aantal patiënten in beide groepen was het gevolg van een verkeerde toewijzing van twee patiënten in de methotrexaatgroep, hetgeen ontdekt werd bij het verbreken van de behandelingscode nadat de laatste patiënt het onderzoek voltooid had. Twee patiënten uit beide groepen voltooiden dit deel van het onderzoek niet: één patiënt overleed door cardiorespiratoire insufficiëntie, bij twee patiënten trad er een renale crisis op en bij één patiënt waren er bijwerkingen op de trialmedicatie. Bij het verbreken van de behandelingscode aan het einde van de studie bleek dat na 24 weken een significant groter aantal patiënten die behandeld waren met methotrexaat (n=8, 53%) gunstig te reageren in vergelijking met de placebo behandelde patiënten (n=1, 10%, p=0.03). Negen patiënten die onvoldoende gereageerd hadden op placebo werden van week 24 tot week 48 behandeld met 15 mg methotrexaat per week en zeven patiënten die onvoldoende gereageerd hadden op 15 mg methotrexaat met 25 mg methotrexaat per week. Twee patiënten uit de laatste groep overleden tijdens de tweede 24 weken van het onderzoek: één ten gevolge van progressie van de sclerodermie, de ander overleed acuut, vermoedelijk ten gevolge van een myocardinfarct. Aan het einde van het onderzoek bleken 15 van de 22 (68%) patiënten die het onderzoek hadden voltooid en gedurende ten minste 24 weken behandeld waren met methotrexaat te voldoen aan de respons criteria. Op week 48 bleek er in deze groep een significante verbetering te zijn opgetreden in TSS (p=0.04), VAS (p=0.02) en grijpkracht van de rechter hand (p=0.02); er trad geen verslechtering op in de onderzochte inwendige orgaanfuncties. Tevens bleek dat vrouwen significant beter reageerden op behandeling met methotrexaat dan mannen (p=0.05) en dat de gemiddelde C4 waarden in het begin van de studie

significant hoger waren in de patiënten, die gunstig reageerden op methotrexaat. Bij de patiënten die niet reageerden op de behandeling met methotrexaat konden vaker, zij het niet significant, antitopoisomerase I antistoffen worden aangetoond (p=0.06). Tijdens dit onderzoek traden slechts bijwerkingen op bij patiënten, behandeld met methotrexaat. Deze bijwerkingen bestonden uit hevige hoofdpijn na elke injectie bij één patiënt, waardoor methotrexaat gestaakt diende te worden; passagère leverfunctiestoornissen bij zes patiënten en pancytopenie bij één. Deze laatste twee bijwerkingen verdwenen 2 tot 4 weken na het staken van de methotrexaat en traden niet opnieuw op na hervatting van de methotrexaat.
De conclusie van deze studie was, dat methotrexaat een effectieve behandeling is voor sclerodermie, en dat de bijwerkingen, hoewel ze relatief vaak voorkwamen, over het algemeen niet ernstig waren en verdwenen na staken van de methotrexaat.

In Hoofdstuk V worden de effecten onderzocht van verschillende doseringen methotrexaat op de proliferatie en synthese van glycosaminoglycanen van in kweek gebrachte, uit de huid geïsoleerde fibroblasten van patiënten met sclerodermie en gezonde controles. Uit eerder onderzoek is bekend dat fibroblasten van sclerodermie patiënten in verhoogde mate collageen, glycosaminoglycanen en fibronectine produceren. Wij brachten fibroblasten in kweek van negen patiënten en negen gezonde vrijwilligers; deze fibroblasten werden vervolgens gedurende 72 uren gekweekt in media met verschillende concentraties methotrexaat. De synthese van glycosaminoglycanen per fibroblast werd bepaald na incubatie van de fibroblasten met [^{3}H]-glucosamine en [^{35}H]-sulfaat. Er bleek een negatieve correlatie te bestaan tussen de hoogte van de methotrexaat concentratie en het aantal fibroblasten zowel bij patiënten als bij controles, en er werd een positieve correlatie gevonden tussen hoogte van de methotrexaatconcentratie en de synthese van glycosaminoglycanen per fibroblast, eveneens zowel bij patiënten als bij controles. Hieruit werd geconcludeerd dat de gunstige effecten van methotrexaat op de fibrosering van de huid bij patiënten met sclerodermie zeer waarschijnlijk niet het gevolg zijn van een rechtstreekse remming van de glycosaminoglycaansynthese van fibroblasten.

Hoofdstuk VI beschrijft de expressie van een cDNA clone in Escherichia Coli, die 695 aminozuren van het humane topoisomerase I (topoI) codeert. Dit recombinante eiwit (HTopoA eiwit) bleek te reageren met 96% van de anti-HeLa topoI-positieve sera van patiënten met een bindweefselaandoening. Alle serummonsters van patiënten met een sclerodermie die reageerden met HeLa topoI reageerden ook met het HtopoA eiwit. Serummonsters van sclerodermiepatiënten waarin geen antitopoisomerase I antilichamen aantoonbaar waren, reageerden niet met het HTopoA eiwit. Er konden ten minste drie verschillende epitopen op het HTopoA eiwit benoemd worden en aangetoond werd dat de concentratie antitopoisomerase I antilichamen gericht tegen deze epitopen in tijd kan fluctueren.

Hoofdstuk VIIa behandelt het effect van plasmaferese op de synthese van antitopoismerase I antilichamen, waargenomen bij een patiënt met ernstige sclero-

dermie, die in een periode van 29 dagen 11 maal een plasmaferese onderging. Na elke plasmaferese bleek de concentratie van IgG- en IgA-antitopoisomerase I antistoffen, gemeten met een ELISA met HtopoA als antigeen, gemiddeld 40% resp. 51% gedaald te zijn. Binnen 1 tot 5 dagen na elke plasmaferese bleken de antitopoisomerase I antistoftiters te stijgen tot 80% - 125% van de waarden verkregen vóór plasmaferese. Hieruit werd de conclusie getrokken, dat bij de behandeling van sclerodermie met plasmaferese rekening gehouden moet worden met een rebound van antitopoisomerase I antistoffen, en de concentratie van deze antistoffen niet gebruikt kan worden om een eventueel effect van de plasmaferese te meten, zoals in de literatuur wordt geopperd.

In Hoofdstuk VIIb wordt het effect onderzocht van lage doseringen methotrexaat op de concentratie van antitopoisomerase I antistoffen. In serum van 16 patiënten, die deelnamen aan het dubbelblinde placebo gecontroleerde onderzoek, konden antitopoisomerase I antistoffen worden aangetoond. Bij aanvang, na 24 en 48 weken werden hiervan de titers bepaald met een ELISA met HtopoA als antigeen. Deze titers daalden zowel bij patiënten die behandeld werden met methotrexaat als met placebo, en zowel bij patiënten die gunstig reageerden op de behandeling met methotrexaat als patiënten die niet reageerden. Significante verschillen tussen de verschillende groepen werden niet waargenomen. Wij concludeerden dat het niet zinvol was om antitopoisomerase I antistoftiters te vervolgen tijdens behandeling met methotrexaat van patiënten met een sclerodermie.

Ten slotte worden in Hoofdstuk VIII de resultaten van een onderzoek beschreven, waarin 46 patiënten met anti-(U1)snRNP antistoffen, die beschouwd worden als kenmerkend voor het ziektebeeld mixed connective tissue disease (MCTD), gedurende ten minste 5 jaren zijn vervolgd. Bij eerste poliklinick bezoek bleek 28% van deze patiënten te voldoen aan criteria voor systemische lupus erythematosus, sclerodermie of reumatoïde artritis, zoals opgesteld door de American Rheumatism Association. Aan het einde van dit follow-up onderzoek voldeed 67% van de patiënten aan deze criteria, of aan een combinatie hiervan. MCTD blijkt dus in de meerderheid van de gevallen een beginstadium van een geclassificeerde bindweefselaandoening te zijn en de term MCTD kan derhalve beter vervangen worden door unclassified connective tissue disease (UCTD).

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Het is een voorrecht om werkzaam te zijn op de afdeling reumatologie van het Academisch Ziekenhuis St Radboud in Nijmegen, niet in het minst door de prettige sfeer die er heerst op de werkvloer. Hieraan wordt door een ieder op specifieke wijze bijgedragen. Alle collega's en medewerkers; bedankt en houden zo!
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Tot slot: een promoticonderzoek uitvoeren tijdens de groeifase van je gezin vereist een kanjer op het thuisfront. *Bedankt Elianne.*

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 11 februari 1954 te Haps. In 1972 behaalde hij het diploma Gymnasium β aan het Elzendaalcollege te Boxmeer. Hij studeerde geneeskunde en culturele antropologie aan de Katholieke Universiteit Nijmegen. In 1981 legde hij het artsexamen af. Vanaf 1 november 1981 volgde hij de opleiding Interne Geneeskunde in het Ziekenhuis Ziekenzorg te Enschede (opleider: Dr. S.G.T. Hulst) en werd in 1986 ingeschreven als internist.
Vervolgens werd de opleiding tot reumatoloog gevolgd op de afdeling reumatologie van het Academisch Ziekenhuis, St Radboud in Nijmegen (opleider: Prof.dr. L.B.A. van de Putte), welke in 1989 voltooid werd. Het onderzoek in dit proefschrift beschreven werd verricht tussen 1989 en 1993.
Sedertdien is hij als stafmedewerker verbonden aan de afdeling reumatologie.

STELLINGEN

behorende bij het proefschrift
Systemic sclerosis: Effects of treatment with methotrexate

I

Methotrexaat is een effectieve behandeling voor sclerodermie.

II

De effectiviteit van methotrexaat bij sclerodermie berust niet op een rechtstreekse remming van de glycosaminoglycaansynthese van fibroblasten.

III

Het is niet zinvol om antitopoisomerase I antistoftiters te bepalen ter beoordeling van de effectiviteit van een behandeling bij sclerodermie.

IV

De term mixed connective tissue disease (MCTD) dient vervangen te worden door unclassified connective tissue disease (UCTD).

V

De alom heersende opvatting dat een operatieve wond bij sclerodermiepatiënten slecht geneest, is onjuist.

VI

Door het verder inkorten van de studieduur zal de student zich niet meer met "buitenschoolse" activiteiten kunnen bezighouden. Hierdoor worden bij aanvang van de studie gratis oogkleppen aangeboden.

VII

Streven naar perfectie mag een doel zijn, nimmer een obsessie.

VIII

Kennis van tekstverwerkingsprogrammatuur bij artsen heeft in niet geringe mate bijgedragen aan de toename van vakliteratuur in het afgelopen decennium.

IX

Door de welkome opmars van het vrouwelijk geslacht in de specialistische gezondheidszorg, zullen artsen van het mannelijk geslacht in de toekomst wellicht met broeder worden aangesproken.

X

Hardnekkige verkeersovertreders, die het verkeer als een spel beschouwen, kunnen slechts gestraft worden door ze hun speeltuig te ontnemen.

XI

Wanneer de r in de maand verschijnt zijn borstrokken en lange onderbroeken beslist noodzakelijke kledingstukken om buiten de reguliere werktijden werkzaamheden op je eigen werkkamer in het Academisch Ziekenhuis St Radboud uit te voeren.

Nijmegen, 10 november 1994.

Frank van den Hoogen

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Δ Systemic Sclerosis

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