Development of connective tissue disease in patients presenting with Raynaud's phenomenon: a six year follow up with emphasis on the predictive value of antinuclear antibodies as detected by immunoblotting

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SUMMARY Eighty five patients referred because of Raynaud's phenomenon (RP) were followed up for six years. Every two years they were screened for signs and symptoms of connective tissue disease (CTD) according to a protocol, and serum was stored. Initially, 30 patients had primary RP, 16 had one symptom of CTD ('possible CTD'), 18 had two or more symptoms ('probable CTD'), and 21 had definite CTD (14 of whom had scleroderma). Most of the symptoms were related to scleroderma. There was an insidious progression to scleroderma or CRST syndrome (calcinosis, Raynaud's phenomenon, sclerodactyly, telangiectasia): 11 of 46 patients with primary RP or possible CTD developed probable scleroderma (two or more symptoms but not fulfilling all criteria), and seven of 13 patients with probable scleroderma developed definite scleroderma or CRST. The presence of distinct antinuclear antibodies (ANAs) as detected by immunoblotting in patients with primary RP and possible CTD at the start of the study was associated with the evolution of symptoms of CTD ($\chi^2=5.7$, $p<0.01$). In patients initially with primary RP or possible CTD the antibody specificities of ANAs as determined by immunoblotting had prognostic value for the development of certain disease entities: anticentromere (CR-19) for CRST (sensitivity 60%, specificity 98%) and antitopoisomerase I (Scl-70 or Scl-86) for scleroderma or probable scleroderma (sensitivity 38%, specificity 100%).

Key words: anticentromere antibodies, anti-Scl-86.

Raynaud's phenomenon (RP) has been reported to occur in 20-30% of otherwise healthy young women.¹ Its overall prevalence has been estimated as approximately 10%.² Although in most cases it is a benign condition (idiopathic or primary RP), it may be secondary to an underlying disorder, especially one of the connective tissue diseases (CTDs). In addition, it may precede other manifestations of a CTD by many years. RP has been reported to be the presenting sign in 50-70% of

patients with scleroderma^{3 4} and in 15% of patients with systemic lupus erythematosus (SLE).⁵ When the prevalence of RP and that of the CTDs are considered one would expect the phenomenon to be secondary only in a minority of the patients. In a previous study, however, we demonstrated a high incidence of secondary disorders in patients referred to an outpatient clinic because of their RP.⁶ This is probably explained by the selection of more severe cases as we also found that the severity of RP correlated with the extent of systemic involvement in the individual patient.⁶ Thus the search for an underlying CTD as well as follow up studies are relevant for patients presenting with RP to the clinician. In addition, it would be useful to have tests

Accepted for publication 25 January 1988.

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with predictive power for the evolution of a CTD in those patients.

Most of the CTDs associated with RP are serologically characterised by the presence of anti-nuclear antibodies (ANAs) of diverse specificities.⁷ Some of these specificities are marker antibodies for certain diseases, such as antibodies to a nuclear protein (topoisomerase I or Scl-70/-86) specific for scleroderma,⁷⁻¹⁰ and anticentromere antibodies, directed against a 19 kilodalton nuclear protein (CR-19), characteristic for the CRST syndrome (calcinosis, Raynaud's phenomenon, sclerodactyly, telangiectasia).¹¹

The recently developed immunoblotting technique allows the detection of more than one of these specificities in a serum sample.¹² The presence of distinct ANAs in the serum of a patient presenting with RP may help in differentiating between primary and secondary RP. In addition, it could have prognostic value for the eventual development of a specific CTD.

In the present report we describe a six year follow up study of 85 patients who were referred to our clinic because of RP. Every two years these patients were screened for clinical and laboratory features of a CTD, and serum samples were stored. We also evaluated the predictive value of ANAs and their specificities (as determined by immunoblotting) for the eventual development of a specific CTD.

Patients and methods

PATIENTS

Of 91 original patients, 85 (50 women, 35 men) were available for evaluation at six years. One patient had died and five were lost during follow up. All patients had been referred to the outpatient department because of RP. The diagnosis of RP was based on a typical history and on plethysmographic patterns during cold provocation or warming up, or both.¹³ Patients using drugs known to provoke the phenomenon were excluded, as were those with large vessel obstructive arterial disease, a history of trauma to the vessels, or a thoracic outlet syndrome.

CLINICAL STUDIES

Patients were evaluated every two years, starting in 1978, over a period of six years. In all patients a careful history and physical examination was performed according to a protocol, with special attention given to signs and symptoms of CTDs, such as sclerodactyly, digital scars, telangiectasias, facial scleroderma, etc. In addition, the following studies were done: chest x rays; pulmonary function tests (vital capacity, forced expiratory volume in one second, total lung capacity, diffusing capacity for

carbon monoxide); barium swallow studies in the horizontal position; urine analysis, serum creatinine, full blood count, and creatine phosphokinase. When indicated, more studies including biopsies were done. Pulmonary involvement was considered present when diffusing capacity was less than 80% of predicted value in the absence of obstructive airway disease. At each visit serum was collected for the detection of ANAs and part of it was stored at -80°C. At the end of the study period these serum samples were thawed and assessed for ANAs by immunoblotting. Clinical diagnoses were made by CK and AW, who followed up the patients throughout the study without prior knowledge of the results of ANA testing by immunoblotting.

DIAGNOSTIC CRITERIA

A diagnosis of SLE, rheumatoid arthritis (RA), or scleroderma was made according to the American Rheumatism Association criteria for those diseases. CRST was diagnosed when all of the syndrome's symptoms were present. Sjögren's syndrome was diagnosed on the presence of xerostomia (characteristic sialogram and histology) and xerophthalmia (positive Schirmer test). A diagnosis of probable scleroderma was made when at least two of the following symptoms were present in a patient not fulfilling criteria for the diagnosis of scleroderma: digital scars, sclerodactyly, contractures of the fingers, telangiectasia, decreased pulmonary diffusing capacity or oesophageal hypomotility. RP was considered part of possible CTD when the phenomenon was accompanied by only one sign or symptom of a CTD. The patient was considered to have primary RP when no sign or symptom of a CTD was detected.

SEROLOGICAL STUDIES

ANAs were detected by indirect immunofluorescence using monolayers of human fetal fibroblasts as substrate. An ANA result was considered significant when positive at a dilution of 1:40. Antibodies to extractable nuclear antigens were detected by counterimmunoelectrophoresis according to Kurata and Tan¹⁴ using a crude extract from rabbit thymus acetone powder (Pel Freeze, Rogers, Arkansas) and reference sera showing identity with the corresponding CDC (Centre for Disease Control, Atlanta) references (anti-U1-RNP, anti-Sm, and anti-SS-B respectively).

In the immunoblotting experiments a nuclear protein fraction from HeLa S3 cells was used for the detection of antibodies against U1-RNP antigens (the 70 kilodalton, A and C proteins associated with U1-snRNA), Sm antigens (the B'/B and D proteins), the SS-B antigen (a 50 kilodalton protein),

the Scl-86 antigen, the centromere (CR-19) antigen, and histone.¹⁵ Protein blots containing cytoplasmic HeLa proteins were used for the detection of antibodies against SS-A (a 59 kilodalton protein) and Jo-1.¹⁵ The identity of antigens on immunoblots was established with reference sera from the CDC (see above) and from the laboratories of Dr E Tan (La Jolla, USA), Dr C Bunn (London, UK), Dr R M Bernstein (Manchester, UK), and Dr E Penner (Vienna, Austria). When appropriate, specificity was also tested by RNA immunoprecipitation with antibodies eluted from the antigen band on the blot.¹⁶ Gel electrophoresis and protein blotting were performed as described previously.¹⁷

STATISTICS

Haldane's test was used to compare the ages of onset between the several groups of patients. The association between the presence of ANAs and the eventual development of signs/symptoms of a CTD was evaluated by the standard χ^2 test with Yates's correction.

Results

CLINICAL AND SEROLOGICAL FINDINGS AT THE ONSET OF THE STUDY

Eighty five patients were followed up throughout the study period. At their initial visit 30 patients (35%) had no signs or symptoms of an underlying CTD. Three of them (10%) had a positive ANA test by indirect immunofluorescence (IIF) and 14 (47%) by immunoblotting. Sixteen patients (19%) were considered as possible CTD, having only one sign or symptom—namely, decreased pulmonary diffusing capacity (six), sclerodactyly (three), flexion contractures of the fingers (three), telangiectasia (two),

oesophageal hypomotility (one), or digital scarring (one). ANAs were detected in five of these patients by IIF (31%) and in six by immunoblotting (38%).

In 18 patients a diagnosis of probable CTD was made, 13 of them having probable scleroderma. These 18 patients had two or more of the following symptoms: sclerodactyly (six), flexion contractures of the fingers (four), digital scarring (five), telangiectasia (seven), oesophageal hypomotility (three), arthralgia (seven), and decreased pulmonary diffusing capacity (seven). ANAs by IIF were positive in 11 cases (61%) and by immunoblotting in 15 (83%).

In 21 patients (25%) an underlying CTD was diagnosed. Fourteen of them had scleroderma, four had SLE, two had RA, and one patient had Sjögren's syndrome. ANAs were present in 10 out of 14 patients with scleroderma by IIF (71%) and in 12 of them by immunoblotting (86%). Six of the seven patients with SLE, RA, or Sjögren's syndrome had a positive ANA test, both by IIF and immunoblotting.

Patients with primary RP had an earlier age of onset of RP (mean age 16.9, range 7–46 years) than patients with possible CTD (mean age 26.3, range 7–51 years) ($p<0.05$), probable CTD (mean age 38.2, range 18–59 years) ($p<0.01$), and definite CTD (mean age 34.9, range 7–60 years) ($p<0.01$). Table 1 summarises these results.

CLINICAL FINDINGS AFTER SIX YEARS OF FOLLOW UP

Twenty two of 30 patients initially with primary RP (73%) did not develop any sign or symptom of a CTD. Two patients developed one symptom (possible CTD), four patients two symptoms of scleroderma (probable scleroderma), and two patients developed probable Sjögren's syndrome (xerophthalmia and arthralgia, and xerostomia with

Table 1 *Diagnosis, age, sex, duration of the phenomenon, and presence of antinuclear antibodies in 85 patients referred because of Raynaud's phenomenon*

Diagnosis	Number of patients	Male/female	Age (mean and range, years)	Age of onset of RP (mean and range, years)	Presence of ANAs	
					IIF	Immunoblotting
Primary RP	30	9/21	40.6 (20–66)	16.9 (7–46)	3	14
Possible CTD	16	7/9	42.4 (20–63)	26.3 (7–51)	5	6
Probable CTD	18	10/8	50.7 (36–64)	38.2 (18–59)		
Probable scleroderma	13				7	12
Probable (undifferentiated) CTD	5				4	3
Definite CTD	21	9/12	45.7	34.9 (7–60)		
Scleroderma	14	6/8	45.5 (23–66)		10	12
SLE	4	2/2	43 (22–59)		4	4
RA	2	1/1	51 (45–57)		1	1
Sjögren's syndrome	1	0/1	48		1	1

ANAs=antinuclear antibodies; RP=Raynaud's phenomenon; CTD=connective tissue disease; SLE=systemic lupus erythematosus; RA=rheumatoid arthritis; IIF=indirect immunofluorescence

Table 2 Diagnosis at presentation, diagnosis at six years' follow up, and presence of antinuclear antibodies as detected by immunoblotting at the time of first presentation in 85 patients with Raynaud's phenomenon

Diagnosis at presentation	Diagnosis at six years follow up	ANAs at first presentation and antigenic specificity*							Remarks	
		Neg.	Scl-86	CR-19	UI-RNP	Sm	SS-A SS-B	Jo-1		Histone
Primary RP (n=30)	Primary RP (n=22)	15	—	—	—	—	—	—	2	5
	Possible CTD (n=2)	—	—	—	—	—	—	—	—	2
	Probable scleroderma (n=4)	1	1	—	—	—	—	—	—	2
	Probable Sjögren's syndrome (n=2)	—	—	—	—	—	1	—	—	1
Possible CTD (n=16)	Possible CTD (n=9)	6	—	—	—	—	—	—	—	3
	Probable scleroderma (n=7)	4	2	1	1	—	—	—	—	—
Probable CTD (n=18) Probable scleroderma (n=13)	Probable scleroderma (n=6)	1	1	—	—	—	—	—	—	4
	Scleroderma (n=2)	—	2	—	1	—	—	—	—	—
Probable (undifferentiated) CTD (n=5)	CRST (n=5)	—	—	3	—	—	—	—	—	2
	Probable CTD (n=2)	1	—	—	1	—	—	—	—	—
	SLE (n=2)	—	—	—	1	—	2	—	1	—
Definite CTD (n=21) Scleroderma (n=14)	RA (n=1)	1	—	—	—	—	—	—	—	—
	Scleroderma (n=9)	2	4	1	1	—	—	—	—	1
	Scleroderma+RA (n=2)	—	1	—	—	—	—	1	—	—
	CRST (n=2)	—	—	1	—	—	—	—	—	1
SLE (n=4) RA (n=2) Sjögren's syndrome (n=1)	Scleroderma+SLE+RA (n=1)	—	—	—	1	1	—	—	—	—
	SLE (n=2)	—	—	—	2	1	—	—	—	—
	SLE+scleroderma (n=2)	—	—	—	1	1	—	—	—	—
	RA+probable scleroderma (n=2)	1	—	—	—	—	—	—	—	1
	Sjögren's syndrome+probable scleroderma (n=1)	—	—	—	—	—	1	—	—	—

ANAs=antinuclear antibodies; RP=Raynaud's phenomenon; CTD=connective tissue disease; SLE=systemic lupus erythematosus; RA=rheumatoid arthritis.
*For the detection of anti-SS-A and anti-Jo-1 cytoplasmic extracts were used.

arthralgia and decreased diffusing capacity respectively).

Nine of 16 patients (56%) with initially only one sign or symptom of a CTD did not have a change in clinical findings during follow up. The remaining seven patients developed additional symptoms suggestive of scleroderma (probable scleroderma).

Six of 13 patients initially with probable scleroderma (46%) did not have a change in clinical symptoms, two patients developed scleroderma, and the remaining five patients developed CRST syndrome.

Five patients had probable undifferentiated CTD at the onset of the study. Two of them did not develop additional symptoms, two patients developed SLE, and one patient RA at the end of the follow up period.

Fourteen patients initially had already a diagnosis of scleroderma. Nine of them had a more or less stable pattern of disease during follow up. In two patients calcinosis developed, resulting in the diagnosis of CRST syndrome, two patients also fulfilled criteria for RA in addition to scleroderma at the end of the study, and one patient developed an overlap syndrome of scleroderma, RA, and SLE. Two of four patients with SLE initially had stable disease, and two of them showed a tendency towards scleroderma during follow up. Two patients with a diagnosis of RA and the one patient with Sjögren's syndrome at the onset of the study also showed a tendency towards scleroderma during follow up. Table 2 summarises these data.

PREDICTIVE VALUE OF ANAs AS DETECTED BY INDIRECT IMMUNOFLUORESCENCE AND IMMUNOBLOTTING

The presence of ANAs at presentation in patients with primary RP and possible CTD was not associated with the development of clinical symptoms of CTD after six years of follow up when ANAs were determined by conventional IIF ($\chi^2=1.3$, NS). A

significant association was found, however, when ANAs were determined by immunoblotting ($\chi^2=4.9$, $p<0.05$). When the analysis was restricted to ANAs of distinct specificities as detected by immunoblotting the presence of ANAs was also associated with the evolution of symptoms of CTD after six years ($\chi^2=5.7$, $p<0.01$; Table 3).

Next, we studied the diagnostic potential of the antigenic specificities with respect to the presence or future development of discrete clinical syndromes. Antibodies against the Scl-86 antigen were present in five out of 14 patients with a diagnosis of scleroderma at their first presentation, in one out of 30 patients with primary RP, in two out of 16 with possible CTD, and in three out of 18 with probable CTD. These last six patients initially with anti-Scl-86 antibodies but without a diagnosis of definite scleroderma all developed probable or definite scleroderma. Thus specificity in the group of patients without a definite CTD at presentation for the development of scleroderma or probable scleroderma was 100%. As to its sensitivity, anti-Scl-86 antibodies were present in both patients who developed scleroderma and in three out of 11 patients who developed probable scleroderma, resulting in a sensitivity of 38%.

Antibodies against the CR-19 (centromere) antigen were initially present in four of seven patients who developed CRST syndrome during the study period, resulting in a sensitivity of 57%. In addition these antibodies were present in one patient with sclerodactyly who developed telangiectasia and flexion contractures of the fingers, and in one patient with an initial diagnosis of scleroderma who had not yet developed CRST. In the group of patients without a definite diagnosis of CTD at their initial presentation, the presence of anti-CR-19 had a sensitivity of 60% and a specificity of 98% for the development of CRST.

Antibodies against the SS-A/SS-B antigens, detected equally by counterimmunoelectrophoresis

Table 3 Association between the presence of ANAs at presentation (as determined by indirect immunofluorescence (IIF), by immunoblotting considering any reactivity as a positive result, and by immunoblotting considering only reactivities with a defined protein as a positive result) and the development of clinical symptoms of CTD in patients presenting with primary Raynaud's phenomenon or possible CTD

		IIF*		Immunoblotting,** all reactivities		Immunoblotting,*** defined specificities	
		ANAs present	ANAs absent	ANAs present	ANAs absent	ANAs present	ANAs absent
Development of clinical symptoms at six years' follow up	Yes	4	11	10	5	5	10
	No	4	27	10	21	2	29

* $\chi^2=1.33$, NS; ** $\chi^2=4.87$, $p<0.05$; *** $\chi^2=5.66$, $p<0.01$.

and immunoblotting, were present in the patient with an initial diagnosis of Sjögren's syndrome, but also in one out of two patients who presented with primary RP and had a diagnosis of probable Sjögren's syndrome at follow up. In addition, these antibodies were initially present in both patients with probable CTD who later developed SLE.

Anti-U1-RNP antibodies were detected in nine patients with RP by immunoblotting and in seven patients by counterimmunoelectrophoresis. These antibodies were present in five of seven patients with a diagnosis of SLE at follow up, two of them having also developed sclerodermatous changes. In addition, anti-U1-RNP antibodies were present in two patients with, ultimately, a diagnosis of scleroderma, and in two patients with probable scleroderma and undifferentiated CTD at follow up. Anti-Sm antibodies, detected in only one case by counterimmunoelectrophoresis, were detected by immunoblotting in three out of seven patients who, ultimately, fulfilled the criteria for SLE. In two cases anti-Sm antibodies were accompanied by anti-U1-RNP antibodies. Table 2 summarises these data.

COMPARISON OF BLOTTING PROFILES FROM SERA INITIALLY AND AT FOLLOW UP

As mentioned, serum samples were collected and frozen at presentation in 1978 but also every two years when the patients presented for evaluation. We compared the antibody profiles of the samples taken from each individual patient and found that they were remarkably similar. In a few cases minor bands of undefined specificity had disappeared or appeared in the more recent serum sample. Bands of defined identity (Sm, U1-RNP, SS-B, SS-A, Scl-86, CR-19, and incidentally occurring bands of histone and Jo-1) when present in the follow up serum had been present in all cases already in the 1978 sample (data not shown). This observation indicates that in all patients included in this study the appearance of specific autoantibodies occurred some time before the initial clinical presentation.

Discussion

We describe the six years' clinical follow up of 85 patients who were referred because of their RP. A moderate incidence of underlying disorders (25%), especially scleroderma, was found at their first presentation, as described in our previous report detailing most of the patients involved in the present longitudinal study.⁶ In addition, 40% of the patients had at least one sign or symptom suggestive of CTD, in particular symptoms suggestive of scleroderma. During a follow up period of six years, however, none of 30 patients with primary RP and none of 16

patients with only one sign or symptom suggestive of CTD developed definite CTD. Nevertheless, 27% (8/30) of the patients with primary RP and 44% (7/16) of those with another symptom of CTD in addition to RP at the onset of the study developed one or more additional signs/symptoms, especially of scleroderma.

These data illustrate the insidious progression of the disease in most of the patients with RP who develop scleroderma. Indeed, a diagnosis of definite scleroderma or CRST syndrome was made at follow up only in patients with a diagnosis of probable scleroderma at their first presentation. From a diagnostic point of view, our data invalidate Allen and Brown's criterion that RP may be considered as primary when an underlying disorder has not developed within two years after the first manifestation of RP.¹⁸ It is also apparent that the underlying disorder in most patients developing clinical symptoms is scleroderma or the CRST syndrome. Probably, RP is the first manifestation in these patients of a disease process affecting the microvasculature.^{19,20} Our clinical data are, in general, in agreement with those of previous studies dealing with the evolution of primary RP to CTD.²¹⁻²³

The age of onset of RP was different in our patients with primary RP than in those with one or more symptoms of CTD. Thus the probability of developing CTD appears to increase as the age of onset of RP rises.

Interestingly, most of our patients with a CTD different from scleroderma at the onset of the study also developed sclerodermatous changes during follow up. This is in agreement with the findings that patients with SLE/scleroderma overlap syndromes present with SLE-like symptoms and progress to a more scleroderma-like syndrome.²⁴

We also evaluated the predictive value of ANAs for the development of a CTD. We used not only the conventional indirect immunofluorescence technique on human fibroblasts, but also immunoblotting on HeLa cell proteins. The latter technique allows the detection of more than one antigenic specificity in a serum sample. Thus we detected antibodies against Scl-86 in two sera with, in addition, anti-U1-RNP antibodies. We showed that the presence of ANAs was associated with the development of additional signs or symptoms of CTD in patients with none or only one symptom at their first presentation. This association was present only when ANAs were detected by immunoblotting. When ANAs were considered positive only in the presence of antibodies against proteins of defined specificity, a stronger association was found. Apparently, by identifying specific antigenic determinants the immunoblotting technique is sensitive

and specific for the detection of evolving connective tissue diseases.

The immunoblotting technique proved to be useful for the detection of antibodies associated with scleroderma—namely, antibodies against the Scl-86 and the CR-19 antigen. Anti-Scl-86 antibodies were highly specific for the presence or future development of scleroderma as has been described earlier.⁸ The predictive power of anti-CR-19 with respect to the future development of CRST, including calcinosis, was also apparent from our study: calcinosis was present in none of the patients at the onset of the study but developed later on in four of six patients with anti-CR-19 at their first presentation. Our data on the diagnostic sensitivity and specificity of anti-Scl-86 and anti-CR-19 are in agreement with those of other studies.²⁵⁻²⁷

Antibodies to U1-RNP, originally described as a prerequisite for the diagnosis of an SLE/scleroderma overlap syndrome designated as mixed connective tissue disease,²⁸ were indeed indicative of the development of SLE/scleroderma overlap, though these antibodies were also present in the sera of some patients with scleroderma lacking SLE-like characteristics.

We observed that antibody specificities in the sera of the patients hardly changed during a six year period. Specificities characteristic for clinical disease entities were already detectable at the start of the study. Thus the presence of antibodies against Scl-86, CR-19, SS-A/SS-B, or U1-RNP has prognostic value for specific diagnoses.

In conclusion, a slowly progressive CTD, in most cases scleroderma, can be diagnosed in many patients who present at an outpatient clinic because of severe Raynaud's phenomenon. In patients presenting with primary RP the presence of ANAs, as detected by immunoblotting, has discriminative power for the future development of connective tissue diseases. The antigenic specificities of the antinuclear antibodies are indicative of the clinical entity that will develop, whether scleroderma, CRST syndrome, or other connective tissue diseases.

This work was supported by a grant (No 28-1217) from the 'Praeventiefonds', The Hague, The Netherlands.

References

- Olsen N, Nielsen S L. Prevalence of primary Raynaud phenomenon in young females. *Scand J Clin Lab Invest* 1978; **37**: 761-4.
- Maricq H R, Weinrich M C, Keil J E, LeRoy C E. Prevalence of Raynaud phenomenon in the general population. *J Chronic Dis* 1986; **39**: 423-7.
- Tuffanelli D L, Winkelmann R K. Systemic sclerosis: a clinical study of 727 cases. *Arch Dermatol* 1961; **84**: 359-67.
- Bennett R, Bluestone R, Holt P J L, Bywaters E G L. Survival in scleroderma. *Ann Rheum Dis* 1971; **30**: 581-8.
- Fessel W J. Systemic lupus erythematosus in the community. *Arch Intern Med* 1974; **134**: 1017-35.
- Kallenberg C G M, Wouda A A, The T H. Systemic involvement and immunological findings in patients presenting with Raynaud's phenomenon. *Am J Med* 1980; **69**: 675-80.
- Tan E M. Autoantibodies to nuclear antigens (ANA). Their immunobiology and medicine. *Adv Immunol* 1982; **33**: 167-240.
- Van Venrooij W J, Stapel S O, Houben H, et al. Scl-86, marker antigen for diffuse scleroderma. *J Clin Invest* 1985; **75**: 1053-61.
- Shero J H, Bordwell B, Rothfield N F, Earnshaw W C. High titres of autoantibodies to topoisomerase I (Scl-70) in sera from scleroderma patients. *Science* 1986; **231**: 737-40.
- Maul G G, French B T, van Venrooij W J, Jimenez S A. Topoisomerase I identified by scleroderma 70 sera. *Proc Natl Acad Sci USA* 1986; **83**: 5145-9.
- Guldner H H, Lakomek H J, Bautz F A. Human anti-centromere sera recognize a 19.5 kD nonhistone chromosomal protein from HeLa cells. *Clin Exp Immunol* 1985; **78**: 13-20.
- de Rooij D J, Habets W J, Stapel S O, van de Putte L B A, van Venrooij W J. Clinical significance of antibodies against nuclear antigens as determined by immunoblotting. *Scand J Rheumatol [Suppl]* 1985; **56**: 93-7.
- Wouda A A. Raynaud's phenomenon. Photoelectric plethysmography of the fingers of persons with and without Raynaud's phenomenon during cooling and warming up. *Acta Med Scand* 1977; **201**: 519-23.
- Kurata N, Tan E M. Identification of antibodies to nuclear acidic antigens by counterimmunoelectrophoresis. *Arthritis Rheum* 1976; **19**: 574-80.
- Van Venrooij W J, Habets W J, de Rooij D J, van de Putte L B A. The molecular composition of nuclear and cytoplasmic antigens: a review. In: Brooks P M, York J R, eds. *Rheumatology-85*. Amsterdam: Elsevier, 1985: 175-80.
- Habets W J, Berden J, Hoch S O, van Venrooij W J. Further characterization and subcellular localization of Sm and U ribonucleoprotein antigens. *Eur J Immunol* 1985; **15**: 992-7.
- Habets W J, de Rooij D J, Hoet M H, van de Putte L B A, van Venrooij W J. Quantitation of anti-RNP and anti-Sm antibodies in MCTD and SLE patients by immunoblotting. *Clin Exp Immunol* 1985; **79**: 417-23.
- Allen E V, Brown G E. Raynaud's disease: a critical review of minimal requisites for diagnosis. *Am J Med Sci* 1932; **183**: 187-200.
- Campbell P M, Le Roy E C. Pathogenesis of systemic sclerosis: a vascular hypothesis. *Semin Arthritis Rheum* 1975; **4**: 351-68.
- Kallenberg C G M, Wouda A A, The T H. Systemic autoimmune disease and Raynaud's phenomenon: a common, immunologically mediated, vascular basis? *Neth J Med* 1984; **27**: 322-6.
- Gifford R W, Hines E A. Raynaud's disease among women and girls. *Circulation* 1957; **16**: 1012-21.
- Gerbrach D D, Steen V D, Ziegler G L, Medsger Th A, Rodnan G P. Evolution of primary Raynaud's phenomenon (Raynaud's disease) to connective tissue disease. *Arthritis Rheum* 1985; **28**: 87-92.
- Harper F E, Maricq H R, Turner R E, Lidman R W, LeRoy E C. A prospective study of Raynaud's phenomenon and early connective tissue disease. *Am J Med* 1982; **72**: 883-8.
- Nimelstein S H, Brody S, McShane D, Holman H R. Mixed connective tissue disease: a subsequent evaluation of the original 25 patients. *Medicine (Baltimore)* 1980; **59**: 239-48.
- Kallenberg C G M, Pastoor G W, Wouda A A, The T H. Antinuclear antibodies in patients with Raynaud's phenomenon: clinical significance of anticentromere antibodies. *Ann Rheum Dis* 1982; **41**: 382-7.
- Tan E M, Rodnan G P, Garcia I, Moroi Y, Fritzler M J, Peebles C. Diversity of antinuclear antibodies in progressive systemic sclerosis: anti-centromere antibody and its relationship to CREST syndrome. *Arthritis Rheum* 1980; **23**: 617-25.

- 27 Catoggio L J, Bernstein R M, Black C M, Hughes G R V, Maddison P J. Serological markers in progressive systemic sclerosis: clinical correlations. *Ann Rheum Dis* 1983; **42**: 23–7.
- 28 Sharp G C, Irvin W S, Tan E M, Gould G C, Holman H R. Mixed connective tissue disease an apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractable nuclear antigen (ENA). *Am J Med* 1972; **52**: 148–59.