IMMUNOBIOLOGY OF ANTI-NUCLEAR ANTIBODIES IN RHEUMATIC DISEASES

WINAND J.A. HABETS

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PROEFSCHRIFT

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Prof. Dr. L.B.A. van de Putte

Co-referent: Dr. W.J. van Venrooij.

In gedachtenis aan Rob

Voor mijn ouders

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

It is the primary task of the immune system to protect the body from harm caused by infection with foreign agents and therefore it must be able to discriminate between self and nonself. Failure of this discriminatory mechanism results in autoimmunity; a malfunction of the immunesystem in which the body attacks its own tissues [1 2]. Many rheumatologic disorders are autoimmune in nature and almost all rheumatic diseases are therefore characterized by the spontaneous occurrence of circulating autoantibodies [3 4 5].

It has long been thought that B-lymphocytes capable of reaction to self antigens were eliminated during ontogeny, and that self-reactive B-cell clones in autoimmune patients had escaped this mechanism (Burnett's forbidden clone theory). From studies of animal models of autoimmune diseases it is now clear, however, that these self-reactive B-cells are normally present in healthy individuals but in a suppressed or inactivated state. The production of autoantibodies is therefore assumed to be a result of a defective suppression or a direct stimulation of these self-reactive B-cells [6]. A simplified schematical representation of the regulation of B-cell activity is shown in figure 1. The B-cell shown contains membrane receptors, two of which are capable to respond to signals from another class of lymphocytes, the T-helper and T-suppressor cells. A third receptor is formed by a specific immunoglobulin, capable of binding antigen. In general, binding of antigen (together with some other factors) triggers the immune respons followed by the differentiation of B-cells into plasma cells. T-helper cells interact with B-cells to amplify the production of antibody, T-suppressor cells act thereby as a control mechanism. The mechanism(s) by which the suppression of self-reactive B-cells in autoimmune patients is overruled is not yet understood. In many autoimmune diseases however, alterations in the function and number of immunoregulatory T-cells are common [6].

As far as rheumatic diseases are concerned, depressed T-suppressor cell function has been described in systemic lupus erythematosus (SLE) [7] [121], juvenile rheumatoid arthritis [8] and progressive systemic sclerosis [9] [122]. An increased T-helper cell function is also found in patients suffering from this latter syndrome and in patients with drug-induced lupus, as well as in mice with a genetic predisposition for an SLE-like syndrome, the MRL-lpr/lpr mice [51] [120] [6].

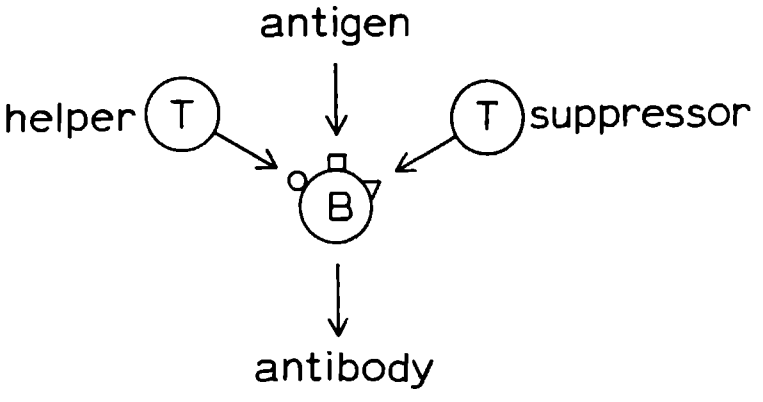

FIGURE 1 Schematic representation of the forces acting on B-cells.

Genetic factors have also been suggested to play a major role in human autoimmune diseases [10]. These theories are substantiated by the finding of familial aggregations, especially for rheumatoid arthritis (RA), SLE and Sjoegren's syndrome [11] [12], and by the high rate of concordance of SLE in identical twins [13]. Even more convincing for the possible involvement of an underlying genetic predisposition is the finding of an association between RA and the DR4 phenotype of the human major histocompatibility complex [14]. Other MHC antigens are also linked to particular rheumatic diseases; Sjoegren's syndrome is associated with DR3 [15] and SLE is linked to DR2 and DR3 [16]. All these connections however, are not absolute and therefore other (environmental) factors are suspected to play an important role. Hormones are thought to be involved

because of the fact that rheumatic diseases in general are more common in women [38]. Another interesting observation in this respect is the protective effect of oral contraceptives against the development of rheumatoid arthritis [36 37].
Viruses form another, most intriguing group of candidates to be involved in the autoimmune processes of rheumatic diseases [10]. In a very recent review, Notkins et al., considered four possible mechanisms of virus-induced autoimmunity [17].

1 The virus alters the appearance of the host cell, or component of it, in such a way that the immunesystem reacts to this host antigen as if it were foreign [18 19]. A possible mechanism for this phenomenon could be the incorporation of host antigen into the virus envelope or by the sudden release of sequestered antigen caused by infection and lysis of host cells. Rigorous prove for such a mechanism has not yet been provided.

2 The virus acts directly on the immune system. A virus capable of infecting immunoregulatory lymphocytes could destroy or stimulate certain subpopulations and in this way trigger the production of autoantibodies. Numerous examples can be found in literature in which a virus infection is followed by autoantibody production [20]. Most of these autoantibodies, however, share the common feature that they are directed against ubiquitous autoantigens such as DNA, IgG, phospholipids, erythrocytes and lymphocytes [10]. Certain drugs (methyldopa, procainamide) [21 22] or microorganisms (malaria infection) [23 24 25] are also capable to elicit the production of this kind of autoantibodies, whereas the same effect can be obtained by in vitro stimulation of lymphocytes from normal persons by non-specific mitogens [26]. Therefore it is assumed that these kinds of autoantibodies form a separate group whose production is an inherent property of the normal immune system [10].
How to explain the production of the very specific autoantibodies directed against minor intracellular antigens, often observed in rheumatic diseases? For this aim two additional possibilities are considered:

3 A mechanism referred to as "molecular mimicry". The idea is that certain antibodies raised against viral antigens may crossreact with host molecules [17]. A monoclonal antibody against a phosphoprotein of measles virus and another against a herpes virus protein have recently been shown to crossreact with the intermediate filament protein vimentin [27]. A further example for this theory has been presented by Woodruff [28]; a monoclonal antibody raised against 65 Coxsackie B4 virus reacted with mouse myocardium cells in an indirect immunofluorescence test. Infection of mice with a Coxsackie B4 virus can result in a severe myocarditis, and therefore this crossreaction may indicate a pathogenic effect of anti-virus antibodies.

4 As a reaction to antibodies elicited against viral antigens, the immune system is able to produce the so-called anti-idiotype antibodies which in turn could react with host cell components (see figure 2) [29]. Experimental data indeed show that some anti-idiotype antibodies are capable of reacting both with e.g. reovirus receptors on the surface of lymphocytes as well as with monoclonal anti-reovirus antibodies [30].

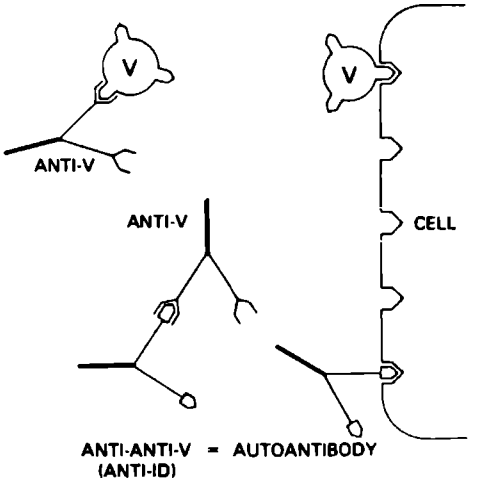

FIGURE 2 How autoantibodies may be anti-idiotypes of anti-viral antibodies.

As far as information is available, any of the mechanisms discussed above may be involved in the pathogenesis of rheumatic diseases. New insights in the role of known and still unknown viruses is to be expected from hybridoma and recombinant DNA technology, while new methods for studying immunoregulation will further elucidate the complex mechanism of autoantibody production. It may be expected that such investigations will ultimately lead to adequate therapies for rheumatic diseases.

1.2 AIM OF THIS STUDY

Diagnosis of rheumatic diseases is difficult because of the very diverse pattern of clinical symptoms [4] and therefore often based upon serological findings [3]. This thesis deals primarily with two aspects of the serology of rheumatic diseases:

1 As pointed out, knowledge about the exact autoantibody composition of the patients' serum can be of use in diagnosis, while insight in the molecular nature and cellular function of the target antigens may lead to a better understanding of the pathogenesis of rheumatic diseases.

2 The use of human autoantibodies as tools in molecular biology.

These aspects will now be discussed in more detail.

1.3 CLINICAL-SEROLOGICAL CORRELATIONS OF ANTI-NUCLEAR ANTIBODIES.

A Diagnostic relevance of anti-nuclear antibodies.

In rheumatic diseases several clinical entities can be distinguished. Many of them are syndromes with overlapping clinical features and only the expression of a certain combination of symptoms is determinative for the diagnosis. It is, therefore, not seldomly seen that one and the same patient evolves from one diagnosis to another in the course of the disease. Serology can often be helpful in this classification. Since the discovery of the LE-cell phenomenon by Hargraves et al., [31] later identified to be

caused by anti-DNA/histone antibodies, [32] [33] [34] [35] many other anti-nuclear antibodies (ANA) have been described [3]. The most interesting from a diagnostical point of view are the so-called "marker" antibodies which are more or less specific for a certain syndrome or disease. Table I presents the most important correlations between clinical diagnosis and presence of specific ANA in the patients' serum [119].

Clinical Diagnosis	"Marker" antibodies reactive with:
Rheumatoid arthritis	-IgG (Rheumatoid factor) -RANA (EBNA(?)) -APF antigen
Sjoegren's syndrome	-SS-A/Ro -SS-B/La
Systemic lupus erythematosus	-native DNA -ssDNA -Sm -histones -SS-A/Ro
Mixed connective tissue disease (MCTD)	-(U1)RNP
Progressive systemic sclerosis	
-diffuse	-Scl-70/Scl-86
-CREST	-centromere/kinetochore
Poly/dermatomyositis	-Jo-1
-scleroderma overlap	-PM-1 -Ku

TABLE 1. Correlations between diagnosis and presence of ANA. For references see text.

The most important serologic finding in RA is the elevated rheumatoid factor titer, present in over 75% of patients. Rheumatoid factors are mostly IgM immunoglobulins which react with the Fc portion of IgG and in this way form a circulating 22S immunoglobulin complex in the patients' serum. As far as ANA are concerned, RA is characterized by a very high incidence (more than 90%) of antibodies against rheumatoid arthritis nuclear antigen (RANA) [39] [40] [41]. Presence of anti-RANA antibodies, however, is not specific for RA because of its high incidence in a.o. SLE, Sjoegren's syndrome, and even normal controls [88]. RANA can be detected by indirect immunofluorescence (I.I.F.) only in nuclei of Epstein-Barr virus (EBV) infected cells, such as WiL-2 cells [42]. A recent study suggests that RANA might be identical with the independently identified Epstein-Barr nuclear antigen (EBNA), an EBV encoded, slightly polymorphic protein with a molecular weight of about 80,000 Dalton [43] [44]. This finding lend additional support to the hypothesis (primarily based on clinical findings) that EBV might be involved in the pathogenesis of RA [45] [46] [47] [48].

Further, the high incidence (50%) of the so-called antiperinuclear factor (APF) is noteworthy. The presence of this antibody is extremely specific for RA [49] [50], but because its laboratory test (I.I.F. on buccal mucosa cells) hardly acquires any international attention, the nature of the antigen as yet remains obscure.

Patients with Sjoegren's syndrome can be subdivided clinically into patients with the sicca complex (xerophtalmia and xerostomia, primary Sjoegren's syndrome) and patients with these symptoms in combination with rheumatoid arthritis (SS-RA). Because of this close association with RA, the high incidence of anti-RANA antibodies in this latter syndrome is not surprising. More characteristic for primary Sjoegren's syndrome is the incidence of antibodies against SS-A/Ro and SS-B/La antigens which are reported to occur in about 70% and 60% of the cases, respectively [42] [52] [53] [54]. The latter of these antibody systems is more specific for primary Sjoegren's syndrome whereas anti-SS-A/Ro antibodies also occur in SLE and scleroderma. The molecular nature of SS-A/Ro and SS-B/La

antigens will be discussed below.

Systemic Lupus Erythematosus (SLE) and drug-induced Lupus Erythematosus (drug-induced LE).

Antibodies to DNA are considered as a hallmark for SLE, and can be divided primarily into two subgroups; one exclusively reactive with single stranded (ss) DNA and the other directed against both ssDNA and double stranded (ds) DNA [62] [63] [64] [65]. Usually, the latter specificity is referred to as anti-native DNA. Monospecific anti-dsDNA has been described in only a very few cases [64] [66] [67]. Both anti-ssDNA and -native DNA are common in SLE, whereas only anti-ssDNA occurs in procainamide-induced LE [3]. An additional common feature of both diseases is the presence of antibodies against histones [55]. These are of IgG and IgM class and directed against the bimolecular complex of histone H2A and H2B [56] [57] [58]. Antibodies in hydralazine-induced disease are predominantly IgM in nature and directed against the H3-H4 complex [3].
Anti-Sm has originally been described as an additional marker antibody for SLE, with an incidence of over 30% [59]. Several large scale studies performed in Europe reported a much lower incidence of about 3-10% [60] [61]. Whether this discrepancy is caused by technical factors or by variations in the populations examined, is as yet not entirely clear.

Mixed Connective Tissue Disease (MCTD).

It is still a matter of dispute whether MCTD has to be considered as a distinct entity, but originally it has been described as an overlap syndrome with features of SLE, progressive systemic sclerosis and poly/dermatomyositis [68] [69]. The diagnosis is further based on the presence of a high titer of anti-(U1)RNP antibodies, which explains the incidence of 100% of this type of antibody in the serum of MCTD patients. Anti-(U1)RNP sera always contain antibodies against one or more out of three antigenic proteins called 70K, A and C (see below). Anti-70K antibodies almost exclusively occur in sera from MCTD patients, whereas anti-(U1)RNP sera from patients with SLE mostly contain anti-A and/or anti-C

antibodies (chapter 2, [70] [108]).

Progressive Systemic Sclerosis

Two major forms of PSS can be distinguished, both with their own marker antibody. A diffuse form with multi-system involvement characterized by vascular abnormalities, atrophy of parenchymal structures in skin and various internal organs and by fibrous tissue deposition [71]. A second form of PSS is called the CREST-variant, characterized by calcinosis, Raynaud's phenomenon, (O)esophagitis, sclerodactyly and telangiectasia. PSS is a slowly progressive and often fatal disorder, the CREST variant is believed to have a more benign course and a more favorable prognosis [74]. Little is known about etiology and pathogenesis and no treatment is yet available [72] [73]. The presence of an anti-centromere/kinetochore antibody (ACA) is highly correlative with the CREST form of scleroderma [75] [76] [77]. Noticeable is the presence of anti-centromere antibodies in sera from patients with Raynaud's phenomenon [61]. Some of these patients have acquired clinical characteristics indicating a developing scleroderma, since the first blotting tests were performed (C. Kallenberg, personal communication). The possible prognostic significance of ACA has already been suggested earlier [74] [77].

An antibody against Scl-70 appears to be characteristic for progressive systemic sclerosis [78]. Using the immunoblotting method our group found that all Scl-70 positive sera, as judged by immunodiffusion, contained antibody against a rather insoluble nuclear matrix protein ofapproximately 86,000 molecular weight [79], but no reaction with a 70,000 molecular weight protein could be detected. It is possible that sera of scleroderma patients contain a tandem antibody, one to Scl-70 and the other to Scl-86. However, another in our view more likely possibility is that both antigens are recognized by the same antibody, which means that both antigens have an extremely close chemical relationship.

Several antibody systems directed against proteins associated with tRNA have been detected in these diseases. One of them, designated Jo-1, has been shown to react with histidyl-tRNA synthetase [80]. This antibody seems to be very specific for and occurs in a relatively high incidence in polymyositis patients with pulmonary fibrosis [81 82]. It is noteworthy in this respect that some picornaviruses are also capable of reacting with tRNA synthetase molecules. Based upon clinical findings in some cases of polymyositis a pathogenic role for a coxsackie virus has been hypothesized [83]. Antibodies to PM-1 and Ku have been described to occur at a high incidence in a polymyositis/scleroderma overlap syndrome [84 85].

B Pathogenic roles for ANA

Recent data indicate that in some cases ANA can also serve as a marker for the activity of a disease (chapter 3 [89 109]) or even as an early symptom for the development of a certain syndrome [74 77 79]. These and similar observations raise the question what exact role, if any, do ANA play in the pathogenesis of a disease. A most obvious role is the formation of circulating immune complexes which then deposit in kidneys and brain and in this way might contribute to the pathogenic defects observed in these organs [92]. It is not very likely, however, that this is the only mechanism involved in the pathogenesis of rheumatic diseases, because of the diversity of clinical symptoms. Another interesting theory is the one postulated by a.o. Faaber et al., [90] that crossreactive binding of anti-DNA antibodies to glycosaminoglycanes e.g. of the glomerular basement membrane can lead to tissue injuries and other clinical features of SLE.

Similar theories have been postulated for other ANA, but no extracellular crossreactive antigens have been described as yet for these antibodies. Some workers therefore presuppose the penetration of cellular and nuclear membranes for the pathogenic effect of the specific antibody. This phenomenon has only once been ob-

served in vivo for anti-(U1)RNP antibodies, penetrating T-lymphocytes via their Fc receptors [91]. This however is as yet the only example and should therefore be interpreted with care. Penetration of somatic cells by antibodies has not been described in vivo and can also hardly be achieved under in vitro conditions (E. Mariman, personal communication). Moreover, it is very difficult to understand how the sometimes very severe tissue injuries observed in some rheumatic diseases, could be attributed to penetration or destruction of some non-regulatory cells by specific antibodies. Therefore it seems unlikely that binding of ANA to their nuclear antigen in situ plays a direct role in pathogenesis.

A finding which emphasizes their distinct character is, that in contrast to DNA and histones, most nuclear antigens listed in table I are fairly good immunogens. For some it has even been shown that, upon immunization, they can elicit antibodies idiotypically identical to autoantibodies present in patients' sera [93]. This is a very interesting observation, because it indicates that the production of at least some ANA in patients is triggered by the release of nuclear antigen itself, rather than by action of a crossreactive agent. However, a random cell lysis cannot be the only cause for the production of ANA taken into account the specificity of some patients' sera for only one autoantigen. To get insight in this mechanism it is necessary to investigate the molecular nature and normal cellular function of nuclear antigens.

1.4 BIOCHEMICAL CHARACTERIZATION OF NUCLEAR ANTIGENS.

Sm and (U1)RNP antigens

Anti-Sm and anti-(U1)RNP sera can be selected by analysis of immunoprecipitates obtained from ^{32}P-labeled extracts of cultured cells [94]. Anti-Sm sera precipitate small nuclear (sn)RNA species U1, U2, U4, U5 and U6 (U1-U6), whereas anti-(U1)RNP sera exclusively react with U1 RNPs. Some sera contain both specificities

and are referred to as anti-Sm/RNP (chapter 6, [110]). Protein analysis of immunoprecipitates obtained with anti-(U1)RNP sera revealed that U1 snRNPs are complexed with at least nine differently sized proteins (70K, A, B′, B, C, D, E, F and G, [95 97 98 101]) while U1 RNP-depleted Sm-precipitates contain B′, B, D, E, F and G polypeptides [96], as well as some other minor species (see below). Association of these polypeptides with the various snRNAs is schematically represented in figure 3.

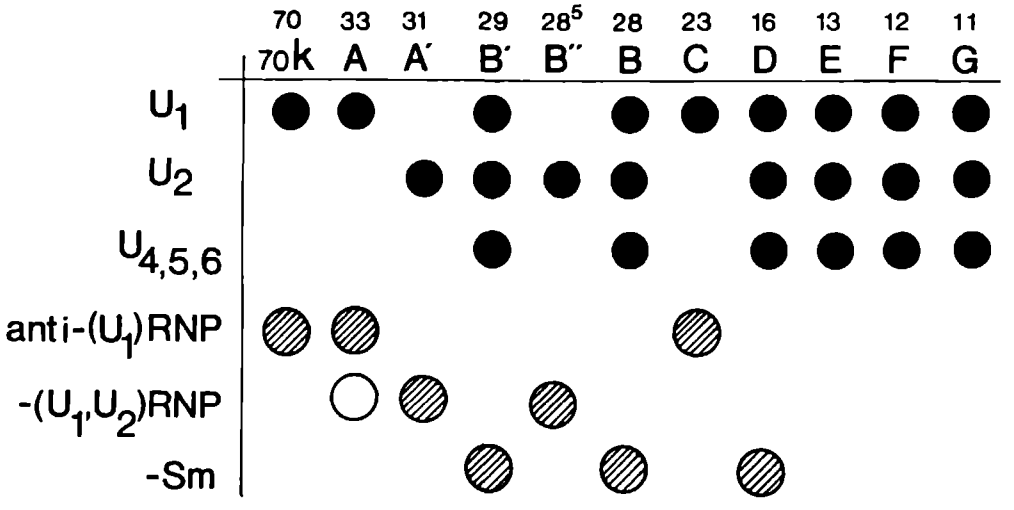

FIGURE 3 Association of proteins with snRNAs

Closed circles indicate the presence of a polypeptide in the respective snRNP particle. Shaded circles indicate the presence of antibodies against the respective polypeptides in the various sera. The open circle indicates cross-reactivity of anti-B′′ antibodies with the A antigen.

When tested by immunoblotting all anti-(U1)RNP sera recognize at least one of the U1 RNA-specific antigens 70K, A and C, but surprisingly some also showed a weak but evident reaction with the (U1-U6) associated B′/B antigens (chapter 6, [110]). This finding can be explained by assuming that most anti-(U1)RNP sera contain trace amounts of anti-Sm antibodies, not detectable in the (very sensitive) RNA-immunoprecipitation assay. Another possibility is that some epitopes on B′ and B antigens become available for immu-

noreaction due to the various treatments in the blotting procedure, while these epitopes are buried inside the snRNP particle under the physiological conditions of the immunoprecipitation assay.

Anti-70K, anti-A and anti-C antibodies contained in anti-(U1)RNP sera were found to be monospecific (chapter 5, [111]). When immunoreactive antibodies are eluted from their respective antigen bands on immunoblots and reassessed on separate blot strips, they react selectively with the corresponding protein antigen from which they had been eluted. Moreover, when these same antibody fractions were used for RNA immunoprecipitation they all reacted exclusively with U1 snRNPs. A different situation is encountered when anti-Sm antibodies are eluted from immunoblots. Anti-B′/B antibodies precipitate U1-U6 RNAs and crossreact with the D antigen and vice versa (chapter 5, [111]). Crossreactivity of B′/B and D antigens with the same monoclonal antibody has been observed previously [99 95]. These findings lend additional support to the conclusion that 70K, A and C antigens are U1 RNP-specific and that B′/B and D antigens are common in U1-U6 RNPs (see also figure 3). As it became clear that U1 and U2 snRNP particles play a crucial role in the processing of pre-mRNA into mRNA, a rapidly increasing interest developed for such monospecific antibodies. With their use it is now possible to unravel details about snRNP primary structure (chapter 8, [114]) and their exact molecular appearance (chapter 7, [113]).

The U2 snRNP antigen

Antibodies directed against U2 RNP-specific polypeptides occur at a low incidence in various types of autoimmune diseases. Such sera allowed the identification of two antigenic polypeptides specific for U2 RNA, designated A′ and B′′ with approximate molecular weights of 32,000 and 28,500, respectively (figure 3, [99] chapter 5, [111]). Purification of antibodies from nitrocellulose blots revealed that antibodies directed against the B′′ antigen crossreact with the U1 RNA specific A antigen. Because of this crossreactivity these sera precipitate also U1 RNA from a ^{32}P-labeled HeLa cell extract (chapter 5, [111]).

The SS-B antigen.

The SS-B (or La) antigen is an antigen of 50,000 molecular weight associated with precursors of a number of small RNAs synthesized by RNA polymerase III, including Ro RNAs (chapter 4, [100] [101] [112]). The antigen is very susceptible to proteolytic degradation resulting in two major antigenic breakdown products of about 40,000 and 25,000 molecular weight. A major part of the SS-B antigen is present in a readily extractable or soluble form. The rest is found in an insoluble form tightly associated with the nuclear matrix (chapter 4, [112]).

The SS-A/Ro antigen

The SS-A (or Ro) antigen is a polypeptide of 59,000 molecular weight [107] associated with a number of small cytoplasmic RNAs (Y-RNAs, [102, 101]). The SS-A antigen is predominantly localized in the cytoplasm and more stable as compared to the SS-B antigen. Anti-SS-A antibodies are mostly found in sera from patients with Sjoegren's syndrome or SLE and in most cases (90%) accompanied by anti-SS-B antibodies.

The centromere antigen

Recently Moroi and coworkers [75] reported on an antibody, the anticentromere/kinetochore antibody, which is found predominantly in the sera of patients with the CREST syndrome. The centromere antigen is not extractable by physiological saline solutions, but can be dissolved in SDS-containing sample buffer allowing analysis by immunoblotting. In an investigation with more than 30 sera showing the typical anti-centromere immunofluorescent pattern, all sera recognized a 19,000 molecular weight protein on HeLa nuclear protein blots [107 61]. These findings are in agreement with the recently published results from Guldner et al. [103] but do not agree with blotting data from Earnshaw et al. [104] and Cox et al. [105]. At the moment we do not have a satisfying explanation for this discrepancy.

The Scl-86 antigen

Sera from patients with progressive systemic sclerosis have been found to inhibit adenovirus DNA replication in an in vitro system [86]. The mechanism seemed to involve the binding by an immunoglobulin G of a factor necessary for elongation. Among the strongly inhibitory sera were those containing anti-Scl-86 antibodies. Two-dimensiomal immunoblotting analysis of anti-Scl-86 sera subsequently revealed that Scl-86 in extracts of mouse 3T6 cells coincided with topoisomerase I, an enzym catalysing DNA relaxation as required for elongation. Moreover, affinity purified anti-Scl-86 antibodies were able to inhibit topoisomerase I activity in a specific in vitro assay [87 123].

The Jo-1 antigen.

The Jo-1 antigen is a polypeptide with an approximate molecular weight of 50,000 associated with histidyl-tRNA [106]. The antigenic component is identical with the PL-1 antigen [60] and has recently been identified as histidyl-tRNA synthetase [80].
When analysed by immunoblotting, the Jo-1 antigen exhibits a slightly lower electrophoretic mobility as compared to the SS-B antigen, and we calculated its molecular weight to be 54,000 Da [107]. Jo-1 is predominantly present in cytoplasmic extracts of (HeLa) cells as would be expected from its function.

The 56K antigen

The 56K antigen was detected in the routine immunoblotting screening of 400 sera from patients with connective tissue diseases and occurs at a low incidence (approximately 3%) in a variety of diseases [61 107]. The antigen is predominantly present in cytoplasmic extracts but a significant moiety is also associated with the nuclear matrix, as is the case with the SS-B antigen [112]. However, we consider the 56K antigen to be a cytoplasmic antigen because, unlike anti-SS-B antibodies, most anti-56K sera produce a diffuse cytoplasmic immunofluorescence pattern on both HeLa and HEp-2 cells. Comparison by immunoblotting

using high resolution gels revealed that the 56K antigen is distinct from vimentin, an intermediate filament polypeptide with a similar molecular weight, which recently has been described as an autoantigen [115].

Proliferating cell nuclear antigen (PCNA).

Antibody to PCNA can be found at a very low incidence in patients with SLE. The antigen has been associated with cellular proliferation by indirect immunofluorescence [116] and in freshly stimulated lymphocytes it appears first in the nucleolus, later moving to the nucleoplasm [117]. The antigen, as detected by immunoblotting, is a protein with molecular weight of 35,000 Dalton and in two-dimensional gels it coincides with cyclin [118].

In this chapter several nuclear and cytoplasmic molecules have been described which are characterized with the help of autoimmune sera. This list cannot be fully exhaustive, neither is it possible to discuss here the entire literature on this subject. Rather it was tried to give an overview how research on autoantibodies can have an influence on practical rheumatology and immunology and on a more fundamental level can add new dimensions to investigations towards the cellular function and structure of their target antigens.

CONCLUDING REMARK

The discovery of a therapy must be the ultimate goal of all investigations in rheumatic diseases. Studies dealing with the characterization of autoantigens as the ones described in this thesis, might not have direct clinical implications but nevertheless they have already proven their usefulness in diagnosis and, as many examples in history show, an improved diagnosis is often a forerunner of an improved therapy.

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CHAPTER 2

ANTIBODIES AGAINST DISTINCT NUCLEAR MATRIX PROTEINS ARE CHARACTERISTIC FOR MIXED CONNECTIVE TISSUE DISEASE

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Antibodies against distinct nuclear matrix proteins are characteristic for mixed connective tissue disease

W J HABETS,* D J DE ROOIJ,‡ M H SALDEN,† A P VERHAGEN,* C A VAN EEKELEN,* L B VAN DE PUTTE‡ & W J VAN VENROOIJ* **Department of Biochemistry, †Division of Hematology and ‡Division of Rheumatic Diseases Department of Internal Medicine University of Nijmegen, The Netherlands*

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SUMMARY

Specific nuclear proteins, separated according to their molecular weight (mol wt) by polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to nitrocellulose sheets, are able to bind antibodies in sera from patients suffering from different types of connective tissue diseases Antibodies against a characteristic set of nuclear protein antigens are found in sera from patients with mixed connective tissue disease (MCTD) Screening of 21 MCTD sera revealed a typical immunoblot pattern with major protein antigens of mol wt 70,000 (20/21) (not identical with the Scl-70 antigen characteristic for scleroderma), mol wt 31,000 (17/21), two proteins around mol wt 23,000 (15/21) and two around mol wt 19,000 (10/21) The 70,000, 23,000 and 19,000 antigens appeared to be rather insoluble nuclear proteins (i e components of the nuclear matrix) On behalf of their structural character they were present in nuclei from several types of cells but only in low amounts detectable in salt extracts of thymus acetone powder The presence of antibodies directed against the mol wt 70,000 antigen correlated strongly with the diagnosis of MCTD This 70,000 antigen is not identical with the RNP antigen, a soluble ribonuclease sensitive ribonucleoprotein, since antibodies against nuclear RNP can be separated from anti-nuclear matrix antibodies by affinity chromatography using immobilized thymus salt extract The distinct character of soluble nuclear RNP and structural nuclear matrix antigens is further supported by the fact that from 14 other anti-RNP sera obtained from patients with systemic lupus erythematosus (SLE), only three contained antibodies against the mol wt 70,000 protein Since the immunoblot pattern obtained with MCTD sera mostly was clearly distinguishable from the patterns obtained with sera from patients with related connective tissue diseases our results suggest that the immunoblotting technique might be useful as a diagnostic tool and support the concept of MCTD as a distinct entity

Keywords mixed connective tissue disease nuclear matrix immunoblotting ribonucleoprotein marker antibody

INTRODUCTION

Mixed connective tissue disease (MCTD) has been described as an apparently distinct rheumatic disease syndrome, characterized by a combination of features similar to those of systemic lupus erythematosus (SLE), systemic sclerosis (scleroderma) and polymyositis Serologically, MCTD is characterized by sometimes extremely high titres of antibody to ribonucleoprotein (RNP) (Sharp *et*

Correspondence Dr W J Habets, Department of Biochemistry, University of Nijmegen, Geert Grooteplein noord 21, P O Box 9101, 6500 HB Nijmegen, The Netherlands

al., 1972, 1976). The protein component of this antigenic RNP complex, purified by affinity chromatography, has been described to consist of at least 7 different proteins with molecular weights (mol. wt) ranging from 9,000 to 40,000 (Lerner & Steitz, 1979; Takano, Agris & Sharp, 1980; Lerner *et al.*, 1981; Kinlaw, Dusing-Swartz & Berget, 1982; Matter *et al.*, 1982).

Antigenic activity of RNP as demonstrated by immunodiffusion or counterimmunoelectrophoresis assays, can be destroyed by either ribonuclease (RNAase) or protease (Sharp *et al.*, 1976; White, Gardner & Hoch, 1981), suggesting that the formation of the immunoprecipitate is dependent on the integrity of both ribonucleic acid (RNA) and protein, although the naked protein contains at least some antigenic sites (White & Hoch, 1981). RNP is further characterized as a set of soluble ribonucleoproteins, since it is present in salt extracts of (thymus) nuclei (Kurata & Tan, 1976).

. Recently it was shown by immunofluorescence that sera from MCTD patients also contain antibodies against other nuclear proteins which are components of a rather insoluble deoxyribonuclease (DNAase), RNAase and high salt resistant proteinaceous structure, generally referred to as nuclear matrix (Salden *et al.*, 1982). To investigate whether the occurrence of these antibodies is a more general phenomenon, the antibody composition of various patient sera was investigated using the immunoblotting technique (Towbin, Stachelin & Gordon, 1979). This technique seems to be very useful because of its capacity to demonstrate antigenic activity in a complex mixture of cellular components and, at the same time, providing information about the exact number and size of molecules that contain antigenic target sites. This latter aspect is a direct improvement when compared to affinity chromatography, in which no distinction can be made between molecules containing antigenic sites and components only associated with, or complexed to, antigens. In this study the immunoblotting technique is used as a routine assay to screen sera from patients with MCTD and other connective tissue diseases for the presence of antibodies against nuclear proteins.

MATERIALS AND METHODS

Patients. Sera from 102 patients with anti-nuclear antibodies (ANA) were used in this study. Most patients were seen in the Departments of Rheumatic Diseases of the University Hospital of Nijmegen and the St Maartens-kliniek in Nijmegen. In addition, 16 sera of SLE and MCTD patients with anti-RNP antibodies were obtained from the University Hospital of Groningen and from the Central Laboratory of the Blood Transfusion Service in Amsterdam, nine sera of patients with Sjögren's syndrome from the University Hospital of Leuven (Belgium), three sera of SLE patients with anti-Sm antibodies from the University of Montpellier (France), two anti-Scl-70 sera of patients with systemic sclerosis from the Royal National Hospital, Bath (England) and serum of one patient with MCTD from the Hospital Ziekenzorg, Enschede.

Patients with MCTD had clinical features of SLE, systemic sclerosis and polymyositis and anti-RNP antibodies (Sharp *et al.*, 1972), patients with SLE satisfied the preliminary American Rheumatism Association criteria for this disease and did not have features diagnostic of other connective tissue disorders (Cohen *et al.*, 1971). Patients with systemic sclerosis satisfied the preliminary criteria for systemic sclerosis (Masi & Rodnan, 1981). Patients with polymyositis had proximal muscular weakness, elevated muscle enzymes, abnormal muscle biopsy and electromyogram (Bohan *et al.*, 1977). Patients with Sjögren's syndrome had keratoconjunctivitis sicca or salivary gland abnormalities with anti-SS-B antibodies. Rheumatoid arthritis patients had classical or definite disease (Ropes *et al.*, 1958).

All sera showed a positive nuclear immunofluorescent staining pattern when acetone fixed cryosections of rat liver tissue and monolayers of cultured cells were used as substrate. Clinical and serological characteristics of the 21 MCTD patients are presented in Table 1. All 21 sera from patients suffering from MCTD showed a speckled nuclear immunofluorescent pattern with nucleoli negative and antibodies to RNP when tested by counterimmunoelectrophoresis (CIE) using a saline extract of rabbit thymus powder (Pel Freez, Rogers, Arkansas) generally referred to as extractable nuclear antigen (ENA) as antigen source (Kurata & Tan, 1976), two of 21 showed anti-DNA antibodies in the Crithidia assay (Aarden *et al.*, 1975), nine of 21 rheumatoid factor using the

Table 1. Clinical and serological characteristics of 21 patients with MCTD

Characteristics	%
Polyarthralgia/polyarthritis	100
Raynaud's phenomenon	90
Myalgia/myopathy	62
Swollen hands	55
Fever	43
Decreased pulmonary diffusion capacity	41 (17)*
Myositis (elevated CPK, abnormal EMG or muscle biopsy)	33
Skin rash (e g butterfly, sunlight sensitivity)	29
Decreased oesophageal motility	28 (18)
Sclerodermatous changes	19
Lymphadenopathy/splenomegalie	19
Sicca syndrome	10
Serositis	10
Neurological abnormalities	5
Renal disease	5
Laboratory features	
Positive for ANA, speckled pattern	100
Positive for RNP (CIE)	100
Positive for (U_1)RNP precipitation	100
Positive for anti-dsDNA (crithidia assay)	10
Hypocomplementemia	19
Positive for rheumatoid factor (Waaler–Rose, Latex)	43

* Figures in parentheses denote No of patients tested

Waaler–Rose and Latex fixation tests (Cats & Klein, 1970) (Table 1) Three out of 36 sera from SLE patients were found to contain exclusively anti-Sm antibodies in CIE whereas 14 of 36 SLE sera contained anti-RNP antibodies Two of these contained weak anti-Sm activity as well Sera were, when not immediately used, stored in liquid N_2

Reference sera Reference sera were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, and from the Division of Rheumatic Diseases, University of Colorado Health Sciences Center, Denver, USA, and used in the CIE assay Blotting data of these sera were not used since diagnostic parameters were not available

Cells HeLa S3 cells were maintained in suspension culture as described (van Eekelen & van Venrooij, 1981) They were shown not to contain Epstein–Barr virus and regularly performed mycoplasma tests were always negative

Preparation of HeLa cell total nuclear protein fraction All procedures were carried out at 0–4°C unless stated otherwise

Cells were harvested on frozen NKM (130 mM NaCl, 5 mM KCl, 1 5 mM $MgAc_2$), pelleted by centrifugation (5 min at 800*g*), washed once with NKM and pelleted again The cells were resuspended in cold buffer 1 (10 mM NaCl, 10 mM Tris-HCl pH 7 4, 1 5 mM $MgCl_2$) containing 0 5 mM PMSC (phenyl methyl sulphonyl chloride) to inhibit endogenous proteolytic activities. After addition of a mixture of sodium deoxycholate (DOC) and Tween 40 (0 5% and 1%, respectively), the cells were homogenized by 10 strokes of a motor driven teflon pestle in a Potter–Elvehjem type tissue homogenizer. The nuclei were pelleted (5 min at 800*g*), washed once with buffer 1 containing 0 5 mM PMSC and resuspended in buffer 2 (110 mM NaCl, 10 mM Tris-HCl pH 7 4, 1 5 mM $MgCl_2$,

0·5 mM PMSC) at a density of 10^8 nuclei/ml. To degrade nucleic acids, the nuclei were incubated with 500 μg/ml DNAase 1 and 100 μg/ml RNAase A for 1 h at 20°C. These nuclease treated nuclei are referred to as total nuclear protein fraction.

Preparation of HeLa cell nuclear matrices. HeLa cell nuclei were treated with DNAase 1 (500 μg/ml) for 1 h at 20°C. Chromatin fragments and RNP complexes were removed by sedimentation through a 1 M sucrose layer in buffer 2 followed by an extraction with 0·4 M $(NH_4)_2SO_4$ (van Eekelen & van Venrooij, 1981). The matrices were resuspended in buffer 1, treated with RNAase (100 μg/ml) for 30 min at 20°C and dissolved completely in sample buffer for gel electrophoresis (see below). Morphological and biochemical characteristics of nuclear matrices isolated in this way have been described (van Eekelen *et al.*, 1982).

Gel electrophoresis and protein blotting. Samples for polyacrylamide gel electrophoresis (PAGE) in sodium dodecylsulphate (SDS) were prepared by dissolving the protein fraction in sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0·1 M Tris-HCl pH 6·8). To ensure complete dissociation of the protein complexes, the samples were heated for 3 min at 100°C followed by gel electrophoresis in SDS on 13% polyacrylamide slab gels (1 mm thick, 10 × 16 cm). Proteins were loaded over the entire width of the gel (1·5 mg of protein per gel) and separated according to their molecular weight essentially as described by Laemmli (1970). After a 4 h run at 20 mA per gel, replicas of the gels were made on nitrocellulose (i.e. blotting) by transferring the proteins electrophoretically using a Bio-Rad trans-blot cell. Transfer was performed overnight at room temperature and at 60 V/0·3 A in 192 mM glycine, 25 mM Tris pH 8·3 and 20% methanol. After transfer the blots were dried and stored at room temperature.

Detection of antigens. The protein blots were cut from top to bottom into strips of about 7 mm and treated with pre-incubation buffer (3% bovine serum albumin [BSA], 350 mM NaCl, 10 mM Tris-HCl pH 7·6, 0·5 mM PMSC) for 3 h at 20°C to saturate additional protein binding sites on the nitrocellulose. Incubation with diluted serum (mostly 1:50) was performed overnight in buffer 3 (0·3% BSA, 150 mM NaCl, 10 mM Tris-HCl pH 7·6, 0·1 mM PMSC, 1% Triton X-100, 0·5% DOC and 0·1% SDS). After extensive washing with buffer 3 (3 × 10 min) IgG immunecomplexes were detected by incubating the blots for 2 h with ^{125}I-labelled protein A (spec. act. 1 mCi/mg) in buffer 3 (2 μCi in 20 ml) then washed again with buffer 3 (3 × 10 min) and water (3 × 10 min), dried under a lamp and exposed to X-ray film for 2–16 h at −70°C using Ilford intensifying screens.

In some blotting experiments ^{125}I-labelled anti-human IgG or horse radish peroxidase conjugated anti-human IgG was used instead of protein A. The results were the same.

Absorption of anti-nuclear RNP antibodies. Twenty milligrams of a saline extract of rabbit thymus powder (ENA) which has been shown to contain the soluble RNP antigens (Kurata & Tan, 1976) was immobilized on 3 g (wet weight) of cyanogen bromide activated Sepharose (Pharmacia). Antibodies against nuclear RNP were absorbed by repeated incubation of 1 ml 1:10 diluted serum in BTP (0·5% BSA, 0·5% Triton X-100, in 10 mM PBS, pH 7·5) with 3 ml ENA-Sepharose for 5 h at 20°C under continuous shaking. The supernatant was tested for remaining anti-nuclear RNP activity by counterimmunoelectrophoresis (CIE), ELISA and immunoblotting. The retained anti-RNP antibodies were eluted from the affinity column with 1 M acetic acid and subsequently neutralized with 1 M Tris.

RESULTS

Sera from 35 patients with anti-RNP antibodies (21 MCTD patients and 14 SLE patients) and 67 other ANA positive sera (see Materials and Methods) were tested for the presence of antibodies against nuclear proteins using the immunoblotting technique.

The immunoblotting assay

The assay was routinely performed as described in the Methods section using as source of antigens the nuclear proteins of HeLa S3 cells. Special care was taken to ensure that all DNA and RNA was degraded completely by the incubation with DNAase I and RNAase A. Control experiments (van Eekelen *et al.*, 1982) showed that about 0·5% of the DNA and less than 5% of the nuclear RNA

resisted the nuclease treatment probably as a result of protection by proteins. This heterogeneous mixture of nucleic acid fragments, however, is dissociated from their protecting proteins by the subsequent boiling in SDS. Therefore, under the electrophoretical conditions used in our assay these DNA and RNA fragments are in no way able to contribute to the distinct pattern of protein bands found (Fig. 1, lanes a and b). To prove the proteinaceous character of the antigens more unequivocally, the nuclear protein fraction was, after nuclease treatment, incubated with pronase. After gel electrophoresis and blotting of this mixture, no antigenic activity was detectable anymore (not shown). After electrophoretic transfer of the proteins, the nitrocellulose sheet can be used for the detection of antigens (Fig. 1, lanes c and d). The binding of proteins to nitrocellulose was always sufficient for recognition by antibodies. Binding of antibodies to slowly migrating high molecular weight proteins as large as mol. wt 120,000 could easily be detected when sera in a dilution of 1:50 were used.

When the total nuclear protein fraction was blotted and this blot was incubated with an MCTD serum a pattern of antigens as shown in Fig. 1c was obtained. As described in the Materials and Methods section, the total nuclear fraction can be subdivided in a nuclear matrix fraction and an $(NH_4)_2SO_4$–sucrose fraction. This lattern fraction contains most if not all of the chromatin and high

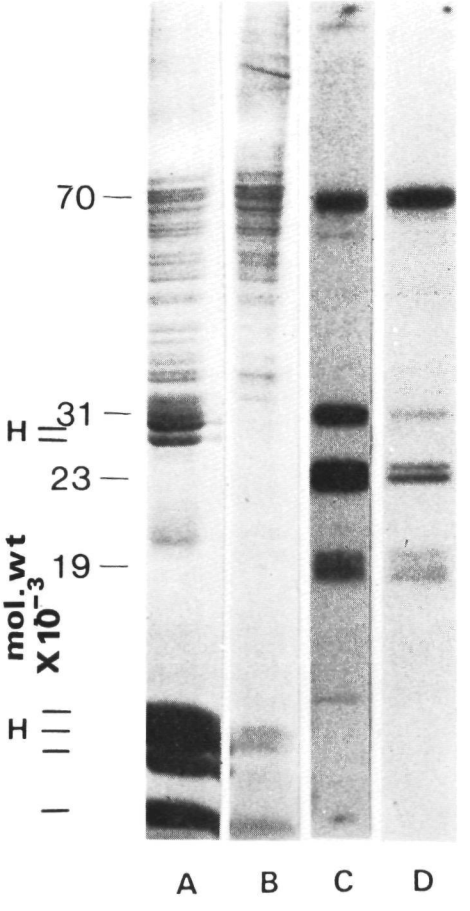

Fig. 1. Detection of nuclear and nuclear matrix antigens with an MCTD serum. HeLa S3 nuclear fractions, treated with DNAase I and RNAase A as described in the Materials and Methods section were completely dissolved in SDS containing sample buffer, heated for 3 min at 100°C and subjected to SDS-PAGE. Electrophoretic transfer of the proteins to nitrocellulose sheets and detection of the antigens was performed as described in the Materials and Methods section. (A) Coomassie brilliant blue stained pattern of total nuclear proteins of HeLa S3 cells; H = Histones. (B) Coomassie brilliant blue stained pattern of nuclear matrix proteins. (C) Immunodetection of nuclear antigens after blotting of lane A and incubation with MCTD serum No. 19. (D) Immunodetection of nuclear matrix antigens after blotting of lane B and incubation with MCTD serum No. 19.

salt extractable RNP structures while the matrix fraction contains matrix components and matrix associated proteins resisting the high salt wash (van Eekelen *et al.*, 1982). The nuclear antigens recognized by the MCTD serum are predominantly present in the matrix fraction (lane d) and have mol. wts of 70,000, 23,000 and 19,000, respectively.

The main advantages using the protein blotting technique for the detection of antibodies are its sensitivity and remarkable reproducibility. Serum from a patient used as a reference for a period of more than 1 year always showed the same characteristic immunoblot pattern, independent from the fact that nuclear protein fractions were used from various batches of HeLa S3 cells. It is by virtue of this reproducibility that we became aware of the characteristic set of antigens recognized by antibodies in sera from patients suffering from MCTD.

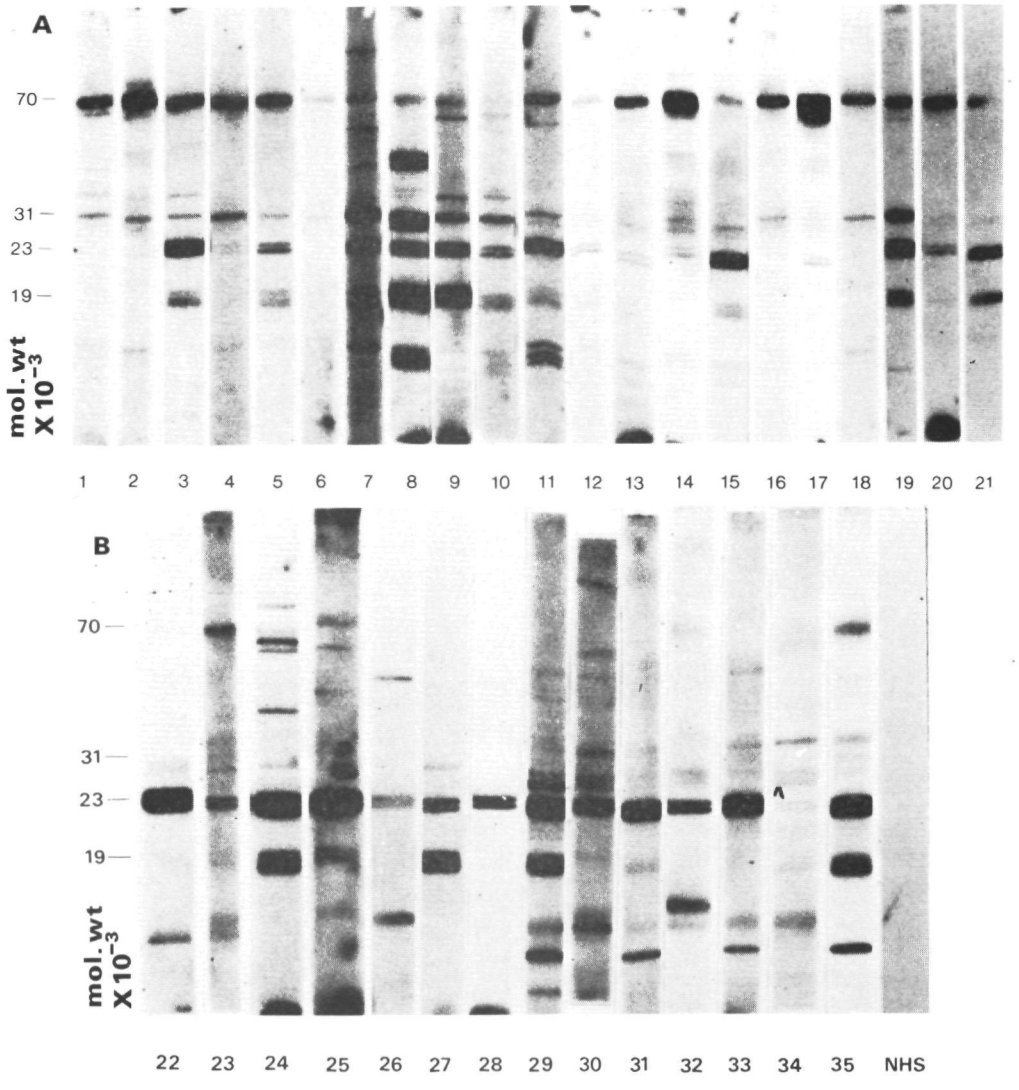

Fig. 2. Immunoblot patterns of patients sera. (A) MCTD sera. Protein blots of a total nuclear protein fraction from HeLa cells were pepared as described in the Methods section. One entire protein blot was cut into strips and each strip was incubated with serum of a patient suffering from MCTD (clinical and serological parameters of these patients are presented in Table 1). Detection of IgG–antigen complexes was performed by incubation with ^{125}I-labelled protein A. (B) Anti-RNP containing SLE sera. Protein blots were incubated with anti-RNP sera obtained from 14 SLE patients (see Methods section). NHS: protein blot incubated with normal human serum obtained from a pool of 40 healthy individuals.

Correlation between MCTD diagnosis and presence of specific antibodies

Comparing the protein blot patterns of sera from MCTD patients (Fig 2A) and SLE sera containing anti-RNP activity (Fig 2B) it was striking that the presence of the mol wt 70,000 band correlated strongly with the diagnosis of MCTD (Table 2) As is shown in Fig 2, 95% of the MCTD sera (20 of 21) recognized the mol wt 70,000 protein, whereas of the remaining 14 anti-RNP sera, all from SLE patients, only three (21%) did recognize the mol wt 70,000 antigen Other commonly found target antigens for antibodies in the MCTD and SLE anti-RNP sera tested were a doublet at mol wt 23,000 (80%, 28 of 35), two proteins around mol wt 19,000 (43%, 15 of 35) and a single protein with mol wt 31,000 (69%, 24 of 35) The presence of multiple other (low molecular weight) antigen bands is particularly evident in the SLE sera Antibodies against the mol wt 23,000 doublet, probably identical to the C proteins of Lerner *et al* (1979) were also found in anti-Sm sera (three of three, Table 2)

Table 2. Occurrence of antibodies against MCTD associated antigens in various sera

Diagnosis	Number of patients	Molecular weight $\times 10^{-3}$ 70	31	23	19
MCTD	21	20 (95%)	17 (81%)	15 (71%)	10 (48%)
SLE anti-RNP	14†	3 (21%)	7 (50%)	13 (93%)	5 (36%)
anti-Sm	3	0	2 (67%)	3 (100%)	0
other	19	1 (5%)	6 (32%)	7 (37%)	1 (5%)
all	36	4 (11%)	15 (42%)	23 (64%)	6 (17%)
PM*	8	0	3 (38%)	1 (13%)	1 (13%)
RA	10	0	0	0	0
PSS	14	0	0	3 (21%)	0
SS	13	0	1 (8%)	0	0

* PM = polymyositis, RA = rheumatoid arthritis, PSS = progressive systemic sclerosis, SS = Sjogren's syndrome

† Two of these sera contained some anti-Sm activity as well

Other sera

Although the majority of other investigated ANA positive sera (including sera from patients suffering from Sjogren's syndrome, $n = 13$, progressive systemic sclerosis, $n = 14$, systemic lupus erythematosus, $n = 22$, polymyositis, $n = 8$ and rheumatic arthritis, $n = 10$) contained antibodies against distinct nuclear proteins (data not shown), the pattern of the target antigens of these sera (number and molecular weights of antigen bands) always was clearly distinguishable from the MCTD type pattern shown in Fig 2A (see also Table 2)

Presence of the MCTD associated antigens in other cell types

An important and often neglected point in the discussion about the character of nuclear antigens has been the nature of the antigen preparation used in various studies The fact that antigens might be present both in a soluble (Kurata & Tan, 1976, Sharp *et al*, 1976) and an insoluble form (White, Billings & Hoch, 1982, Salden *et al*, 1982) has prompted us to use in our blotting assay the total nuclear protein fraction containing structural as well as soluble components of the HeLa cell nucleus In Fig 1 we have shown that the insoluble nuclear matrix fraction, after dissolving it in SDS containing buffer, contained the mol wt 70,000, 23,000 and 19,000 proteins as the major antigens when an MCTD serum was used Since these structural proteins constitute only a small fraction of the total cellular protein and thereby are rather insoluble in physiological salt solutions, it is not unexpected that the concentration of these antigens in the commonly used salt extracts of thymus powder is very low as compared with total thymus powder (Fig 3, lanes F and G) Better

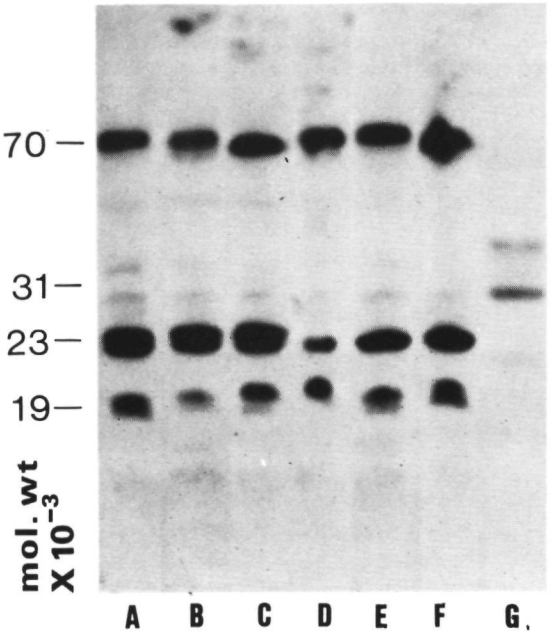

Fig. 3. Occurrence of MCTD associated antigens in various cell lines and tissues. Purified nuclei from (A) HeLa S3 cells, (B) human lymphoblasts in culture (Rosenfeld *et al.*, 1977), (C) cultured hepatoma cells (Alexander *et al.*, 1976) and (E) hamster lens cells (Bloemendal *et al.*, 1980) were prepared as described for HeLa cells in the Methods section. Nuclei from rat liver tissue (D) were prepared as described (Blobel & Potter, 1966). The nuclear protein fractions from these nuclei were prepared by DNAase I and RNAase A digestion followed by SDS solubilization as described in the Methods section. Total rabbit thymus powder (F) was sonicated prior to DNAase I and RNAase A digestion and SDS solubilization. Salt extractable nuclear proteins (G) were isolated from rabbit thymus powder using the procedure of Kurata & Tan (1976). Equivalent amounts of all protein samples were used in the protein blotting assay. All strips were incubated with serum from MCTD patient No. 3 and after ^{125}I-labelled protein A treatment autoradiographed for 16 h.

sources of these antigens, therefore, are purified nuclei from either cultured cells or tissues. Fig. 3, lanes A to E, show the antigens recognized by an MCTD serum in nuclear proteins from various types of cultured cells and rat liver tissue. It is interesting to note here that, for example, in HeLa and lymphocyte nuclear proteins there is a double mol.wt 23,000 antigen band, whereas in rat liver and hamster lens nuclear proteins this antigen appears as a single polypeptide. This phenomenon might be caused by post-synthetic modification of the 23,000 protein which occurs in one type of cell but not in another.

MCTD-70 is not identical with Scl-70

Because of the similarity in molecular weight it was investigated whether the MCTD 70,000 antigen could be identical with the Scl-70 antigen that is often found in sera from patients with progressive systemic sclerosis (Douvas, Achten & Tan, 1979). When Scl-70 sera, which had been shown to react positively in immunodiffusion and immunofluorescence tests (Catoggio *et al.*, 1982) were examined in the protein blotting assay, they did not recognize an antigen of mol. wt 70,000. This strongly suggests that the Scl-70 and the MCTD-70 antigens are not identical. Moreover, none of the 14 sera from scleroderma patients showed antibodies against an mol. wt 70,000 protein in the blotting assay. It is possible that the antibodies against Scl-70 recognize a delicate three-dimensional conformation of the antigen *in situ* that is destroyed during our preparation procedure.

MCTD associated nuclear matrix antigens are distinct from nuclear RNP

The distinct character of nuclear RNP and the MCTD associated nuclear matrix antigens is already indicated by the fact that from 14 anti-RNP sera (all obtained from SLE patients) only three did

recognize the mol. wt 70,000 nuclear matrix antigen. Moreover, none of the matrix antigens were detectable in a saline extract of rabbit thymus (ENA, Fig. 3 lane G). To prove the distinct character of the nuclear matrix antigens more unequivocally the following experiment was performed. An MCTD serum was passed over an ENA-Sepharose column (see Materials and Methods) to remove the antibodies against nuclear RNP. To make sure that the absorption was quantitative, the non-bound antibody fraction was tested in counter-immunoelectrophoresis, by immunoblotting and in a very sensitive ELISA technique, always using ENA as the substrate. No reactivity towards soluble nuclear RNP antigens was detectable anymore (not shown). However, anti-nuclear matrix antibodies were still present in the non-bound antibody fraction as is shown in Fig. 4 lane B. When the retained anti-nuclear RNP antibodies were eluted from the affinity column, they did not show any reactivity towards the nuclear matrix antigens (Fig. 4 lane C) but appeared to be enriched in activity towards polypeptides with mol. wts of 9,000–14,000.

Summarizing we conclude that MCTD sera recognize a specific set of nuclear protein antigens of mol. wt 70,000, 31,000, 23,000 and 19,000. Some of these proteins are also recognized by other anti-RNP sera (Table 2 & Fig. 2B). However, the presence of antibodies against a mol. wt 70,000 antigen, mostly in combination with (some of) the other three antigens is highly characteristic for MCTD.

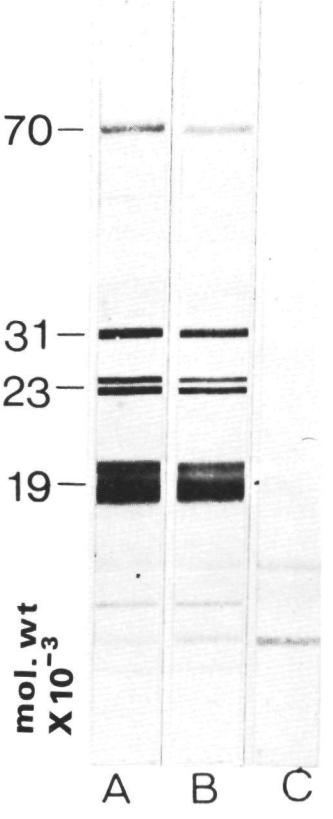

Fig. 4. Detection of separated anti-RNP and anti-nuclear matrix antibodies. Serum from MCTD patient No. 19 was depleted from antibodies against nuclear RNP by absorption on an ENA–Sepharose affinity column. The non-bound fraction and the eluate were tested by immunoblotting. Three identical strips of the same protein blot of a HeLa total nuclear protein fraction were incubated with: (A) Serum before absorption; (B) serum after absorption (non-bound fraction); (C) purified anti-nuclear RNP antibodies (eluate). The strips were incubated for 1 h with horse radish peroxidase conjugated anti-human IgG and peroxidase activity was detected by incubating the washed blots in PBS (10 mM phosphate buffer in 0·9% NaCl, pH 7·5) containing 4-chloro-1-naphtol (0·5 mg/ml) and H_2O_2 (0·06%).

DISCUSSION

The use of the immunoblotting technique for the detection of specific antigens recognized by antibodies in autoimmune sera has several advantages Because not only the soluble but also the insoluble antigens are dissolved in SDS before gelelectrophoresis, more complete information is obtained about the number and size of the antigens In contrast to affinity chromatography or immunoprecipitation, in the immunoblotting technique the antigenic complexes are first separated into their individual constituents, so that only the antigenic sites bearing polypeptides are detected Moreover, the method is extremely sensitive (Towbin *et al*, 1979) and reproducible as has been pointed out already in the Results section Finally the blotting technique can be used for the screening of large groups of patient sera

The group of 21 MCTD patients examined in this study is a rather heterogeneous one as far as their clinical characteristics are concerned (Table 1) All 21 MCTD sera, however, showed a speckled nuclear immunofluorescent pattern, a finding not only characteristic for MCTD sera but also often found when, for example, SLE sera are used Furthermore, all 21 sera contained precipitating antibodies to (U_1)RNP when tested in counterimmunoelectrophoresis and immunoprecipitation studies (Table 1) The antibodies to RNP have been described as being characteristic for MCTD (Sharp *et al*, 1972) although they can be found also (in somewhat lower titres and frequency) in sera from patients suffering from SLE, rheumatoid arthritis, Sjogren's syndrome and progressive systemic sclerosis (Notman, Kurata & Tan, 1975) Although all ANA positive sera tested with immunoblotting contained antibodies against a great variety of nuclear proteins, the antigen patterns obtained with sera from MCTD patients were surprisingly similar In particular the presence of antibodies against a mol wt 70,000 antigen in almost all MCTD sera was striking and allowed us to distinguish them easily among immunoblot patterns of about 100 sera from patients with various types of connective tissue diseases, even when these sera contained anti-RNP activity (Table 2 & Fig 2)

It is commonly agreed upon that the soluble RNP complex precipitated by anti-RNP sera contains at least seven proteins in the range of mol wt 9,000–40,000 (Lerner & Steitz, 1979, Takano *et al*, 1980, Sri-Widada *et al*, 1982, Lenk, Maizel & Crouch, 1982, Matter *et al*, 1982, Kinlaw *et al*, 1982) although some reports suggest the presence of mol wt 70,000 and 65,000 proteins as well (White *et al*, 1982, Takano *et al*, 1981)

However, these soluble antigens are distinct from the nuclear matrix antigens since we have demonstrated here than an MCTD serum, completely depleted from anti-nuclear RNP antibodies still contains anti-nuclear matrix activity The fraction containing the antibodies against nuclear RNP showed reactivity towards polypeptides with mol wts of 9,000–14,000 present in a total (soluble as well as insoluble) nuclear protein fraction from HeLa cells Antibodies against such low molecular weight proteins in this region are commonly found in anti-RNP sera (White *et al*, 1981, Sri-Widada *et al*, 1982, Lenk *et al*, 1982) It is interesting to note further that these low molecular weight proteins are recognized in a higher frequency by RNP sera obtained from SLE patients (11 of 14) than by the MCTD sera

In summary we conclude that the presence of antibodies against a mol wt 70,000 nuclear matrix antigen is strongly correlated with the diagnosis of MCTD (Table 2) suggesting that this disease indeed might be considered as a distinct entity Our results further demonstrate the immunoblotting technique to be a powerful diagnostic tool

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CHAPTER 3

QUANTITATION OF ANTI-RNP AND ANTI-Sm ANTIBODIES IN MCTD AND SLE PATIENTS BY IMMUNOBLOTTING

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Quantitation of anti-RNP and anti-Sm antibodies in MCTD and SLE patients by immunoblotting

W. J. HABETS,* D. J. DE ROOIJ,† MARGOT H. HOET,* L. B. VAN DE PUTTE† & W. J. VAN VENROOIJ** *Department of Biochemistry and †Division of Rheumatic Diseases, Department of Internal Medicine, University of Nijmegen, Nijmegen, The Netherlands*

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SUMMARY

A quantitative immunoblotting assay (QIBA) for the determination of specific antibody titres in human autoimmune sera is described. In this assay, a total HeLa nuclear protein fraction, immobilized on nitrocellulose blot strips, was used as source of antigens and immunoreactive species of autoantibodies were quantitated by an enzyme linked second antibody procedure. Besides being more discriminative, QIBA appeared to be up to 500 times more sensitive than immunodiffusion or immunoelectrophoresis. In this study we used 21 sera from patients with SLE or MCTD for a quantitative analysis of their specific autoantibody content. Within this group, a very diverse spectrum of antibody populations was observed; anti-RNP sera appeared to contain, among others, high titred antibody versus 70K and 31K polypeptides while all ($n=6$) anti-Sm sera recognized a 25kD protein doublet. In a follow-up study of two MCTD patients significant flares in specific antibody content could be observed.

Keywords immunoblotting Sm/RNP SLE/MCTD quantitation

INTRODUCTION

Autoantibodies directed against intracellular (mostly nuclear) antigens are frequently found in association with rheumatic diseases (Tan, 1982). Some of these antibodies are characteristic for certain syndromes and therefore of diagnostic value. For example, anti-Sm and high titres of anti-RNP antibodies are found to be associated with systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD), respectively (Sharp *et al.*, 1972, 1976). Much effort has been made to elucidate the clinical significance of the presence of these anti-Sm or anti-RNP antibodies (Williamson, Pennebaker & Boyle, 1983, Field Munves & Schur 1983, Barada *et al.*, 1981, Sharp *et al.*, 1972, 1976) but until now no clear correlations have been found.

Attempts to elucidate the molecular composition of the Sm and RNP antigens were more successful. Both appeared to be a ribonucleoprotein complex consisting of one or more small nuclear RNA species, termed U1, U2, U4, U5 and U6, and a set of five to eight nuclear proteins (Lerner & Steitz 1979). The target for the anti-RNP antibody reaction has been shown by non-immunological isolation procedures to consist of one single RNA species (U1 snRNA) and a set of eight different nuclear polypeptides varying in mol. wt from 70,000 to 9,000 daltons (D) (Kinlaw, Robberson & Berget, 1983, Hinterberger, Pettersson & Steitz, 1983). Polypeptides 70K, A and C (of 70, 31 and 19 kD, respectively) are unique for the U1 snRNA particle and therefore it can be assumed that the U1-RNP specific epitopes are formed by at least one of these proteins. The

Correspondence: Dr W. J. Habets, Department of Biochemistry, University of Nijmegen, Geert Grooteplein noord 21, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

other five proteins B,D,E,F and G (mol wt 25, 13, 11, 10 and 9 kD, respectively) are also found to be associated with the U2, U4, U5 and U6 snRNA which are among the targets of the anti-Sm reaction It is not clear yet which of these five proteins carry the Sm specific determinant(s) but the 25 kD B-B′ doublet and the 13 kD D protein are common targets when anti-Sm sera are tested in immunoblotting (White, Billings & Hoch, 1982, Billings *et al*, 1982)

Quantitation of these antibodies has, until now, only been performed using conventional semi-quantitative techniques as counter-immunoelectrophoresis (CIE) (Field Munves & Schur, 1983, Barada *et al*, 1981) or haemagglutination (HA) (Sharp *et al*., 1976, 1972) which both are only capable of measuring the total amount of reactive antibody The assay we present here has a higher discriminative power in that it measures the amount of antibody directed against each antigenic polypeptide separately This assay makes it possible to investigate whether there are correlations between the quantity of these specific antibodies and distinct clinical features. We tested 21 MCTD and SLE sera and followed two MCTD patients in a longitudinal study for a period of 2 years

MATERIALS AND METHODS

Patients. Most patients with MCTD or SLE were seen in the St Radboud hospital or the St Maartensclinic in Nijmegen, and diagnoses were reached as described (Habets *et al*, 1983a, 1983b). Clinical characteristics of these patients are presented in Table 1.

Table 1. Clinical and laboratory features of patients with MCTD or SLE

Characteristics	Code	%
Polyarthralgia/polyarthritis	A	100
Raynaud's phenomenon	B	89
Swollen hands	C	58
Myalgia/myopathy	D	52
Skin rash (e g butterfly, sunlight sensitivity)	E	37
Fever	F	32
Myositis (elevated CPK, abnormal EMG or muscle biopsy)	G	26
Sclerodermatous changes	H	21
Vasculitis	I	21
Pleuro/pericarditis	J	16
Sjogren's syndrome	K	11
Lymphadenopathy	L	11
Renal disease	M	5
CNS involvement	N	5
Decreased pulmonary diffusion capacity	O	38 (16)*
Decreased oesophageal motility	P	35 (17)
Laboratory features		
Positive for ANA, speckled pattern		100
Positive for RNP (CIE)		68
Positive for Sm (CIE)		21
Positive for Sm + RNP (CIE)		11
Hypergammaglobulinaemia	Q	58
Leuco/thrombopenia	R	47
Positive for rheumatoid factor (Waaler–Rose, Latex)	S	42
Hypocomplementaemia	T	32
Positive for anti-dsDNA (Farr assay)	U	32

* Figures in parentheses denote number of patients tested.

Reference sera. Sera containing anti-nuclear antibodies (ANA) were selected essentially as described (Habets *et al.*, 1983b, Kurata & Tan, 1976). Reference anti-RNP, anti-Sm and anti-SS-B sera were a gift of the Centers for Disease Control, Atlanta, Georgia, USA. Anti-Scl-70 reference sera (Douvas, Achten & Tan, 1979) were a kind gift of Dr E. Penner (Vienna).

Culturing and labeling of HeLa S3 cells. HeLa S3 cells were grown in suspension cultures as described (van Eekelen & van Venrooij, 1981). They were shown to contain no Epstein–Barr or Adenovirus and regularly performed mycoplasma tests were always negative.

RNA was labelled for 48 h with 3 μCi/ml ^{3}H-uridine and 1 μCi/ml ^{3}H-cytidine at a density of $0{\cdot}5 \times 10^6$ cells/ml.

Preparation of antigenic protein fractions. Extractable nuclear antigen was prepared by a 4 h extraction at 4°C of rabbit thymus powder (Pel-Freez, Arkansas, USA) with PBS (3mM NaH_2PO_4, 7 mM Na_2HPO_4, 0·9% NaCl, pH 7·6) containing 0·5 mM PMSC (phenyl-methyl-sulphonyl chloride) to inhibit proteolytic activity followed by centrifugation for 30 min at 20,000*g* (Kurata & Tan, 1976).

Hela nuclear protein fraction for blotting analyses was prepared as described earlier (Habets *et al.*, 1983b).

Qualitative immunoblotting. Transfer of proteins from 13 or 15% polyacrylamide gels onto nitrocellulose sheets was performed as described (Habets *et al.*, 1983b). After transfer the blots were dried, cut into strips and stored at room temperature. Immediately before use additional protein

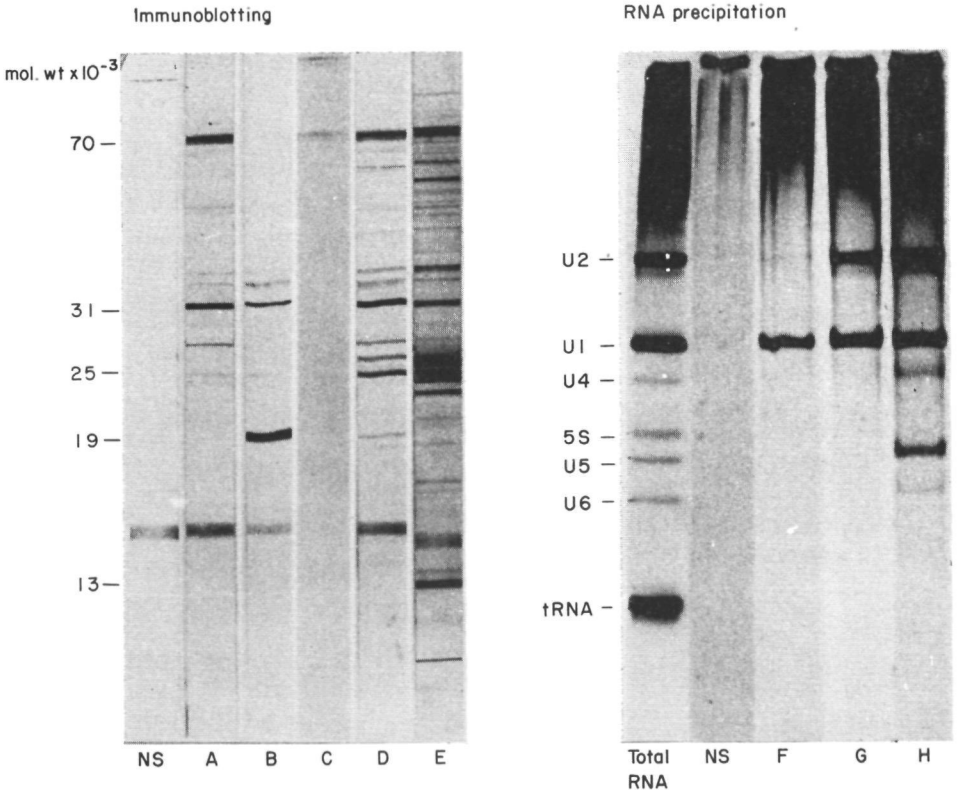

Fig. 1. Characterization of sera by qualitative immunoblotting and RNA precipitation analysis. Immunoblotting: identical strips from one HeLa nuclear protein blot were incubated with 1:100 diluted normal human serum (NS), three different 1:100 diluted human anti-RNP sera (lanes A, B and D), a 1:50 diluted monoclonal anti-70K RNP culture supernatant (White *et al.*, 1982, lane C) and a human anti-Sm serum, 1:100 diluted (lane E). RNA precipitation: polyacrylamide gel analysis of RNA precipitated from a nuclear supernatant of HeLa cells prepared as described by Hinterberger *et al.* (1983) either directly extracted with phenol (lane total RNA) or immunoprecipitated with normal human serum (lane NS), anti-(U1)RNP specific serum (lane F), anti-RNP, U1 plus U2 specific serum (lane G) and anti-Sm serum (lane H). Identity of snRNA was verified by precipitation with anti-2,2,7 trimethylguanosine specific antibody (Bringmann *et al.*, 1983).

binding sites on the strips were blocked by incubation in buffer A (3% bovine serum albumin [BSA], 350 mM NaCl, 10 mM Tris-HCl, pH 7·6) for 1 h followed by a 2 h incubation in 1 : 100 diluted serum in buffer B (0·3% BSA, 150 mM NaCl, 10 mM Tris-HCl, pH 7·6, 1% Triton X-100, 0·5% DOC and 0·1% SDS). After extensive washing (3 × 10 min) in PBS containing 0·5%. (Triton X-100, the strips were incubated for 1 h in 1 : 1,000 diluted horseradish peroxidase labelled human IgG heavy and light chain specific second antibody (Nordic, Tilburg, The Netherlands) in PBS containing 0·5% Triton X-100 and 0·5% BSA (referred to as PTB buffer) and washed again. Immune complexes on the strips were visualized by incubation in substrate solution 1 (PBS containing 0·5 mg/ml 4-chloro-1-naphtol and 0·012% H_2O_2).

Quantitative immunoblotting. Several (mostly 15) identical strips were cut from nitrocellulose blots prepared as described above. In all quantitative experiments two strips from every new sheet of nitrocellulose were always tested with the same reference serum sample as an internal standard. Specific antibody titres were determined by incubating the strips with appropriate dilutions of the test serum in buffer B for 2 h. Then the strips were washed three times for 10 min in PBS containing 0·5% Triton X-100, followed by incubation with horseradish peroxidase labelled anti-human IgG diluted 1 : 1,000 in PTB buffer. The antigen–antibody complexes formed on the strip with the highest serum concentration were visualized by a 2 min incubation in substrate solution 1. The corresponding regions on the other strips were then cut out and incubated separately in 0·5 ml substrate solution 2 (50 mM PO_4 buffer pH 6·0 containing 0·8 mg/ml 5-aminosalicylic acid and 0·024% H_2O_2) in a 24 well multidish (NUNC). After 30 min the extinction at 492 nm (E492) of this substrate solution was determined in a Titertek multiscan. From the results obtained with the reference anti-RNP serum a standard dose–response curve for each of the specific antibody populations was established (see Results section). Shapes of the dose–response curves of 10 patients sera tested, closely fitted with the standard curves. Therefore, binding values for other sera were read directly from these standard curves and expressed in arbitrary units (AU) that we define here as the product of the multiplication factor (MF) read from the standard curve and the corresponding dilution factor. The multiplication factors in the standard curves are chosen in such a way that the standard anti-RNP serum contains 100 AU/ml of all three of the antibody populations measured (see also Fig. 2).

For the longitudinal studies all the serum samples from one patient were tested with identical blot strips originating from one transfer to ensure optimal reproducibility. As a consequence of this modification, standard deviations never exceeded 15% as was established by incubation of a 1 : 500 diluted serum sample with 10 identical blot strips.

RESULTS

The specific autoantibody composition of 21 anti-Sm and/or anti-RNP sera, as such characterized by CIE, was established by qualitative immunoblotting and RNA precipitation analysis.

Qualitative laboratory parameters

With purified HeLa S3 nuclei as antigen source a remarkable diversity was observed between the various sera tested. Most anti-RNP sera (11 out of 13) recognized a 70,000 D antigen and six of these recognized in addition an antigenic polypeptide of 31 kD (70K and 31K, Fig. 1, lane A). Two sera recognized exclusively the 31K and 19K proteins called A and C in the Steitz nomenclature (Lerner & Steitz, 1979, Fig. 1, lane B). Eight sera showed a (weak) reaction with a 25K antigen doublet identical with the B-B′ protein doublet of the Steitz nomenclature (Fig. 1, lane D). The six anti-Sm sera tested, on the other hand, recognized this doublet as most prominent antigens (Fig. 1, lane E). As a reference also the blot pattern of a monoclonal anti-RNP serum is shown (Fig. 1, lane C, Billings *et al.*, 1982).

The possibility that this multiple band pattern is caused by cross-reaction of one antibody type with more than one of these polypeptides can be ruled out, because antibodies eluted from one polypeptide band do not react with one of the other bands as was shown by Guldner, Lakomek & Bautz (1983) and confirmed by us (data not shown). Moreover, the monoclonal anti-70K antibody

exclusively recognizes the 70,000 D antigen Therefore it is clear that at least the 70K,A,B and C antigen bands represent distinct polypeptides and are not breakdown products of each other as occasionally has been suggested (White *et al*, 1982, Wooley, Zukerberg & Chung, 1983)

A comparable heterogeneity as described above for the protein antigens was also observed when the results from RNA precipitation analysis of the 21 anti-Sm and anti-RNP sera were compared Within the anti-RNP group ($n = 13$) three reaction types were observed exclusively anti-U1 ($n = 6$, Fig 1, lane F, anti-U1 plus U2 ($n = 1$, Fig 1, lane G) and anti-U1,2,4,5,6 ($n = 6$, Fig 1, lane H) Anti-Sm sera ($n = 6$) or anti-RNP plus anti-Sm sera ($n = 2$) all precipitated U1,2,4,5,6 (see also Table 2)

Sensitivity of immunoblotting

One major advantage of immunoblotting lies in its ability to detect insoluble antigens, as is illustrated by the recognition of the mol wt 70,000 nuclear matrix associated protein (Salden *et al*, 1982, Habets *et al*, 1983b, Fritzler, Ali & Tan, 1984) Antigens from this nuclear substructure cannot be detected in double diffusion assays as has been shown previously (Habets *et al*, 1983a)

The detection level of the immunoblotting assay was determined as follows Varying amounts of human IgG were spotted directly onto small nitrocellulose squares The total amount of protein was immediately bound as was verified by control experiments with ^{125}I-labelled human IgG After incubation with goat anti-human IgG, bound IgG was quantitated as described in the Materials and Methods section using 5-amino salicylic acid as substrate When performed in this way, amounts of 8 ng IgG could easily be detected

Standard serum curves

We have chosen for the quantitation of antibodies versus the 70K and 31K (U1)RNP associated antigens and the 25K doublet, the most prominent target for the anti-Sm antibodies These antibodies are of IgG class, and no reaction could be detected when peroxidase linked anti-IgA, IgM or IgD was used instead of anti-IgG as the second antibody

Three separate dose–response curves were produced for the antibodies versus the 70K, 31K and 25K antigens by incubating eight identical nitrocellulose strips, prepared as described in the Materials and Methods section, with serial dilutions of a standard serum The amount of antibody bound to one distinct polypeptide band was then quantitated by an incubation with peroxidase

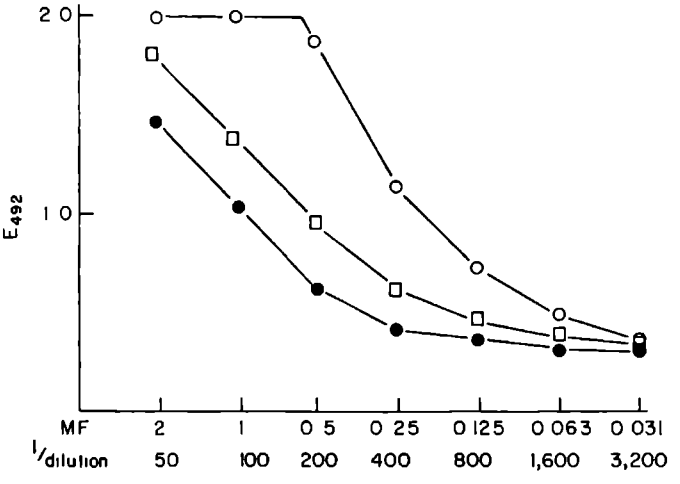

Fig. 2. Standard dose–response curves of three different antibody populations (anti-70K = ○, anti-31K = ●, anti-25K = □) in an anti-RNP reference serum Seven identical protein blot strips were probed with serial dilutions of an anti-RNP reference serum, followed by excision of the regions at mol wt 70,000, 31,000 and 28,000 D Bound IgG on these fragments were quantitated as described in the Materials and Methods section MF = multiplication factor See text for details

Table 2. Clinical and immunoblotting data from individual patients

		Clinical features*																			CIE			Quantitative immunoblotting†		
Patient	Diagnosis	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	RNP	Sm	RNA-Prec.	Anti-70K	Anti-31K	Anti-25K
A1	MCTD	A	B	C					H	I						O	P	Q					+		U1	48	200	36
D1	MCTD	A	B	C	D	E	F	G		I		K				O		Q	R		T	U	+		U1	100	150	120
G15	MCTD	A	B	C													P						+		U1	8	50	10
K3	MCTD	A	B	C								K						Q	R	S			+		U1	60	160	40
K4	MCTD	A	B	C	D																		+		U1	16	36	24
T2	MCTD	A	B	C	D			G	H							O							+		U1	32	100	20
S2	MCTD	A	B	C					H										R	S			+		U1+U2	95	84	48
C3	MCTD	A	B		D	E														S			+		U1–U6‡	48	500	150
D4	MCTD	A	B			E	F								N					S			+		U1–U6	60	230	50
H13	MCTD	A	B	C	D			G										Q	R	S	T		+		U1–U6	120	90	60
S3	MCTD	A	B		D	E	F											Q		S			+		U1–U6	97	110	240
W1	MCTD	A	B	C	D											O	P	Q	R	S			+		U1–U6	400	72	46
W2	MCTD	A	B		D		F	G									P	Q					+		U1–U6	28	100	40
B16	SLE/MCTD	A	B		D		F				J						P	Q	R		T	U	+	+	U1–U6	450	1,000	400
D2	MCTD	A	B	C	D			G	H	I	J		L					Q				U	+	+	U1–U6	300	500	300
C12	SLE	A	B			E	F			I								Q	R	S	T	U		+	U1–U6	82	200	520
H11	SLE	A				E										O					T	U		+	U1–U6	14	120	600
M13	SLE	A	B	C												O	P		R					+	U1–U6	17	280	108
R11	SLE	A				E					J		L	M				Q	R		T	U		+	U1–U6	14	104	100
CDC REF Sm§																								+	U1–U6	70	350	>1,600
A'DAM REF Sm§																								+	U1–U6	30	300	600
Control sera																												
NS ($n=10$)																							NEG	NEG	NEG	< 11	< 35	< 20
SS-B ($n=10$)																							NEG	NEG	ND¶	< 19	< 40	< 20
Scl-70 ($n=10$)																							NEG	NEG	ND	< 20	< 30	< 20

* In code, see Table 1.
† In arbitrary units.
‡ U1–U6 denotes the precipitation of U1, U2, U4, U5 and U6 snRNPs.
§ No clinical data available.
¶ ND = not determined.

linked human IgG specific second antibody, followed by excision of the antigen band concerned, and incubation of this fragment in a substrate solution (see Materials and Methods). The colorimetric change at 492 nm of this substrate solution after 30 min appeared to be a measure for the amount of anti-70K, 31K and 25K antibody in the diluted serum sample (Fig. 2). As a consequence standard curves can be used for the quantitation of these antibodies in various patients sera.

Patients sera

We quantitated the specific antibody content of 51 different sera (21 anti-RNP or anti-Sm, 10 anti-SS-B, 10 anti-Scl-70 and 10 normal human sera). From triplicate analysis of every serum sample the average anti-70K, 31K and 25K antibody content was calculated and expressed in AU. Significant differences in antibody content could be observed between the sera tested; for the antibodies against the 70K protein, none of the SS-B, Scl-70 or normal human sera showed binding values above 20 AU/ml, while from the anti-RNP group only two sera contained less than 20 AU/ml of anti-70K antibody. These two sera decorated exclusively the 31K and the 19K band in the qualitative immunoblotting procedure (see Fig. 1, lane D). Normal binding values for the 31K protein are somewhat higher than for the 70K protein, but the group of anti-Sm and anti-RNP sera had much higher antibody titres against this antigen when compared to the anti-SS-B/anti-Scl-70/ normal control group. High anti-25K activity was mainly found in the anti-Sm group.

Other laboratory parameters such as RNA precipitation analysis from the anti-Sm/RNP group are presented in Table 2 together with some clinical features.

Longitudinal studies

In a follow-up study we determined the titres of three distinct antibody populations in sera from 2 MCTD patients collected during a period of 2 years. Therefore every serum sample was analysed in duplicate with identical protein blot strips at three different dilutions chosen in an appropriate range. Binding values were read directly from the standard curves (see Materials and Methods). The fluctuations of the separate antibody populations, expressed in arbitrary units, are shown in Figs 3 & 4.

Patient K3, a 77 year old woman had since 1974 episodes of Raynauds phenomenon, diffuse swelling of the hands, polyarthralgia, leucocytopenia and thrombocytopenia. During follow-up most symptoms subsided, Raynauds phenomenon and arthralgia remained. All three antibody populations were present in low titres and showed a rather stable course during follow-up as is shown in Fig. 3.

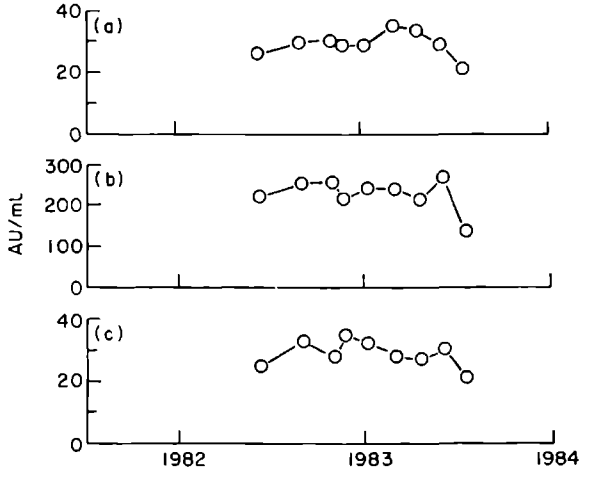

Fig. 3. Longitudinal anti-70K (a), 31K (b) and 25K (c) antibody profiles of MCTD patient K3. AU = arbitrary units.

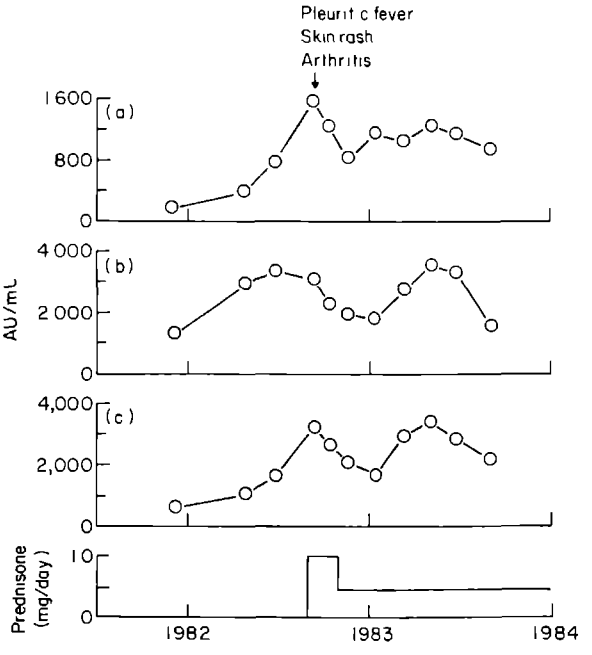

Fig. 4. Longitudinal anti-70K (a), 31K (b) and 25K (c) antibody profiles of MCTD patient D2

Patient D2, a 32 year old woman developed in 1980 Raynauds phenomenon, polyarthritis, swollen hands and severe polymyositis treated with high doses of prednisone At the start of follow-up myositis symptoms subsided, mid-1982 she had a flare in disease activity with arthritis, pleuritis, fever, Raynauds phenomenon and cutaneous vasculitis Antibody populations were present in rather high titres and profiles showed a fluctuating course with two peaks during the period of follow-up (Fig 4), from which the first coincided with a flare in disease activity Treatment with low dose of prednisone had a stabilizing effect on her symptoms and all three antibody profiles showed a decline after start of medication While the patient was still under treatment with prednisone, the second peak in the antibody profile was not accompanied by a clinical flare Fluctuations in the antibody profiles were not reflected in total serum IgG patterns and could not be detected by CIE (data not shown)

DISCUSSION

The purpose of our investigation is to establish a possible correlation between the presence and titre of autoantibodies and clinical features of patients with rheumatic diseases We therefore developed a QIBA as described in this study From a group of 300 anti-nuclear antibody containing sera we selected 21 with antibodies to Sm, RNP or both, using CIE and immunodiffusion (ID) Almost all patients from which these sera were obtained were diagnosed by one of us (DJdeR) and their files were screened for the clinical features shown in Table 1

It has recently become clear that the anti-Sm and anti-RNP reaction is directed against several distinct nuclear polypeptides, all complexed *in vivo* in RNA–protein particles (Hinterberger *et al*, 1983, Kinlaw *et al*, 1983) When tested by qualitative immunoblotting, all anti-RNP sera recognized two to four different antigenic polypeptides with mol wt of 70, 31, 25 and 19 kD All anti-Sm sera ($n=6$) contained antibodies against a 25kD doublet and five recognized also a 13K single polypeptide With the QIBA we were able to quantitate antibody populations against each of these antigenic polypeptides separately Other laboratory parameters were determined as well using

conventional techniques as CIE or ID (for a review see Tan, 1982) and the more recently described RNA precipitation technique (Lerner & Steitz, 1979, Fisher *et al.*, 1983).

Anti-RNP and anti-Sm specificity as established by CIE correlated with the diagnosis of MCTD and SLE, respectively. Among the 13 anti-RNP sera however, only six appeared to be exclusively anti-(U1)RNP, while one showed anti-U1 plus U2 activity, a finding not reported earlier in MCTD. The remaining six anti-RNP sera also contained some anti-U2,4,5,6 activity which correlated in five cases with elevated (> 40 AU/ml) anti-25K levels in QIBA. These samples were negative for anti-Sm antibodies in CIE and immunodiffusion, using RNAase digested extractable nuclear antigen from rabbit thymus. Presence of low titres of anti-Sm antibodies in MCTD has been reported earlier (Williamson *et al.*, 1982) using haemagglutination (HA) techniques. In this respect one should realize that the association of anti-RNP and anti-Sm antibodies with MCTD and SLE, respectively, has been established using conventional techniques as CIE or ID, and that this concept might need some reconsideration now that more sophisticated and more sensitive techniques have become available.

The antigen of 70,000 D (70K) seems to be a triplet of bands (see Fig. 1) reactive with patients sera as well as with the monoclonal anti-70K antibody. Whether this represents a genuine *in vivo* situation or is a result of *in vitro* modification of the antigen is subject of present investigations. Considerable variations in anti-70K activity (from 8 to 400 AU/ml) were observed within our population of 21 patients, but high levels were mainly found in MCTD patients (see Habets *et al.*, 1983b). High anti-25K activity (> 100 AU/ml) was always found in sera exhibiting anti-Sm activity in CIE, while high anti-31K activity was found in SLE as well as in MCTD patients. Anti-(U1)RNP was found equally distributed in mild and severe cases of MCTD, this in contrast with the findings of Assens *et al.* (1982) who reported a correlation between presence of these antibodies and a mild course of disease.

Fisher *et al.* (1983) have suggested that some antibodies in anti-Sm and anti-RNP sera could be directed against SDS sensitive epitopes. Since immunoblotting is based on SDS-PAGE, at least some of these sites are destroyed during preparation of the blot strips. As a consequence it might be possible that the lack of any apparent correlation between distinct clinical features and levels of antibodies measured in QIBA is caused by this phenomenon.

To investigate whether such a correlation would exist within the same patient we followed two patients with MCTD in a longitudinal study. One of them, having a stable course of disease showed almost no significant fluctuations (see Fig. 3) with respect to the three antibody populations measured. Antibody profiles of the other, clinically unstable patient, showed two peaks from which one coincided with a flare in disease activity that was treated with low dose of prednisone. Thereafter, while still on medication, the second peak in the antibody profiles did not correlate with a recognizable change in clinical symptoms. Most interesting was the observation that profiles of the anti-70K, anti-31K as well as anti-25K antibodies appeared to be of an identical shape which could point towards a coupled mechanism of autoantibody production.

The unique aspect of our study is the separate quantitative determination of anti-70K, 31K and 25K antibodies in anti-RNP and anti-Sm sera and the possibility of measuring titres of these antibodies in longitudinal studies. Our results with a group of 21 patients with anti-Sm and/or anti-RNP antibodies show a great quantitative diversity in the subpopulations of these antibodies which together are responsible for the Sm or the RNP precipitation reaction. The three subpopulations of antibodies we investigated showed no apparent correlation with diagnosis or clinical parameters but peaks in longitudinal antibody profiles were found to correlate with a flare in disease activity in one patient. This finding might lend support to the concept that anti-Sm and anti-RNP antibodies play a pathophysiological role in systemic diseases. In this respect clinically useful data might be obtained by quantitating anti-Sm and anti-RNP antibody subpopulations. The quantitative immunoblotting described here is to our knowledge the only technique sufficiently sensitive for such an analysis. Performed on a large scale, QIBA therefore might be an aid in the management of systemic diseases.

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CHAPTER 4

CHARACTERIZATION OF THE SS-B (La) ANTIGEN IN ADENOVIRUS-INFECTED AND UNINFECTED HeLa CELLS

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Characterization of the SS-B (La) antigen in adenovirus-infected and uninfected HeLa cells

Winand J.Habets*, Jan H.den Brok, Agnes M.Th.Boerbooms[1], Levinus B.A.van de Putte[1] and Walther J.van Venrooij

Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, 6525 EZ Nijmegen, and [1]Division of Rheumatic Diseases of the Department of Internal Medicine, University of Nijmegen, Nijmegen, The Netherlands

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The molecular composition and subcellular localization of the antigens recognized by anti-SS-B (La or Ha) antibodies was investigated. Ten anti-SS-B sera were selected by indirect immunofluorescence and by their immunological identity in counter-immunoelectrophoresis (CIE) with an anti-SS-B reference serum. All sera precipitated virus-associated (VA) RNA from cellular extracts of adenovirus-infected HeLa cells. Earlier results had shown that in adenovirus-infected HeLa cells a cellular 50 000 mol. wt. protein was tightly associated with VA RNA *in situ*. Our present results indicate that this 50 000 protein is the only SS-B antigen present in adenovirus-infected as well as in uninfected cells. A major part (>80%) of the SS-B antigen is present in a readily extractable, soluble form. The rest is found in an insoluble form tightly associated with an internal nuclear structure that is mostly referred to as the nuclear matrix. Both forms are very susceptible to proteolytic degradation resulting in at least two distinct breakdown products of mol. wts. 40 000 and 25 000. The cellular 50 000 polypeptide is present in extracts of various types of cells and tissues, indicating that this antigen is very well conserved during evolution. The association of the 50 000 mol. wt. antigen with host- as well as viral-coded RNA polymerase III products also suggests an important function for this protein in the metabolism of these small RNAs.

Key words: adenovirus/autoimmune antibodies/immunoblotting/SS-B antigen/VA RNA

Introduction

Sera from patients with connective tissue diseases often contain antibodies against cellular components consisting of protein associated with small RNA molecules of ~80–200 nucleotides in length (Lerner and Steitz, 1981). The RNA moiety of some of these ribonucleoprotein particles (RNPs) has already been elucidated. Anti-RNP sera [most commonly obtained from patients with mixed connective tissue disease (MCTD)] include antibodies directed against RNP particles containing one discrete small nuclear RNA called U1 snRNA (Lerner and Steitz, 1979). Anti-Sm sera [mostly obtained from patients with systemic lupus erythematosus (SLE)] recognize particles containing various snRNAs namely U1, U2, U4, U5 and U6 (Lerner and Steitz, 1979). The U-snRNAs precipitated by anti-Sm and anti-RNP antibodies are thought to be RNA polymerase II products (Zieve, 1982). A third class of RNP precipitating antibodies is referred to as anti-SS-B (La or Ha) and is often found in sera from patients with Sjögren's syndrome (Akizuki *et al.*, 1977). These antibodies precipitate a great variety of nuclear RNPs in mouse cells and uninfected HeLa cells (Lerner *et al.*, 1981a). The RNA moiety of these RNPs can be separated on acrylamide gels yielding a highly banded pattern of RNAs ranging in size between 80 and 120 nucleotides (Lerner and Steitz, 1981). These small RNAs are all RNA polymerase III products and some of them have been shown to be precursor molecules (Rinke and Steitz, 1982). From extracts of adenovirus-infected HeLa cells the anti-SS-B sera are able to precipitate a virus-associated RNA (VA RNA) of 158 nucleotides length (Lerner *et al.*, 1981a; Franceour and Mathews, 1982), while from Epstein-Barr virus-infected primate cells the virus-encoded EBER I and II RNAs (180 nucleotides) are precipitated (Lerner *et al.*, 1981b). Both VA and EBER RNA are also RNA polymerase III transcripts (Söderlund *et al.*, 1976).

The protein moiety of the RNPs precipitated by the three categories of sera just described is less well documented. It has been established, however, that it is the protein moiety and not the RNA that carries the antigenic determinants (Lerner and Steitz, 1979). The antigens associated with the U-snRNAs have been described as a group of proteins with mol. wts. between 7000 and 70 000 (Lerner *et al.*, 1981b; Takano *et al.*, 1980; White *et al.*, 1981). Recently some apparently conflicting reports have been published on the nature of the SS-B protein antigens. Franceour and Mathews (1982) found a 45 000 mol. wt. protein to be antigenic while Matter *et al.* (1982) showed that anti-SS-B sera recognize polypeptides of mol. wts. 55 000 and 45 000. Venables *et al.* (1983) also found two antigenic SS-B proteins with mol. wts. of 40 000 and 29 000, and Lieu *et al.* (1982) detected a major 30 000 mol. wt. SS-B antigen. van Eekelen *et al.* (1982a) however, showed that VA RNA in the intact cell is tightly associated with only one protein with a mol. wt. of 50 000. This suggests that, at least in adenovirus-infected cells, this protein is the antigen recognized by anti-SS-B antibodies able to precipitate VA RNA. Here we show that the SS-B antigen both in adenovirus-infected and in uninfected HeLa cells is a cellular 50 000 mol. wt. protein that is very susceptible to proteolytic degradation. This instability might be the reason for the apparently conflicting results obtained so far.

Results

Specificity of the anti-SS-B sera

To define patient sera as anti-SS-B, they had to fulfill each of the following four criteria. (I) Positive nuclear staining in indirect immunofluorescence on HeLa monolayer cells. (II) Anti-SS-B positive reaction in a counter-immunoelectrophoresis (CIE) assay, i.e., (a) RNase-insensitive, trypsin-sensitive precipitation reaction with a rabbit thymus extract, mostly referred to as ENA (extractable nuclear antigen) as substrate (not shown). (b) Immunological identity with a reference anti-SS-B serum and no (partial) identity with RNP or Sm reference sera (not shown). (III) Precipitation of VA

*To whom reprint requests should be sent.

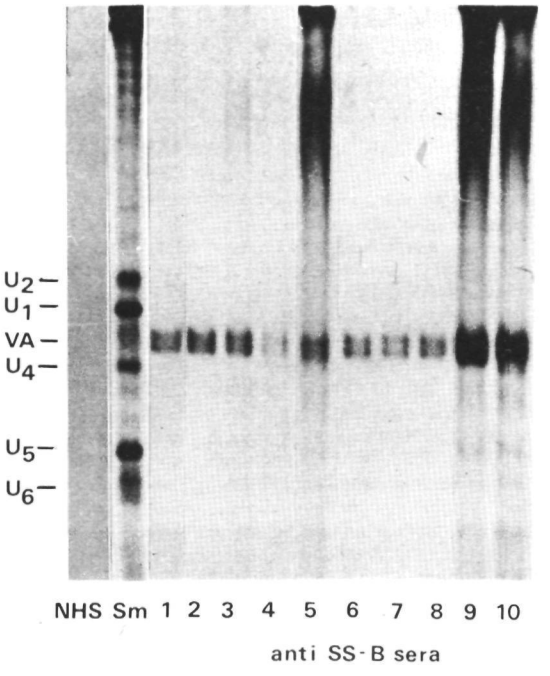


Fig. 1. Characterization of anti-SS-B sera by RNA precipitation. For the precipitation with normal human serum (NHS) and anti-SS-B sera, an extract from adenovirus-infected HeLa cells was used as source of antigens. U-snRNAs were precipitated from an uninfected HeLa cell extract (see Materials and methods). The figure shows a fluorogram of immunoprecipitated [^{3}H]uridine-labeled RNAs using NHS, reference anti-Sm serum, and 10 anti-SS-B sera selected by immunofluorescence and CIE as described in the text.

RNA from adenovirus-infected HeLa cells (Figure 1). (IV) No U-snRNAs precipitated from uninfected HeLa cells (not shown).

Ten sera, at first selected via criteria I and II, were subsequently shown to fulfill the other criteria as well. These anti-SS-B sera were obtained from patients mostly diagnosed as SLE, using the 1982 revised criteria for the classification of SLE, (Tan *et al.*, 1982), or as secondary Sjögren's syndrome (Table I). For the diagnosis of Sjögren's syndrome the patient had to have at least two of the following clinical features of the sicca complex: xerophthalmia, xerostomia or salivary gland enlargement.

Cellular localization of the SS-B antigen using the indirect immunofluorescence technique

It is known that the SS-B antigen(s) are primarily localized in the nucleus when tested by indirect immunofluorescence on various types of cells (Tan, 1982). In HeLa cells, a speckled nuclear fluorescent pattern is observed with slight cytoplasmic staining depending on the particular serum tested. Since anti-SS-B sera are able to precipitate VA RNA from a cytoplasmic extract of adenovirus-infected cells, we investigated whether SS-B specific antigens could be detected in the cytoplasm of HeLa cells late after infection with adenovirus. Indirect immunofluorescence studies were performed on HeLa S3 monolayers 14 h after infection. Control experiments using anti-total adenovirus protein antibodies raised in rabbits,

Table I. Clinical and serological features of 10 patients with anti-SS-B antibodies

Patient number	Sex	Age at testing	Diagnosis[a]	Anti-DNA antibodies
1	M	33	UCTD	–
2	F	57	SS/arthralgia	–
3	M	36	SLE	–
4	F	45	SS/SLE	–
5	F	42	SLE	+
6	F	60	SS/RA	–
7	F	82	RA	–
8	F	41	UCTD	–
9	F	31	SLE	+
10	F	20	SS/RA	–

[a]UCTD, Undifferentiated connective tissue disease; RA, rheumatoid arthritis; SS/SLE, Sjögren's syndrome with systemic lupus erythematosus; SS/RA, rheumatoid arthritis with sicca syndrome.

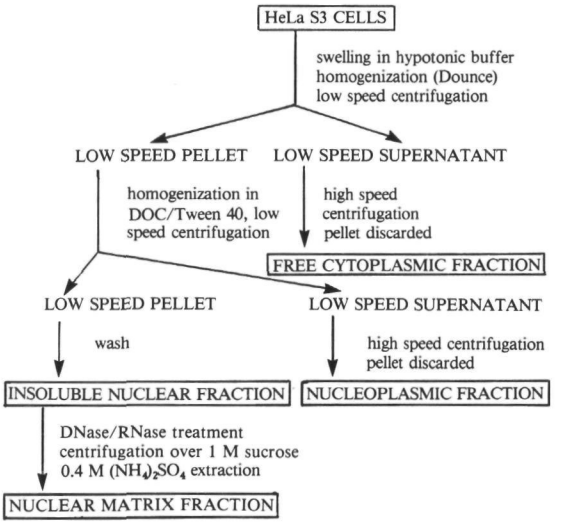
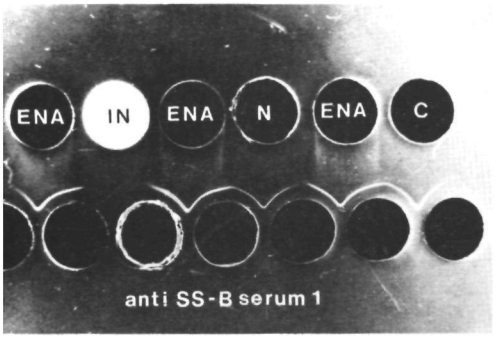


Fig. 2. Isolation scheme of HeLa cell protein fractions.

showed that 95 – 100% of the HeLa cells were infected. Nevertheless, the fluorescence of the SS-B antigen in these infected cells is still predominantly nuclear as it is in uninfected cells (data not shown). With almost all anti-SS-B sera some discrete fine speckles in the cytoplasm of infected cells could be observed. These speckles were not specific for anti-SS-B sera since several normal human control sera showed exactly the same phenomenon. It is possible that this cytoplasmic staining is due to the fact that almost every (human) serum contains, to some extent, antibodies directed against adenovirus proteins, and the fine speckles, therefore, might represent an accumulation of these antigens at specific locations in the cytoplasm of infected HeLa cells. It should be emphasized that the nuclear fluorescence was typical for the anti-SS-B sera; none of the human control sera showed nuclear fluorescence neither in adenovirus-infected nor in uninfected HeLa cells.

These indirect immunofluorescence experiments do not exclude the possibility that the SS-B antigen is located in the



. Detection of SS-B antigens in HeLa cell protein fractions and ENA
unter-immunoelectrophoresis. The cell fractions were prepared as
ed in Figure 2. IN: insoluble nuclear fraction; N: nucleoplasmic frac-
C: cytoplasmic fraction.

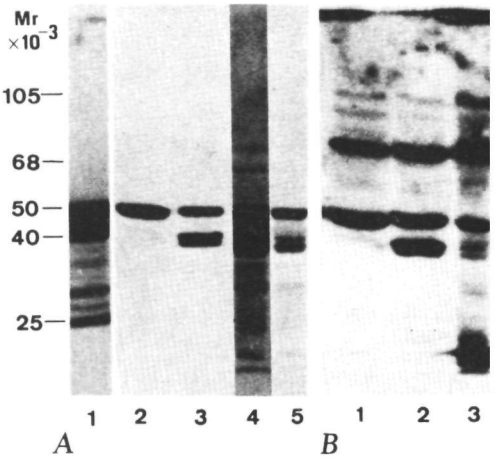


Fig. 4. Detection of SS-B antigens by immunoblotting. **(A)** ENA and uninfected HeLa cell protein fractions were isolated as described in Materials and methods and separated into their individual polypeptic stituents by SDS-PAGE. Subsequently the gel was blotted electrophoretically onto nitrocellulose and probed with anti-SS-B serum no. Immunoreactive species were detected using ^{125}I-labeled protein A. P fractions used were: **lane 1**, ENA; **lane 2**, free cytoplasmic fraction; nucleoplasmic fraction; **lane 4**, insoluble nuclear fraction; **lane 5**, nu matrix fraction. **(B)** Detection of antigens in adenovirus-infected Hel protein fractions. **Lane 1**, free cytoplasmic fraction; **lane 2**, nucleopl fraction; **lane 3**, insoluble nuclear fraction; **lane 4**, nuclear matrix fra

plasm *in vivo*. After serum incubation of the alcohol/
one fixed slides the unbound immunoglobulins and other
m proteins were removed from the preparation by several
ies with barbitone buffer. Even after high speed cen-
gation (15 min, 8000 *g*) the collected washing buffer con-
ed SS-B antigens in very easily detectable amounts, when
d by immunoblotting. This suggests that at least part of
SS-B antigen may not be detected by the indirect immuno-
rescence technique since it can be washed out of the fixed
olayer cells.

ction of SS-B antigens in HeLa cell fractions by CIE and unoblotting

or the localization of antigens a cell fractionation pro-
re was used that resulted in a free cytoplasmic, a nucleo-
mic and an insoluble nuclear fraction (Figure 2). The free
plasmic fraction probably also contains some readily
ble components of the nucleoplasm, due to the Dounce
ogenization procedure in hypotonic buffer. The nucleo-
mic fraction certainly contains cytoskeleton components
bilized during the detergent treatment. Finally, the insolu-
nuclear fraction contains chromatin and nuclear RNA
ein complexes associated with the internal nuclear ultra-
cture generally referred to as nuclear matrix.
hese three cellular fractions were tested by CIE with all
SS-B sera. As is shown in Figure 3, the free cytoplasmic
nucleoplasmic fractions apparently contain (an) identical
gen(s). They also show identical precipitation lines with
gens extracted from rabbit thymus powder (ENA). No
ipitation lines could be observed with the insoluble
ear fraction, indicating that this fraction does not contain
cient amounts of soluble antigens any more. No different
ificities could be detected between sera with and without
DNA antibodies. All sera gave a CIE precipitation pat-
identical to the one shown in Figure 3 (serum no. 1).
or the detection of both soluble and insoluble antigens the
unoblotting technique is very well suited. After
bilization of the antigen-containing fractions in 2% SDS-
aining sample buffer, antigens can be separated accor-
to their mol. wt. by polyacrylamide gel electrophoresis
GE), blotted onto nitrocellulose, and used as a substrate
he immunoreaction (see Materials and methods). For the
unoblotting experiments a nuclear matrix preparation
also used as a source of antigen(s). This fraction was prepared from the insoluble nuclear fraction by subse DNase/RNase treatment and sucrose – high salt extra as described in Materials and methods. After separati PAGE, the four cellular fractions and ENA all sho specific polypeptide pattern. After immunoblotting, these fractions the same 50 000 mol. wt. antigen recognized by SS-B serum no. 1 (Figure 4A). In the plasmic fraction this 50 000 polypeptide seems to be th antigen, in contrast to the other fractions and ENA, appeared also to contain an antigenic polypeptide of mo 40 000. This antigen will be shown to be an *in* breakdown product of the 50 000 protein (see below).

Since all sera precipitate a viral-coded (VA) RNA fro tracts of adenovirus-infected cells, we investigated wl SS-B specific proteins of viral origin exist. The fractio and immunoblotting experiments were repeated with cells 4 and 16 h after infection with adenovirus serot The polypeptide pattern and immunoblotting results ob with the 4 h infected cells were identical with those ob with the uninfected cells (not shown). In the late in cells, however, some additional reactive polypeptides be observed (Figure 4B). These major viral antigens ha proximate mol. wts. of 80 000 and 105 000 and the could be identical to the viral hexon and penton capsi teins. The antibodies against these viral antigens were ob ly not characteristic for anti-SS-B sera since these an were also recognized by antibodies present in normal h control sera (from 20 normal sera tested, 18 containec bodies against the 80 000 protein and three sera showec anti-105 000 activity). We therefore conclude that a adenovirus-infected cells a host-coded 50 000 mol. wt tein is the major antigen recognized by anti-SS-B antib

Table II. Distribution of VA RNA, protein and SS-B antigen in various cell fractions

	Uninfected cells			Adenovirus-infected cells		
	C[d]	N	IN	C	N	IN
		%			%	
Protein[a]	40	36	24	47	31	22
VA RNA[b]	–	–	–	70	14	16
SS-B antigen[c]	20	64	16	10	84	6

[a]Cells were labeled with [^{35}S]methionine as described (van Eekelen and van Venrooij, 1981). Total ^{35}S radioactivity in TCA-precipitable material in each cell fraction was used for the calculation of protein distribution.
[b]Adenovirus 2-infected cells were labeled with [^{3}H]uridine (2 μCi/ml) from 14–16 h after infection. Cell fractions were prepared as described in the methods section. Total RNA (van Eekelen and van Venrooij, 1981) was separated on 10% polyacrylamide slab gels in 8 M urea/40 mM Tris-acetate pH 7.8, 0.1% SDS, 2 mM EDTA. The VA RNA in the gel, visualized by fluorography, was solubilized in 0.5 ml Soluene (Packard) for 24 h at 55°C and measured in a liquid scintillation counter.
[c]The SS-B antigen was quantitated as described in Materials and methods.
[d]C = free cytoplasmic fraction; N = nucleoplasmic fraction; IN = insoluble nuclear fraction.

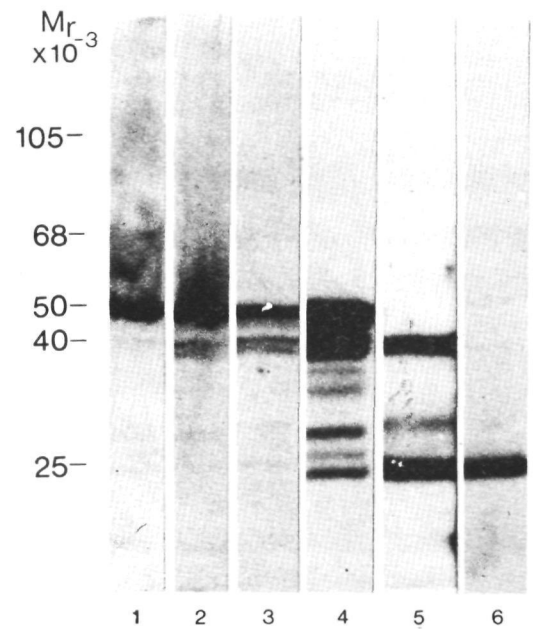


Fig. 5. *In vitro* degradation of the SS-B antigen from rabbit thymus. Rabbit thymus acetone powder (Pel Freez) was extracted at 4°C with PBS containing 0.5 mM PMSC to inhibit proteolytic activities. Aliquots were removed at different time intervals and centrifuged for 5 min at 10 000 *g* at 4°C. Both pellet and supernatant were analysed after SDS gel electrophoresis by immunoblotting. **Lane 1**, total rabbit acetone thymus powder; **lane 2**, pellet after *10 min extraction*; **lane 3**, supernatant after *10* min extraction; **lane 4**, supernatant after 4 h extraction (ENA); **lane 5**, ENA after 2 month storage at –20°C; **lane 6**, ENA after incubation at 37°C overnight.

Most of the SS-B antigen is present in the nucleoplasmic fraction

To obtain more quantitative information about the distribution of the SS-B antigen in the various cell fractions, a semi-quantitative immunoblotting analysis (see Materials and methods) was carried out. The results, obtained with various batches of cells, clearly show that the majority of the SS-B antigen in uninfected as well as in infected cells was present in the nucleoplasmic fraction (Table II). These results indicate that the bulk of the SS-B antigen is released from the nucleus by the DOC-Tween 40 treatment (see Materials and methods). Using adenovirus-infected cells we also quantitated the relative amounts of VA RNA in each cell fraction. About 70% of the VA RNA was found in the free cytoplasmic fraction which contains only 8–12% of the total cellular content of SS-B antigen. Immunoprecipitation studies have shown that VA RNA is almost quantitatively associated with the SS-B antigen (Lerner *et al.*, 1981a; our unpublished observations). In other words, ~8–12% of the SS-B antigen in the free cytoplasmic fraction is probably sufficient to complex 70% of the VA RNA (Table II). Since about eight times as much SS-B antigen is present in the nucleoplasmic fraction, this strongly suggests that at least part of the SS-B antigen is present in the nucleus in an uncomplexed, i.e., a not RNA-associated form.

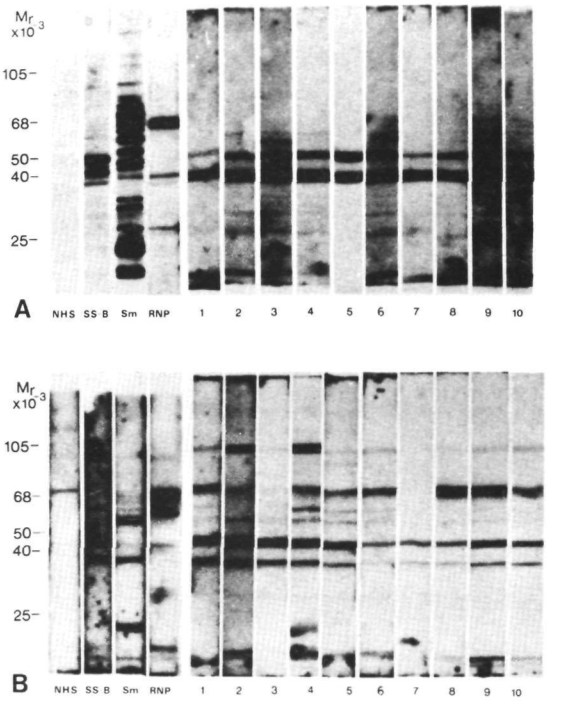


Fig. 6. Analysis by immunoblotting of anti-SS-B sera and reference sera. **(A)** The protein blot from one gel loaded over the entire width with a nuclear protein fraction of uninfected HeLa cells, was cut into strips and probed with various anti-SS-B sera **(lanes 1–10)** and reference anti-SS-B, anti-RNP and anti-Sm sera. NHS: normal human serum. **(B)** as **A**, but now a nucleoplasmic extract from adenovirus-infected cells was used as source of antigens.

In vitro breakdown of the SS-B antigens

Because all sera recognized always both the 50 000 and 40 000 polypeptides with about the same relative efficiency, the possibility that they were related to each other was investigated. When commercially available acetone extracted rabbit thymus powder was sonicated, solubilized in 2% SDS-

containing sample buffer and tested by immunoblotting, only the 50 000 antigen was detectable (Figure 5, lane 1). ENA, prepared by extraction of this thymus powder with phosphate buffered saline (PBS), contained additional 40 000 and 25 000 antigens (Figure 5, lane 4) although extraction was performed under sterile and RNase-free conditions with 0 5 mM phenylmethylsulphonyl chloride (PMSC) as an inhibitor of proteolysis added to the extraction buffer In fact, this breakdown occurs very rapidly, since a 10 min extraction already induces the presence of the 40 000 antigen (Figure 5, lane 3). The pellet remaining after 10 min extraction was washed twice with PBS but nevertheless still contained the 40 000 as well as the 50 000 antigen (Figure 5, lane 2). The breakdown of the SS-B antigen into a 25 000 polypeptide occurs at a somewhat slower rate, but is completed after incubation of the ENA overnight at 37°C (Figure 5, lanes 5 and 6).

It should be pointed out that the 40 000 and 25 000 polypeptides are discrete breakdown products carrying the SS-B specific determinant(s). Some other breakdown products might also exist which do not carry an antigenic determinant and are therefore not recognized in immunoblotting experiments

All anti-SS-B sera recognize the same protein antigen

From earlier work we know that a particular type of human auto-antibodies (for example anti-RNP or anti-Sm), although precipitating a specific set of small RNAs, may recognize a greatly varying set of proteins (Habets *et al*, 1983) We therefore investigated whether such a diversity also existed among the anti-SS-B sera As is shown in Figure 6A, all anti-SS-B sera, including the SS-B reference serum, only recognized the 50 000 mol wt antigen and its breakdown products in an insoluble nuclear fraction from uninfected HeLa cells

This same cellular 50 000 antigen was also recognized by all these sera in adenovirus-infected cells (Figure 6B) Such a specific recognition of only one antigen by a class of human auto-immune sera is in contrast with the findings reported for anti-RNP and anti-Sm sera. With such sera, mostly several antigens are detected by the immunoblotting method (Habets *et al*, 1983) The immunoblot patterns of these anti-RNP and anti-Sm sera also depend on the cellular substrate offered (Figure 6A,B) Furthermore, there is also a great variation in antigen patterns within one class (Habets *et al*, 1983), a phenomenon that is also not observed with anti-SS-B sera As discussed above for serum no. 1 (Figure 4), the other SS-B sera also have rather high levels of antibodies against adenovirus-coded proteins Sera 2 and 4 show a strong reaction against the 105 000 mol. wt protein, and eight out of 10 anti-SS-B sera recognize the 80 000 mol wt adenoprotein. As expected, all sera (10 anti-SS-B, the three reference sera and 20 tested normal human control sera) reacted positively in an enzyme-linked immunosorbent assay that measured the anti-adenovirus protein antibody titer (A van Loon, personal communication)

The RNA binding 50 000 mol. wt. SS-B antigen is evolutionarily highly conserved

Various types of RNA molecules can be precipitated by anti-SS-B sera when extracts from either uninfected mouse cells or human cells are used as a substrate. For this reason it was concluded that the SS-B RNPs were evolutionarily not as highly conserved as, for example, the U-snRNPs (Lerner *et al*, 1981a). Our results show that in rabbit thymus extracts and human HeLa cells only one protein antigen is recognized by anti-SS-B sera (Figure 4). This 50 000 mol wt protein is also the only antigen recognized by anti-SS-B sera in other types of cells and tissues like rat liver, hamster lens, human lymphoblasts in culture (Rosenfeld *et al.*, 1977) and cultured hepatoma cells (Alexander *et al*, 1976) (data not shown) Since in all these types of cells the breakdown products of the 50 000 antigen are also identical, it seems that the protein part of the SS-B antigen is very highly conserved.

Discussion

We have shown that the SS-B antigen is a cellular protein with a mol wt. of 50 000. This protein is the only SS-B specific antigen in uninfected as well as in adenovirus-infected cells Several RNA species, in particular precursors of RNA polymerase III transcripts, can be precipitated by anti-SS-B sera by virtue of their association with the SS-B specific antigen. For this reason, we assume that the 50 000 mol. wt antigen is associated with all these RNA species. This assumption is supported by the fact that VA RNA specifically recognizes a 50 000 mol wt protein in a cytoplasmic extract immobilized on nitrocellulose sheets (van Eekelen *et al.*, 1982a) Furthermore, VA RNA has been shown to be associated with a 50 000 mol wt protein in the intact cell after RNA-protein cross-linking *in vivo* by u.v irradiation of adenovirus-infected HeLa cells (van Eekelen *et al*, 1982a) All these results strongly point to the fact that SS-B specific RNAs (including EBER and the RNA polymerase III precursors), at least for some time during their life cycle are associated *in vivo* with the 50 000 protein

The 50 000 SS-B antigen is very susceptible to proteolytic degradation Even after 10 min extraction of rabbit thymus powder, a 40 000 degradation product, derived from the 50 000 antigen, can be detected in the ENA extract as well as in the residual pellet (Figure 5). After a 4 h extraction, both the 40 000 and a 25 000 degradation product are detectable. These degradation products are not found in cytoplasmic extracts of HeLa cells except when detergents are used. We assume that the detergent treatment releases proteolytic activities by permeabilizing or dissolving cytoplasmic structures like lysosomes. In rabbit thymus powder, the source of ENA, such structures are already severely damaged and permeabilized by the acetone extraction and proteolytic activities might therefore have been released. The rapid rate of degradation of the SS-B antigen explains why it has been described as a protein with mol wt varying from 29 000 to 45 000 (Franceour and Mathews, 1982, Matter *et al.*, 1982; Venables *et al*, 1983; Lieu *et al*, 1982)

The quantitation of the SS-B antigen in the various cell fractions showed that most of the SS-B antigen is present in the nucleoplasmic fraction Because immunofluorescence experiments clearly indicate that the SS-B antigen is located in the nucleus, we conclude that the majority of the SS-B antigen is readily extracted from the nucleus by simple detergent treatment Nevertheless, since the detergent-extracted nuclei, even after repeated washing, still contain ~6–16% of the total cellular amount of SS-B antigen (Table II) we must also conclude that part of the SS-B antigen is present in the nucleus in a rather insoluble form. Removal of the chromatin and most of the nuclear RNA by nuclease treatment and high salt extraction does not remove these residual SS-B antigens. It is therefore probable that this portion of the SS-B antigen is

associated with the nuclear matrix (compare Figure 4), a nuclear ultrastructure that is involved in the processing of various RNA transcripts like pre-5S RNA and pre-mRNA sequences (Ciejek *et al*, 1982, Mariman *et al*, 1982; Ross *et al*, 1982). The free cytoplasmic fraction of uninfected as well as infected cells also contains 10–20% of the SS-B antigen. It might be possible that these antigens are extracted from the nucleus during homogenization of the cell suspension in hypotonic medium. However, since the SS-B antigen in adenovirus-infected cells is associated *in vivo* with VA RNA (Lerner *et al*, 1981a, van Eekelen *et al.*, 1982a) and VA RNA has a cytoplasmic localization (Table II) and most probably a cytoplasmic function as well (Thimmappaya *et al*, 1982), one would expect some of the SS-B antigen to be present in the cytoplasm of infected cells Indirect immunofluorescence experiments that point to an exclusive nuclear localization do not contradict such a statement because we have observed that soluble SS-B antigens are easily extracted from the acetone-fixed cells during the subsequent washing with buffer solutions. It is therefore possible that cytoplasm-localized SS-B antigens not associated with structural cell components are not detected by the indirect immunofluorescence assay because of their extreme solubility

Another point of interest is the fact that the amount of SS-B antigen in the cytoplasmic fraction of adenovirus-infected cells (8–12% of the total cellular amount) is sufficient to complex ~70% of the cellular VA RNA (Lerner *et al*, 1981a, our unpublished observations) The nucleoplasmic fraction, however, contains about eight times as much SS-B antigen and only a small part of the VA RNA. These data suggest strongly that part of the SS-B antigen in the nucleus of adenovirus-infected cells is present in an uncomplexed, not RNA associated form.

Materials and methods

Sera

Sera from patients [mostly with SLE or Sjogren's syndrome associated with rheumatoid arthritis (RA) or SLE] were obtained from the St Radboud Hospital in Nijmegen They were first screened for anti-nuclear antibodies (ANA) by the indirect immunofluorescence test on HeLa cell monolayers essentially as described (van Eekelen *et al*, 1982b)

All ANA-containing sera were then screened for anti SS-B antibodies by CIE using a saline extract (ENA) of rabbit thymus powder (Pel Freez, Rogers, AR) as antigen source (Kurata and Tan, 1976) The sera which showed an RNase insensitive, trypsin-sensitive precipitation reaction, were tested for identity with anti SS-B, anti Sm and anti RNP reference sera Using these criteria, we selected ~10 anti-SS-B sera with which this study was performed All these sera were anti Sm and anti RNP negative Only two contained anti-DNA antibodies as tested by the *Crithidia* assay (Aarden *et al*, 1975)

Reference sera were obtained from the Centers for Disease Control, Atlanta, GA

Culturing and infection of cells

HeLa S3 cells were grown in suspension and monolayer cultures as described (van Eekelen and van Venrooij, 1981) They were shown not to contain Epstein-Barr virus and regularly performed mycoplasma tests were always negative

HeLa S3 cells were infected with purified adenovirus serotype 2 as described (van Eekelen *et al*, 1981) The percentage of infected cells, 14 h after infection (mostly 95–100%), was determined by indirect immunofluorescence with an anti-total adenovirus-protein serum [kindly provided by F Asselbergs (Asselbergs *et al*, 1983)]

RNA immunoprecipitation

HeLa S3 suspension culture cells were labeled with 2 μCi/ml [^{3}H]uridine at a density of 0 5 x 10^6 cells/ml for 24 h Nuclear supernatants were prepared by the following procedure carried out at 0–4°C Cells were harvested at 800 *g* for 5 min on frozen NKM [130 mM NaCl, 5 mM KCl, 1 5 mM $Mg(Ac)_2$], washed once with NKM, pelleted and resuspended in reticulocyte suspension buffer (RSB, 10 mM Tris (pH 7 4) 1 5 mM $MgCl_2$ 10 mM NaCl 0 5 mM PMSC) Cells were then broken by 15 strokes in a Dounce homogenizer and the crude nuclei were pelleted at 800 *g* (5 min) The crude nuclei were resuspended in RSB and sonicated 2 x 15 s with a Branson sonifier at setting 2 After centrifugation (5400 *g* 5 min) this nuclear supernatant was used as source of antigens for the immunoprecipitation of U snRNPs

Extraction of antigens from adenovirus infected cells was performed slightly differently because most of the VA RNA is present in the cytoplasm Labeling from 14–16 h after infection and harvesting procedures of cells were identical but then the cell pellet was resuspended in RSB A mixture of sodium deoxycholate (DOC) and Tween 40 was added (final concentrations 0 5% and 1%, respectively), cells were broken by brief shaking on a vortex shaker and pelleted at 2000 *g* for 5 min The supernatant was used for the immunoprecipitation of VA RNA

In a typical RNA precipitation experiment, 15 μl of IgG selected by protein A Sepharose affinity chromatography was incubated at 0°C for 20 min with the nuclear or cellular extract from 50 x 10^6 cells Then 200 μl 10% protein A-Sepharose in PBS (3 mM NaH_2PO_4, 7 mM Na_2HPO_4 0 9% NaCl, pH 7 6) was added and the mixture was left on ice for 20 min Immune complexes were isolated by centrifugation, and washed 5 times with PBS The precipitated RNAs were extracted with phenol and analysed on 10% polyacrylamide slab gels in 8 M urea/40 mM Tris acetate pH 7 8/0 1% SDS/2 mM EDTA After electrophoresis overnight, gels were prepared for fluorography

Preparation of HeLa cell fractions

The fractionation procedure (Figure 2) was carried out at 0–4°C unless otherwise stated A hypotonic swelling method was used to prepare cytoplasmic extracts Cells were harvested on frozen NKM, washed with NKM, resuspended in hypotonic buffer (RSB) and left on ice for 5 min The cells were then disrupted in an all glass Dounce homogenizer (Type B, seven strokes) followed by low speed (1000 *g* 5 min) centrifugation to pellet the nuclei The supernatant was clarified by high speed centrifugation (8000 *g*, 15 min) and is referred to hereafter as free cytoplasmic extract The crude nuclei were resuspended in RSB and a DOC/Tween 40 mixture (final concentrations 0 5% and 1%, respectively) was added followed by homogenization with 10 strokes of a motor-driven Teflon pestle in a Potter Elvehjem tissue homogenizer The homogenate was centrifuged at low speed, the supernatant was then clarified by high speed centrifugation (8000 *g*, 15 min) and is referred to hereafter as nucleoplasmic fraction The low speed pellet was washed twice with RSB, resuspended in RSB and is further referred to as insoluble nuclear fraction

In some experiments this insoluble nuclear fraction was subfractionated in to a chromatin fraction and a nuclear matrix fraction (Figure 2) For this purpose the insoluble nuclear fraction was resuspended in HRSB [110 mM NaCl, 10 mM Tris (pH 7 4), 1 5 mM $MgCl_2$ and 0 5 mM PMSC] at a density of 10^8 nuclei/ml and treated with DNase I and RNase A (500 μg/ml and 100 μg/ml, respectively) for 1 h at 20°C Fragmented chromatin and RNP complexes were removed by sedimentation through a 1 M sucrose layer in HRSB (2000 *g*, 10 min) followed by an extraction with 0 4 M $(NH_4)_2SO_4$ The pellet was washed and resuspended in RSB and is referred to hereafter as nuclear matrix fraction Morphological and biochemical characteristics of nuclear matrices isolated in this way have been described (van Eekelen *et al*, 1982b)

Gel electrophoresis and protein blotting

Samples for PAGE in SDS were prepared by dissolving the protein fraction in sample buffer (2% SDS, 10% glycerol, 5% β mercaptoethanol, 0 1 M Tris HCl pH 6 8) To ensure complete dissociation of the protein complexes, the samples were heated for 3 min at 100°C followed by gel electrophoresis in SDS on 10% polyacrylamide slab gels (1 mm thick, 10 x 16 cm) In most experiments proteins were loaded over the entire width of the gel (1 5 mg protein per gel) and separated according to their mol wt essentially as described (Laemmli, 1970) After a 4 h run at 20 mA per gel, replicas of the gels were made on nitrocellulose (i e, blotting) by transferring the proteins electrophoretically using a Bio-Rad trans blot cell Transfer was performed overnight at room temperature and at 60 V/0 3 A in 192 mM glycine, 25 mM Tris pH 8 3 and 20% methanol After transfer the blots were dried and stored at room temperature

Detection of antigens

The protein blots were cut from top to bottom into strips of ~7 mm and treated with pre-incubation buffer [3% bovine serum albumin (BSA), 350 mM NaCl, 10 mM Tris HCl pH 7 6, 0 5 mM PMSC] for 3 h at 20°C to saturate additional protein binding sites on the nitrocellulose Incubation with diluted serum (mostly 1 50) was performed overnight in buffer 1 (0 3% BSA, 150 mM NaCl, 10 mM Tris HCl pH 7 6, 0 1 mM PMSC, 1% Triton X-100, 0 5% DOC and 0 1% SDS) After extensive washing with buffer 1 (3 x

10 min) IgG immune complexes were detected by incubating the blots for 2 h with ^{125}I labeled protein A (sp act 1 mCi/mg) in buffer 1 (2 μCi in 10 ml) then washed again with buffer 1 (3 x 10 min) and water (3 x 10 min), dried under a lamp and exposed to X ray film for 2–16 h at −70°C using Ilford intensifying screens

In some blotting experiments, ^{125}I labeled anti human IgG was used instead of protein A The results were the same (not shown)

Quantitation of the SS-B antigen

For the quantitation of the SS-B antigen in the various cell fractions, serial dilutions of the antigen containing fractions were subjected to PAGE and subsequently blotted onto nitrocellulose For these experiments we used the horizontal protein blotting procedure as described by Vaessen *et al* (1981), that resulted in a >90% efficient transfer of the proteins (This percentage was calculated from transfers carried out with [^{35}S]methionine-labeled cell fractions) Protein blots containing serial dilutions of the various cell fractions were incubated with excess anti SS-B serum for 2 h and then washed with PBS containing 0 5% Triton X100 Immune complexes were detected either with horseradish peroxidase labeled anti human IgG (Nordic, Tilburg, The Netherlands) or with ^{125}I labeled protein A as described (Habets *et al* , 1983) Results were read in several ways In the case of the peroxidase-labeled second antibody the highest dilution that gave a signal above background was determined visually In the case of the ^{125}I-labeled protein A the autoradiograms were scanned and quantitated as described (van Eekelen *et al* , 1982c) Both ways of reading gave identical results

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Note added in proof

Recent results of the group of S O Hoch and of J Stefano (Abstracts presented at the Cold Spring Harbor meeting on RNA processing, May 1983) also showed that a polypeptide of 50 000 mol wt is the only SS-B specific antigen

CHAPTER 5

AUTOANTIBODIES TO RIBONUCLEOPROTEIN PARTICLES CONTAINING U2 SMALL NUCLEAR RNA

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Autoantibodies to ribonucleoprotein particles containing U2 small nuclear RNA

Winand Habets, Margot Hoet, Peter Bringmann[1], Reinhard Lührmann[1] and Walther van Venrooij

Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, 6525 EZ Nijmegen, The Netherlands, and [1]Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 63–73, D-1000 Berlin 33, FRG

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Autoantibodies exclusively precipitating U1 and U2 small nuclear ribonucleoprotein (snRNP) particles [anti-(U1,U2)RNP] were detected in sera from four patients with autoimmune disorders. When tested by immunoblotting, these sera recognized up to four different protein antigens in purified mixtures of U1–U6 RNP particles. With purified antibody fractions eluted from individual antigen bands on nitrocellulose blots, each anti-(U1,U2)RNP serum precipitated U2 RNP by virtue of the recognition of a U2 RNP-specific B″ antigen (mol. wt. 28 500). Antibodies to the U2 RNP-specific A′ protein (mol. wt. 31 000) were found in only one serum. The B″ antigen differs slightly in mol. wt. from the U1–U6 RNA-associated B/B′ antigens and can be separated from this doublet by two-dimensional gel electrophoresis, due to its more acidic pI. In immunoprecipitation assays, the purified anti-B″ antibody specificity also reacts with U1 RNPs which is due to cross-reactivity of the antibody with the U1 RNA-specific A protein, as demonstrated by immunoblotting using proteins from isolated U1 RNPs as antigenic material. Thus the A antigen not only bears unique antigenic sites for anti-A antibodies contained in anti-(U1)RNP sera, it also shares epitopes with the U2 RNP-specific B″ antigen.

Key words: autoantibodies/snRNP/antibody-elution/immunoblotting/ U2 snRNA

Introduction

Sera from patients with connective tissue diseases often contain antibodies against nuclear RNA-protein complexes (Tan, 1982; Lerner and Steitz, 1981). The RNA moiety of some of these antigenic complexes has been identified as U snRNAs, a discrete class of stable and highly conserved small nuclear RNAs (for reviews, see Steitz *et al.*, 1983; Reddy and Busch, 1983). U1 RNPs have been shown to function in the splicing of pre-messenger RNAs in *in vitro* systems (Yang *et al.*, 1981; Padgett *et al.*, 1983; Krämer *et al.*, 1984). A similar function has been postulated for U2 RNA (Ohshima *et al.*, 1981), but experimental evidence is still lacking. U4 RNA has been proposed to be involved in the 3′ processing of pre-mRNA (Berget, 1984). All of the snRNAs are commonly found to be associated with hnRNP particles (Sekeris and Niessing, 1975; Gallinaro and Jacob, 1979). Some of the snRNAs (U1, U2 and U4) could even be cross-linked to hnRNA by a psoralen derivative (Calvet and Pederson, 1981; Calvet *et al.*, 1982). All of these considerations suggest that the nucleoplasmic U-snRNAs play closely related functions in mRNA processing or transport.

Anti-Sm antibodies react with the complete set of U1, U2, U4, U5 and U6 (U1–U6) RNP particles whereas anti-(U1)RNP sera specifically precipitate particles containing U1 RNA, the most abundant species of snRNAs (Lerner and Steitz, 1979). Anti-(U2)RNP activity has recently been found in a patient with a scleroderma-polymyositis overlap syndrome (Mimori *et al.*, 1984). In contrast, with anti-Sm and anti-(U1)RNP antibodies which are rather common in sera from patients with SLE and MCTD, respectively, exclusive anti-(U2)RNP specificity is very rarely found (Pettersson *et al.*, 1984; Mimori *et al.*, 1984).

At least 10 polypeptides are associated with snRNAs from which U1 RNP particles contain at least nine, with mol. wts. of 70 000 (70 K), 33 000 (A), 29 000 (B′), 28 000 (B), 22 000 (C), 16 000 (D) and a triplet around mol. wt. 12 000 (E, F and G, Lerner and Steitz, 1979; Brunel *et al.*, 1984; Billings and Hoch, 1984; Pettersson *et al.*, 1984). Polypeptides 70 K, A and C have recently been reported to be present only in U1 RNPs (Pettersson *et al.*, 1984; Billings and Hoch, 1984) whereas B/B′, D, E, F and G are associated with U1–U6 RNAs. Immunopurified U2 RNPs contain at least one additional polypeptide call-

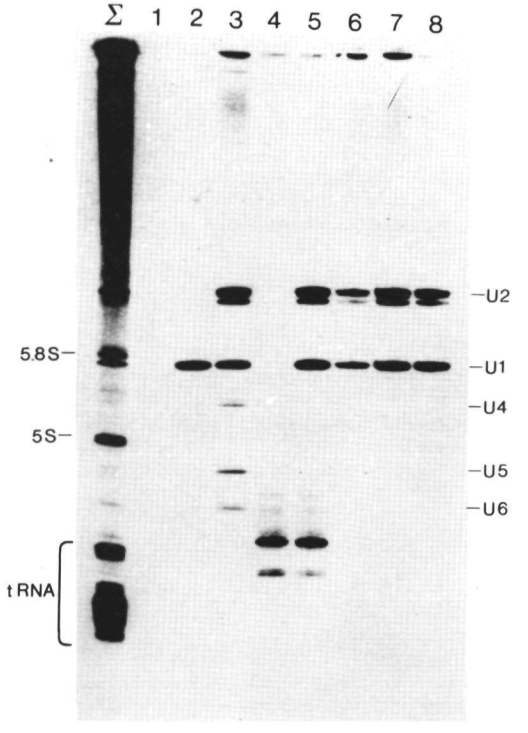

Fig. 1. Polyacrylamide gel fractionation of ^{32}P-labeled immunoprecipitated RNAs. Precipitates were obtained from a total HeLa cell extract (**lane Σ**) using normal human serum (**lane 1**), anti-(U1)RNP serum (**lane 2**), anti-Sm serum (**lane 3**), anti-La serum (**lane 4**) and four different anti-(U1,U2)RNP sera from patients B25, G18, V26 and P21, respectively (**lanes 5–8**).

Table I. Immunodiffusion and immunofluorescence data obtained with the four anti-(U1,U2) RNP sera, and patients' clinical data

Patient	Sex	Age	Diagnosis[a]	Immunofluorescence[b]			Immunodiffusion[c]		
				cyt	nucl	nucleol	Sm	(U1)RNP	La
B25	F	52	RA + SS	−	++	−		+	+
G18	F	19	MCTD	−	++	+/−		+	
V26	F	50	SLE	+	++	+/−	+	+	
P21	F	34	SLE	−	++	+/−		+	

[a]RA = rheumatoid arthritis; SS = Sjögrens syndrome; MCTD = mixed connective tissue disease; SLE = systemic lupus erythematosus.
[b]cyt = cytoplasmic immunofluorescence pattern; nucl = fine speckled nuclear immunofluorescence pattern; nucleol = nucleolar immunofluorescence pattern; +/− denotes a signal at the limit of detection. + denotes a standard signal; ++ denotes a strong signal.
[c]+ Indicates a (partial) identity with a reference serum in immunodiffusion tests.

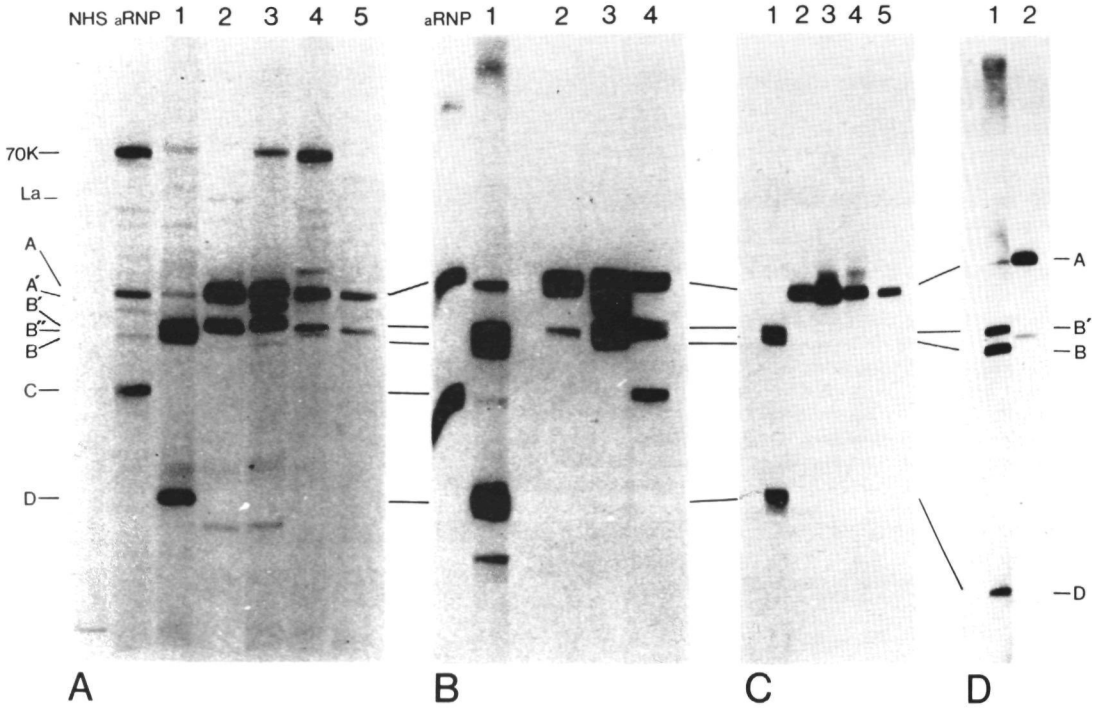

Fig. 2. Immunoblot analysis of anti-(U1,U2)RNP sera. Nitrocellulose blots contained HeLa total nuclear proteins (**panels A** and **D**), anti-m_3G purified U1−U6 snRNPs (**panel B**) or purified U1 snRNPs (**panel C**). All blot strips presented in one panel originate from the same blot (see Materials and methods). Strips were probed with normal human serum (NHS), anti-(U1)RNP serum (aRNP), anti-Sm serum (**lanes 1**) or the anti-(U1,U2)RNP sera B25 (**lanes 2**), V26 (**lanes 3**), G18 (**lanes 4**) and P21 (**lanes 5**). Immunoreactive proteins were detected using ^{125}I-labeled protein A. The protein blots shown in **panel D** differ from those in **panels A−C** in that they originate from high resolution (40 cm) 13% polyacrylamide gels. The blot strip incubated with serum P21 from **panel B** was lost due to an experimental error and could not be repeated because more serum was not available. Sera V26 and G18 also recognized the 70-K antigen on (U1)RNP blots (**panel C**) as visualized on autoradiograms after a four times longer exposure. However, the C antigen recognized by serum G18 (**panel B, lane 4**) could not be detected in these longer exposures, probably due to some loss of this antigen during the further purification of U1 RNPs on DEAE-Sepharose.

ed A′ (Mimori *et al.*, 1984) or P27 (Kinlaw *et al.*, 1982, 1983), both migrating just below the A antigen upon polyacrylamide gel electrophoresis (PAGE).

Here we describe four human autoimmune sera which selectively react with snRNPs U1 and U2. Precipitation of U2 RNPs is due to the presence of two antibody specificities reactive with U2 RNP-specific polypeptides of mol. wt. 31 000 (A′) in one serum and of mol. wt. 28 500 (B′′) in all four sera. Interestingly this latter antibody specificity cross-reacts with the U1 RNP-specific A protein demonstrating that both antigens share at least one common epitope.

Results

RNA immunoprecipitation

From 400 sera of patients with connective tissue diseases, four sera were selected that precipitated snRNP species U1 and U2 from a ^{32}P-labeled HeLa cell extract (Figure 1, lanes 5−8). As a reference, sera with previously described specificities are shown; anti-(U1)RNP (lane 2) and anti-Sm antibodies (lane 3) precipitate U1 RNA and U1−U6 RNAs, respectively, whereas anti-La antibodies (lane 4) precipitate RNAs of ~95 nucleotides representing tRNA precursors. Immunoprecipitates obtained from

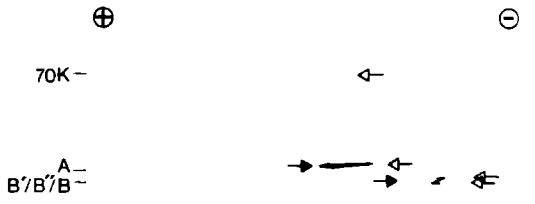

Fig. 3 Two-dimensional protein blots Total proteins from nuclear extracts of HeLa cells were separated in the first dimension by non-equilibrated pH gradient electrophoresis (NEPHGE, + = acidic − = basic side) which was followed by separation in the second dimension by SDS PAGE (15%) (O Farrell *et al* 1977) Immunostaining was performed with anti (U1,U2)RNP serum B25 and peroxidase conjugated anti human IgG (filled arrow) Subsequently the same blot was incubated with equal amounts of anti (U1)RNP and anti Sm sera followed by staining with peroxidase conjugated anti human IgG (open arrow)

^{32}P-labeled Ehrlich ascites mouse cells revealed identical patterns (not shown), supporting the observations that the structure of the U snRNA-associated proteins are highly conserved during evolution Serum from patient B25 also contains anti-La antibodies (Figure 1, lane 5), whereas the other three anti-(U1,U2)RNP sera (sera G18, V26 and P21) exclusively precipitate U1 and U2 RNA Even upon a six times longer exposure of autoradiograms, no other discrete low mol wt RNA species could be detected in immunoprecipitates obtained with these sera

Immunodiffusion and immunofluorescence data obtained with the four anti-(U1,U2)RNP sera, together with some clinical data of the patients are listed in Table I

Immunoblotting

Identical nitrocellulose strips from electroblots of proteins from a total nuclear extract of HeLa cells (Habets *et al* , 1983a) were probed with the four anti-(U1,U2) sera As is shown in Figure 2A, these sera share reactivity with two polypeptides, one of which is located in the region of the B/B′ antigens (and which will be termed B″ hereafter), the other very heavily stained antigen being identical with the U1 RNA-associated A antigen (see below) Sera G18 and V26 also contain antibodies against the 70 K U1 RNA-specific antigen while serum V26 reacts with a single polypeptide of mol wt 31 000 (A′) as well Serum B25 reacts with the La antigen [(mol wt 50 000) Habets *et al* , (1983b)] consistent with the finding that B25 precipitated LaRNAs from ^{32}P-labeled HeLa extracts (see Figure 1, lane 5)

To investigate which of the antigens recognized by these anti-(U1,U2)RNP sera actually were associated with snRNAs, purified (U1 − U6) snRNP particles, isolated by immuno-affinity chromatography with anti-2,2,7-trimethylguanosine (m_3G) IgGs, were used as a source of antigen for immunoblotting These experiments demonstrated that the B″, A′ and A antigens identified by anti-(U1,U2)RNP sera indeed represent proteins of snRNP particles (Figure 2B) Intensities of individual bands on the autoradiograms vary among the blots obtained with total nuclear extract and purified snRNPs This is probably due to differences in the concentration of the respective proteins in these two sources of antigenic material Figure 2C shows the immunoblotting patterns of the same sera when purified U1 RNP particles were used as source of antigen In this case the four sera only recognized the A antigen and no reaction with the proteins A′ or B″ could be observed

These results demonstrated that the B″ antigen is not contained in purified U1 RNP particles and suggested further that the B″ protein is distinct from the proteins B and B′, a notion which is corroborated by the data shown in Figure 2D In a high resolution gel the B″ protein can be shown to have an electrophoretic mobility in between the U1 − U6 RNA-associated proteins B and B′

Two-dimensional protein blots

Two-dimensional (2D) gels from a total nuclear HeLa extract were blotted onto nitrocellulose sheets, probed with serum B25 and immunostained with peroxidase-conjugated anti-human IgG Due to the limited amount of antigen that can be applied to the first dimension polyacrylamide gel (NEPHGE), only the dominant antigens A and B″ could be visualized on 2D blots (Figure 3, filled arrow) The same blot was subsequently reacted with an anti-Sm and an anti-(U1)RNP serum Antigens recognized by these sera are marked by an open arrow in Figure 3 The A antigen recognized by serum B25 appeared as a heterogeneous smear of slightly basic pI and no extra spots showed up in this region after incubation with an anti-(U1)RNP serum The same result was obtained when the order of incubation of the 2D blots with the sera was reversed, indicating that the same A polypeptide(s) are recognized by anti-(U1)RNP and the anti-(U1,U2)RNP sera When probed with an anti-Sm serum, the B/B′ antigens always appeared as a quadruplet of spots on 2D blots Subsequent incubation of the blot with an anti-(U1,U2)RNP serum showed that the B″ antigen had a more acidic pI, corroborating the idea that the B″ antigen is distinct from the B/B′ proteins

Reactivity of antibody specificities eluted from individual protein bands of immunoblots

To trace the antibodies that contribute to the precipitation of U2 snRNA, antibodies bound to individual bands on nitrocellulose blots were eluted and directly used for immunoblotting and immunoprecipitation of snRNPs from extracts of ^{32}P-labeled HeLa cells

As a control, we purified antibodies from a blot incubated with a serum containing anti-Sm as well as anti-(U1)RNP antibodies In agreement with earlier observations (Habets *et al* , in preparation), purified anti-70 K, anti-A and anti-C antibodies precipitated exclusively U1 RNP, whereas anti-B/B′ and anti-D antibodies recognized U1 − U6 RNPs (Figure 4A, lanes 8 − 14) When protein blot strips were probed with each of the above purified antibody fractions, antibodies eluted from protein bands 70 K, A and C reacted selectively with the corresponding protein antigen from which they had been eluted Antibodies eluted from protein bands B, B′ or D, however, reacted with all of the three antigens on immunoblots demonstrating that these antigens share common epitopes (Figure 4A, lanes 1 − 7)

Antibody specificities contained in serum B25 were also purified from the several reactive regions on a total nuclear protein blot Figure 4B shows that antibodies eluted from the mol wt 50 000 region precipitate La RNAs (lane 7) and that anti-A antibodies from this particular serum selectively react with U1 RNP (lane 8) Surprisingly, anti-B″ antibodies precipitated both U1 and U2 RNPs (lane 9) This was unexpected in view of our finding that the B″ protein is not contained in purified U1 snRNP particles (see Figure 2C) On inspection of the immunoblots obtained with purified anti-B″ antibodies, however, the co-precipitation of both U1 and U2 RNPs by this antibody specificity can be explained anti-B″ antibodies cross-react with the U1 RNP-specific A protein (Figure 4B, lane 4) Thus these two pro-

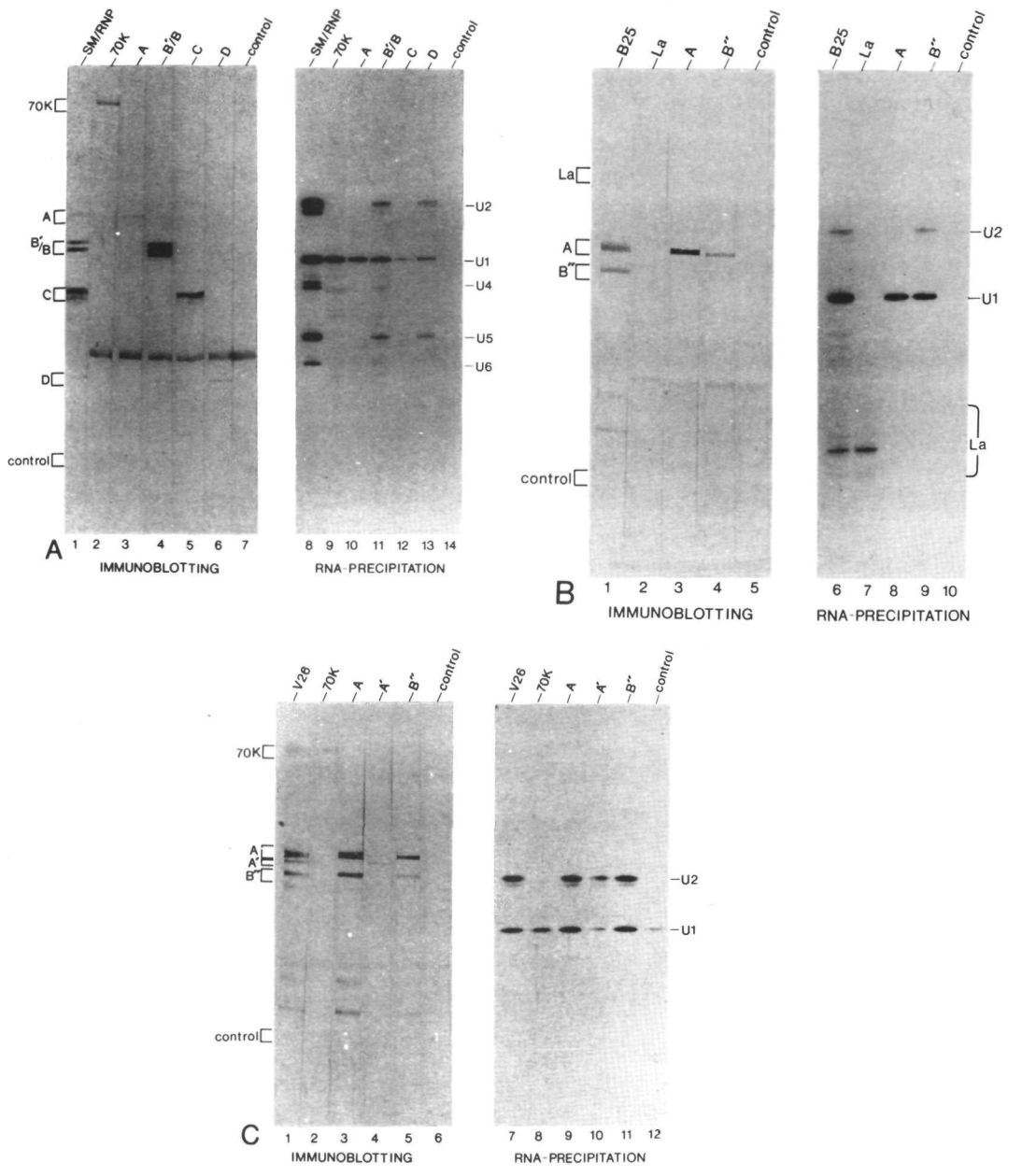

Fig. 4. Purification of antibodies from nitrocellulose blots. HeLa total nuclear protein blots were incubated with an anti-Sm/RNP serum (**panel A**), anti-(U1,U2)RNP sera B25 (**panel B**) or V26 (**panel C**). The reactive regions marked on the left of each panel were excised and bound antibodies were eluted (Smith and Fisher, 1984). These fractions were re-used for immunoblotting or RNA precipitation as indicated on the top of each lane. Immunoblots of purified antibodies were stained for 15 min whereas strips incubated with whole serum (**lanes 1**) were stained for 5 min.

teins must share at least one common epitope. Purified anti-A antibodies react exclusively with the A antigen (Figure 4B, lane 3) and eluted anti-La antibodies show a monospecific reaction with the La antigen of mol. wt. 50 000 (lane 2). No reaction was found when a control region of the nitrocellulose blots was extracted and the eluted fraction was used for immunoblotting and RNA immunoprecipitation (lanes 5 and 10).

The same elution experiments were also performed with serum V26. Anti-70 K antibodies again precipitated exclusively U1 RNP (Figure 4C, lane 8), but in contrast with the findings in serum

B25, anti-A antibodies purified from serum V26 precipitated U1 and U2 RNA (Figure 4C, lane 9) and reacted with A as well as B'' on protein blots (Figure 4C, lane 3) Purified anti-A' antibodies precipitated predominantly U2 RNPs (Figure 4C, lane 10) in accordance with the previous demonstration that the A' protein is unique to U2 RNP particles (Mimori *et al* , 1984) The co-precipitation of the low amounts of U1 RNPs observed with the anti-A' antibody is most probably due to contamination with anti-A antibodies from the nearby A protein band In fact some reaction with the A protein is also observed with the A'-specific antibody fraction on immunoblots (Figure 4C, lane 4) Such dual contamination can be avoided if proteins are separated in high resolution gels (data not shown) Anti-B'' antibodies reacted in the same way as those purified from serum B25, i e , they selectively precipitate snRNPs U1 and U2 (Figure 4C, lane 11) and cross-react with the A antigen on protein blots (Figure 4C, lane 5)

Discussion

This paper describes a novel autoantibody specificity reacting with proteins associated with snRNAs U1 and U2 which we detected in four out of 400 sera of patients with connective tissue diseases The four sera precipitated selectively the snRNAs U1 and U2 which excluded the presence of anti-Sm antibodies, known to react with proteins common to all nucleoplasmic snRNPs (Lerner and Steitz, 1979, Pettersson *et al* , 1984) When total proteins of purified U1 – U6 snRNPs were used as antigenic material for immunoblotting studies, all four sera showed reactivity with the U1 RNP-specific A protein and a second polypeptide of mol wt 28 500 (B'' protein, Figure 2) This protein could be shown to be distinct from the B and B' antigens and not to be contained in purified U1 RNP particles (Figure 2C), indicating that the B'' protein is unique to U2 RNPs and that precipitation of U2 RNPs was due to B''-specific autoantibodies in the four sera In this respect it was surprising that purified B''-reactive antibodies not only precipitated U2 RNPs, but also recognized U1 RNPs (Figure 4B, lane 9, 4C, lane 11) This suggested that co-precipitation of U1 RNPs was either due to interaction of U1 with U2 RNPs *in vitro*, or due to cross-reaction of B''-reactive antibodies with one of the U1-specific polypeptides The latter possibility is the more likely one as we could demonstrate cross-reactivity of anti-B'' antibodies with the A polypeptide (Figure 4B, lane 4, 4C, lane 5) At least with serum V26 the reverse was also found to be true, i e , antibodies eluted from the A band cross-reacted with the B'' antigen and precipitated both U1 and U2 RNPs (Figure 4C, lane 9) This clearly demonstrates that both polypeptides have at least one antigenic determinant in common

It should be pointed out that the A antigen also bears antigenic sites which are unique to this protein and which are recognized by anti-A antibodies contained in anti-(U1)RNP sera The presence of this antibody specificity in serum B25 would explain our finding that the purified A-reactive antibody fraction did not cross-react with the B'' polypeptide though this serum contains antibodies reacting with antigenic sites common to both proteins (Figure 4B, lane 3) It would further suggest that recognition of the respective antigenic sites on the A polypeptide by the two classes of autoantibodies is mutually exclusive

The observed immunological cross-reactivity between the polypeptides A and B'' is a further example to demonstrate structural relatedness between snRNP proteins encompassed in distinct snRNP particles It has previously been shown that the proteins B, B' and D react with the same monoclonal antibody, indicating that these antigens also share common epitopes (Mimori *et al* , 1984, Pettersson *et al* , 1984)

We characterized the B'' antigen as a 28 500 mol wt polypeptide migrating in between the B' and B antigen upon PAGE, while exhibiting a more acidic pI upon 2D gel electrophoresis This antigen is probably identical with a third B polypeptide, occasionally observed as a very faint band in ^{35}S-labeled immunoprecipitates obtained with anti-Sm sera (Billings and Hoch, 1984, Pettersson *et al* , 1984) Further fractionation of these U1 – U6 snRNP precipitates indeed revealed that this third B polypeptide co-purified with U2 RNP (Hinterberger *et al* , 1983, Mimori *et al* , 1984)

Sequential immunoaffinity chromatography utilizing successively the anti-m_3G antibody, anti-(U1,U2)RNP serum V26 and a patient anti-(U1)RNP serum enabled us to select highly pure U1 and U2 RNP moieties Protein analysis of these purified fractions support the conclusions drawn above, the A protein is exclusively contained in the U1 RNP fraction while B'' is specific for U2 RNP (P Bringmann and R Luhrmann, in preparation)

Very recently, the anti-(U1,U2)RNP serum P21 described in this paper was used to determine the binding site of specific snRNP proteins on mutant U2 RNAs (Mattaj and De Robertis, 1985) From their results and the immunoblotting profiles of serum P21 (Figure 2, lanes 5) it can be deduced that it is most probably the B'' antigen which interacts with the two 3' loops of the U2 RNA molecule

All these results strongly suggest that the B'' antigen is a unique polypeptide specifically associated with U2 RNA The question of whether this protein is a primary translation product or a result of post-translational modification (from one of the other snRNP-associated proteins) can only be answered unequivocally when the genes coding for snRNP proteins have been cloned

One anti-(U1,U2)RNP serum also decorated a polypeptide on snRNP immunoblots migrating just below the A protein (referred to as A') Recently, Mimori *et al* (1984) used a human autoimmune serum (Ya) to identify this A' protein as a methionine-deficient, exclusively U2 RNA-associated polypeptide We have several reasons to assume that the A' antigen recognized by serum V26 is identical with the polypeptide identified by the Ya serum Both antigens exhibit identical mol wts when sera V26 and Ya were probed on blots (not shown) and, as is the case with the Ya serum, serum V26 recognizes the A' antigen only on protein blots containing U1 – U6 RNPs and not in purified U1 RNP blots Moreover, anti-A' antibodies purified from serum V26 recognize almost exclusively U2 RNP particles

In contrast with anti-(U1)RNP and anti-Sm antibodies, the occurrence of anti-(U1,U2)RNP antibodies does not seem to be syndrome-specific (Table I), and is therefore of little diagnostic value However, these sera might serve as an important tool in the fractionation of snRNP into its individual constituents and so greatly facilitate the investigations on their function and structure

Materials and methods

Reference sera

Immunological identity of sera was tested by immunodiffusion and counter immunoelectrophoresis using extractable nuclear antigen (ENA, Kurata and Tan, 1976) and reference sera obtained from the Centers of Disease Control (CDC) in Atlanta, GA Human sera were further obtained from patients treated at the St Radboud Hospital in Nijmegen, the Hospital Stadsmaten in Enschede and the Dijkzigt Hospital Rotterdam, The Netherlands, and from the Centre Hospitalier, Luxembourg Ya serum was a kind gift of Dr T Mimori (New Haven, CT) Clinical data of the patients from which anti (U1,U2)RNP sera were obtained are listed in Table I Monoclonal anti Sm and anti-(U1)RNP antibodies

(Billings and Hoch, 1984 Billings *et al* , 1982) were used as references in RNA immunoprecipitation assays (see below)

Preparation of radiolabeled cell extracts

HeLa S3 cells were maintained in suspension culture as described (van Eekelen and van Venrooij 1981)

For RNA analyses, 1 5 x 10^8 HeLa cells were labeled for 15 h with [^{32}P] phosphate (30 μCi/ml) at 5 x 10^5 cells/ml in phosphate free essential medium supplemented with 10% dialysed fetal calf serum After harvesting, cells were washed twice with PBS (20 mM Na phosphate pH 7 3, 130 mM NaCl) and resuspended in 3 ml sonication buffer (100 mM NaCl, 1 mM EDTA, 20 mM K phosphate buffer pH 7 5) The suspension was sonicated 2 x 60 s at setting 3 of a Branson sonifier and clarified by centrifugation at 10 000 *g* for 30 min The supernatant was used either immediately for immunoprecipitation assays or stored at − 70°C

RNA immunoprecipitation assay

Analysis of immunoprecipitated RNA was based on the method described by Matter *et al* (1982) Non specifically binding material in ^{32}P-labeled cell extracts was pre adsorbed by incubation with one volume of 10% protein A Sepharose in IPP and two volumes of IPP (500 mM NaCl, 10 mM Tris pH 7 5, 0 05% Nonidet P40) for 1 h at 4°C Meanwhile, 10 μl serum was adsorbed on 250 μl 10% protein A Sepharose in PBS and washed five times with IPP After centrifugation, the pellet was incubated with 400 μl of the pre-adsorbed ^{32}P-labeled cell extract by end over-end rotation for 1 h at 4°C, and washed five times with IPP After the final wash, the pellet was re-suspended in NET 2 (150 mM NaCl, 50 mM Tris HCl pH 7 4 0 05% Nonidet P40) and 20 μl of 10% SDS was added After phenol extraction, RNAs were ethanol precipitated separated by electrophoresis on 10% polyacrylamide gels containing 7 M urea, 0 1 M Tris borate pH 8 3 and 2 mM EDTA and detected by autoradiography

Affinity purification of snRNPs

Anti-m_3G affinity columns were used to purify U1 – U6 RNPs from nuclear extracts of HeLa cells (Bringmann *et al* , 1983, 1984) From this fraction U1 RNPs were purified according to Hinterberger *et al* (1983) using DEAE-Sepharose chromatography

Immunoblotting

For blotting analyses either purified snRNPs or a total nuclear protein fraction (Habets *et al* , 1983a, 1983b) was used An appropriate amount of protein was dissolved in 2% SDS-containing sample buffer and loaded over the entire width of a 13% polyacrylamide gel (Laemmli, 1970) After electrophoresis, gels were blotted overnight onto nitrocellulose in a Bio-Rad trans blot cell at 60 V/0 3 A in 192 mM glycine, 25 mM Tris pH 8 3 and 20% methanol After transfer, the blots were dried and cut from top to bottom into strips of ~7 mm Individual strips were saturated with 3% BSA and incubated with serum as described (Habbets *et al* 1983a, 1983b) Antigen bound antibody specificities were visualized either with [^{125}I]protein A or with horseradish peroxidase-conjugated second antibody (Habets *et al* , 1985)

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CHAPTER 6

FURTHER CHARACTERIZATION AND SUBCELLULAR LOCALIZATION OF Sm AND U1 RIBONUCLEOPROTEIN ANTIGENS

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Winand J. Habets[△],
Jo H. M. Berden[+],
Sallie O. Hoch[○] and
Walther J. Van Venrooij[△]

Departments of Biochemistry[△] and Nephrology[+], University of Nijmegen, Nijmegen and the Agouron Institute[○], La Jolla

Further characterization and subcellular localization of Sm and U1 ribonucleoprotein antigens*

Sera from patients with systemic autoimmune diseases often contain antibodies against small nuclear ribonucleoprotein (snRNP) particles. Anti-Sm antibodies react with the entire set of U1, U2, U4, U5 and U6 (U1–U6) RNP particles whereas anti-(U1)RNP sera specifically recognize particles containing U1 RNA. Here we performed semi-quantitative immunoblotting using 16 human anti-Sm, 15 human anti-(U1)RNP sera and two mouse monoclonal antibodies to establish which snRNA-associated proteins carry antigenic determinants. Almost every (15/16) human anti-Sm sera recognized epitopes present on a 28-kDa (B/B′) protein doublet and on a 16-kDa (D) polypeptide. Nine anti-(U1)RNP sera also recognized the B/B′ doublet, but in all cases a much stronger reaction was observed with one or more of the specifically U1 RNA-associated 70 kDa, A or C antigens. With affinity-purified antibody fractions eluted from individual antigen bands on nitrocellulose blots it is shown that the anti-Sm-reactive polypeptides B/B′ and D contain common epitopes. We also report the finding of one human anti-Sm serum with exclusive specificity for the B/B′ doublet and a mouse monoclonal anti-Sm antibody recognizing only the D protein, indicating that these antigens also carry unique epitopes. In immunoprecipitation assays, purified anti-B/B′ and -D antibodies react with (U1–U6) RNP while purified anti-70 kDa, anti-A and anti-C antibodies precipitate exclusively U1 RNP particles. Finally, we established the subcellular localization of Sm and U1 RNP antigens using a biochemical cell fractionation procedure. Part of the 70 kDa and B/B′ antigens were found in a nuclease and high salt-resistant nuclear substructure, usually referred to as nuclear matrix, while the A and D antigens could be extracted completely from HeLa nuclei by ribonuclease treatment and subsequent high salt extraction.

1 Introduction

One of the characteristics of systemic autoimmune diseases is the presence of circulating autoantibodies directed towards nuclear antigens (reviewed in [1, 2]). Although these autoantibodies mostly show a wide variety in disease specificity, some of them are quite restricted to a certain syndrome (*e.g.* anti-Sm in systemic lupus erythematosos (SLE) patients, anti-(U1)RNP, in patients with mixed connective tissue disease (MCTD) [3] and anti-Scl-86 in scleroderma [4]), and can therefore be used as a marker antibody [2].

In recent years more precise chemical analyses of the various antigens recognized by these autoantibodies have been performed. Many of them recognize ribonucleoprotein (RNP) particles and several of such RNP classes have been described so far. Anti-Sm antibodies recognize RNP particles containing the small nuclear RNA (snRNA) species U1, U2, U4, U5 and U6 (U1–U6) whereas anti-(U1)RNP sera precipitate particles exclusively containing U1-snRNA [5]. Anti-SS-B (anti-La) antibodies react with RNP particles containing RNA polymerase III products [6], and anti-SS-A (anti-Ro) antibodies recognize a subset of SS-B particles containing a few discrete cytoplasmic RNA [7, 8].

Since the protein components of all these RNP particles are required for antigenicity, the immunoblotting technique seems to be ideally suited for examining the antigens recognized by these sera [9]. With this technique a large number of anti-SS-B and anti-SS-A sera have already been analyzed and shown to recognize predominantly a single 50-kDa and 60-kDa protein, respectively [10–12]. In contrast, immunoblot analyses of anti-(U1)RNP and anti-Sm sera show a much greater variation in antigenic polypeptides [13–23] although the prevalence of antibodies against a 70-kDa protein in anti-(U1)RNP sera [14–17] and against B/B′ and D proteins in anti-Sm sera [24] has been noted.

Our results show that most human anti-Sm sera recognize B/B′ and D antigens simultaneously. This seems to support a previous hypothesis that B/B′ and D antigens exclusively contain common epitopes [25]. However, B/B′ as well as D antigens also carry unique antigenic determinants, as is shown by their individual recognition by one patients' anti-Sm serum and a mouse monoclonal antibody.

All anti-(U1)RNP sera recognized at least one of the U1 RNA-specific polypeptides although some also reacted (weakly) with the (U1–U6)-associated B/B′ doublet. Using a recently described antibody purification procedure [26] we show that monospecific antibody fractions can be purified from sera containing both anti-(U1)RNP and anti-Sm antibodies. Finally, using a biochemical cell fractionation procedure we were able to establish the subcellular localization of Sm and (U1)RNP antigens.

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Correspondence: Winand J. Habets, Department of Biochemistry, University of Nijmegen, Geert Grooteplein noord 21, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

Abbreviations: **SLE:** Systemic lupus erythematosus. **RNP:** Ribonucleoprotein. **snRNP:** Small nuclear RNP. **snRNA:** Small nuclear RNA. **SDS:** Sodium dodecyl sulfate. **CIE:** Counterimmunoelectrophoresis. **ID:** Immunodiffusion. **PBS:** Phosphate-buffered saline.

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2 Materials and methods

2.1 Sera

Human sera were obtained from patients treated at the St. Radboud Hospital in Nijmegen, and from the Department of Clinical Immunology, University of Groningen, and were tested by counterimmunoelectrophoresis (CIE) and immunodiffusion (ID) for anti-Sm or anti-RNP activity. Immunological identity with reference sera obtained from the Centers of Disease Control (CDC) in Atlanta (anti-Sm reference serum 5, lot 82-0011 and anti-RNP reference serum 4, lot 82-0010) was in all cases established. Additional anti-Sm sera were obtained from Dr. E. M. Tan (Scripps Clinic, La Jolla, CA), and from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam). Mouse monoclonal antibodies 2.73 and 7.13 were prepared from culture supernatants as previously described [17, 27]. Both antibodies were used as standards in immunoblotting and RNA immunoprecipitation assays.

2.2 Antigen substrates, gel electrophoresis and immunoblotting

Extractable nuclear antigen was obtained commercially (Pel-Freez, Rogers, Arkansas) and was also prepared from rabbit thymus acetone powder (Pel-Freez) as described by Kurata and Tan [28]. A nuclear protein fraction from HeLa S3 cells to be used in the immunoblotting experiments was prepared as described [10, 14]. Gel electrophoresis and protein blotting were performed as described previously [10, 14, 29]. In brief, 13% polyacrylamide gels were loaded over the entire width with a nuclear protein fraction of HeLa cells. After electrophoresis, gels were blotted overnight onto nitrocellulose paper and the resulting blots were then cut from top to bottom into 20 identical strips of about 7 mm. Immunodetection of bound IgG on blots was performed as described previously [10, 14, 29].

2.3 Preparation of radiolabeled cell extracts

For use in RNA and protein immunoprecipitation assays, 1.5×10^8 HeLa cells were labeled for 15 h either with ^{32}P-phosphate (30 μCi/ml = 1.15MBq/ml) or with [^{35}S]methionine (5 μCi/ml) at 5×10^5 cells/ml in phosphate- or methionine-free essential medium supplemented with 10% dialyzed fetal calf serum. After harvesting, cells were washed twice with phosphate-buffered saline (PBS) (20 mM sodium phosphate, pH 7.3, 130 mM NaCl) and resuspended in 3 ml sonication buffer (100 mM NaCl, 1 mM EDTA, 20 mM potassium phosphate buffer, pH 7.5). The suspension was sonicated 2×60 s at setting 3 of a Branson sonifier and clarified by centrifugation at $10\,000 \times g$ for 30 min. The supernatant was used either immediately for immunoprecipitation assays or stored at –70 °C.

2.4 RNA and protein immunoprecipitation assays

Analysis of immunoprecipitated RNA and protein was based on the method described by Matter et al. [30]. Nonspecifically binding material in labeled cell extracts was preadsorbed by incubating 1 vol. of 10% protein A-Sepharose in PBS and 2 vol. of IPP (500 mM NaCl, 10 mM Tris, pH 7.5, 0.05% Nonidet-P40) for 1 h at 4 °C. Meanwhile, 10 μl serum was adsorbed on 250 μl 10% protein A-Sepharose in PBS and washed 5 times with IPP. After centrifugation the pellet was incubated with 400 μl of the preadsorbed cell extract by end-over-end rotation for 1 h at 4 °C and washed 5 times with IPP. After the final wash the pellet was resuspended in NET-2 (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Nonidet-P40) and 20 μl of 10% sodium dodecyl sulfate (SDS) was added. After phenol extraction, RNA was ethanol precipitated, separated by electrophoresis on 10% polyacrylamide gels containing 7 M urea, 0.1 M Tris-borate, pH 8.3 and 2 mM EDTA and detected by autoradiography. Proteins were recovered from immunoprecipitates by heating the Sepharose beads at 100 °C in SDS gel sample buffer for 3 min and separated by electrophoresis on 15% polyacrylamide-SDS gels.

3 Results

3.1 Characterization of patients' sera

From our stock of about 400 patients' sera we selected 12 sera that contained anti-Sm activity, 4 sera with both anti-Sm and anti-RNP (Sm/RNP) activity and 27 sera containing anti-RNP activity as detected by CIE and ID using extractable nuclear antigen from rabbit thymus powder as the antigen source. The precipitation lines in ID from these sera were immunologically identical with the precipitation lines obtained with anti-Sm and anti-RNP reference sera. Further characterization of these sera was performed by RNA- and protein-immunoprecipitation analysis. Anti-Sm sera precipitated (U1–U6)RNA (Fig. 1, lane 3) as well as a set of eight differently sized proteins, known to be associated with snRNA (Fig. 1, lane 5).

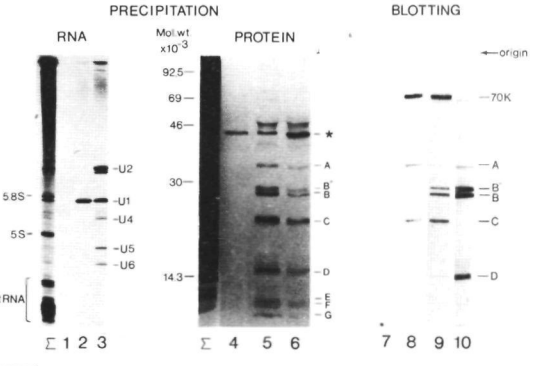

Figure 1. Characterization of human anti-Sm and anti(U1)RNP sera. Precipitation: polyacrylamide gel fractionation of immunoprecipitated ^{32}P-labeled RNA (lanes 1–3) or ^{35}S-labeled proteins (lanes 4–6). Precipitates were obtained from a total HeLa cell sonicate (lanes Σ), using normal human serum (lanes 1 and 4), anti-(U1)RNP serum (lanes 2 and 6) or an anti-Sm serum (lanes 3 and 5). Positions of mol. wt. markers run in an adjacent lane are indicated at the left of each panel. Asterisk indicates a nonspecifically precipitated polypeptide probably identical with actin. Blotting: identical blot strips originating from the same blot were incubated with; lane 7: normal human serum; lane 8: anti-(U1)RNP serum; lane 9: anti-RNP/Sm serum; lane 10: anti-Sm serum.

Anti-RNP sera precipitated the same set of proteins (Fig. 1, lane 6) confirming the original findings of Lerner and Steitz [5]. The 70-kDa U1 RNA-associated polypeptide is not visible in these precipitates because of its methionine deficiency [22, 31]. Twelve of 27 anti-RNP sera as defined by CIE also precipitated (U1–U6)RNA, probably due to a minor anti-Sm activity not detectable in our CIE or ID assays. The remaining 15 anti-RNP sera exclusively precipitated U1 snRNA (Fig. 1, lane 2) and will be further referred to as anti-(U1)RNP sera.

3.2 Immunoblotting patterns of anti-Sm and anti-(U1)RNP sera

A nuclear extract of HeLa cells, previously shown to contain the full complement of snRNA-associated proteins [14, 29], was used as a source of antigen for immunoblotting experiments. A typical example of such an immunoblotting analysis is shown in Fig. 1. An anti-(U1)RNP serum recognizing the 70-kDa, the A and the C protein is shown in lane 8, while our standard anti-Sm serum recognizes the B/B′ doublet and the D protein (lane 10). The immunoblotting pattern of a serum containing both specificities is shown in lane 9.

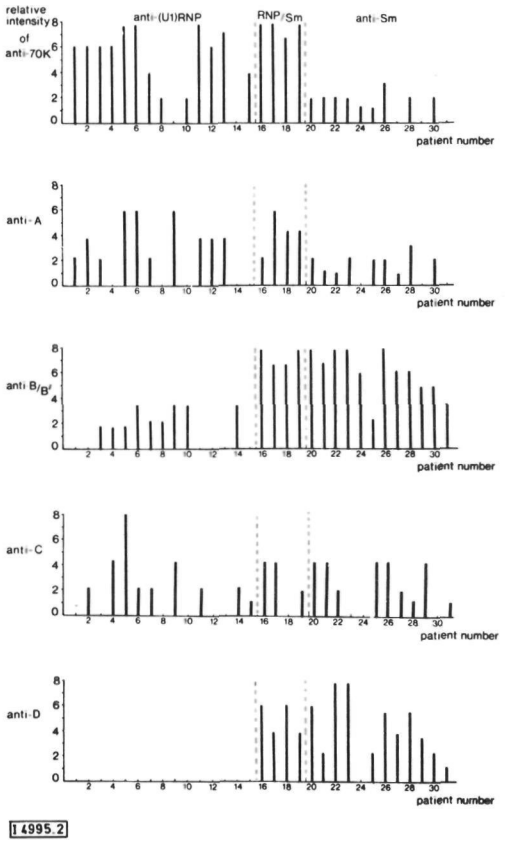

Figure 2. Semi-quantitative analysis of presence of anti-70 kDa, A, B/B′, C antibodies in patients' sera. Identical blot strips were autoradiographed for 24 h after serum incubation and detection of bound IgG using ^{125}I-labeled protein A. Relative intensities of the respective bands were estimated independently by three investigators. Mean values of their readings are depicted on an arbitrary scale from 0 to 8. Fourty normal human sera all scored negative (0).

We reported recently that the intensity of the immunoreaction on blots can be interpreted semi-quantitatively [29]. Here we used a modification of this method in a comparison of anti-(U1)RNP and anti-Sm sera concerning the 70-kDa, A, B/B′, C and D antigens. The intensity of the reaction of anti-(U1)RNP and anti-Sm sera with these antigens was scored on an arbitrary scale of 0 to 8 (Fig. 2).

Almost all (87%) anti-(U1)RNP sera recognized the 70-kDa protein, 67% showed reactivity towards the A antigen whereas 60% recognized the C polypeptide. A weak but evident reaction with the B/B′ antigens was observed in 9 cases (60%) while no anti-D activity could be detected in these sera. All anti-Sm sera (#20–31) showed a strong reaction with the B/B′ antigen doublet. All but one (serum #24, see below) anti-Sm sera reacted with the D antigen and 9 (75%) contained anti-A and anti-C antibodies. Eight anti-Sm sera contained antibodies against a 70-kDa protein, but, upon close examination, this antigen band exhibited a slightly different shape and electrophoretic mobility as compared with the U1 RNA-associated 70-kDa antigen (Fig. 1, lane 10, see Sect. 4). Reaction with this antigen was nevertheless scored as anti-70-kDa activity in Fig. 2.

These results demonstrate that the 70-kDa antigen is recognized predominantly by anti-(U1)RNP sera whereas presence of anti-D antibodies is most characteristic for anti-Sm sera. Anti-B/B′ activity can be found both in anti-(U1)RNP and anti-Sm sera, but high levels are only present in anti-Sm sera. In a separate set of experiments we analyzed anti-Sm antibodies in the sera from 24 MRL/Mp-*lpr*/*lpr* (MRL/l) mice [32]. All sera tested contained strong anti-B/B′ and anti-D activity on HeLa nuclear protein blots (data not shown).

3.3 Immunological relation between Sm and RNP antigens

Evidence has been provided [23, 33] that the 70-kDa, A, B, C and D antigens represent distinct polypeptides but several immunoreactive breakdown products have been described, in particular from the 70-kDa antigen [15, 16, 34]. To investigate whether certain antibodies could show a cross-reactive binding, antibodies reactive with specific antigen bands on nitrocellulose blots were eluted [26] and their binding was reassessed on a new blot. After incubation of a blot with a patients' serum containing both anti-Sm and anti-(U1)RNP antibodies (#17), the blot was immunostained with peroxidase-conjugated anti-human IgG and antibodies were eluted from every reactive antigen. When fractions eluted from the U1 RNP-specific 70-kDa, A or C polypeptide bands were subsequently incubated with separate blot strips, purified antibodies only reacted with the antigen they were eluted from (Fig. 3A). The specificity of these purified antibodies is further substantiated by the observation that every individual antibody fraction precipitated exclusively U1 RNA from a ^{32}P-labeled HeLa cell extract (Fig. 3A).

A different situation was encountered with the B/B′ and the D antigens. As is shown in Fig. 3A, antibodies eluted from the B/B′ region cross-reacted with the D proteins as did purified

anti-D antibodies with B/B′ antigens. Elution experiments with a pure anti-Sm serum confirmed this cross-reactivity (Fig. 3B). Both anti-D and anti-B/B′ antibodies precipitated U1–U6 RNA from a ^{32}P-labeled HeLa cell extract (Fig. 3A).

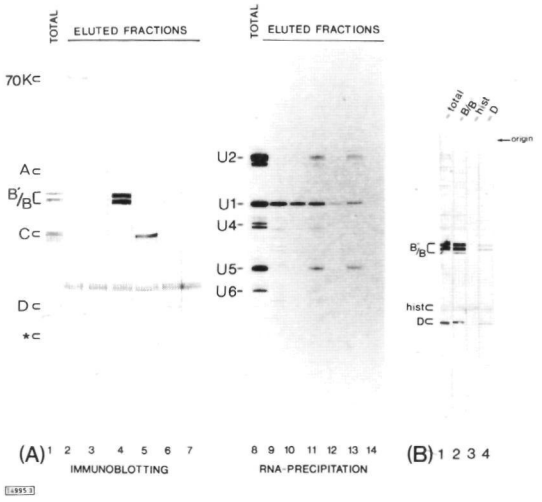

Figure 3. Affinity purification of antibodies from nitrocellulose blots. (A) A HeLa nuclear protein blot was incubated with anti-Sm/RNP serum #17 (lane 1). After peroxidase staining, the regions marked on the left of lane 1 were excised from one blot over a length of 10 cm and reactive antibodies were eluted [26]. These eluates were then used either for RNA immunoprecipitation (lanes 8–14) or to probe separate strips from the same blot (lanes 1–7). The following eluted fractions were used: lanes 2 and 9: anti-70 kDa; lanes 3 and 10: anti-A; lanes 4 and 11: anti-B/B′; lanes 5 and 12 anti-C; lanes 6 and 13: anti-D. Lanes 7 and 14 show control experiments performed with fractions eluted from the region indicated by an asterisk. Immunoblots of purified antibodies were stained for 15 min, while strips incubated with whole serum (lanes 1) were stained for 5 min. (B) As (A) using an anti-Sm serum (#23). Antibodies eluted from B/B′ and D antigens (lanes 2 and 4) precipitated U1–U6 RNA (not shown). hist = Histone protein.

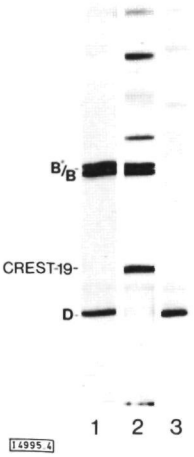

Figure 4. Immunoblotting patterns of anti-Sm sera. Three identical nuclear protein blot strips originating from the same blot were incubated with: lane 1: reference human anti-Sm serum; lane 2: human anti-B/B′-specific serum (#24); lane 3: murine monoclonal anti-D antibody [27]. Serum #24 (lane 2) also contained antibodies against CREST-19, a 19-kDa centrometric antigen [48] as established by its apparent mol. wt. Antibodies eluted from the CREST-19 antigen did not react with any other polypeptide. Unlike anti-B/B′ antibodies contained in other anti-Sm sera (compare lane 1), the anti-B/B′ antibodies from serum #24 cross-react with several higher mol. wt. proteins (lane 2), a phenomenon also observed with the monoclonal anti-D antibody (lane 3).

These results indicate that simultaneous recognition of B/B′ and D antigens is more likely due to cross-reactivity of one type of antibody rather than to the presence of two different antibody populations.

It is therefore of particular interest that we found one anti-Sm serum which exclusively recognized B/B′ antigens and showed no reaction with the D antigen (serum #24, Fig. 4, lane 2). Since also (monoclonal) anti-D antibodies have been described (Fig. 4, lane 3, see also [27]), it can be concluded that both these antigens contain unique epitopes in addition to those commonly recognized by anti-Sm antibodies.

3.4 Subcellular localization

For the subcellular localization of Sm antigens an earlier described cell fractionation procedure was used [4, 35, 36]. Polyacrylamide gel electrophoresis of the various cell fractions clearly shows that this fractionation procedure indeed seems to succeed in separating groups of proteins belonging to physiologically different cell compartments (Fig. 5, CBB). When identical gels were used for immunoblotting with anti-SS-B sera, we found that the SS-B antigen was predominantly localized in the detergent-soluble fraction (Fig. 5, SS-B), although a nonextractable moiety reproducibly appeared in the nuclear matrix fraction [10, 37–39]. Incubation with an

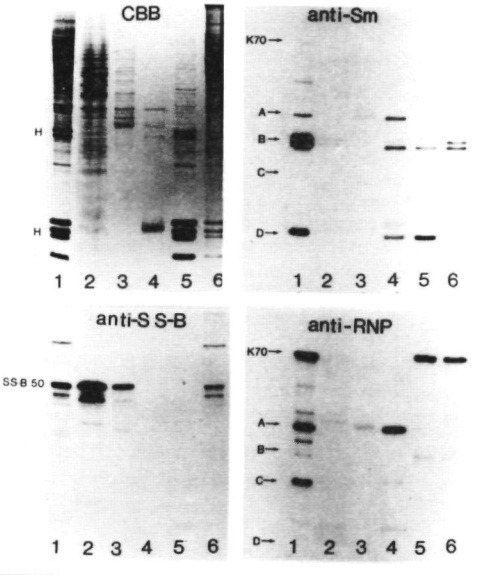

Figure 5. Subcellular localization of antigens. Proteins from several cellular fractions obtained from 3×10^6 HeLa cells [4] were separated on a 13% polyacrylamide gel. Identical gels were stained either with Coomassie brilliant blue (CBB) or blotted onto nitrocellulose and incubated with a human anti-SS-B serum also containing some anti-Scl-86 activity [4], an anti-(U1)RNP serum or an anti-Sm serum. Lane 1: total HeLa nuclear protein extract, H marks histones; lane 2: detergent-soluble fraction; lane 3: DNase-extractable fraction; lane 4: RNase-extractable fraction; lane 5: high salt-extractable fraction; lane 6: residual nuclear matrix fraction.

anti-(U1)RNP serum showed that the 70-kDa antigen is present in the high-salt (= chromatin) fraction and in the insoluble nuclear matrix fraction The A antigen is preferentially released by the ribonuclease (RNase) treatment (Fig 5-RNP, lane 4), while the C antigen is not detected in any specific fraction, a phenomenon most likely due to a loss of antigenicity upon high-salt treatment When an identical blot was incubated with an anti-Sm serum it was found that the D protein is released by RNase A and high-salt treatment In the residual nuclear matrix fraction, no detectable amounts of D protein were present In contrast, the B/B′ doublet is only partly released by these treatments and is still detectable in the nuclear matrix fraction (Fig 5, Sm) Only trace amounts of anti-Sm and anti-(U1)RNP-reactive antigens were detected in the detergent-soluble (predominantly cytoplasmic) fraction, a finding which agrees with their exclusive nuclear location as determined by indirect immunofluorescence and is in accord with the observation that such cytoplasmic extracts do not give precipitation lines in the CIE or ID assays with anti-(U1)RNP and anti-Sm sera (unpublished observation)

4 Discussion

Besides their function as "marker antibody" in human autoimmune diseases [1–4], autoantibodies have proven to be valuable tools in molecular biology, therefore, exact biochemical characterization of autoantigens has become particularly important Recent developments have shown that U snRNP particles, which are the targets of human anti-(U1)RNP and anti-Sm sera, play an important role in the processing of messenger RNA [40–42] In this study we selected anti-Sm and anti-(U1)RNP sera from SLE and MCTD patients, respectively, by RNA and protein immunoprecipitation analysis, the most sensitive techniques currently available [24] Immunoreactive protein antigens were then identified in a sensitive semi-quantitative immunoblotting assay

Our main results show that almost all anti-Sm sera contain high-titered antibodies against the B/B′ and D antigens However, the presence of anti-D antibodies seems more characteristic for anti-Sm sera since they are not found in anti-(U1)RNP sera (Fig 2) Anti-(U1)RNP sera contain antibodies against at least one out of three U1 RNP-specific polypeptides, *i e* the 70-kDa, the A and the C proteins Most characteristic for anti-(U1)RNP sera, however, was the presence of high-titered anti-70-kDa antibodies, while anti-A and anti-C antibodies less frequently occurred (Fig 2)

The finding that almost all anti-Sm sera recognize both the B/B′ and the D antigens can be explained by the observation that these antigens share common epitopes as shown by the cross-reactivity of purified anti-B/B′ and anti-D antibodies (Fig 3) This is in apparent contrast with the findings of Guldner et al [23] who suggested that both antigens have no immunological relationship However, they purified antibodies using KSCN, an agent suspected to cause a dramatic decrease in specific activity of the eluted antibodies, when compared with the much milder elution by pH shock as performed here [26] Besides antibodies recognizing common epitopes on B/B′ and D polypeptides, another antibody specificity reactive with B/B′ or D-specific epitopes (Fig 4) can be present in anti-Sm sera Anti-B/B′ antibodies from the type contained in serum #24 could also be present in other anti-Sm sera and would then contribute for the observed quantitative differences in the ratio of B/B′ *vs* D recognition (Fig 2)

Interestingly, almost all anti-Sm sera tested showed some reactivity with a protein of 70 kDa as well (see also Fig 2) This protein thus could be identical with the 70-kDa U1 RNP-associated antigen, which would mean that these anti-Sm sera all contain a minor anti-(U1)RNP activity However, whereas the U1 RNP 70 kDa protein appears as a very typical triplet polypeptide on HeLa blots [29], the 70-kDa antigen recognized by anti-Sm sera in all cases had another composition, containing polypeptides with a slightly higher electrophoretic mobility (Fig 1, lane 10, Fig 5, anti-Sm, lane 1) Comparison of these antigen populations on two-dimensional gels is now in progress

By several nonimmunological isolation procedures, both B/B′ and D antigens have been shown to be associated with U1, U2, U4, U5 and U6 snRNA [22, 24, 43] It is therefore puzzling that some, exclusively U1 snRNA-precipitating sera contain antibodies against the B/B′ antigens, although this activity is generally much weaker than the anti-B/B′ activity from anti-Sm sera (Fig 2) Two-dimensional gel separation followed by immunoblotting established that the B/B′ proteins recognized by anti-(U1)RNP sera are identical with the antigens recognized by anti-Sm sera (data not shown) Therefore, it is possible that these anti-(U1)RNP sera contain trace amounts of anti-Sm antibodies not detectable in the (very sensitive) RNA immunoprecipitation assay Another acceptable explanation is that the epitopes of B/B′ antigens which are recognized on blots by these anti-(U1)RNP sera are not available for reaction in intact snRNP particles but are buried inside the snRNP structure Antibodies directed against these epitopes would thus not be able to contribute to the precipitation of RNA from ^{32}P-labeled cell extracts

Cell fractionation studies showed that like the 70-kDa antigen [14], part of the B/B′ antigens is associated with an insoluble nuclear substructure called the nuclear matrix [35–38] The lower band of this doublet, the B antigen, is preferentially released from HeLa nuclei by RNase A treatment and high-salt wash Attachment of B/B′ and 70-kDa antigens to the nuclear matrix is in accord with recent immunofluorescence studies showing that U1 as well as U1–U6 snRNP particles are associated with this nuclear substructure [38, 44] These B/B′ and 70-kDa antigens might therefore function in snRNP-nuclear matrix association The D proteins, on the other hand, can more easily be extracted from nuclei These findings are in agreement with the observations that extracts from several tissues contain predominantly the readily extractable D antigen [13] as well as the single B protein [14, 45] In accord with recent observations made with purified snRNP [33], we found that the U1 RNA-associated A antigen was also released from isolated nuclei by RNase A treatment Only trace amounts of snRNP antigens were detectable in the detergent-soluble (predominantly cytoplasmic) fraction This possibly reflects the *in vivo* situation (see also [46, 47]) since snRNP proteins are made in the cytoplasm and have been shown to be important for the transport of mature U snRNA to the oocyte nucleus [46]

Fisher et al showed that B/B′ and D antigens are translated from differently sized messenger RNA [33] Thus, these antigens are probably distinct polypeptides and not *e g* degradation products of one higher mol wt antigen, as occasionally has been suggested [34] Since both antigens, however, share common epitopes it would still be possible that their messengers share common nucleotide sequences or are even (partly)

transcribed from the same genomic region Molecular cloning of genes encoding for B/B and D antigens might provide definite answers to such questions

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CHAPTER 7

MONOSPECIFIC ANTIBODIES REVEAL DETAILS OF U2 snRNP STRUCTURE AND INTERACTION BETWEEN U1 AND U2 snRNPs

Iain W. Mattaj[1], Winand J. Habets and Walther J. van Venrooij.

[1]European Molecular Biology Laboratory, Meyerhofstrasse 1, Heidelberg FRG.

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Summary

Monospecific antibodies directed against several U small nuclear ribonucleoprotein (U snRNP) particle proteins were affinity purified from a patient anti-(U1,U2)RNP serum. These were used to demonstrate that: 1. Proteins equivalent to the mammalian U2 snRNP specific A′ and B′′ proteins are present in Xenopus laevis oocytes. 2. Both proteins A′ and B′′ have the same structural requirements for binding to U2 snRNA. 3. Proteins B, B′ and D have the same structural requirement for binding to U2 snRNA. 4. Using very high specific activity RNA probes it is possible to detect a fraction of either U1 or U2 snRNA precipitable by antibodies directed against proteins specific for the other U snRNP, indicating an interaction between U1 and U2 snRNPs. The structural requirements of this interaction were studied for the U2 snRNP. All changes made to U2 snRNA or snRNP struture resulted in loss of the interaction with U1 snRNP.

Since the discovery by Lerner and Steitz (1979) that sera from certain patients suffering from autoimmune disorders contain antibodies directed against ribonucleoprotein particles, these antibodies have been extensively used in studies of the structure and function of these RNPs (for reviews see Lerner and Steitz, 1981; Busch et al., 1982; Mattaj, 1984 and Brunel et al., 1986). The most extensively studied of the RNPs have been the U snRNPs. There is now strong evidence for an involvement of U1, U2 and U7 snRNPs in the processing of mRNA precursors (Hernandez and Keller, 1983; Padgett et al., 1983; Bozzoni et al., 1984; Kramer et al., 1984; Krainer and Maniatis, 1985; Black et al., 1985; Galli et al., 1983; Strub et al., 1984) which lends additional interest to study of the structure of the RNP particles themselves.

Studies on the particle proteins have been complicated by the difficulty of purifying the particles by standard biochemical fractionation methods, and by the number, at least 11, of different proteins associated with the U snRNAs (Kinlaw et al., 1982, 1983; Hinterberger et al., 1983; Pettersson et al., 1984; Mimori et al., 1984). Recently developed purification methods based on the use of antibodies to the unique tri-methyl cap structures of the U snRNAs have enabled purification in good yields of pure U snRNPs for the first time (Bringmann et al., 1983, 1984), and this in turn has enabled the preparation of monospecific antibodies to various U snRNP proteins by affinity purification from serum (Habets et al., 1985a).

A complementary method of analysis of U snRNP structure, based on the analysis of mutant U2 snRNAs, their interaction with U2 snRNP proteins, and the correlation of these interactions with nuclear accumulation of the RNAs (De Robertis et al., 1982; Zeller et al., 1983) has recently been developed (Mattaj and De Robertis, 1985). We have now combined the two methods to further analyse the RNA-protein interactions involved in U2 snRNP structure. This analysis

has led us to the conclusion that epitopes on the U2 snRNP specific proteins A′ and B′′ are highly conserved in evolution, as are other U snRNA-associated proteins, and that a small fraction of the U2 snRNPs exists in a complex with U1 snRNPs.

U2 snRNP specific proteins in Xenopus oocytes

By a variety of methods two U2 snRNP specific proteins, named A′ and B′′, have been identified in mammalian cells. In Xenopus oocytes it has been possible to detect, by immunoblotting and immunoprecipitation, proteins equivalent to the common U snRNP proteins B, D, E, F and G and the U1 snRNP specific proteins 70K, A and C and even putative tissue-specific variants of some of these proteins (Zeller et al., 1983; Fritz, 1983; Fritz et al., 1984). It has not been possible, however, to identify unequivocally U2 snRNP specific proteins in Xenopus by these methods (Fritz, 1983), although their existence can be inferred since anti-(U1,U2)RNP sera immunoprecipitate specifically U1 and U2 snRNPs from Xenopus oocytes (Mattaj and De Robertis, 1985).

We used the anti-(U1,U2)RNP serum V26 (Habets et al., 1985a) to try to detect proteins equivalent to A′ and B′′ in oocyte extracts, in the experiment whose results are shown in Fig. 1A. In lanes 1 and 5 the reaction of V26 with proteins 70K, A, A′ and B′′ in HeLa nuclear extracts is shown. The lack of obvious equivalent bands in oocyte extracts can be seen in lanes 2-4. In order to increase the sensitivity of detection we next purified U snRNPs from oocytes using anti-tri-methyl cap antiserum (Bringmann et al., 1983, 1984), and stained these with whole and fractionated V26 serum. V26 was fractionated into monospecific antibodies by elution of antibodies bound to specific bands on Western blots of HeLa extracts (Habets et al., 1985a). Figure 1B lanes 1 and 2 show the staining patterns obtained using the anti-(U1,U2)RNP sera B25, which recognizes the HeLa A and B′′ proteins, and V26, which recognizes HeLa 70K, A, A′ and B′′ proteins (Habets et al., 1985a). Reaction of these antisera with the purified Xenopus oocyte U snRNP proteins allows the identification of equivalents to protein A (33,000 Da), A′ (32,500 Da) and a triplet of proteins of around 26-28,000 Da equivalent to B′′. It is not clear whether these three bands correspond to different proteins or are modifi-

cations or degradation products of a single protein. We do not detect a band equivalent to the HeLa 70K protein. This must be due to lack of sensitivity since monospecific anti-70K antibodies immunoprecipitate Xenopus oocyte U1 snRNP (see below).

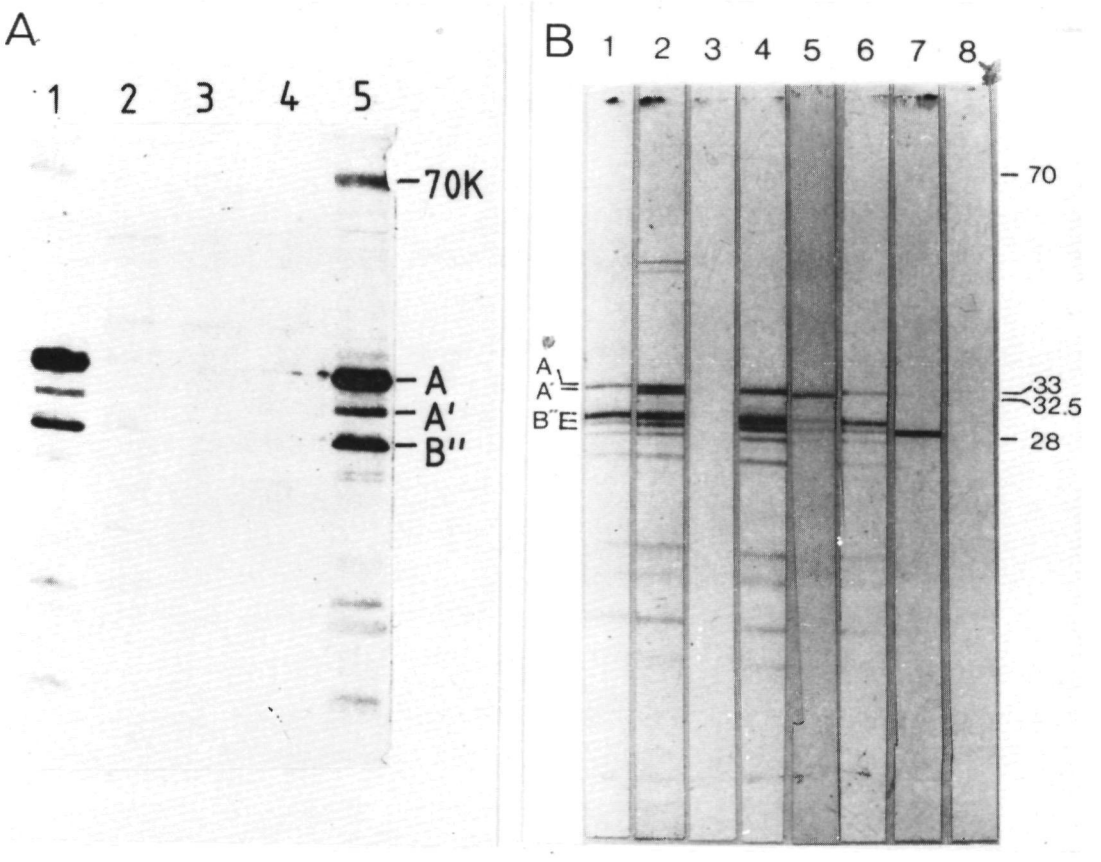

FIGURE 1 Immunoblot analysis of Xenopus proteins with anti-(U1,U2)RNP sera.

Panel A: Analysis of total Xenopus oocyte 12,000g supernatant with V26 serum.
The U snRNP proteins 70K, A, A´ and B´´ are indicated. Lanes 1 and 5; HeLa nuclear extract. Lanes 2, 3 and 4; Xenopus oocyte 12,000g supernatant. The approximate amounts of protein loaded were 40, 20 and 10 ug. In comparison to the HeLa controls, there is no evidence for specific staining of particular U snRNP proteins.
Panel B. Analysis of Xenopus oocyte U snRNPs purified with anti-trimethyl cap antiserum.
The antisera and monospecific antibodies used were: lane 1; serum B25, lane 2; serum V26, lane 3; affinity purified anti-70K lane 4; affinity purified anti-A, lane 5; affinity purified anti-A´, lane 6; affinity purified anti-B´´, lane 7; anti-Sm serum, lane 8; non-immune control serum. The position of the stained bands equivalent to HeLa A, A´ and B´´ are indicated.

Staining of the purified U snRNP proteins with monospecific antibodies (Fig. 1B, lanes 3-6) shows that the cross-reativity between antibodies to proteins A and B′′, already observed in HeLa extracts (Habets et al., 1985a) is also present in Xenopus. The common epitope on these proteins has thus been conserved in evolution. The lack of cross-reactivity of antibodies to A′ and 70K allow monospecific anti-(U2)RNP and anti-(U1)RNP antibodies to be prepared.

An anti-Sm serum which recognizes proteins B and B′ as well as D in HeLa extracts (Habets et al., 1985b) stains a single band in the region of protein B (27,000 Da) in Xenopus U snRNPs (Fig. 1B, lane 7). A single band of this size has previously been observed using other anti-Sm antisera to stain Xenopus oocyte extracts (Fritz et al., 1984). It is not clear whether this band is a single protein or two proteins which are not resolved. The lack of reactivity of anti-Sm serum with any of the triplet of B′′ bands underlines the difference between these different "B" proteins.

In conclusion these results demonstrate the existence in Xenopus oocyte U snRNPs of proteins equivalent to the U2 snRNP-specific A′ and B′′. The patterns of immunological crossreactivity, A and B′′ cross-reacting while A and A′ and B and B′′ do not cross react, is identical to that observed in mammalian cell U snRNPs.

Binding requirements of proteins B and B′ on U2 snRNA

A method for analysing protein-RNA interactions in RNPs has recently been developed and used to analyse U2 snRNPs. Genes coding for wild-type Xenopus laevis U2 snRNA (Mattaj and Zeller, 1983) or for a series of mutants called ΔA-ΔE in which portions of the RNA sequence have been changed (Mattaj and De Robertis, 1985) were injected into Xenopus oocyte nuclei. The immunoprecipitability of these RNAs was then tested with various antibodies. In this way the RNA sequences required for the binding of particular antigens could be determined. Previous experiments have shown that proteins D, E, F and G protect a short region of the U2 SNRNP against micrococcal nuclease digestion (Liautard et al., 1982) and that

this region of U2 RNA is required in order that the RNA be immunoprecipitated by anti-Sm antisera (Mattaj and De Robertis, 1985). Since most anti-Sm antisera recognize at least proteins B, B′ and D it was not possible to determine what were the binding requirements of proteins B, B′ and D separately.

Recently an anti-Sm antiserum called M13 which recognizes proteins B and B′ but not D has been described (Habets et al., 1985b). We compared the immunoprecipitation of the U2 mutants with this serum with their immunoprecipitation by antiserum K22, which recognizes proteins B, B′ and D, as well as the U1 snRNP specific A and C proteins (Habets et al., 1985b). The transcripts of the wild-type and mutant genes are shown in Fig. 2, panel A. Their immunoprecipitation by K22 antiserum is shown in Fig. 2, panel B. This confirms that an anti-Sm antiserum recognizing B, B′ and D immunoprecipitates wild type U2 and all the mutants with the exception of ΔC, from which at least part of the Sm binding site has been deleted (Mattaj and De Robertis, 1985).

M13 antiserum immunoprecipitates exactly the same U2 mutants as K22 (Fig. 2, compare panels B and C). Since this antiserum recognizes only B and B′ this indicates that these two proteins require the Sm binding site to bind to U2 snRNA. Since we already know from the results of Liautard et al. (1982) that one or more of the proteins D, E, F and G interact directly with this region of U2. This indicates that B and B′ most likely bind to the U2 snRNP through protein-protein rather than protein-RNA interactions. This will be discussed further below.

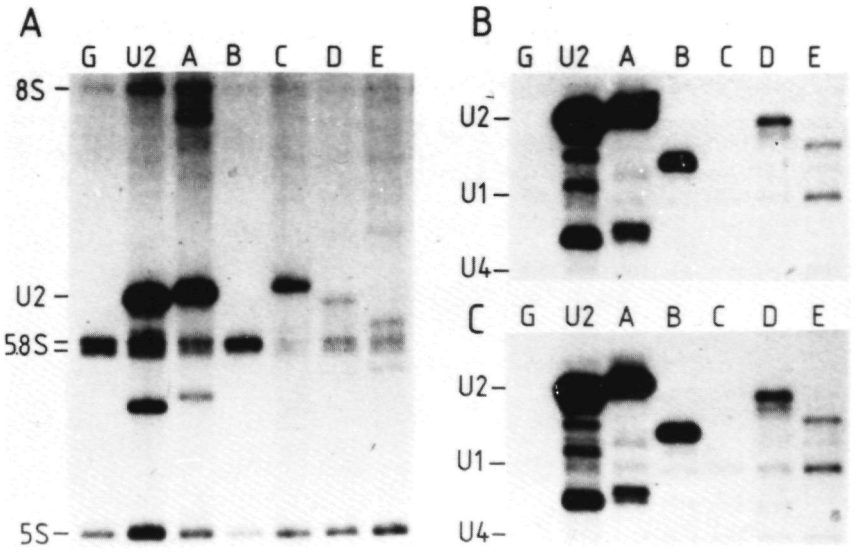

FIGURE 2. Immunoprecipitation of wild-type and mutant U2 snRNAs with anti-Sm antisera.
The two antisera used were K22, which recognizes U snRNP proteins B, B′, D, A and C, and M13 which recognizes only B and B′ (Habets et al., 1985b). The wild-type U2 and mutants ΔA-ΔE have been described (Mattaj and Zeller, 1983; Mattaj and De Robertis, 1985). Xenopus oocytes were microinjected with α-^{32}P GTP alone (lanes G) or in combination with either wild-type U2 DNA (lanes U2) or one of the mutant DNAs ΔA-ΔE (lanes A-E).
Panel A: total transcription products from microinjected oocytes. The positions of 8S, U2, 5.8S and 5S RNAs are indicated. Note that on this gel ΔB transcripts co-migrated with 5.8S RNA. Panel B; RNA precipitated by K22 antiserum. Panel C; RNA precipitated by M13 antiserum.

Binding requirements of A′ and B′′ on U2 snRNA

A previous analysis of binding both common U snRNP proteins and U2 snRNA specific proteins (Mattaj and De Robertis, 1985), could not distinguish which of the two U2 snRNP proteins A′ and B′′ were being studied. The monospecific anti-A′ and anti-B′′ antibodies (described above) were therefore used to further the analysis of U2 snRNP structure.

The results presented in Fig. 3 represent immunoprecipitated RNAs from control oocytes or from oocytes injected with either wild-type or a mutant U2 gene. The antibodies used are either whole V26 serum or monospecific antibodies prepared by elution from HeLa

protein immunoblots. It is important to note that the control used is serum eluted from part of the immunoblot showing no specific staining. In other words it shows the small but detectable amount of V26 serum sticking non-specifically to the nitrocellulose (Fig. 3, compare panels 1 and 2). The RNAs precipitable by V26 serum are shown in Fig. 3, panel 2.

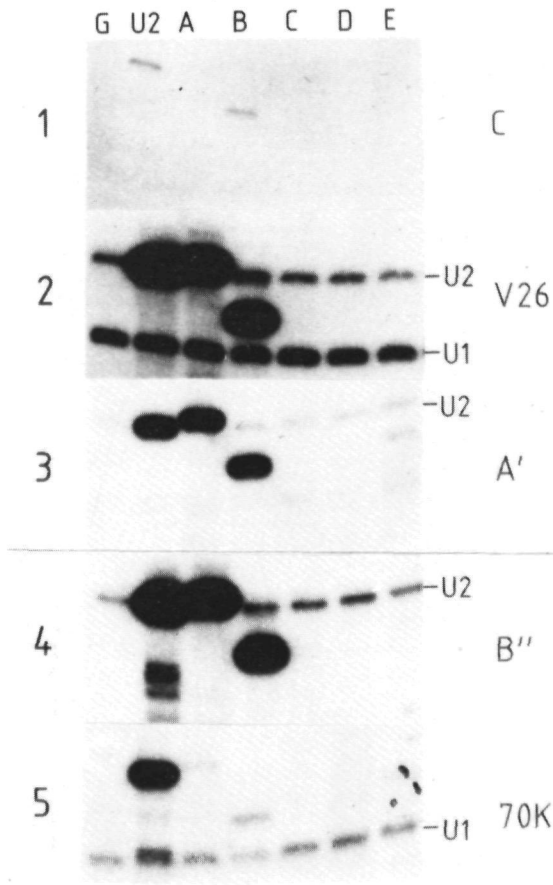

FIGURE 3. *Immunoprecipitation of* mutant and wild-type U2 snRNAs with anti-(U1-U2)RNP and monospecific antibodies.
Xenopus oocytes were microinjected with α-^{32}P GTP alone (lane G) or in combination with either wild-type U2 DNA (lane U2) or one of the mutant U2 DNAs ΔA-ΔE (lanes A-E).
Extracts of oocytes were then immunoprecipitated with either whole V26 serum or with affinity purified monospecific antibodies to 70K, A′ or B′′. These were purified by elution of antibodies from immunoblots of HeLa nuclear proteins (Habets et al., 1985a). As a control non-specifically sticking antibodies were eluted from a blanc region of the immunoblot. Panel 1; control elution, panel 2; whole V26 serum, mock-eluted, endogenous U1 and U2 are indicated, panel 3; anti-A′, endogenous U2 is indicated, panel 4; anti-B′′, endogenous U2 is indicated, panel 5; anti-70K, endogenous U1 is indicated.
No endogenous U1 is detectable in anti-B′′ (or anti-A, not shown) immunoprecipitates in spite of the crossreaction of these antibodies with A. This is probably due to the antibodies having a lower affinity for A than for B′′.

Endogenous U1 and U2 RNA are precipitated, as are wild-type U2, and RNAs transcribed from the U2 mutants ΔA and ΔB. RNAs transcribed from mutants ΔC-ΔE are not immunoprecipitated by this serum. This result is identical to that previously obtained using another anti-(U1,U2)RNP serum called Pick (Mattaj and De Robertis, 1985a) or P21 (Habets et al., 1985a) which apparently recognized protein B'' but not A' (Habets et al., 1985a). In order to test which parts of the U2 sequence were specifically required for binding A' and B'' we next tested these two monospecific antibodies separately. The results (Fig. 3, panels 3 and 4) show that both these proteins have the same structural requirements for binding to U2. They require the binding site for the Sm antigen, deleted in ΔC (Mattaj and De Robertis, 1985) and the sequences 3' to this site deleted in ΔD and ΔE. As expected from their immunological cross-reactivity, monospecific anti-A and anti-B'' antibodies behaved identically in these assays (not shown).
The structural information gleaned from these results is incorporated into the schematic model of U2 snRNP structure presented in Fig. 5.

Interaction between U1 and U2 snRNPs

An unexpected result was that monospecific anti-70K antibodies immunoprecipitated not only endogenous U1, but also a significant amount of wild-type U2 RNA from oocytes injected with the wild-type gene (fig. 3, panel 5). That this immunoprecipitation of U2 is not due to unspecific binding is shown by comparison of control and anti-70K precipitations (Fig. 3, panels 1 and 5). The amount of mutants ΔA and ΔB precipitated by non-specifically bound V26 antiserum and by monospecific anti-70K is similar. The amount of wild-type U2 precipitated by the 70K antibodies is clearly above the control value, and indicates an indirect precipitation of wild-type U2 snRNPs through interaction with U1 snRNPs. We are able to detect this interaction due to the very high specific activity of U2 snRNA in oocytes injected with U2 genes. The specific activity of the endogenous RNA does not allow this detection (Fig. 3, panel 5, lane G). The fact that none of the mutant U2 snRNAs is precipitable with anti-70K antibodies indicates that all

of the changes to U2 snRNA structure made in mutants ΔA-ΔE and the resulting changes in the composition of the U2 snRNPs, prevent interaction with U1 snRNPs.

As a further check on the interaction between U1 and U2 we carried out the reciprocal experiment. X.1.U1.3, a minor U1 snRNA gene from Xenopus whose structure and sequence have been described (Zeller et al., 1984; Mattaj et al., 1985) was injected into oocytes in order to obtain high specific activity U1 snRNA (Fig. 4, panel A). Monospecific antibodies were then used to analyse immunoprecipitable RNAs in control and injected oocytes.

The results from control oocytes show the expected pattern (Fig 4B). Whole V26 serum (lane 2) precipitates both U1 and U2, anti-70K only U1 detectably (lane 3) and anti-A′ only U2 detectably (lane 4). Anti-A and anti-B′′ precipitate as expected both U1 and U2 (not shown). When the specific activity of U1 is increased by gene injection, the immunoprecipitation pattern observed is that given in Fig. 4, panel C. V26 precipitates endogenous U2 plus U1 (lane 2), anti-70K only detectably U1 (lane 3), while anti-A′ precipitates endogenous U2 and a small amount of U1 (lane 4). Comparison with the control (lane 1) indicates that the U1 precipitation is significant, arising from interaction with U2 snRNP rather than non-specific contamination with whole V26 serum. This result together with those presented above suggest that a small fraction of U1 and U2 snRNPs exist in a coprecipitable form. The possible basis of this interaction will be discussed below.

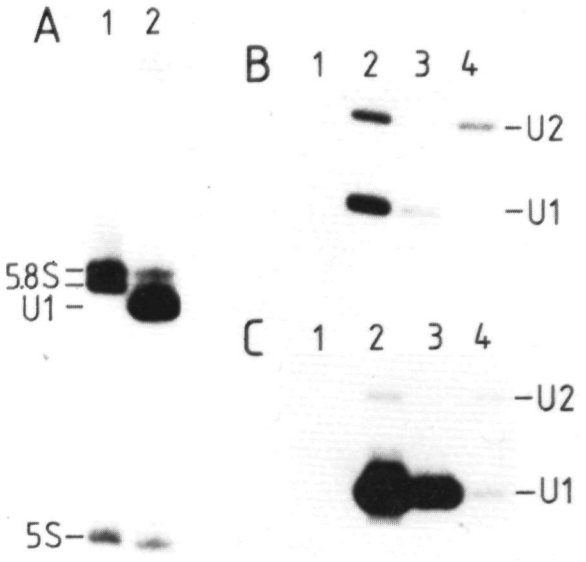

FIGURE 4. Immunoprecipitation of U1 RNA.
Xenopus oocytes were injected either with α-^{32}P GTP alone or together with DNA of the clone X.1.U1.3. Total RNA extracted from these oocytes is shown in panel A. Lane 1; GTP injection, lane 2; X.1.U1.3 injection. Extracts of the injected oocytes were then immunoprecipitated with monospecific anti-A´ or anti-70K antibodies eluted from immunoblots of HeLa nuclear extracts or with mock-eluted whole V26 serum or non-specifically sticking serum from a blank region of the immunoblot. Panel B; GTP injected oocytes, panel C; X.1.U1.3 injected oocytes. Lanes 1; control elution, lanes 2; whole V26, lanes 3; anti-70K, lanes 4; anti-A´.

The results presented here, together with previous results obtained by the same method (Mattaj and De Robertis, 1985) can be combined with results from micrococcal nuclease digestion experiments in which the regions of U2 snRNA protected against nuclease in U2 snRNPs were determined (Liautard et al., 1982; Reveillaud et al., 1985) and results obtained from the study of assembly of U snRNP particles *in vivo* (Fisher et al., 1985). In this way a consistent model of RNA protein interactions in the U2 snRNP particle can be obtained. This is diagrammed schematically in Fig. 5. Proteins D, E, F and G, which associate with one another in the absence of RNA to form a 6S particle (Fisher et al., 1985), bind to and protect a region near the centre of the U2 RNA molecule from micrococcal nuclease digestion (Liautard et al., 1982).

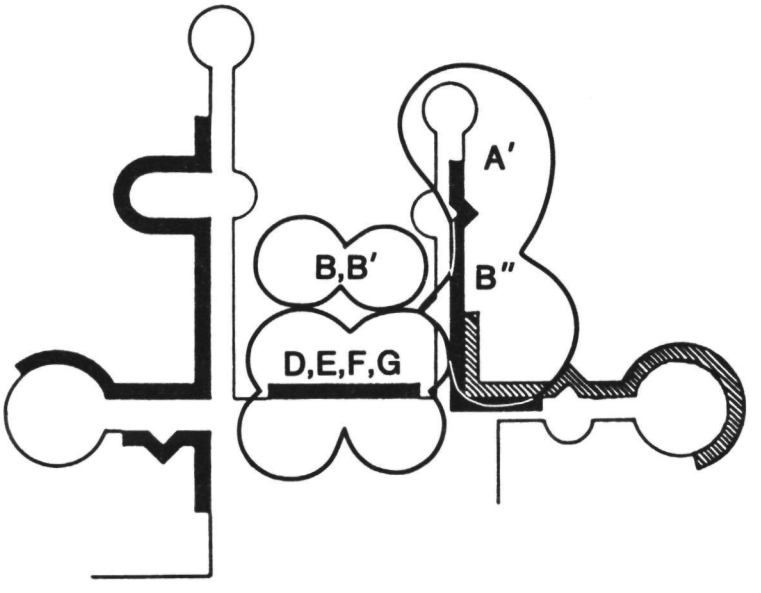

FIGURE 5. Schematic representation of the U2 snRNP.

The U2 snRNP secondary structure is based on data from Reddy et al. (1981), Branlant et al. (1982), and Black et al. (1985). The shaded blocks represent the sequences altered in mutants ΔA-ΔE (Mattaj and De Robertis, 1985).

This region is required in order that the RNA be precipitated by anti-Sm antibodies which recognize proteins B, B′ and D (Mattaj and De Robertis, 1985) or B and B′ alone (this paper). The logical conclusion is that one or more of proteins D, E, F and G bind directly to this region of U2, and that B and B′ are attached to this particle through protein-protein interactions.

The U2 snRNP specific proteins A′ and B′′ bind only to wild-type U2 and mutants ΔA and ΔB. In other words they require for binding not only that the Sm binding site was present, but also RNA structure 3′ to this. They therefore appear to interact both with RNA in the 3′ half of the U2 RNA molecule directly and with proteins bound to the Sm binding site. The interaction of these proteins with the 3′ half of the U2 RNA molecule is likely to be the cause of the protection of this region against micrococcal nuclease digestion of the U2 RNPs observed by Reveillaud et al. (1985). The results are combined in the model of U2 snRNP structure shown in Fig. 5. The model of course is based upon several simplifying assumptions, of which perhaps the most unjustified is that the proteins are all present in all particles in equal molar ratio. It may well be that there exist several different U2 snRNPs which differ in their protein composition, and thus are structurally, or even functionally, distinct. For example in any given U2 snRNP only protein A′ or B′′ might be present.

The interaction of U1 and U2 snRNPs is of interest given that both of these RNAs are involved in the removal of introns from mRNA precursors in splicing (Hernandez and Keller, 1983; Padgett et al., 1983; Bozzoni et al., 1984; Kramer et al., 1984; Krainer and Maniatis, 1985; Black et al., 1985). This process occurs in a particle which has been called the spliceosome (Brody and Abelson, 1985; Grabowski et al., 1985; Frendewey and Keller, 1985). The interaction which we see is however unlikely to represent the immunoprecipitation of splicing complexes since our immunoprecipitations are carried out in 0.5M NaCl. At this ionic strength spliceosomes are at least partially dissociated (Grabowski et al., 1985), and in fact earlier studies of U1 interaction with hnRNPs showed that U1 dissociates from hnRNP at roughly 0.15M NaCl (Zieve

and Penman, 1981). It is therefore more likely that we are studying a direct U1-U2 snRNP interaction, although this may well represent a sub-component of the spliceosome. It is interesting that none of the mutant U2 snRNAs is capable of forming an RNP which can interact with U1 RNP. This indicates that the interaction is not a simple one involving one binding site, but that it requires structural integrity of the U2 RNP. It will be of interest to study the entry of the mutant U2 snRNAs into splicing complexes, which may reveal whether the U1-U2 interaction is a step along the pathway to spliceosome formation.

Materials and Methods

Immunoblotting and preparation of monospecific antisera

Whole oocyte extracts (Fritz et al., 1984), HeLa nuclear extracts (Habets et al., 1983) and oocyte U snRNPs purified by affinity chromatochraphy on anti-trimethyl cap antibody columns (Bringmann et al., 1983, 1984) were analysed as described (Habets et al., 1985a,b). Monospecific antisera were eluted (Smith and Fisher, 1984) from high resolution immunoblots of HeLa nuclear extract (Habets et al., 1985a,b).

Oocyte microinjection and immunoprecipitation

Oocytes were injected, fractionated and extracted, and the extracted RNAs were analysed as described in Mattaj and De Robertis (1985).

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CHAPTER 8

SUMMARY / SAMENVATTING

Sera from patients with rheumatic diseases often contain antibodies that react with various normal cellular components. Some of these autoantibodies are very characteristic for the disease in which they occur and therefore frequently used as a "marker" and help in diagnosis. Apart from a diagnostic and possible prognostic value, human autoantibodies are useful tools in molecular biology. In 1979 Lerner and Steitz first showed that some sera from patients with SLE contained antibodies against small nuclear ribonucleoprotein (snRNP) particles participating in the processing of mRNA precursors. Since then, sera from patients with various autoimmune syndromes were screened with modern biochemical techniques, one of the most prominent being immunoblotting. The use of this technique allowed a more accurate determination of a patients antibody profile, in particular when more than one specificity was present.

Chapter 2 of this thesis describes an immunoblotting study upon the presence of marker antibodies in sera from patients with mixed connective tissue disease (MCTD). The results indicate that so-called anti-70K antibodies (70K is a protein exclusively present on U1 snRNP particles) are quite specific for this disease.

To investigate whether anti-70K antibodies might also have a prognostic value, we developed a quantitative immunoblotting assay (QIBA, chapter 3). This assay enabled us to follow patients longitudinally with respect to the exact autoantibody composition of their serum. The results showed that in a patient with a rather fluctuating disease activity an exacerbation of disease coincided with a peak in his anti-70K antibody level. No correlation could be found between severeness of the disease and anti-70K level when 21 sera of SLE and MCTD patients were examined in a one point study, i.e. one serum sample from every individual patient taken at an arbitrary stage of the disease (chapter 3).

Chapters 4, 5 and 6 deal with the molecular characterization of the autoantigens SS-B, (U1,U2)snRNP and Sm, respectively.

SS-B or La is a cellular antigen with a molecular weight of about 50,000 Daltons, in most cases recognized by antibodies in sera from patients with Sjoegren's syndrome. In adeno- and Epstein-Barr virus infected cells this antigen associates with several small virus-encoded RNAs whereas in uninfected cells a great variety of polymerase III-transcribed RNAs binds to it. The SS-B antigen has been proposed to function as an RNA polymerase III transcription factor, whereas others suspect that it might be an RNA modifying enzyme because of its association with precursor RNAs. No conclusive evidence has as yet been provided for any of these possibilities.

Anti-(U1,U2)RNP antibodies, described in chapter 5 are a relatively new autoantibody specificity that greatly have facilitated the investigations towards the structure of U2 RNP. As described in chapter 5, U2 RNA is associated with at least two unique proteins, one of which shares a common epitope with the U1 RNA specific A protein. Both these RNAs are involved in the splicing process of pre-mRNA; U1 has been shown to bind to the 5' splice-site of pre-mRNAs in in vitro systems, U2 is proposed to associate with the so-called branch point of the lariat-shaped intron, an intermediate in the cascade of splicing events.

Binding requirements for RNA-protein interactions in U2 RNP particles are described in chapter 7. These investigations also revealed that a small portion of the U2 RNPs in vivo exists in a complex with U1 RNP. This association may represent a functional subunit of the spliceosome, a particle in which the splicing event takes place.

Chapter 6 describes the characterization of other U-snRNP-associated proteins. Some of these proteins are common in at least U1, U2, U4, U5 and U6 (=U1-U6) RNPs (U3 RNA is located in the nucleolus). Antibodies directed against any of these proteins precipitate U1-U6 RNA from (radiolabeled) cell extracts, whereas anti-(U1)RNP sera react exclusively with one of the three U1 RNP specific proteins. Some of these antigens appeared to be associated with the so-called nuclear matrix, a nuclear substructure on

which RNA transcription takes place. This attachment of snRNP associated proteins provides another argument in favor of the hypothesis that splicing occurs while pre-mRNAs are still matrix-associated.

In het serum van patienten met reumatische aandoeningen worden vaak antistoffen gevonden die gericht zijn tegen verschillende komponenten uit cellen van het eigen lichaam. Sommige van deze autoantistoffen zijn karakteristiek voor de ziekte waarbij ze voorkomen en worden daarom gebruikt als een zogenaamde "marker" bij de diagnose van een patient. Aan enkele autoantistoffen kan ook een prognostische betekenis worden toegekend. Daarnaast zijn humane autoantistoffen een hulp bij de bestudering van verschillende molekulair-biologische processen. In 1979 toonden Lerner en Steitz voor het eerst aan dat sommige sera van patienten met systemische lupus erythematodes (SLE) antistoffen bevatten die gericht waren tegen zogenaamde "small nuclear ribonucleoprotein particles" (snRNP), deeltjes bestaande uit eiwit en RNA die betrokken zijn bij het rijpingsproces van boodschapper RNA. Sindsdien zijn talloze sera van patienten met autoimmuunziekten onderzocht met behulp van moderne biochemische methoden. Met name de immunoblotting-techniek maakte het mogelijk om van iedere patient een zogenaamd antistof-profiel te bepalen, wat vooral nuttig is als er meerdere autoantistoffen tegelijk in een serum aanwezig zijn.

Hoofdstuk 2 van dit proefschrift beschrijft een onderzoek naar de aanwezigheid van "marker"-antistoffen in sera van patienten met "mixed connective tissue disease" (MCTD). De resultaten hiervan tonen aan dat het voorkomen van zogenaamde anti-70K antistoffen (70K is een eiwit dat alleen voorkomt in U1 snRNP partikels) specifiek lijkt te zijn voor deze ziekte.

Om een mogelijk prognostische waarde van deze anti-70K antistoffen te kunnen onderzoeken hebben we een kwantitatieve immunoblotting methode ontwikkeld (QIBA, hoofdstuk 3). Deze test stelde ons in staat om het antistof-profiel van een patient longitudinaal te vervolgen. De resultaten wezen erop dat bij een patient met een nogal fluctuerende ziekteactiviteit een plotselinge verergering van de ziekte samenviel met een anti-70K piek in zijn antistofprofiel. Geen samenhang kon worden ontdekt tussen de ernst van de ziekte en anti-70K antistof nivo in een onderzoek met eenmalig afgenomen serum van SLE en MCTD patienten

De hoofdstukken 4, 5 en 6 behandelen de molekulair-biologische karakterisering van respectievelijk de autoantigenen SS-B, (U1,U2)RNP en Sm.
SS-B, ook wel La genoemd, is een cellulair antigeen met een molekuulmassa van ongeveer 50.000 Dalton waartegen vooral patienten met Sjoegren's syndroom antistoffen bezitten. In adeno- en Epstein-Barr virus geinfecteerde cellen associeert dit antigeen met verschillende kleine door het virus gekodeerde RNAs, terwijl in ongeinfecteerde cellen een grote verscheidenheid aan polymerase III transcripten met het SS-B antigeen is gecomplexeerd. Sommige onderzoekers hebben daarom geponeerd dat het SS-B antigeen een polymerase III transcriptiefactor zou zijn terwijl anderen vermoeden dat het een RNA-modificatie enzym zou kunnen zijn vanwege de gevonden associatie met zogenaamde "precursor"-moleculen. Tot nu toe is echter geen overtuigend bewijs gevonden voor deze theorieen.

Hoofdstuk 5 beschrijft een relatief nieuwe soort autoantistof, anti-(U1,U2)RNP, waarmee bijvoorbeeld onderzoek kan worden gedaan naar de structuur van U2 RNP. Zoals in hoofdstuk 5 wordt beschreven is U2 RNA geassocieerd met tenminste twee unieke eiwitten, waarvan een een gemeenschappelijk epitoop heeft met het U1 RNA-specifieke A eiwit. Deze beide RNAs zijn betrokken bij het rijpingsproces van de voorloper van boodschapper RNA, het pre-mRNA. Van U1 RNA is in in vitro systemen aangetoond dat het bindt aan de zogenaamde "5' splice site" van pre-mRNAs, terwijl U2 zou binden aan de zogenaamde "branch point" van het lasso-vormige intron, een intermediair in het pre-mRNA rijpingsproces.

Factoren die belangrijk zijn voor de interactie tussen eiwit en RNA in U2 RNP partikels worden beschreven in hoofdstuk 7. Tevens wezen de resultaten van dit onderzoek uit dat een klein gedeelte van de U2 RNPs in vivo geassocieerd is met U1 RNPs. Hierdoor zou een functionele eenheid gevormd kunnen worden die een rol vervult in het zogenaamde "spliceosome", een complex waarin het mRNA rijpingsproces zich afspeelt.

Hoodstuk 6 beschrijft de karakterisering van andere met U-snRNP geassocieerde eiwitten. Sommige van deze eiwitten komen voor in zowel U1, U2, U4, U5 als U6 (U1-U6)RNPs. Antistoffen, gericht tegen deze eiwitten precipiteren allen U1-U6 RNA uit (radioactief gelabelde) celextracten, terwijl anti-(U1)RNP antistoffen alleen reageren met een van de drie eiwitten specifiek voor U1 RNPs. Sommigen van deze antigenen bleken geassocieerd met de zogenaamde kernmatrix, een structuur in de celkern waaraan onder andere RNA transcriptie plaatsvindt. Deze associatie vormt een extra argument voor de hypothese dat het rijpingsproces van pre-mRNAs zich afspeelt terwijl het pre-mRNA gebonden is aan de kernmatrix.

Future outlook

Elucidation of the primary structure of autoantigens has come within reach now a variety of well-characterized autoimmune sera enable the large scale isolation of several autoantigens. Recombinant DNA technology allows the identification of genes coding for autoantigens and provides tools to study their regulation, whereas monoclonal antibodies raised against autoantibodies or their target antigens can provide insight in the mechanisms involved in autoimmunity.

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Curriculum vitae

Winand Habets werd geboren op 21 juli 1955 te Kerkrade.

In 1974 behaalde hij het Atheneum-B diploma aan het Antonius Doctor College te Kerkrade.

In datzelfde jaar begon hij met de studie biologie aan de Katholieke Universiteit van Nijmegen waar hij in 1978 slaagde voor het kandidaatsexamen. De doctoraalstudie omvatte de bijvakken biochemie (Dr. W.J. van Venrooij, Prof. Dr. H. Bloemendal) en biogeologie (Drs A.J. Kempers, Prof. Dr. D. Teunissen) en het hoofdvak microbiologie (Dr. D.B. Janssen, Prof. Dr. Ir. G.D. Vogels). Tijdens deze periode werd een eerste graads lesbevoegdheid biologie behaald evenals een diploma C-deskundigheid stralingshygiene.
In december 1981 werd het doctoraal examen biologie afgelegd.

Sinds 1982 is hij werkzaam als wetenschappelijk medewerker op het laboratorium voor biochemie aan de Katholieke Universiteit van Nijmegen. Daar werd met financiele steun van het "Praeventiefonds" en het "Reumafonds" het in dit proefschrift beschreven onderzoek uitgevoerd in de werkgroep van Dr. W.J. van Venrooij. In 1984 werden het ANA symposium te Leuven en de Bertine Koperberg Conference te Oosterbeek bezocht en in 1985 het Colloquium Protides of the Biological Fluids te Brussel. Tevens werden werkbezoeken afgelegd aan de groepen van Dr. R. Luhrmann (Berlijn) en Dr. E. Penner (Wenen). Tijdens de promotieperiode werd een bijdrage geleverd aan het practisch biochemie-onderwijs aan eerste en tweede jaars studenten medicijnen en werd tevens de functie van plaatselijk veiligheidsdeskundige m.b.t. stralingshygiene vervuld.

Vanaf juni 1985 is hij op bovengenoemde afdeling werkzaam op een door het "Reumafonds" gesubsidieerd project dat de verdere karakterisering van autoantigenen bij reumatische ziekten tot doel heeft.

STELLINGEN

I

Bij zijn conclusie dat U1 en U2 snRNPs op identieke plaatsen in de celkern gelocaliseerd zijn gaat Spector er ten onrechte van uit dat het door hem gebruikte serum antistoffen bevat die uitsluitend gericht zijn tegen U2 snRNPs.

Spector, D.L. (1984) Biol. Cell, 51, 109-112.

II

Bepaling van circulerende auto-antistoffen in het serum van patienten met reumatische aandoeningen middels immuunfluorescentie op levercoupes is ontoereikend en dient te worden vervangen door een bepaling met een groter differentierend vermogen.

Dit proefschrift

III

De conclusie van Fritzler et al. dat sera van MCTD patienten antistoffen bevatten tegen hnRNP kan niet gebaseerd worden op de door hen gepresenteerde resultaten.

Fritzler, M.J., Rashid Ali, Tan E.M. (1984) J. Immunol. 132, 1216-1222.

IV

Goede reumaserologie omvat ook bepaling van de anti-perinucleaire factor.

V

De eerste door Gazit et al. voorgestelde initiatie site in exon 1 van het humane c-sis proto-oncogen zal in vivo niet gebruikt kunnen worden.

Gazit, A., Igarashi, H., Chiu, I-M., Srinivasan, A., Yaniv, A., Tronick, S.R., Robbins, K.C., Aaronson, S.A. (1984) Cell 39, 89-97.

VI

De regionale localisatie van een aantal anonieme chromosoom 19 "markers" die Brook et al. vinden uit studie aan somacelhybriden is onjuist.

Brook, J.D., Shaw, D.J., Thomas, N.S.T., Meredith, A.L., Corvell, J., Harper, P.S. (1986) Cytogenet. Cell Genet. 41, 30-37.

VII

Het ontbreekt Ozaki et al. aan de juiste controle experimenten om te mogen concluderen dat er eiwitsynthese in de kern van de ooglens plaatsvindt.

Ozaki, L., Jap, P., Bloemendal, H., (1985) Exp. Eye Res. 41, 569-575.

VIII

De door Crabbe gepresenteerde homologie tussen c-myc en β- en γ-crystallines getuigt niet van inzicht in de evolutionaire verwantschap van deze ooglenseiwitten.

Crabbe, M.J.C., (1985) FEBS Letters 181, 157-159.

IX

De exponentiele toename in het gebruik van computers leidt tot een verarming van de nederlandse taal.

X

Het is niet zinvol om iedere radiologische werker van een film-badge te voorzien.

XI

Gezien de bruikbaarheid van immunoblotting voor het aantonen van antistoffen tegen onder andere LAV/HTLV III getuigt de benaming "Golden Blot" van commercieel inzicht.

Brada, D., Roth, D. (1984) Anal. Biochem. 142, 79-83.

XII

Het omkopen van stemgerechtigden bij (2^{e} kamer) verkiezingen is blijkbaar alleen te veroordelen wanneer dat op kleine schaal gebeurt.

Nijmegen, 22 mei 1986
Winand Habets.