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A multiple sample immunoblotting system (MSIS) for the intrinsic detection of antinuclear autoantibodies

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A multiple sample immunoblotting system (MSIS) is described. The MSIS permits the detection and classification of antinuclear antibodies (ANA) according to the pattern of antigenic polypeptides recognized in four different extracts: calf thymus whole tissue, nuclear extract, and two ammonium sulphate fractions of the nuclear extract. The procedure permits the classification of anti-RNP, anti-SS/B and anti-Sm, and the detection of new ANAs in sera from SLE patients. The reaction patterns presented are specific and unambiguous, and make the use of validated control sera unnecessary.

Key words: Antinuclear antibody; Autoimmune disease; Anti-RNP; Anti-Sm; Anti-SS/B; Immunoblotting

Introduction

Autoimmune diseases are characterized by multiple abnormalities of the immune system including the production of antibodies reacting against self antigens. These autoantibodies attract increasing interest in clinical medicine as tools aiding the diagnosis and prognosis of autoimmune disorders (Weiss et al., 1983).

Different techniques have been developed to detect ANAs and to determine their specificities. Indirect immunofluorescence and passive haemagglutination only detect major specificities (Tuffanelli, 1983), or need laborious enzymatic treatments of the antigenic extracts in order to differentiate between specificities. Immunodiffusion (ID) and counterimmunoelectrophoresis (CIE) are also currently used. The most specific test is CIE (Kurata and Tan, 1976; Lakomek et al., 1983) but monospecific reference sera are needed to test for lines of identity with unknown sera and, in general, only major specificities can be determined.

In recent years, several groups have developed immunoblotting methods to detect and classify ANAs (Guldner et al., 1983; Habets et al., 1983). Immunoblotting provides a simple and sensitive method to recognize antigenic polypeptides present in tissue samples and extracts. However, its use for ANA determination is hindered by the complexity of the nuclear antigenic samples and the autoimmune sera used. Current immunoblotting methods classify sera by the reaction against only one antigenic extract blotted onto the nitrocellulose; and therefore, only the molecular weight of the antigenic polypeptides recognized in a simple nuclear extract is used to determine ANAs. This is a very limited approach taking into account the relatively high number of nuclear proteins. Moreover, some of the known nuclear antigens (Jo-1, SS/B) show the same molecular weight when tested by immunoblotting (Elkon and Jankoski, 1985).

For similar reasons, the currently used immunoblotting methods cannot be directly applied

Abbreviations: BSA, bovine serum albumin; MSIS, multiple sample immunoblotting system; SDS, sodium dodecyl sulphate; PMSF, phenylmethylsulphonyl fluoride.

to the definition of new ANA specificities, this being an important limitation since unidentified ANAs account for a high percentage of total ANAs (Maini et al., 1985).

In this paper, we present a new multiple sample immunoblotting system (MSIS), as a tool for the detection of known ANAs, such as anti-RNP, anti-SS/B and anti-Sm, and for the definition of new ones in sera from patients suffering from autoimmune diseases. As an example, we present a complete study of a series of sera from patients suffering from systemic lupus erythematosus (SLE).

This system permits the classification of sera based not only on the molecular weight of the antigenic polypeptides, but also on these polypeptides and/or their degradation products, detected in various extracts and nuclear fractions blotted on the same nitrocellulose sheet. The procedure yields reproducible and easily identificable patterns for each class of ANA, even when polyspecific sera are tested. Continuous use of validated control sera for pattern comparison is not required.

Materials and methods

Human sera and patients

84 serum samples from 59 patients were obtained from the Servei d'Immunologia de l'Hospital Clínic i Provincial, Barcelona, Spain. All sera were from patients who fulfilled at least four of the A.R.A. (American Rheumatism Association) criteria for SLE.

Various anti-RNP, anti-Sm and anti-SS/B reference sera were generous gifts of Dr. Carmina Gutiérrez (Servicio de Inmunología, Clínica Puerta de Hierro, Madrid, Spain), Dr. P.J.W. Venables (Kennedy Institute of Rheumatology, London, U.K.), Dr. P.J. Maddison (Royal National Hospital for Rheumatic Diseases, Bath, U.K.), and the Center for Disease Control (Atlanta, GA 30333, U.S.A.). All sera were stored at −80°C.

Sample preparation

Four types of samples were used as antigenic sources for immunoblotting: whole calf thymus, a soluble nuclear extract obtained from calf thymus nuclei (coded as HSS nuclear extract), and two fractions obtained from the HSS nuclear extract by sequential precipitation with ammonium sulphate using 25%–60%, and 70%–90% saturated solutions (coded as 60% ASS and 90% ASS fractions respectively).

Tissue and fractions were prepared as follows: whole calf thymus was powdered at liquid nitrogen temperature, thawed and sonicated in electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.001% bromophenol blue, 5% 2-mercaptoethanol, 1 mM PMSF) at 75 mg tissue/ml, and stored at −80°C. The HSS nuclear extract was obtained from calf thymus nuclei, isolated and homogenized in a hypotonic medium as described by Sharp et al. (1972). The nuclear material, extracted with PBS buffer, was centrifuged at $12000 \times g$ for 10 min, the supernatant was then centrifuged at $113000 \times g$ for 90 min, and the new supernatant (the HSS nuclear extract) was used as an antigenic source after extensive dialysis against water and lyophilization.

Ammonium sulphate fractionation of HSS nuclear extracts was performed at 4°C by sequential precipitation at 25%, 60%, 70% and 90% saturation of ammonium sulphate buffered with sodium carbonate (0.8 mg sodium carbonate per g of ammonium sulphate). Precipitates were recovered by centrifugation at $10000 \times g$ for 60 min, were resuspended in PBS buffer and then dialyzed against either PBS buffer or water. Samples were either lyophilized and stored at −80°C until use or, alternatively, the lyophilized powder from HSS nuclear extract and ammonium sulphate fractions were dissolved in electrophoresis sample buffer (see above) at 10 mg/ml of total protein (determined as described by Lowry et al., 1951) sonicated and stored at −80°C for a period of time no longer than a month.

Identification of sera

All sera were tested and classified by CIE against HSS nuclear extract by their immunological identity with anti-RNP, anti-SS/B and anti-Sm reference sera according to Kurata and Tan (1976).

Protein blotting

15% SDS polyacrylamide slab gel electrophoresis was performed as described by Laemmli (1970).

Lysozyme (14.3 kDa), soybean trypsin inhibitor (21 kDa), ovalbumin (45 kDa) and bovine serum albumin (66 kDa) were used as molecular weight standards. Gels were stained with 0.4% Coomassie brilliant blue R. The molecular weight of the autoantigens was determined from blots as described by Durán et al. (1984).

Samples from whole thymus, HSS nuclear extract, 60% ASS fraction and 90% ASS fraction, were resolved in contiguous slots by polyacrylamide gel electrophoresis, and transferred to nitrocellulose sheets (BA 85, Schleicher and Schull, 0.45 μm pore size) at 70 V for 8 h in a Bio-Rad Trans-blot device as previously described (Renart et al., 1979). These nitrocellulose sheets were preincubated in PBS buffer containing 3% BSA and 0.05% Nonidet P-40 for 2 h, and then overnight with serum diluted 1/40 in the same buffer. After washing for 1 h with 1 M NaCl in 10 mM phosphate buffer pH 7.5, strips were incubated for 3–4 h with ^{125}I-protein A (8.8 mCi/mg, New England Nuclear) at 1 μCi and 10 ml incubation buffer per 4 sample sheet, washed again with 1 M NaCl in 10 mM phosphate buffer pH 7.5, dried, and autoradiographed for 7–15 h with intensifying screens.

Nitrocellulose paper sheets with transferred antigenic extracts could be stored at 4°C for months and be reincubated several times with different sera.

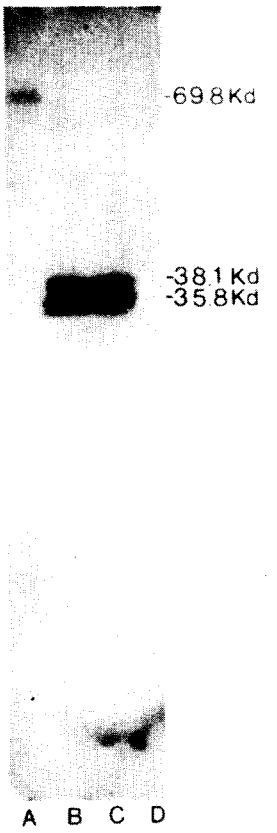

Fig. 1. Pattern of antigenic polypeptides recognized by an anti-RNP specific serum from a human patient with SLE. A: whole calf thymus; B: HSS nuclear extract; C: 25%–60% ASS fraction; D: 70%–90% ASS fraction. Numbers show molecular weight in kDa.

Results

Patterns obtained for anti-RNP, anti-Sm and anti-SS/B using the MSIS

We designed an immunoblotting system characterized by the simultaneous use of several different antigenic samples, blotted onto the same nitrocellulose sheet in blocks of four: calf thymus whole tissue, HSS nuclear extract and two fractions of the precipitation of the HSS nuclear extract with saturated ammonium sulphate (60% ASS and 90% ASS). These nitrocellulose sheets coated with multiple samples were used for immunodetection.

To test the specificity of the new reaction MSI system, 64 sera were used, including 48 sera previously classified by CIE as positive (RNP, Sm, SS/B and other unknown ANAs) and 16 negative sera.

After testing all the anti-RNP sera and comparing the common reactive antigenic polypeptides using the method described, the patterns illustrated in Fig. 1 were obtained. A positive reaction was evident against the 69.8 kDa antigen in whole thymus (lane A), and also against a discrete number of polypeptides ranging from 35.8 to 38 kDa in both the HSS nuclear extract and 60% ASS fraction (lanes B and C). None of the sera tested displayed any reaction with the 90% ASS fraction (lane D). Low molecular weight spots in lanes C and D are blotting artefacts.

The same approach was used to define the specific anti-SS/B pattern (Fig. 2). Sera showed reactions with the 52 kDa SS/B antigen in whole tissue (lane A), with one polypeptide of 47.1 kDa

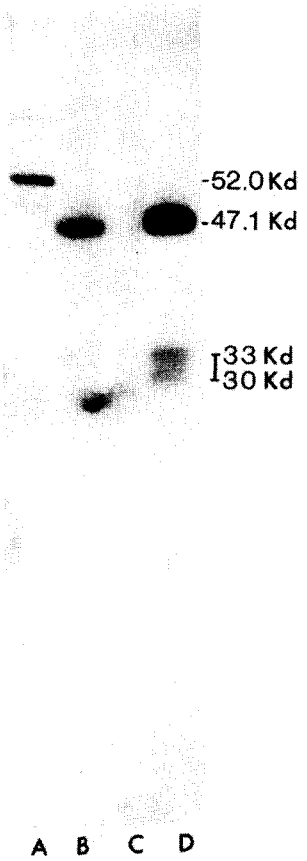

Fig. 2. Pattern of antigenic polypeptides recognized by an anti-SS/B-specific serum from a human patient with SLE. The symbols and samples are the same as for Fig. 1.

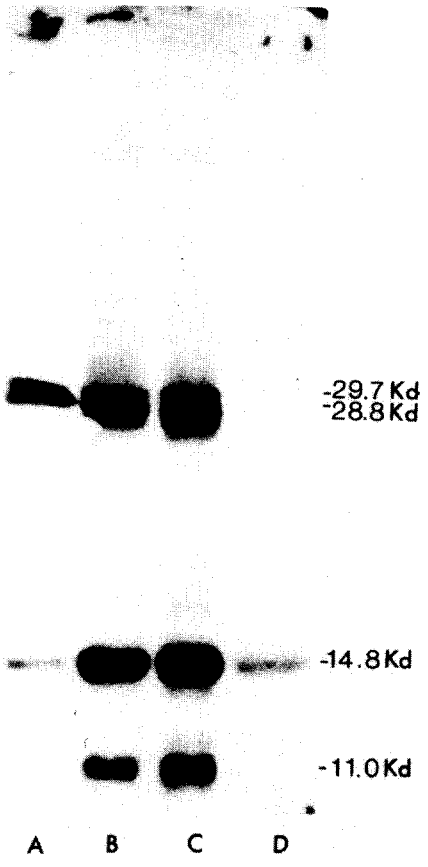

Fig. 3. Pattern of antigenic polypeptides recognized by an anti-Sm-specific serum from a human patient with SLE. The symbols and samples are the same as for Fig. 1.

in the HSS nuclear extract, and a band of 47.1 kDa and a group of polypeptides from 30 to 33 kDa in the 90% ASS fraction. No reaction was observed using the 60% ASS fraction (see Fig. 2, lanes B, C, and D).

When this series of sera was tested by CIE, only one serum could be classified as anti-Sm and was in fact anti-RNP plus anti-Sm. This serum was tested by the MSI system and displayed the RNP pattern, together with a supplementary 14.8 kDa band using both the HSS nuclear extract and the 60% ASS fraction (not shown). Fig. 3 shows the pattern obtained with an anti-Sm reference serum (from the Center for Disease Control, Atlanta). As can be seen, this serum displayed a strong reaction against 11 kDa, 14.8 kDa and 28.8–29.7 kDa antigenic polypeptides in the HSS nuclear extract and the 60% ASS. While the 14.8 kDa polypeptide showed a weak signal in the whole tissue and in the 90% ASS, and the 28.8–29.7 doublet was clearly recognized in the thymus slot but not in the 90% ASS, the 11 kDa antigen could only be detected in the two fractions previously mentioned.

Another seven sera in this series, not having any of the known specificities when tested by CIE, also displayed the same reaction against the 14.8 kDa antigenic polypeptide in the HSS nuclear extract and the 60% ASS fraction. One serum also reacted with the same antigenic polypeptide in the whole tissue. Furthermore, three of these seven sera recognized the 28.8–29.7 kDa doublet in whole tissue but not in the other three extracts. No sera in this series showed any reaction with the 11 kDa Sm antigenic polypeptide in any of the extracts (see Table I).

TABLE I

Sm ANTIGENIC POLYPEPTIDES RECOGNIZED BY IMMUNOBLOTTING WITH SERA FROM SLE PATIENTS

Serum number	Serum specificity [a]	Sm antigenic polypeptides (kDa)					
		Whole calf thymus			HSS nuclear extract 25–60% ASS fraction [e]		
		29.7/28.8	14.8	11.0	29.7/28.8	14.8	11.0
L20	RNP + Sm	–	–	–	–	+	–
L22	RNP	+	–	–	–	+	–
L40	WEAK	–	–	–	–	+	–
L57	RNP	?	+	–	–	+ +	–
L72	WEAK [b]	+	–	–	–	+ +	–
L76	RNP	+	–	–	–	+	–
L78	RNP	–	–	–	–	+	–
L81	WEAK [c]	–	–	–	–	+	–
CDC Reference	Sm [d]	+ +	+	–	+ +	+ +	+ +

[a] Determined by CIE.
[b] Serum showing partial identity with anti-RNP reference sera when tested by CIE.
[c] Serum showing partial identity with anti-Sm reference sera when tested by CIE.
[d] See Fig. 3.
[e] All anti-Sm sera tested displayed reaction against the same polypeptides in both fractions.

Pattern specificity

As a control for pattern specificity, another 30 sera from the HSS (+) group were tested. None had previously showed immunological relationship with any of the RNP, Sm or SS/B reference sera when tested by CIE.

Except for the three sera that displayed Sm patterns by MSIS (see Table I), the antigenic polypeptides recognized in all of the fractions employed were heterogeneous in number and molecular weight, with patterns differing from those already described for RNP, Sm or SS/B antigens.

Nevertheless, serum samples from five different patients recognized the pattern of bands shown in Fig. 4, which is defined by two polypeptides, one having an MW 43 000 in whole thymus and with a slightly lower MW in both the HSS nuclear extract and the 60% ASS fraction, and the other of 17 kDa in the same extracts and fractions. This pattern may correspond to a new autoantigen and work is in progress in order to further characterize it.

As negative controls sera from the HSS (−) group were tested. Several of them showed heterogeneous reactions, mainly with whole calf thymus, but also with the HSS nuclear extract and the different ammonium sulphate fractions. No char-

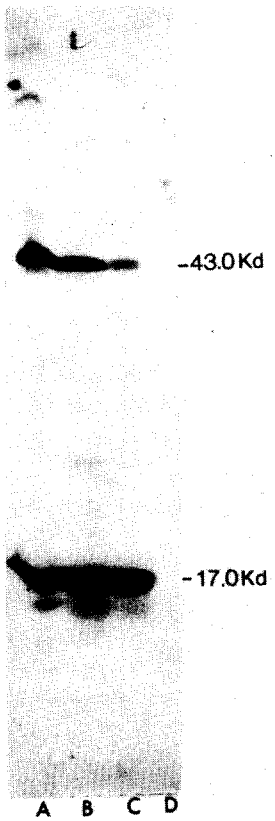

Fig. 4. Pattern of antigenic polypeptides recognized by an anti-Bar 1-specific serum from a human patient with SLE. The symbols and samples are the same as for Fig. 1.

acteristic or reproducible pattern was found with this subset of sera, and none of them exhibited any of the defined patterns displayed by the well characterized ANAs.

Discussion

Immunoblotting is a useful and widely used method for the detection of antigenic polypeptides in unfractionated extracts, but its application to the detection and classification of ANAs is hindered by the confusion which arises when molecular weight is the only criterion used for classification. Furthermore, this criterion seems inadequate for the definition of new ANAs.

To overcome such problems Elkon and Jankowski (1985) proposed an immunoblotting method using extracts previously resolved by a two-dimensional method (polyacrylamide gel electrophoresis and electrofocussing). Although this procedure is useful for discrimination between Jo-1 and SS/B, it appears to be complex, time consuming and difficult to adapt to clinical routine practice.

We have designed a multiple sample immunoblotting system (MSIS) that allows the unambiguous determination of known ANAs, as well as the definition of new ones. This method is based on the use of four different extracts and fractions to detect antigens and their degradation products.

Whole calf thymus, prepared by direct sonication of whole thymus powder thawed in SDS-electrophoresis sample buffer, was included as a control for antigenic integrity and permitted the detection of autoantibodies directed against antigens not present in the other nuclear extracts. The molecular sizes of the RNP, Sm and SS/B antigenic polypeptides were essentially the same as the values already published (see Van Venrooij and Habets, 1985, for a review), showing the absence of proteolysis during preparation of the extract and suggesting that the extract is suitable for the detection and description of new ANAs.

The HSS nuclear extract is our standard extract for CIE, and its inclusion in the MSIS allowed the results obtained with both techniques to be compared. Furthermore, it is enriched in the RNP antigenic polypeptides formed by degradation of the 69.8 kDa RNP antigen (Durán et al., 1984), and the SS/B antigenic polypeptides derived from the 52 kDa SS/B antigen, giving a characteristic and easily identifiable pattern for both specificities. Also, anti-Sm displayed a clear reaction with the 14.8 kDa antigen in this extract although the antigen appears almost absent in whole tissue.

In both ASS fractions, antigenic polypeptides and degradation products were distributed so that specific and unambiguous patterns were obtained.

The patterns described for anti-RNP and anti-SS/B were constant and reproducible, and were displayed by all sera tested in this series (9 anti-RNP, 5 anti-SS/B and 3 anti-RNP plus anti-SS/B). The anti-Sm pattern appeared to be more complex. When the unique anti-Sm serum found by CIE was tested by the MSI system the only specific polypeptide was the 14.8 kDa one. This reaction was also displayed by other sera from this series (see the results section and Table I) as well as by other anti-Sm sera from other series of patients (data not shown), frequently associated with the 28.7–29.8 kDa and/or the 11 kDa anti-Sm antigenic polypeptides, a fact already described by Guldner et al. (1983). Although a strong monospecific reference serum reacted against all four antigenic polypeptides (see Fig. 3) no other sera were found to display the same complete reaction pattern (see Table I). This diversity of reaction patterns may be explained by the fact that at least two different sets of antibodies are involved in the reaction (Guldner et al., 1983).

This MSI system also allows the detection of a wider group of autoantibodies reacting against cellular structures other than nuclei or soluble nuclear extracts, providing an insight into the diversity of autoantibodies present in sera. Because of its reproducibility, MSIS should prove to be a powerful diagnostic tool permitting further studies of clinical correlations.

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References

Durán, N., M. Bach, P. Puigdomènech and P. Palau, 1984, Mol. Immunol. 21, 731.

Elkon, K.B. and P.W. Jankowski, 1985, J. Immunol. 134, 3819.

Guldner, H.H., H.J. Lakomek and F.A. Bautz, 1983, J. Immunol. Methods 64, 45.

Habets, W.J., D.J. De Rooij, M.H. Salden, A.P. Verhagen, C.A. Van Eekelen, L.B.A. Van De Putte and W.J. Van Venrooij, 1983, Clin. Exp. Immunol. 54, 265.

Kurata, N. and E.M. Tan, 1976, Arthritis Rheum. 19, 574.

Laemmli, U.K., 1970, Nature 227, 680.

Lakomek, H.J., N. Sablotni, H.J. Hagedorn and H.L. Kruskemper, 1983, J. Clin. Chem. Clin. Biochem. 21, 621.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, J. Biol. Chem. 193, 265.

Maini, R.N., P.J. Charles and P.J.W. Venables, 1985, Scand. J. Rheumatol. 56 (suppl.), 49.

Renart, J., J. Reiser and G.R. Stark, 1979, Proc. Natl. Acad. Sci. U.S.A. 76, 3116.

Sharp, G.C., W.S. Irwin, E.M. Tan, R.G. Gould and H.R. Holman, 1972, Am. J. Med. 52, 148.

Tuffanelli, D.L., 1983, Dermatol. Clin. 1, 517.

Van Venrooij, W.J. and W.J. Habets, 1985, Scand. J. Rheumatol. 56 (suppl.), 32.

Weiss, R.A., H.S. Mogavero, D.R. Synkowski and T.T. Provost, 1983, Ann. Allergy 51, 135.