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T B Okarma, ... , J A Krueger, H R Holman

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### **Research Article**

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# Analysis of Speckled Fluorescent Antinuclear Antibody Test Antisera Using Electrofocused Nuclear Antigens

THOMAS B. OKARMA, JUDITH ANN KRUEGER, and HALSTED R. HOLMAN, *Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, Divisions of Gerontology and Immunology, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305*

**ABSTRACT** Antibodies to different components of the extractable nuclear antigen (ENA) have been thought to be serological markers for clinical subsets of rheumatic diseases. However, incomplete characterization and standardization of antigenic components such as ribonucleoprotein (RNP), Sm, and SS-B (Ha), and the multiplicity of autoantibodies produced by different patients have confounded correlations between autoantibody specificity and disease subsets.

This study describes the preparative separation of the antigens Sm, RNP, and SS-B (Ha) by electrofocusing and their use in a rocket electrophoretic assay that in one step identifies and quantifies the multiple reactivities of patient sera exhibiting the speckled FANA pattern. Preparative electrofocusing generates milligram quantities of these antigens with retention of their immunologic and biochemical characteristics, facilitating further study of their biological properties and relationships to disease subsets.

## INTRODUCTION

The production of different antinuclear autoantibodies by patients with rheumatic disease undergirds the assumption of an autoimmune pathogenesis. Certain of the antibodies correlate with diagnosis: anti-DNA antibody with systemic lupus erythematosus (SLE),<sup>1</sup> and anti-IgG antibody with rheumatoid arthritis. Furthermore, antibodies to a crude nucleoplasm extract, called the extractable nuclear antigen (ENA), which yield a speckled pattern on the fluorescent antinuclear antibody test (FANA), have been associated with particular clinical subsets of SLE (1); these include antibodies to Sm with classical systemic lupus erythematosus (2), to ribonucleoprotein (RNP) with mixed connective

tissue disease (3), and to SS-B (Ha) with the sicca syndrome (4).

These associations, however, have been challenged (5-9). The lack of accord may be due to variation in preparations of ENA, in the array of autoantibodies present in the patients' sera, and in assay methods. Clinical-correlative studies have used impure and uncharacterized saline nuclear extracts as antigen source, many components of which react with sera of rheumatic disease patients. Common assays (hemagglutination, immunodiffusion, or counterimmunoelectrophoresis) do not clearly differentiate between specificities in reactive sera; specificity is inferred from cross reactivity with "reference" sera or loss of reactivity after enzyme treatment. Understanding the relationships between serological reactivities and their pathogenic, prognostic, and therapeutic implications will require (a) sufficient quantities of purified antigens, (b) demonstration of homogeneity of antibody specificity among sera that react with particular nucleoplasm components, and (c) a quantifiable assay that distinguishes between the multiple antigenic specificities that yield the speckled FANA pattern.

This paper describes the preparative separation of three components of ENA. We show that different individuals with similar antibody specificities react with an identical set of peptides and we illustrate the use of these separated antigens in a simple rocket assay that differentiates anti-ENA specificities and titers in one step.

## METHODS

**Preparation of nuclei.** Calf thymus nuclei were prepared according to a modification of the method of Allfrey et al. (10). Rat liver nuclei were obtained according to Teng et al. (11).

**Nuclear fractionation.** ENA was prepared from purified calf thymus or rat liver nuclei either by a modification of the method of Sharp et al. (3) or by pooling successive saline/EDTA-Tris buffer extraction supernates of homogenized nuclei according to Hwang and Hwang (12). Chromatin from calf thymus nuclei was prepared according to a modification of the method of Levy et al. (13).

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<sup>1</sup> Abbreviations used in this paper: ENA, extractable nuclear antigen; FANA, fluorescent antinuclear antibody test; RNP, ribonucleoprotein, SLE, systemic lupus erythematosus.

**Analytical electrofocusing.** Analytical electrofocusing in thin-layer polyacrylamide gels was performed according to LKB manufacturer's instructions (application note 250, December 1977. LKB Instruments, Inc., Rockville, MD) with modification.

Protein staining and preservation followed LKB application note RB423 and nucleic acid staining used the procedure of McMaster and Carmichael (14). Electrofocused acrylamide strips were frozen by rapid immersion in liquid nitrogen using the support tray of the LKB staining kit and were stored at  $-20^{\circ}\text{C}$  in an airtight container with desiccant. A typical run produced 24 identical electrofocused strips, one of which was stained for protein, another stained for nucleic acid, and the remaining 22 snap frozen for subsequent crossed immunoelectrophoresis.

**Crossed immunoelectrophoresis.** Crossed immunoelectrophoresis used frozen Pagplate strips according to LKB application note 269 with modifications (15). The resulting precipitin patterns were visualized by dehydration and staining according to the procedure of Weeke (16).

**Preparative electrofocusing.** Preparative flat bed electrofocusing using granulated gel was performed according to the LKB application note 198. The antigenic specificity contained in each separate fraction was determined by rocket immunoelectrophoresis.

**Immunological determinations.** Rocket immunoelectrophoresis and counterimmunoelectrophoresis were performed according to Weeke (16) and Kurata and Tan (17) using whole patient antiserum.

The Ouchterlony immunodiffusion technique was performed according to Campbell et al. (18)

The passive hemagglutination technique of Stavisky (19) was used as modified by Sharp et al. (20). Fluorescent antinuclear antibody assays were performed according to Tan (21) using mouse liver as substrate.

Whole unfractionated serum from patients with systemic lupus erythematosus, mixed connective tissue disease, or Sjogren's syndrome obtained from the Immunology Serum Bank were used as reference standards. Samples were inactivated at  $56^{\circ}\text{C}$  30 min and stored at  $-20^{\circ}\text{C}$ . Serums used as anti-ENA standards in this study were negative for DNA antibody by radioimmunoassay and for rheumatoid factor by latex fixation reaction. FANA-negative control sera were obtained from healthy laboratory volunteers.

## RESULTS

**Quantitation of ENA antibody titer in patient sera by rocket immunoelectrophoresis.** A direct linear relationship between immunoprecipitate (rocket) height and antigen concentration has been documented for various antigen-antibody systems with rocket immunoelectrophoresis when antibody concentration in the gel is held constant (16, 22). This linear relationship has previously formed the basis for quantitation of antigens using specific antibody as detecting reagent in this technique. In this study, however, we were seeking to identify the specificities and concentrations of unknown patient antibodies dispersed in the gel that react with known quantities of purified antigen. At constant antigen concentration, immunoprecipitate height is known to be inversely proportional to antibody concentration in the gel, but this relationship has

not been quantitated. In the ENA system, we found an exponential decrease in the height of the immunoprecipitate as the antibody concentration in the gel was increased linearly (Fig. 1). This semilogarithmic relationship is not unique for the ENA system; recalculation of the data of Weeke (16) and Laurell (22) reveals an identical inverse semilogarithmic relationship between antiserum concentration in agarose gel and rocket height when antigen concentration is held constant.

This relationship is represented graphically in the insert of Fig. 1. Semilogarithmic expression of serum concentration in agarose gel plotted against peak height results in linearity. This relationship has been found to hold for all anti-Sm, anti-RNP, and anti-Ha specificities tested. Thus a standard curve can be constructed with reference antisera and antigen, and the antibody concentration of unknown serum samples measured by comparison to the immunoprecipitate heights for known dilutions of the reference sera. Although this technique could quantitate ENA antibody using crude nucleoplasm as antigen, we sought to refine its use by isolating the individual antigenic components, which could then be tested individually in separate wells on the same plate to determine both antigenic specificity and antibody concentration in one step.

**Identification of antigens in electrofocused nucleoplasm by crossed immunoelectrophoresis.** Crossed immunoelectrophoresis of electrofocused nucleoplasm defined the pH range of each antigen system and assessed the uniformity of antibody specificities among antisera of different individuals. Fig. 2 shows the results of crossed immunoelectrophoresis of electrofocused nucleoplasm on agarose plates containing whole antiserum from four different patients. Fig. 2A displays the immunoprecipitate resulting from the electrofocusing of electrofocused nucleoplasm into agarose containing  $25\ \mu\text{l/ml}$  of serum with anti-RNP specificity. The immunoprecipitate is localized to a broad range between pH 5.1 and 6.0 (large dark arrow). The small peak at the application site (open arrow) is presumably an aggregate of denatured antigenic material too large to enter the acrylamide gel; its position always corresponds to the application site and does not reflect the pI of antigenically active material. Fig. 2B displays the immunoprecipitate resulting from a serum with anti-Sm specificity at a concentration of  $100\ \mu\text{l/ml}$ . The immunoprecipitate (large arrow) occupies a pH range of 5.2 to 6.8, considerably overlapping the pH range of the RNP antigen but extending farther toward neutrality.

Fig. 2C displays the pattern resulting from patient serum with specificities for Sm, RNP, and Ha antigens. The Sm/RNP immunoprecipitate extends from pH 5.4

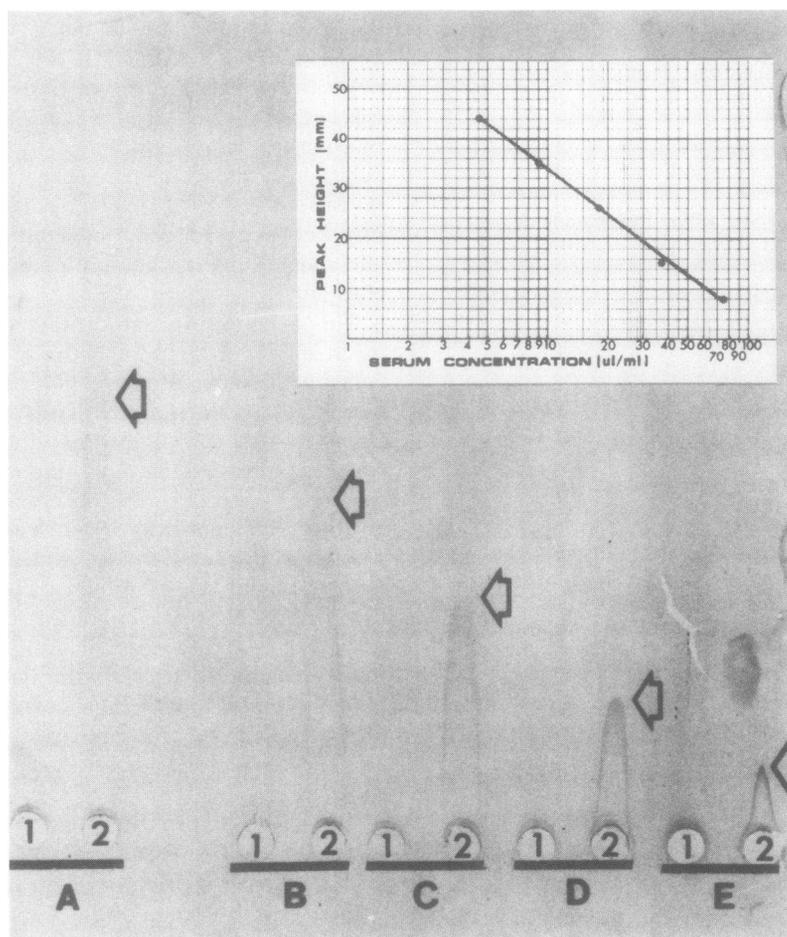
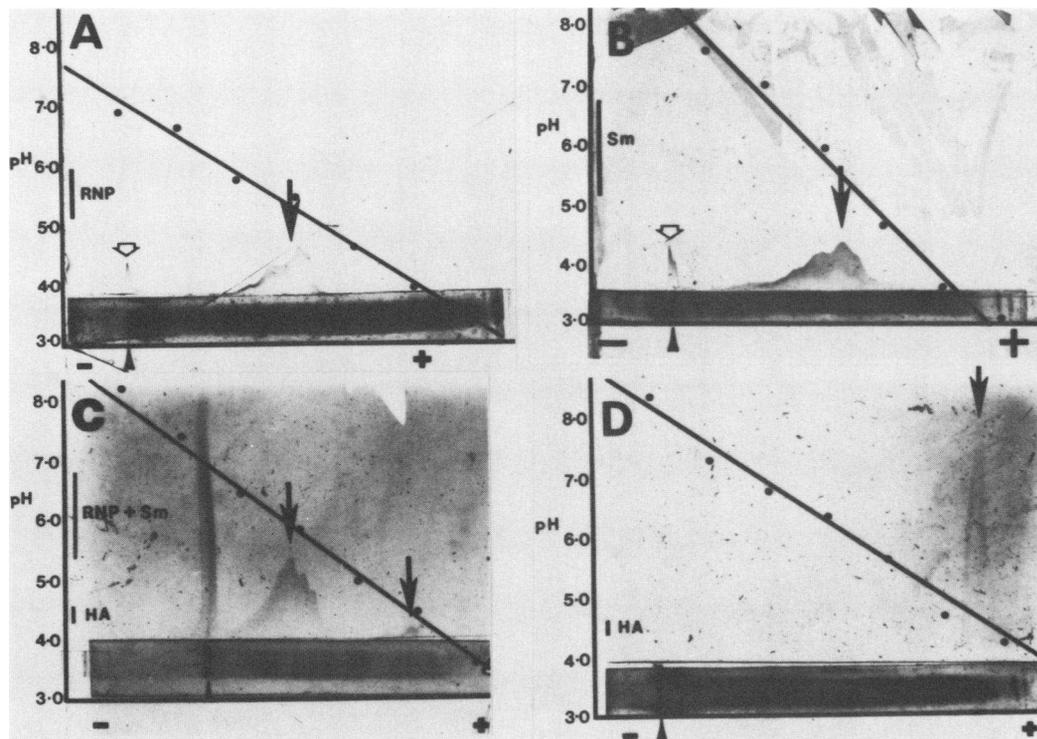


FIGURE 1 Quantitation of patient antibody concentration by rocket immunoelectrophoresis. Tracks A-E contain, respectively, 4.5, 9.0, 18.0, 37.0, and 75.0  $\mu$ l of monospecific RNP antiserum per milliliter of agarose. In each track, well 1 contains 25  $\mu$ l of nucleoplasm at a concentration of 50 mg/ml digested by ribonuclease (which destroys RNP antigenicity); well 2 contains an identical amount of nucleoplasm not exposed to ribonuclease. Rocket immunoprecipitate height decreases as antiserum concentration in agarose is increased. The insert presents these data in graphic form and shows the relationship between peak height and serum concentration to be semilogarithmic.

to 6.8, and the Ha immunoprecipitate is located between pH 4.3 and 4.6 (dark arrows). Fig. 2D shows the precipitate resulting from a patient with only anti-Ha specificity. One peak appears at pH 4.4–4.7 (large arrow). These pH ranges of antigen activity were identical with sera from six patients with anti-RNP specificity, four with anti-Ha specificity, and five with anti-Sm specificity. This technique thus identified the pH range of antigenically active components of nucleoplasm (ENA) and demonstrated uniformity of antibody specificities among these individuals. Negative controls included electrofocused ENA run against normal serum, and strips electrofocused without antigen run against reference anti-Sm and anti-RNP serum.

*Isolation of Sm, RNP, and Ha antigens from nucleoplasm.* With the pH range of antigen activity defined by analytical electrofocusing and crossed immunoelectrophoresis, gram quantities of nucleoplasm were then electrofocused in granulated gel. Preparative electrofocusing of 500 mg of nucleoplasm resulted in recoveries of 15 mg of Sm antigen, 30 mg of Sm/RNP complex, and 10 mg of Ha antigen. The stained paper print of a typical preparative electrofocusing run for 500 mg of nucleoplasm is displayed in Fig. 3. The pH gradient is linear in the pH range, 4.0 to 8.5. Each fraction was assayed for antigen activity using reference antisera in the rocket technique. Appearing in Fig. 3 are the composite results: Ha activity found



**FIGURE 2** Crossed immunoelectrophoretic patterns obtained with patient reference antisera. A. Whole, unfractionated patient antiserum with RNP specificity was applied to a glass plate at a concentration of 25  $\mu\text{l/ml}$  agarose and allowed to congeal. Nucleoplasm at a concentration of 50 mg/ml applied in a total volume of 20  $\mu\text{l}$  was electrofocused in acrylamide and then electrophoresed into the antiserum-containing agarose. Two immunoprecipitate peaks resulted. One peak (denoted by the open arrow) occurred at the application point, and its position was independent of pH. The second immunoprecipitate (large arrow) repeatedly occurred between the range of 5.1 to 6.0. The pH profile of the electrofocused strip is depicted by the graph. B. Nucleoplasm was electrofocused under conditions identical to those in A, and electrophoresed into agarose containing 100  $\mu\text{l/ml}$  of whole unfractionated patient antiserum with Sm specificity. Again, note the artifact peak occurring at the application point (open arrow). The second immunoprecipitate (large arrow) occurred in the pH range of 5.2–6.8. This pH range for antigen activity was reproducible for different antisera exhibiting Sm specificity. C. Electrofocused nucleoplasm was electrophoresed into agarose containing 50  $\mu\text{l/ml}$  of whole, unfractionated patient serum with anti-Sm, anti-RNP, and anti-Ha specificities. Note the nonspecific immunoprecipitate (open arrow) at the application site. The broad immunoprecipitate (large arrow) representing Sm and RNP activity was again found in the range of 5.4 to 6.8. A third peak occurred at a pH of 4.3–4.6 and identified Ha activity. D. Electrofocused nucleoplasm was electrophoresed into agarose-containing patient antiserum monospecific for Ha. Electrofocusing conditions were identical to those for Figs. A–C. A single immunoprecipitate peak can be seen in the pH range of 4.4 to 4.7. Note the absence of the artifact immunoprecipitate at the point of application. It was repeatedly found that application point immunoprecipitates occurred only with antisera with specificity for either Sm or RNP. It was therefore concluded that the application precipitate included aggregates with Sm and RNP activity. Neither the application artifact peaks nor the antigen-antibody precipitates were seen using normal serum or pathological sera nonreactive with nucleoplasm.

only in fractions 22 and 23 with a pH range of 4.5 to 4.9, RNP activity in fractions 15–21 with a pH range of 5.0 to 6.1, and Sm activity in fractions 11–21 with a pH range of 5.0 to 6.8. These values are in close agreement with those obtained using analytical electrofocusing and crossed immunoelectrophoresis (Fig.

2 A–D). Ouchterlony analysis using anti-RNP and anti-Sm sera confirmed the presence of Sm without RNP in fractions 11–14 of nucleoplasm and both Sm and RNP in fractions 15–21.

To verify antigen specificities, fractions 11–21 were each subjected to ribonuclease digestion and retested

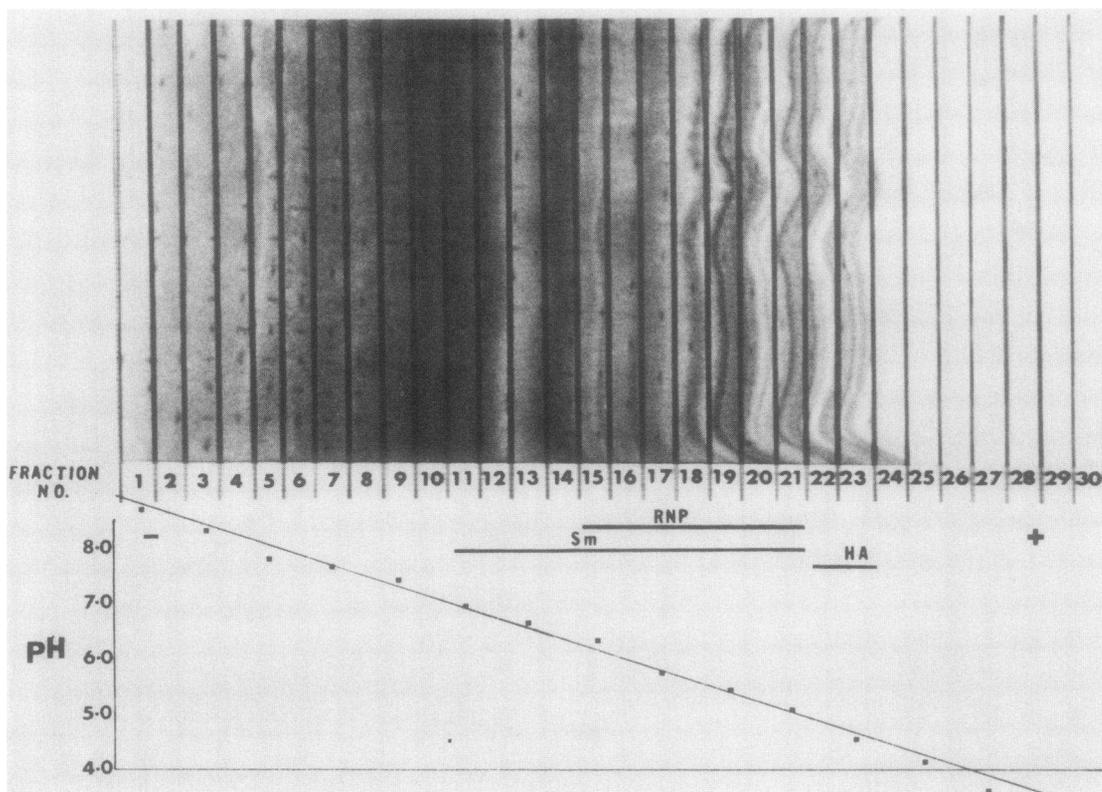


FIGURE 3 Preparative electrofocusing of nucleoplasm. Preparative electrofocusing was performed in granulated gel. A paper print of the electrofocusing protein bands appears at the top of the figure. The gel was divided into 30 fractions and material was eluted in disposable columns, dialyzed, lyophilized, and analyzed for antigen activity by rocket immunoelectrophoresis. The pH gradient is shown below the paper print and the fractions active for RNP, Sm, and Ha antigen activity are shown by the bars. The pH range for antigen specificities obtained in the preparative mode are in good agreement with the pH ranges obtained in analytical crossed immunoelectrophoresis (not shown). The application of 500 mg of nucleoplasm to the granulated gel resulted in a yield of 15 mg of Sm antigen, 30 mg of Sm/RNP complex, and 10 mg of Ha antigen.

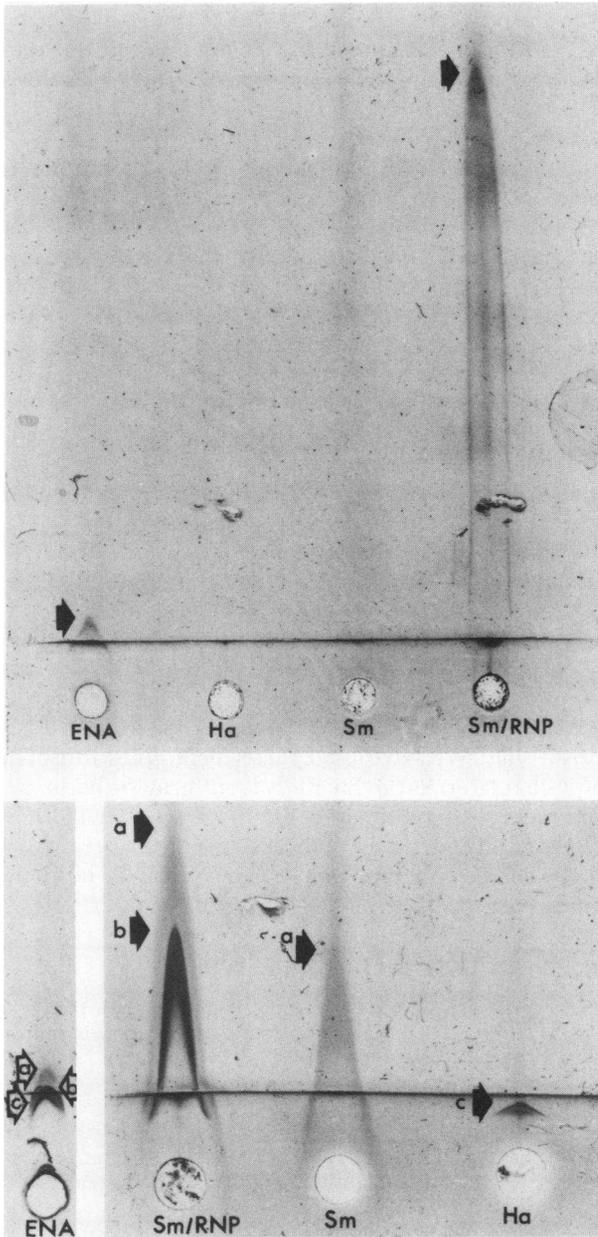
by rocket immunoelectrophoresis. The result was complete loss of reactivity with the RNP antisera but no loss with antibody to Sm. When 500 mg of nucleoplasm was digested with ribonuclease before preparative electrofocusing none of the 30 fractions was active against anti-RNP sera, but fractions within the pH range of 5.1 to 6.4 again reacted with antibody to Sm.

Thus, the preparative and analytical electrofocusing data both demonstrated (a) that Sm activity is present without RNP activity in the pH range of 6.0 to 6.8, (b) that both antigens occur together in the pH range of 5.0 to 6.0, and (c) that SS-B (Ha) antigen can be separated from both Sm and RNP at pH 4.5–4.9.

*Quantitation of antibody concentration and identification of antigenic specificity of patient sera by rocket immunoelectrophoresis using purified antigens.* Having established that rocket immunoelectrophoresis can quantitate antibody concentration using

crude ENA as antigen, we then sought to extend its utility toward the assignment of antigenic specificity by substituting antigens isolated by preparative electrofocusing.

Fig. 4 illustrates the immunoprecipitate patterns obtained with a representative antiserum to RNP (4A) and a serum reactive with all three antigens (Fig. 4B). Both sera gave 1:640 speckled FANA patterns and were negative for anti-DNA by hemagglutination and radioimmunoassay. However, they came from patients with very different clinical manifestations (mixed connective tissue disease in Fig. 4A; SLE with nephritis and malignant hypertension in Fig. 4B). The technique reveals differences between patient sera inapparent by the FANA reaction. The single immunoprecipitate above the native ENA well in Fig. 4A corresponds to RNP antibody as fractions containing only Ha or Sm do not react. Three immunoprecipitates above the na-



**FIGURE 4** Differentiation of speckled FANA antisera by rocket immunoelectrophoresis using antigens purified by preparative electrofocusing. **A.** Nuclear antigens separated by electrofocusing were electrophoresed into agarose containing 50  $\mu$ l/ml of antiserum with specificity for the RNP antigen with a speckled FANA titer of 1:640. Wells contain (left to right): nucleoplasm (ENA) before preparative electrofocusing; Ha antigen (fraction 22 of Fig. 3); Sm antigen (fraction 14 of Fig. 3); both Sm and RNP (fraction 15 of Fig. 3). All antigens were applied at 1 mg/ml in a total volume of 25  $\mu$ l. A single rocket precipitate is seen above the nucleoplasm and Sm/RNP wells only, indicating specificity for RNP. Peak height is increased above the Sm/RNP well because of purification by electrofocusing. Antibody concentration can be derived from a standard curve of antibody

tive ENA well in Fig. 4B correspond to antibodies to (a) Sm, (b) RNP, and (c) Ha antigens. The Sm/RNP fraction gives two precipitates. The Sm and Ha fractions give one each. The increased peak height of the precipitates above the purified antigen wells relative to the peak height above the native ENA wells in Fig. 4A and B indicates increased concentration (purification) of Sm and RNP antigens in those fractions (identical protein loads were applied to each well). The anti-Sm immunoprecipitate decreased in height from the Sm/RNP well to the Sm well in Fig. 4B because of a relatively decreased Sm antigen concentration in that fraction of the granulated gel containing Sm without RNP. The very short Ha immunoprecipitate reflects the extremely high concentration of Ha antibody relative to Sm and RNP antibodies. Dilution of the latter antiserum to a concentration within the linear portion of the standard curve would be required for quantitation of this antibody.

Three to five serum samples obtained from the Serum Bank for each of 10 patients with speckled FANA patterns drawn at different times over 18 mo were analyzed by this technique using purified antigens. Two patients had Sjogren's syndrome, three had SLE, and five overlap syndromes. Antibody concentration varied from 10- to 200-fold between samples from the same patient. No relationship was found between antibody concentration and disease activity. No variation in antibody specificity was detected over the 18-mo period in any patient despite fluctuations in disease activity, changes in drug therapy, and in two cases, progressive renal involvement in association with high-titered antibody to RNP.

## DISCUSSION

Clinical correlative studies in the rheumatic diseases often test the hypothesis that the presence or absence

dilutions vs. peak height for any purified antigen fraction. **B.** Nuclear antigens separated by electrofocusing were electrophoresed into agarose containing 50  $\mu$ l/ml of antiserum with specificities for the RNP, Sm, and Ha antigens that also gave a speckled FANA titer of 1:640, identical to that in A. Wells contain (left to right): nucleoplasm (ENA) before preparative electrofocusing, both Sm and RNP, Sm alone, and Ha antigen. All antigens were applied at 1 mg/ml in a total volume of 25  $\mu$ l. Three rocket immunoprecipitates are seen above the nucleoplasm well, two above the Sm/RNP well and one each above the Sm and Ha wells, indicating specificity for all three antigen systems. The Sm rocket ("a" above the Sm/RNP well) decreased in height from the Sm/RNP well to the Sm well because of a relative decrease in Sm concentration in that fraction of the granulated gel containing Sm without RNP. The anti-Ha concentration is high relative to anti-Sm and anti-RNP, and this serum would have to be diluted further for quantitation of anti-Ha concentration.

of antibodies to certain "self" antigens predicts patient outcome. The relationship between complement fixing, antidouble-stranded DNA antibodies, and nephritis is well recognized and the DNA-anti-DNA immune complex may play an etiologic role in lupus nephritis. These relationships were developed after the DNA antigen was available in large quantities in purified form. Until recently, however (23-27), there has been little quantitative information on the nature of the ENA antigen, either in relation to its biological function or role in disease pathogenesis. The literature is conflicting on the relationship between antibodies to ENA components and disease outcome (1-9); the use of uncharacterized nuclear extracts as antigen sources and multispecific patient antisera certainly contribute to this controversy.

This paper describes the use of ENA antigens partially purified by preparative electrofocusing in a rocket immunoelectrophoretic assay using whole unfractionated patient antisera. Crossed immunoelectrophoresis results in distinctive immunoprecipitate patterns when analytically electrofocused antigens are electrophoresed directly into agarose containing different patient antisera. The patterns were reproducible for the multiple sera from 10 different patients and served to identify the pH range of each antigen. The discriminatory capacity of the technique was verified by Ouchterlony analysis, counterimmunoelectrophoresis using enzyme-treated antigens, and hemagglutination. Cell extracts inactive by the latter methods were nonreactive, as were FANA-negative sera. These data suggest that anti-Sm, anti-Ha, and anti-RNP antisera from different individuals consistently react with the same group of proteins and/or RNA species. Uniformity of antibody specificities between individuals with similar anti-ENA reactivities validates the use of purified antigens for clinical correlative studies.

Antigens separated by preparative electrofocusing retain their immunologic activity and can be generated in milligram quantities for use in clinical correlative studies. Rocket immunoelectrophoresis is based upon the electrophoretic migration of antigens through a gel impregnated with antiserum at a pH that minimizes the charge (migration) of the detecting antibody. As the antigens are driven through the gel they form soluble immune complexes that migrate more slowly than the free antigen. When the complexes aggregate and precipitate, a "rocket" is formed, and its height no longer changes with continued application of current. Unique precipitates are formed for each antigen-antibody system that facilitates the identification of antisera with multiple specificities. Rocket immunoelectrophoresis offers greater resolution and precision than other methods, yet demands technical expertise and standardized antigens for quantitation.

The rocket technique is 100-1,000 times as sensitive as Ouchterlony immunodiffusion but less sensitive than hemagglutination or counterimmunoelectrophoresis. The assay requires 20-50  $\mu$ g of antigen per well with 0.25-0.5 ml whole serum per 10 ml of agarose.

The prospective application of this technique to more precise clinical correlative studies may clarify the relationship between anti-ENA antibodies and disease attributes. Our retrospective analysis did not support an association between disease activity and anti-Sm, anti-RNP, or anti-Ha titer. We found no evidence for a relationship between antibody specificity and either disease severity, response to therapy, or particular end-organ involvement.

Furthermore, preparative electrofocusing techniques also open avenues for accurate characterization of the antigens, study of their biological and pathological roles, and exploration of their potential involvement in eukaryotic gene expression. The RNA components of Sm and RNP antigens (small nuclear RNA-Sn RNA) bear sequence homology to nucleotide sequences across splice junction boundaries of primary RNA transcripts (28). Isolation of these Sn RNAs and their associated peptides in undenatured form would facilitate direct testing of their putative role in RNA processing. We have used these techniques to isolate and characterize the Sn RNP from growth hormone-producing rat pituitary cells *in vitro* and will show elsewhere<sup>2</sup> that these Sn RNPs specifically recognize intervening sequences of the cloned growth hormone gene.

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