

# ANTIBODIES FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS RECOGNIZE DIFFERENT EPITOPES OF A SINGLE HETEROGENEOUS NUCLEAR RNP CORE PROTEIN

## Possible Role of Cross-Reacting Antikeratin Antibodies

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**Antibodies to recombinant heterogeneous nuclear RNP core protein A1 were detected in sera from 27 of 58 patients with rheumatoid arthritis (RA) and from 7 of 31 patients with systemic lupus erythematosus, by immunoblotting and enzyme-linked immunosorbent assay. Protein A1 consists of 2 distinct domains: The N-terminal sequence is identical to a single-stranded DNA binding protein termed UP1, and the C-terminal domain shows a partial homology with keratin. All 7 A1-positive systemic lupus erythematosus sera reacted with UP1, whereas 9 of the 27 A1-positive RA sera did not. In RA, anti-A1 activity was significantly associated with antikeratin antibodies (AKA); these antibodies were present in 23 of 27 A1-positive sera and 10 of 31 A1-negative sera ( $P < 0.01$ ). Immunoabsorption with recombinant protein A1 resulted in a significant reduc-**

**tion of AKA titers in 6 of 10 RA sera tested, suggesting that AKA from RA patients may cross-react with the C-terminal portion of the heterogeneous nuclear RNP protein A1.**

Antibodies to heterogeneous nuclear ribonucleoprotein (hnRNP) particles have been found in patients with mixed connective tissue disease (MCTD) (1), but little is known about the autoantigenic properties of the individual peptides involved. In eukaryotic cells, preprocessed messenger RNA (hnRNA) are found associated with proteins to form hnRNP particles. The majority of the protein component is represented by a family of structurally related polypeptides (hnRNP core proteins), which are basic in charge and rich in the amino acid glycine; these core proteins have been suggested to play a role in RNA splicing (2). One of the main constituents of hnRNP core proteins is a 34-kd protein termed A1. As recently revealed by complementary DNA (cDNA) cloning, this protein consists of 2 distinct domains with different structures (3). The N-terminal domain (residues 2–196) has a sequence identical to that reported for a single-stranded DNA binding protein previously termed UP1 (4). The C-terminal portion is a glycine-rich polypeptide that shows a partial homology with human epidermal keratin (3,4).

In recent studies, the use of purified protein A1 obtained by recombinant DNA techniques has allowed detection of autoantibodies directed toward this protein. Anti-A1 antibodies have been found in 23% and 37% of systemic lupus erythematosus (SLE) patients in 2 different studies (5,6). Furthermore, antibodies reacting with protein A1 were found in sera from patients with adult-onset rheumatoid arthritis (RA) (5)

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and patients with juvenile arthritis (6). Results of the present study indicate that antibodies from patients with SLE and RA may react with different epitopes of protein A1 and that cross-reacting antikeratin antibodies (AKA) may partially account for anti-A1 activity in RA sera.

## PATIENTS AND METHODS

**Patients.** Serum samples were obtained from 31 patients (27 women and 4 men) with SLE, as defined by the American Rheumatism Association (ARA) 1982 revised criteria (7), and 58 patients (47 women and 11 men) with RA, as defined by the ARA 1987 revised criteria (8). The mean age of the SLE patients was 36 years (range 16–62) and that of the RA patients was 45 (range 16–73). Twenty-five healthy volunteers (20 women and 5 men) were studied as a control group; their mean age was 42 (range 21–63). Informed consent was obtained from all subjects.

**Antigen purification procedure.** Heterogeneous nuclear RNP core protein A1 was overexpressed as unfused 34-kd protein in *Escherichia coli* harboring a recombinant expression vector (pRC23) that carries the A1 cDNA coding sequence (3). For the expression of the N-terminal domain (UP1), the C-terminal portion of A1 cDNA was removed from the original plasmid using appropriate restriction endonucleases, and the truncated product was inserted into pRC23 as previously described (9). The expression of recombinant proteins A1 and UP1 was determined by Coomassie blue staining of sodium dodecyl sulfate (SDS)–polyacrylamide gels and by Western blot analysis. The recombinant proteins were purified by affinity column procedures as previously described (9), and the final preparations were found to be homogeneous by SDS–polyacrylamide gel electrophoresis and by amino acid sequencing.

**Rabbit antibodies against hnRNP A1 protein.** A New Zealand white rabbit was injected intramuscularly, 6 times, at 15-day intervals, with 200  $\mu$ g of recombinant hnRNP protein A1 in 0.5 ml of sterile saline solution plus 0.5 ml of Freund's adjuvant (complete for the first immunization and incomplete for the following immunizations) (10). The final titer of anti-A1 antibody was 1:500,000, as determined by enzyme-linked immunosorbent assay (ELISA).

**Immunoblotting technique.** Four micrograms of recombinant hnRNP A1 or 150  $\mu$ g of crude nuclear extract from HeLa cells (11) was mixed in 62.5 mM Tris HCl, pH 6.8, containing 5% 2-mercaptoethanol, 2% SDS, and 0.1% bromophenol blue, heated at 100°C for 5 minutes, and then centrifuged. The supernatant was loaded on a 10% SDS–polyacrylamide gel and run at 30 mA for 4 hours. The gel was blotted onto nitrocellulose as described by Towbin et al (12). The nitrocellulose sheet was cut into strips, washed extensively, and processed as described previously (13).

**ELISA.** Serum antibodies reacting with protein A1 and UP1 peptide were detected and quantitated by ELISA as previously described (14). Briefly, the purified protein was diluted at 2  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6, plated (100  $\mu$ l/well), and incubated at 4°C overnight. Plates were then blocked with 10% fetal calf serum in phosphate buffered

saline (PBS), washed in PBS–Tween 20, and incubated for 2 hours with patient sera diluted 1:100. After additional washings, plates were incubated for 2 hours with peroxidase-conjugated antibodies to all human Ig classes (rabbit anti-human IgA, IgM, IgG,  $\kappa$ ,  $\lambda$ ; Dakopatts, Copenhagen, Denmark), washed, and exposed to the substrate solution as described (14).

The optical density (OD) was measured at 492 nm, using a Titertek multiscan reader (Flow Laboratories, Opera, Italy). Values more than 3 standard deviations above the mean in normal controls were considered positive. In a previously reported study, all but 1 patient serum with OD above this value were shown to react with purified protein A1 by immunoblotting assays, while no patient sera with lower values reacted with this protein, nor did any control serum (5).

**Immunoabsorption.** Two hundred micrograms of purified recombinant protein A1 was diluted in 200  $\mu$ l of patient serum (diluted 1:5 in 150 mmoles/liter PBS, pH 7.5) for 2 hours at room temperature, then incubated overnight at 4°C. After incubation, the samples were centrifuged at 13,000g for 20 minutes at 4°C, and the supernatant was tested by immunofluorescence for the presence of autoantibodies.

**Immunofluorescence studies.** AKA in patient sera were detected by immunofluorescence on human epidermis from skin biopsy samples that had been obtained for lupus band testing or other immunofluorescence studies and had been found to be normal. Unfixed, 5  $\mu$ m-thick cryostatic sections were mounted onto glass slides, incubated for 30 minutes with patient serum diluted in PBS, and washed 3 times in PBS. Fluorescein-conjugated rabbit anti-human Ig antibodies were then added for 20 minutes, prior to 3 additional washings in PBS. To exclude the possibility of in vivo binding of autologous antibodies, a negative control, using PBS alone instead of patient serum, was routinely tested along with each skin biopsy. Distinguishable epidermal fluorescence at a serum dilution of  $\geq 1:10$  was considered positive. No detectable fluorescence was found in controls. Positive samples showed a laminar fluorescence pattern (15) similar to that obtained using a monoclonal mouse anti-human keratin antibody (Dako-Keratin; Dakopatts).

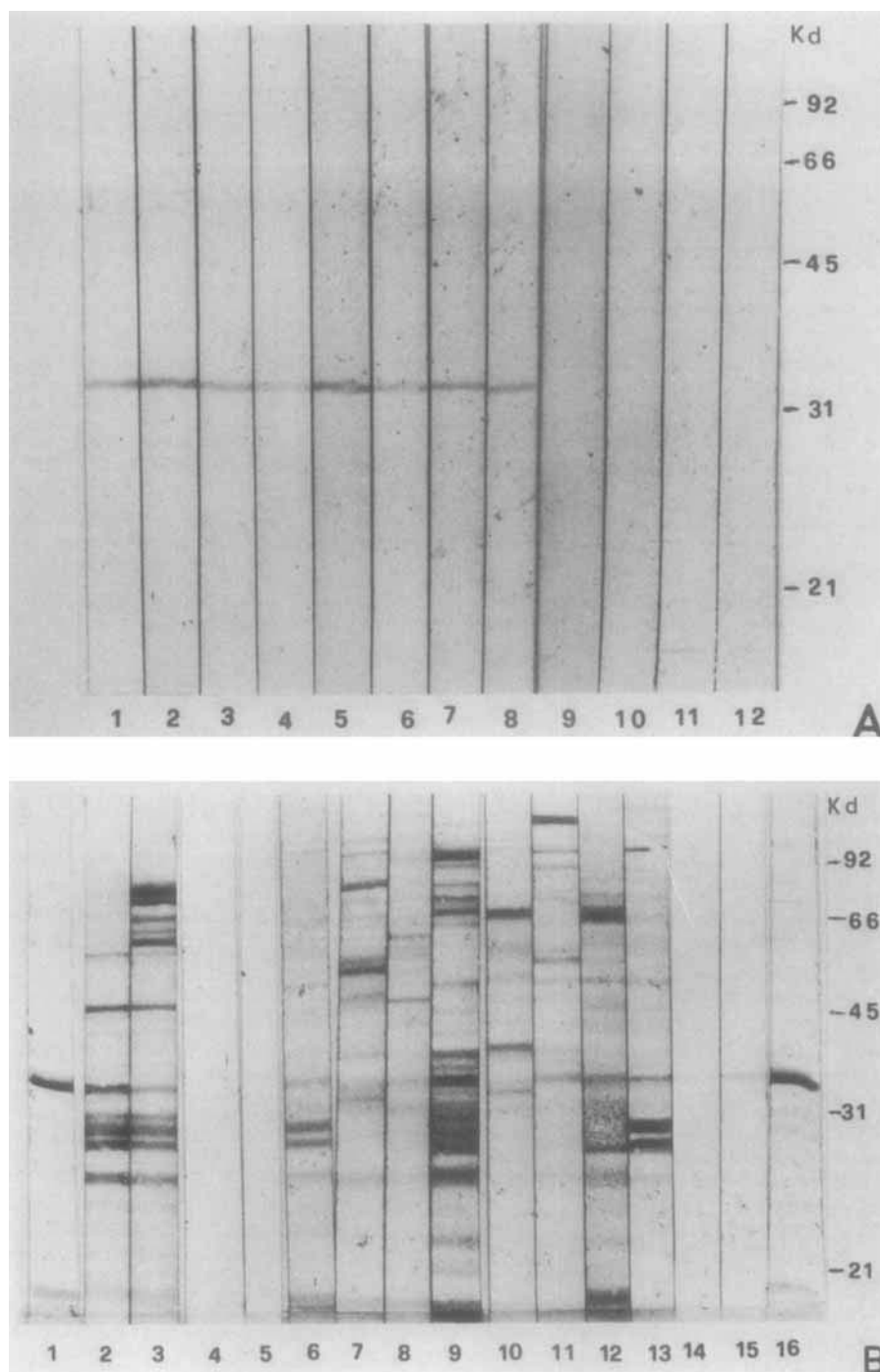
Antinuclear antibodies (ANA) were detected by immunofluorescence on cultured HEp-2 cells, and antibodies to double-stranded DNA (anti-dsDNA) were detected using commercially available *Crithidia luciliae* preparations (Delta Biologicals, Pomezia, Italy). ANA and anti-dsDNA were considered positive when titers were  $\geq 1:80$  and  $\geq 1:10$ , respectively.

**Statistical analysis.** The frequency distributions of various autoantibodies in anti-A1-positive patients and anti-A1-negative patients were compared by chi-square and Fisher's exact test.

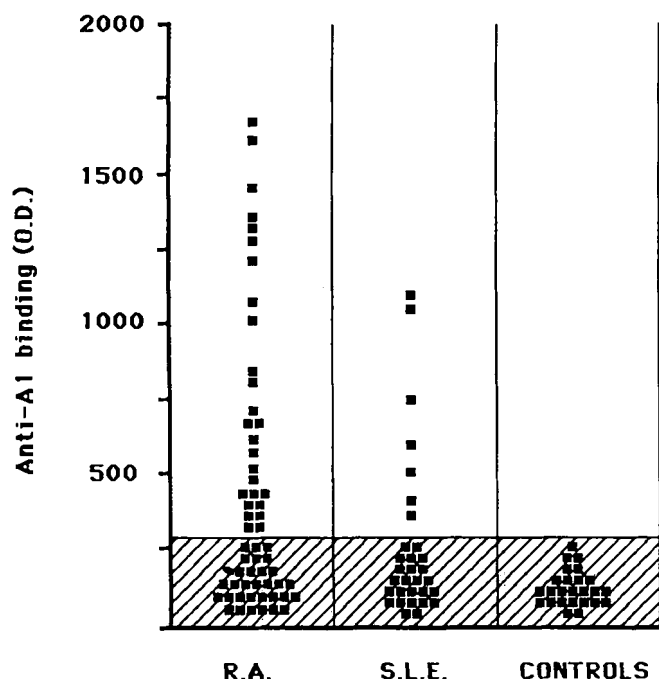
## RESULTS

### Detection and quantitation of anti-A1 antibodies.

Human sera were tested for binding to protein A1 in immunoblot assays using a purified antigen preparation (Figure 1A). Sera reacting with purified protein in



**Figure 1.** Detection of antibodies to heterogeneous nuclear RNP (hnRNP) core protein A1 by immunoblotting assays. **A**, Binding of human sera to purified recombinant protein. Lanes 1–4, rheumatoid arthritis (RA) sera; lanes 5–8, systemic lupus erythematosus (SLE) sera; lanes 9–12, control sera. **B**, Binding of human sera to hnRNP core protein A1 present in crude nuclear extract, as compared with rabbit anti-A1 antibodies. Lanes 1 and 16, rabbit anti-A1 antibody; lanes 2, 6, 8, 11, and 15, positive RA sera; lanes 7 and 14, negative RA sera; lanes 3, 9, 12, and 13, positive SLE sera; lane 10, negative SLE serum; lanes 4 and 5, control sera. The second antibody was directed to rabbit Ig in lanes 1 and 16, and to all human Ig classes in lanes 2–15.



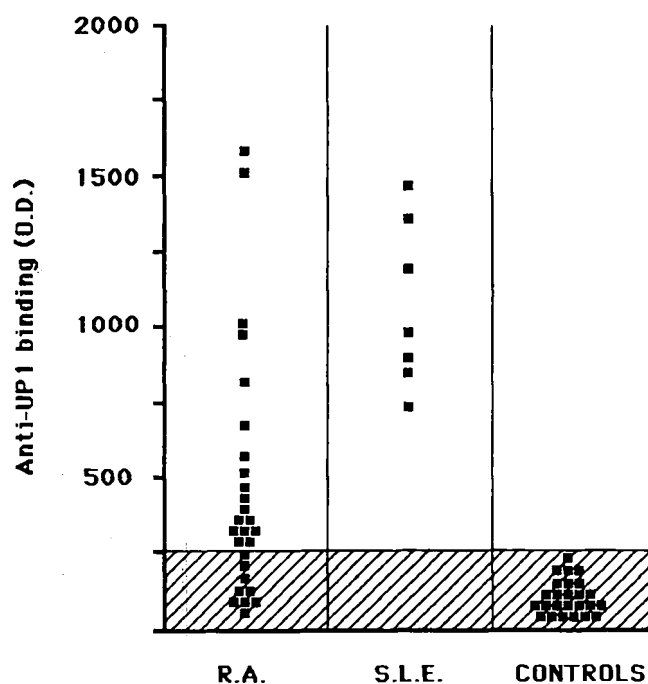
**Figure 2.** Binding of rheumatoid arthritis (R.A.) and systemic lupus erythematosus (S.L.E.) patient sera and normal control sera to purified recombinant heterogeneous nuclear core protein A1, as determined by enzyme-linked immunosorbent assay. Optical density (O.D.) was measured at 492 nm. Shaded area represents the range of values considered negative.

this assay were also found to bind protein A1 in immunoblot assays using crude nuclear extract (Figure 1B). None of the normal control sera reacted with purified recombinant protein or protein A1 in crude nuclear extract.

The same purified antigen preparation used for immunoblotting was used in the ELISA. Positive values were found in 7 of the SLE patients (23%) and in 27 of the RA patients (47%). RA sera showed the highest binding values (Figure 2).

**Binding to the N-terminal domain (UP1).** To further characterize the antibody specificity in SLE and RA patients, all A1-positive sera were tested, by ELISA, for selective binding to the N-terminal region (UP1). All of the A1-positive SLE sera showed a strong reaction with the UP1 peptide, while 9 of 27 A1-positive RA sera did not. The level of UP1 binding was generally higher in the SLE sera (Figure 3).

**Association with ANA and AKA.** Binding to A1 correlated with ANA and AKA positivity in patients with rheumatoid arthritis, but no relationship was found between anti-A1 and rheumatoid factor. In SLE



**Figure 3.** Binding of anti-A1-positive patient sera and normal control sera to the N-terminal region of recombinant hnRNP core protein A1 (UP1). See Figure 2 for definitions.

patients, no correlation was found between anti-A1 and any of the other antibodies tested (Table 1).

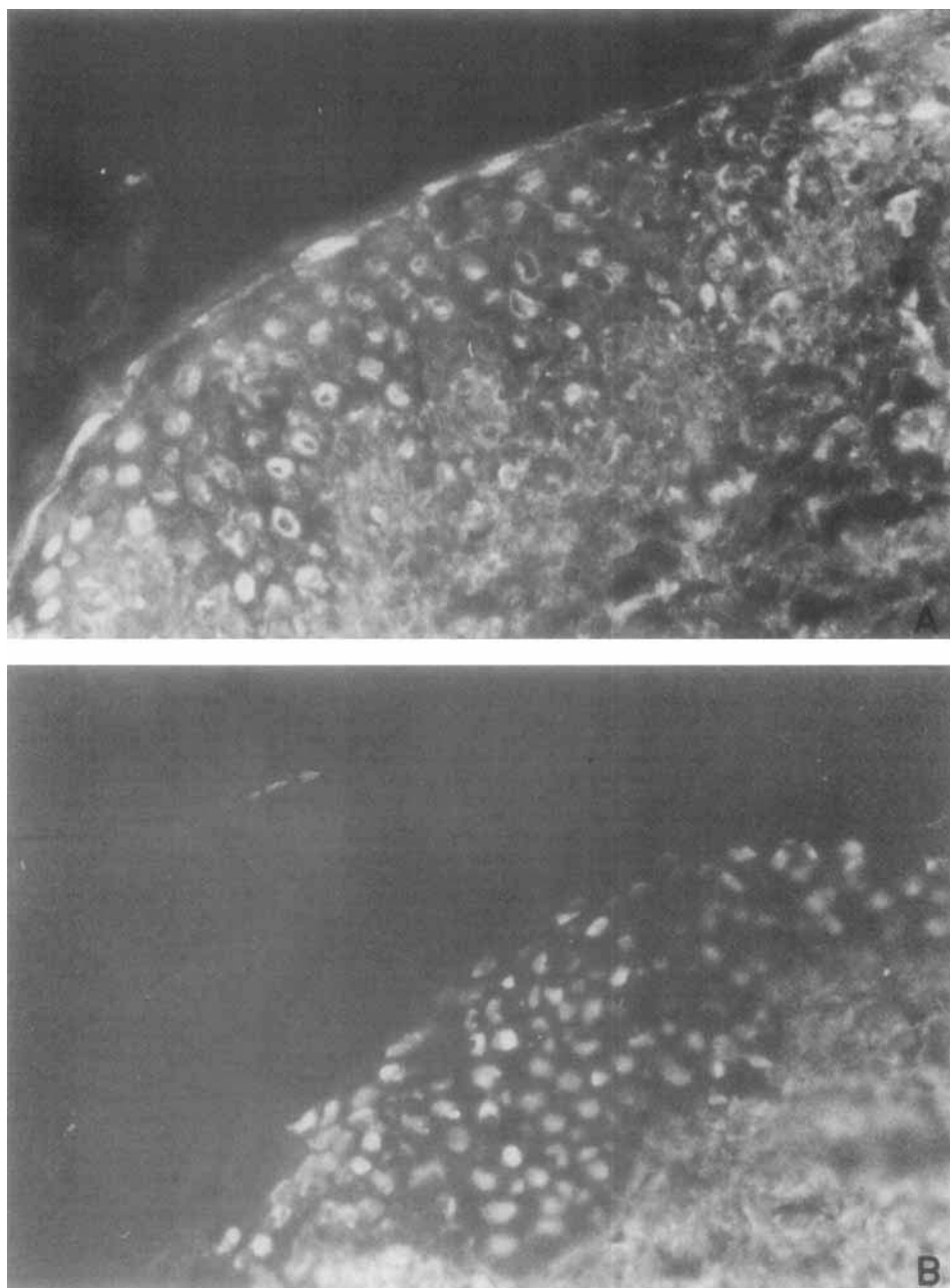
To determine the role of anti-A1 antibodies in ANA-positive RA, ANA titers in 10 ANA-positive sera were measured before and after immunoabsorption with purified recombinant protein A1. None of the sera showed a significant change in ANA titer following immunoabsorption. The same procedure was used to determine whether AKA cross-react with hnRNP protein A1. Six of 10 anti-A1-positive sera had a

**Table 1.** Frequency distribution of antinuclear antibodies, antikeratin antibodies, anti-double-stranded DNA, and rheumatoid factor according to the presence or absence of anti-A1 antibodies\*

	SLE patients			RA patients		
	ANA	AKA	Anti-dsDNA	ANA	AKA	RF†
Anti-A1+	7/7	1/7	3/7	19/27	23/27	23/27
Anti-A1-	24/24	3/24	11/24	8/31	10/31	21/31
P	NS	NS	NS	<0.01	<0.01	NS

\* SLE = systemic lupus erythematosus; ANA = antinuclear antibodies; AKA = antikeratin antibodies; anti-dsDNA = anti-double-stranded DNA; RA = rheumatoid arthritis; RF = rheumatoid factor; NS = not significant.

† By latex fixation.

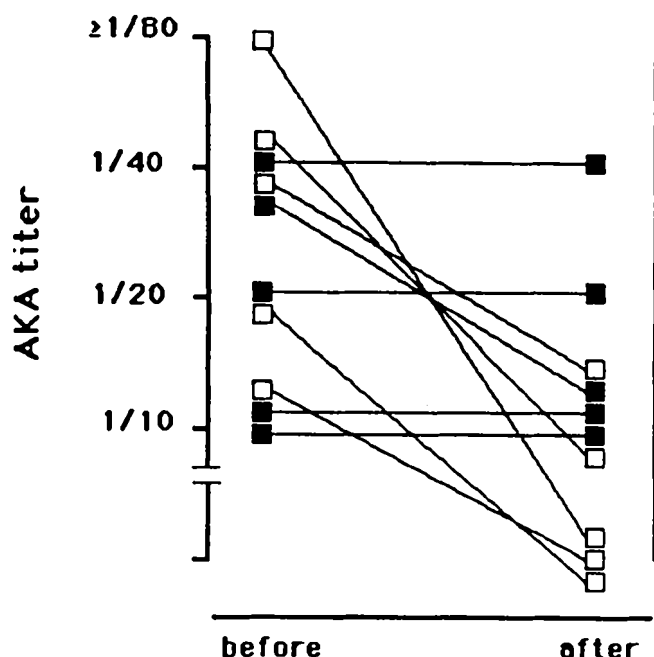


**Figure 4.** Immunoabsorption of an anti-A1-positive rheumatoid arthritis serum with recombinant heterogeneous nuclear RNP core protein A1. **A.** Before immunoabsorption, bright fluorescent staining of the outer epidermal layer (demonstrating antikeratin antibodies), associated with nuclear staining (antinuclear antibodies), is seen. **B.** After immunoabsorption, there is selective disappearance of antikeratin antibodies.

detectable decrease in AKA titer after immunoabsorption with recombinant protein A1 (Figures 4 and 5). The AKA titer decreased in all of the anti-A1-positive sera that were negative for anti-UP1 binding, and in only 1 of 5 sera that reacted with UP1 (Figure 5).

## DISCUSSION

Our results show that sera from patients with SLE and RA do react with hnRNP protein A1. Antibodies from SLE patients are specific for the N-



**Figure 5.** Changes in antikeratin antibody (AKA) titers in 10 anti-A1-positive rheumatoid arthritis sera after immunoabsorption with purified recombinant heterogeneous nuclear RNP core protein A1. ■ = sera with positive UPI binding. □ = sera with negative UPI binding.

terminal domain, which has a sequence identical to that of a previously identified DNA binding protein (4), whereas RA sera bind either the N-terminal or the C-terminal domain, the latter of which is structurally related to human epidermal keratin (3,4). Cross-reacting antikeratin antibodies, often found in RA sera (15,16), can partially account for the high frequency of anti-A1 reactivity in these cases.

In a study by Fritzler et al (1), sera from patients with MCTD were found to react with an hnRNP/RNA antigen, but no data were given about any specific polypeptide involved in anti-hnRNP autoimmunity. In our previous studies (5), sera from patients with connective tissue diseases were shown to react with purified recombinant hnRNP protein A1. In the present study, we found that sera from patients with SLE and RA also react with protein A1 present in crude nuclear extract. These findings strongly support the view that protein A1 can be an important autoantigenic structure within the hnRNP complex (6).

The use of purified recombinant antigens offers a great advantage for studying autoantibody specificity and may enable the identification of cross-reactivity with other proteins because of structural homology of

different molecules (6,16). We used this approach in the present study to further characterize anti-A1 antibodies in 2 different autoimmune conditions, SLE and RA. In SLE, anti-A1 antibodies react with the N-terminal (UPI) domain. Since UPI peptide binds DNA (4,17), as do anti-DNA antibodies, our data further support the hypothesis described by Jensen et al (6), that anti-A1 antibodies may be involved in the idiotype-antiidiotype network of anti-DNA antibodies. Studies on the biologic significance and clinical relevance of anti-UPI antibodies in SLE and related conditions are now in progress.

A very high frequency of anti-A1 antibodies was found in RA. The majority of A1-positive RA patients also displayed positive ANA, but our immunoabsorption results demonstrated that ANA positivity was not due to anti-A1 antibodies. A1 protein is easily degradable (17), and it could be lost during the cell fixation procedure for the ANA fluorescence test. However, immunoblotting results suggest that anti-A1 antibodies are frequently associated with other autoantibodies to nuclear antigens, e.g., histones (Figure 1), which may account for the high frequency of ANA positivity in these patients.

In contrast to SLE sera, a significant percentage of RA sera with anti-A1 reactivity did not recognize UPI. This may be evidence in support of the following hypotheses: 1) Since A1 protein is easily degraded *in vivo* (17), different determinants expressed by degradation may be immunogenic in different conditions (6). 2) Since epidermal keratin and the C-terminal domain of the A1 protein show a partial sequence homology (3,4), anti-A1 activity in RA may be partially due to cross-reacting AKA (5).

It is well known that more than 50% of all patients with RA have circulating AKA (18). We have shown that anti-A1 antibodies are often associated with AKA detected by immunofluorescence assays on human skin. Furthermore, a marked reduction in antikeratin activity is found following immunoabsorption with purified protein A1. It is noteworthy that significant reductions in the AKA titer occurred mainly in those sera that reacted with A1 but not with UPI. This suggests that AKA can actually bind the C-terminal domain of protein A1.

In conclusion, our results indicate that AKA from RA patients may react with a protein belonging to the hnRNP particle, i.e., the A1 protein, which is largely expressed in human nuclei (2,4). A different epitope of the same protein is also recognized by other autoantibodies, which are mainly present in SLE.

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