Double Reactivity Against Actin and α-Actinin Defines a Severe Form of Autoimmune Hepatitis Type 1

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Anti-filamentous actin antibodies characterize autoimmune hepatitis type 1 (AIH-1). Recently, the binding domain of α-actinin on actin was shown to be a predominant epitope. To test this reactivity, an anti-α-actinin enzyme-linked immunosorbent assay was developed, and positivity confirmed by Western blot. Anti-α-actinin antibody was found in 21/50 (42%) of AIH-1 patients, compared with 52/401 (12.9%) of liver disease control patients, and with 6/200 (6%) of blood donors. Anti-filamentous and anti-α-actinin activities were found specifically together in 66% of anti-filamentous-positive AIH-1 patients. This combination of specificities reflected clinical and histological disease activity, short duration and absence of treatment. Finally, using an actin-α-actinin complex assay, the binding of anti-filamentous actin to α-actinin-binding domain on actin was demonstrated, as well as that of anti-α-actinin on the actin-binding domain of α-actinin. Thus, the frequent combination of anti-filamentous and anti-α-actinin antibodies seems to be the hallmark of activity in AIH-1.

KEY WORDS: autoimmunity; anti-smooth-muscle antibodies; active disease; binding domain; anti-ssDNA.

INTRODUCTION

The diagnosis of autoimmune hepatitis (AIH) relies on clinical, serological, and histological criteria (1).

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AIH type 1 (AIH-1) is characterized by anti-filamentous actin (AFA) antibodies (Abs), among anti-smooth-muscle (SMA) Abs (2), and by anti-single-stranded (ss) and anti-double-stranded (ds) DNA Abs, among anti-nuclear Abs (ANA) (3). In contrast, autoAbs either to liver and kidney microsome (LKM1), or to liver cytosol 1 (LC1) (4) are found in AIH type 2 (AIH-2). In addition, anti-soluble liver antigen (Ag)/liver-pancreas (SLA/LP) Ab have also been shown to define a severe form of AIH (5).

AFA Abs have been found not only in 52–85% of AIH-1 cases, but also in non-AIH liver diseases and non-liver diseases (2, 6). They are more frequent in active than in inactive disease (7). Those in AIH-1 are, nevertheless, different, since they target a specific epitope corresponding to the α-actinin-binding domain (BD) located at positions 350–375 of the C terminus of human FA (8–10). AFA may be associated not only with anti-α-actinin Abs but also with anti-myosin, anti-tropomyosin, anti-filamin, anti-troponin, and anti-tubulin Ab (11).

Four spectrin repeats join an EF sequence to an FA-binding site to make up a molecule of α-actinin (12). The spectrin repeat region is required to form its anti-parallel dimer, and hold one FA-binding site at each of its extremities (13). In non-muscle cells, FA associated with α-actinin contributes to cellular motility, cell–cell interaction, and signaling pathways. Disruption of this complex affects cellular viability and cellular regeneration (12, 13). Information on the liver localization of α-actinin is lacking. There appear to be two forms in hepatocytes (14): α-actinin-1 which is exclusive to the cytosol and interacts with hepatitis C virus (HCV) (15) and α-actinin-4 which is present in the cytosol and on the plasma membrane.

In this study, we have investigated whether AFA, which characterize AIH-1, are associated with anti-α-actinin in a consecutive cohort of AIH-1 patients. In addition, the clinical and diagnostic significance of this autoantibody (anti-α-actinin) in AIH-1 was assessed. Our results

provide evidence of an association between these two autoAbs in some patients with active AIH-1. Furthermore, the latter autoAbs from AIH-1 cross-react with ssDNA, unlike those from systemic lupus erythematosus (SLE), which cross-react with dsDNA (16). The practical relevance of our findings is that the association of AFA with anti-α-actinin Ab improves diagnosis and points to active AIH-1.

METHODS

Patients and Controls

Blood was collected from 50 AIH-1 patients (36 females and 14 males; median age 52 years, range 7–80 years) referred to the Medical School Hospital, Larissa, and fulfilling the International AIH Group (IAHG) criteria (1) for definite AIH since they all had an IAHG score above 15 before treatment or above 17 after treatment. Thirty-four of them were tested before the initiation of treatment. Clinically active disease was assessed by the presence of one or more of the following symptoms (17, 18): malaise, fatigue, arthralgia, jaundice, nausea, anorexia, and weight loss, irrespective of the elevation of serum amino-transferases (ASTs).[6] Histologically active disease was considered according to the IAHG report (1). Liver biopsies for 44 AIH-1 patients in close proximity (0–2 months) to the data of serum samples were available for analysis, while HLA status was available in 36 patients.

Disease controls were recruited from Brest University and the Thessaly University Hospitals. The autoimmune liver disease group included 60 patients with primary biliary cirrhosis (PBC), and 41 with primary sclerosing cholangitis (PSC). In the non-autoimmune liver disease group, there were 75 patients with HCV, 75 with chronic hepatitis B, 40 with alcoholic liver disease, 60 with non-alcoholic steatohepatitis, 15 with hepato-cellular carcinoma, 16 with idiopathic hyper-transaminasemia, 9 with idiopathic cholestasis, 5 with Wilson's disease, and 4 with hemochromatosis. All diagnoses were based on standard clinical, biochemical, and histological criteria. Two hundred blood donors' sera served as normal controls. All samples tested by enzyme-linked immunosorbent assay (ELISA) in this study were examined blindly.

Another 80 serum aliquots from healthy volunteers were used to optimize the ELISA in the Brest University Laboratory of Immunology. These sera were obtained from members of staff and residents of a nursing home.

AIH-1 patients, disease controls, and normal controls gave their informed consent. The study was approved by the Ethics committee at the Brest and Thessaly University Medical School Hospitals.

Autoantibody Tests

Anti-α-Actinin Antibody The anti-α-actinin ELISA originally developed by Mason *et al*. (19) was used with our modifications as described previously (20). Limbro microplates (Flow, Irvine, UK) were coated with 10 μg/mL of chicken α-actinin (Sigma, St. Louis, MO) in bicarbonate buffer (pH 9.6), left to evaporate overnight at 37°C, washed three times in phosphate-buffered saline (PBS), blocked with 2% bovine serum albumin in PBS (PBS-BSA) for 1 h at 37°C, and washed with PBS containing 0.05% Tween 20 (PBS-T). Sera were diluted 1:200 in PBS-BSA, and incubated for 90 min at 37°C. Following another three washes, bound Ab was visualized using a horseradish peroxidase (HRP)-conjugated $F(ab')_2$ goat anti-human IgG (Jackson, West Grove, PA). Optical Density (OD) of each sample to α-actinin-free wells was automatically subtracted from the test OD. The sera were scored positive when the OD was greater than 0.15, that is 3 standard deviations (SDs) above the mean of 80 normal sera.

The presence of anti-α actinin Abs was confirmed by Western blotting (WB). As described (16), the α-actinin was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and then electroeluted for 3 h to polyvinylidene difluoride sheets (Biorad, Hercules, CA). The membranes were blocked overnight using 5% nonfat dry milk in PBS, probed with sera diluted 1:50 in PBS-T containing 1% milk, and revealed by biotinylated $F(ab')_2$ fragment of goat anti-human IgG (Zymed, South San Francisco, CA). Samples were then incubated for 1 h with HRP-labeled streptavidin. α-actinin purity was assessed using SDS-PAGE Coomassie F250 stain solution (Biorad).

Anti-Filamentous Actin Antibody An IgG AFA ELISA was adapted from Leibovitch *et al*. (21). Microtiter plates were coated with 100 μL of 10 μg/mL FA (Sigma). Sera were diluted 1:100 in PBS-BSA 1% and incubated for 90 min at 37°C. An HRP-conjugated $F(ab')_2$ goat anti-human IgG (Jackson, West Grove, PA) revealed the Ab binding. The cutoff level for OD was set at 0.200, i.e., 3 SDs above the mean of 80 normal sera.

Other Autoantibodies ANA and SMA were tested following published protocols (22, 23). The identification of

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[6] The IAHG score includes many parameters (sex, IgG increase, positivity for autoantibodies) including biopsy findings.

the vessel (V) and glomeruli/tubuli (G/T) SMA patterns was made according to Bottazzo *et al.* (24) and Vergani *et al.* (25).

Our in-house anti-ssDNA and anti-dsDNA IgG ELISAs were used as described previously (16). Briefly, DNA was denatured by boiling dsDNA for 10 min and cooling on ice for 5 min, and ssDNA coated onto nontreated plates. The cutoff OD for positivity was set at 0.200, that is 3 SDs above the mean of 80 normal sera.

Anti-SLA, anti-LKM1, and anti-LC1 Abs were tested following published protocols (22, 23) and by dot blots (D-Tek Mons, Belgium). Sera with anti-LKM1 and/or anti-LC1 were classified as AIH-2 and excluded from this study.

Actin/α-Actinin Assay

Two AFA + /anti-α-actinin + sera (#1 and 2), three AFA + /anti-α-actinin − sera (#3, 4, and 5), and two AFA − /anti-α-actinin + sera (#6 and 7), all from AIH-1 patients, plus one normal serum (#8) were tested in the AFA and anti-α-actinin ELISAs, after the addition of increasing amounts of α-actinin or FA (0.1–500 μg/mL). The percentage of inhibition was [(OD before inhibition − OD after inhibition)/(OD before inhibition) × 100].

Affinity Purification of Autoantibodies

Based on the results of the AFA and anti-α-actinin Ab assays, four pools of sera (sera #1 plus #2, sera #3 plus #4, sera #6 plus #7, and sera #5 plus #8) were loaded separately on a protein-G-sepharose column (Pharmacia, Uppsala, Sweden), and IgG eluted with 0.1 M HCl–glycin, pH 2.8.

Each IgG preparation was run through a column of 1 mg α-actinin coupled to Hitrap^{TM} NHS-activated sepharose column (Amersham, Aylesbury, England). The α-actinin-column-bound IgG was recovered stepwise with 15 mL of 0.15 M NaCl as the effluent, and 6 M urea – 2 M NaCl as

the eluate. The collected fractions were dialyzed against PBS and the concentrations of IgG measured using the Bradford micro-BCA assay (BioRad), and adjusted to 20 μg/mL in PBS-BSA 1%.

α-Actinin/ssDNA Inhibition Experiments

Two AFA + /anti-α-actinin + /anti-ssDNA + (#1′ and 2′) and another two AFA + /anti-α-actinin-/anti-ssDNA + sera (#3′ and 4′) from patients with AIH-1, plus 1 AFA − /anti-α-actinin − /anti-ssDNA + sera from HCV (#5′) were selected on the basis of their high reactivity to ssDNA. One 200 μL aliquot of serum at a dilution giving 50% of its maximum OD with ssDNA was used, with 0.1–500 μg/mL of FA, α-actinin, ssDNA, or dsDNA as inhibitors. The AFA − /anti-α-actinin − /anti-ssDNA − serum (#6′) served as a negative control. Following a 1-h incubation with the inhibitor, the samples were depleted of immune complexes by precipitation with 2% polyethylene glycol 6.000 (Merck, Fontenay-sous-bois, France) and a 30-min centrifugation at 15.000 × g. Supernatants were then examined in anti-ssDNA, AFA, and anti-α-actinin ELISAs. The percentage inhibition was calculated as described above.

Statistics

Results from triplicate wells were averaged, and expressed as mean ± SD. Comparisons were made using the chi-square test, the Fisher exact test, and the Mann–Whitney U test for unpaired data.

RESULTS

Combinations of Autoantibodies

As depicted in Table I, AFA were detected in 21/50 AIH-1 sera ($P < 10^{-4}$), compared with 32/401 liver disease control sera, and with 1/200 blood donors ($P < 10^{-4}$).

Table I. Anti-Filamentous Actin (AFA) and Anti-α-Actinin IgG, Alone or in Combination, Detected by Enzyme-Linked Immunosorbent Assays in Sera from Patients with Liver Diseases

AutoAbs		Number of positive sera (%)					
AFA	Anti-α-actinin	AIH-1 ($n = 50$)	PBC ($n = 60$)	PSC ($n = 41$)	Viral hepatitis ($n = 150$)	Other liver diseases ($n = 150$)	Blood donors ($n = 200$)
+	+	14 (28)	1 (1.7)[a]	0[a]	0[a]	0[a]	0[a]
+	−	7 (14)	6 (11)	2 (5)	10 (6.7)	14 (9.3)	1 (0.5)*
−	+	8 (16)	14 (23)	7 (17)	18 (12)	12 (8)	6 (3.0)*
−	−	21 (42)	40 (66.3)*	32 (78)[a]	122 (81.3)[a]	124 (82.7)[a]	193 (96.5)[a]

Note. AIH-1: autoimmune hepatitis type 1; PBC: primary biliary cirrhosis; PSC: primary sclerosing cholangitis.
[a]$P < 0.005$ *versus* AIH-1.
*$P < 0.05$.
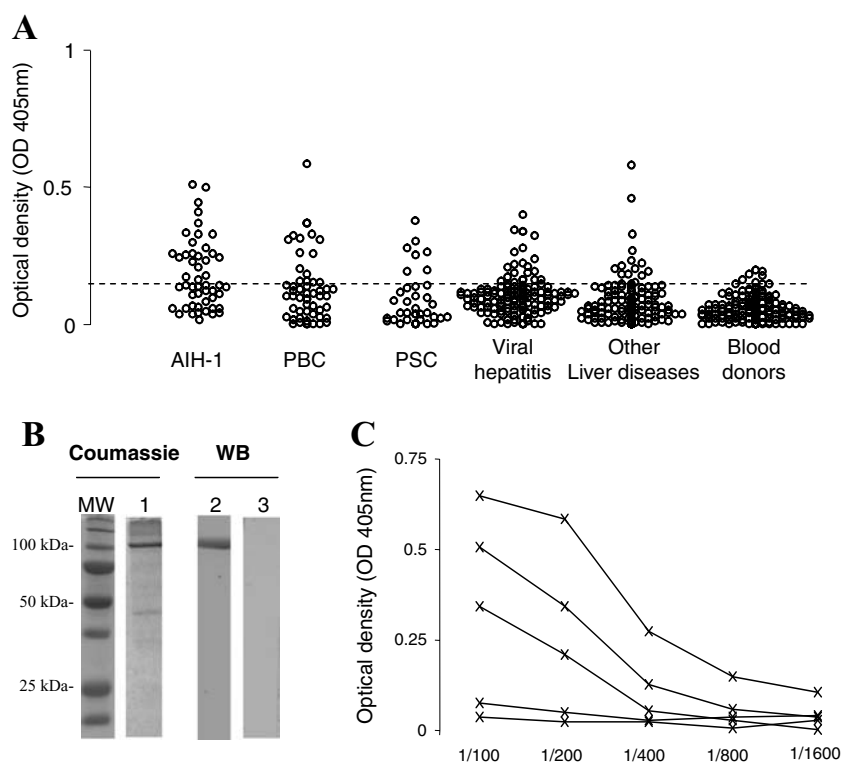
Fig. 1. Anti-α-actinin in patients with autoimmune hepatitis type 1 (AIH-1). (A) α-Actinin-specific IgG levels measured by ELISA. Optical density (OD) values in AIH-1 ($n = 50$), in primary biliary cirrhosis (PBC, $n = 60$); in primary sclerosing cholangitis (PSC, $n = 41$); in viral hepatitis ($n = 150$); in other liver diseases ($n = 150$); and in blood donors ($n = 200$). The *dotted line* represents the positive cutoff. (B) Assessment of α-actinin (100 kDa) purity by gel electrophoresis and protein staining (lane 1). Western blots for anti-α-actin Abs: lane 2, representative AIH-1 sera with anti-α-actinin; lane 3, blood donor sera without anti-α-actinin. (C) Dilution curves of sera containing anti-α-actinin Abs. Three anti-α-actinin + sera (*bold lines*) and two anti-α-actinin − sera (*dashed lines*) are shown in the figure.

AFA titers were significantly higher in patients with AIH-1 (OD = 0.223 ± 0.132) compared to patients with other liver diseases (OD = 0.166 ± 0.108) ($P < 0.01$) and healthy controls (OD = 0.104 ± 0.041) ($P < 0.001$). Both ELISA and WB detected anti-α-actinin Abs in 22/50 AIH-1 sera, compared with 51/401 liver disease controls ($P < 10^{-4}$), and with 6/200 blood donors sera ($P < 10^{-4}$).

AIH-1 patients displayed higher anti-α-actinin ELISA titers (OD = 0.182 ± 0.125) compared to liver disease controls (OD = 0.112 ± 0.110) ($P < 0.001$) and healthy donors (OD = 0.050 ± 0.060) ($P < 0.0001$) (Fig. 1A). Except one, all AIH-1 sera positive for anti-α-actinin by ELISA were positive by WB (Fig. 1B). Sera from patients displaying high titers of anti-α-actinin were examined

Table II.   Anti-Filamentous Actin (AFA) and Anti-α-Actinin and Anti-α-Actinin IgG in Discriminating between Patients with and without Autoimmune Hepatitis Type 1

	Sensitivity	Specificity	PPV	NPV
AFA	0.42 (0.28–0.57)	0.90 (0.86–0.93)	0.38 (0.26–0.52)	0.91 (0.88–0.94)
Anti-α-actinin	0.44 (0.30–0.58)	0.84 (0.80–0.88)	0.29 (0.20–0.41)	0.91 (0.87–0.94)
AFA + /anti-α-actinin +	0.28 (0.17–0.43)	0.99 (0.98–1.00)	0.93 (0.66–0.99)	0.90 (0.86–0.93)

Note. PPV: positive predictive value; NPV: negative predictive value. Values in parentheses indicate 95% confidence interval.

Table III. Clinical, Biological, and Serological Parameters of 50 Patients with Autoimmune Hepatitis 1 (AIH-1)

	AIH-1 ($n = 50$)	AFA + ($n = 21$)	α-Actinin + ($n = 22$)
Age (years)	52.1 ± 19.4	50.0 ± 19.2	50.4 ± 19.2
Female/male	36/14	17/4	13/9
Histological active disease	23/44	14/19*	15/18†
Clinical active disease	27	15*	19†
Developed cirrhosis	20	5	9
Liver death or transplant	7	3	3
No treatment	34	13	19*
Follow-up (months)	44.7 ± 60.7	37.4 ± 63.8	26.6 ± 34.6*
AST (nl, <30 U/L)	155 ± 396	222 ± 566	274 ± 576†
Bilirubin (nl, <1.1 mg/dL)	1.3 ± 2.0	2.6 ± 3.0	1.8 ± 2.9
Albumin (nl, 3.4–4.8 g/dL)	4.3 ± 0.9	4.3 ± 0.9	4.4 ± 1.1
γ-globulin (nl, 0.7–1.7 g/dL)	3.6 ± 1.1	4.0 ± 1.2	4.0 ± 1.3
IgG (nl, 0.5–1.5 g/dL)	1.9 ± 1.1	2.2 ± 0.8	2.2 ± 1.0
HLA DR3	9/36	7/16*	5/16
HLA DR4	8/36	5/16	4/16
ANA	45	19	20
SMA	46	21	22
SMA-G/T +	33	20†	17

Note. AFA: anti-filamentous actin antibody (Ab); AST: amino-transferase; ANA: anti-nuclear antibodies; SMA: smooth muscle antibodies; -T: peritubular staining; -G: glomerular staining; nl: normal limit. Liver biopsy and HLA status were available for 44 and 36 patients, respectively.
*P<0.05; †P<0.0005.

with regard to their capacities to bind α-actinin at different sera dilution (Fig. 1C). Sera containing both AFA and anti-α-actinin Abs were found in 14/21 AFA + AIH-1 patients, compared with 1 of 401 liver disease controls, a patient with PBC (P<10^{-4}). Specificity and the positive predictive value of AFA-plus-anti-α-actinin Abs were 99 and 93%, respectively, performing better than each assay alone (Table II).

High levels of anti-ssDNA Abs (0.300 ± 0.298) were found in 25 positive AIH-1 sera, whilst low levels of anti-dsDNA (0.066 ± 0.126) Abs were seen in 3 positive

AIH-1 sera. Anti-SLA/LP Abs were positive in 5 of 50 individual AIH-1 sera.

Association Between Clinical, Biological, and Serological Parameters

AIH-1 activity ($P = 0.03$), HLA DR3 positivity ($P = 0.02$), and correlation with SMA-G/T pattern ($P = 0.0002$) were the only distinguishing features between AFA-positive and AFA-negative AIH-1 patients (Table III). Anti-α-actinin Ab were highly significantly associated with histological (15/18 vs. 3/18) and

Table IV. Clinical, Biological, and Serological Parameters of AIH-1 Patients with Antibodies to AFA and α-Actinin

	Whole population ($n = 50$)		Untreated	
	AFA + and α-actinin + ($n = 14$)	Others ($n = 36$)	AFA + and α-actinin + ($n = 11$)	Others ($n = 23$)
Age (years)	47.4 ± 18.8	53.9 ± 19.5	49.0 ± 17.6	57.1 ± 19.5
Female/male	11/3	25/11	8/3	14/9
Histological active disease	12/13$^{\$}$	11/31	10/11*	9/18
Clinical active disease	13†	14	10*	12
Follow-up (months)	23.4 ± 36.1*	53.0 ± 66.5	14.3 ± 23.6*	57.1 ± 19.5
AST	314 ± 681†	93 ± 180	328 ± 760*	125 ± 219
HLA DR3	4/10	5/26	2/8	1/16
HLA DR4	4/10	4/26	2/8	1/16
ANA	12	33	9	22
SMA	14	32	11	20
SMA-G/T +	14$^{\$}$	19	11$^{\$}$	11

Note. AFA: anti-filamentous actin antibody (Ab); AST: amino-transferase; ANA: antinuclear antibodies; SMA: smooth muscle antibodies; -T: peritubular staining; -G: glomerular staining. Biopsy and HLA status were referred to the number of tested patients.
*P<0.05; $^{\$}$$P$<0.005; †$P$<0.0005.

clinical (19/22 vs. 3/22) AIH-1 activity ($P = 8 \times 10^{-4}$ and $P = 5 \times 10^{-5}$, respectively). In addition, shorter duration of disease ($P = 0.03$), higher AST levels ($P = 3 \times 10^{-4}$), and absence of treatment ($P = 0.015$) characterized the anti-α-actinin Ab-positive AIH-1 patients.

Since immunosuppressive therapy may affect anti-α-actinin positivity (Table III), the analysis of the AFA + /anti-α-actinin + subgroup of AIH-1 patients was performed in the whole population of AIH-1 patients as well as in the untreated patients (Table IV). This study revealed that the combined AFA/anti-α-actinin Ab positivity was associated with the clinical and histological activity ($0.02 < P < 5 \times 10^{-4}$), short disease duration ($P = 0.002$; $P = 0.04$, respectively), and an SMA-G/T pattern ($P < 0.05$) either in the whole AIH-1 population or in the untreated group of AIH-1 patients (Table IV).

Relationship between AFA and Anti-α-Actinin Antibodies

In AIH-1 patients, AFA were associated with anti-α-actinin in 14/21 AFA-positive sera ($P = 0.006$). This may be due to the coexistence of two independent populations of auto-Abs, or due to a subpopulation of one of these autoAbs that cross-reacts with the Ag of the second autoAb, while the other subpopulation does not. To distinguish between these two possibilities, AIH-1 sera were incubated with increasing amounts of α-actinin and tested in the AFA ELISA (Fig. 2A). A total of 500 μg/mL of α-actinin, reduced the FA binding by 65–71% in sera #1, 2, and 5. Similarly, 0.1–10 μg/mL of α-actinin reduced α-actinin binding in sera #1, 2, 6, and 7 (Figs. 2A and 4C).

FA influences the binding of anti-α-actinin Ab (Fig. 2B), since the anti-α-actinin reactivity of sera #1 and 2 was reduced by this form of actin in a dose-dependent manner up to 60%. However, sera #6 and 7 (AFA-/anti-α-actinin +) were unaffected, and those of sera #3 and 4, but not #5, were increased (Figs. 2B and 4D).

*Interaction between Actin and Actinin Blocks Antibody Binding*

Sera were affinity-purified on an α-actinin column to distinguish the binding properties of the AFA from those of anti-α-actinin Abs. Cross-reactivity between AFA and anti-α-actinin Abs of purified fractions were tested by ELISA. AFA + /anti-α-actinin + sera and AFA + /anti-α-actinin- sera effluents of α-actinin affinity column reacted with FA not with α-actinin (Fig. 3A). Conversely, the eluates of AFA + /α-actinin + sera and AFA − /α-actinin + sera reacted with α-actinin, but not with FA (Fig. 3B). These results suggest that, in AIH-1, AFA do
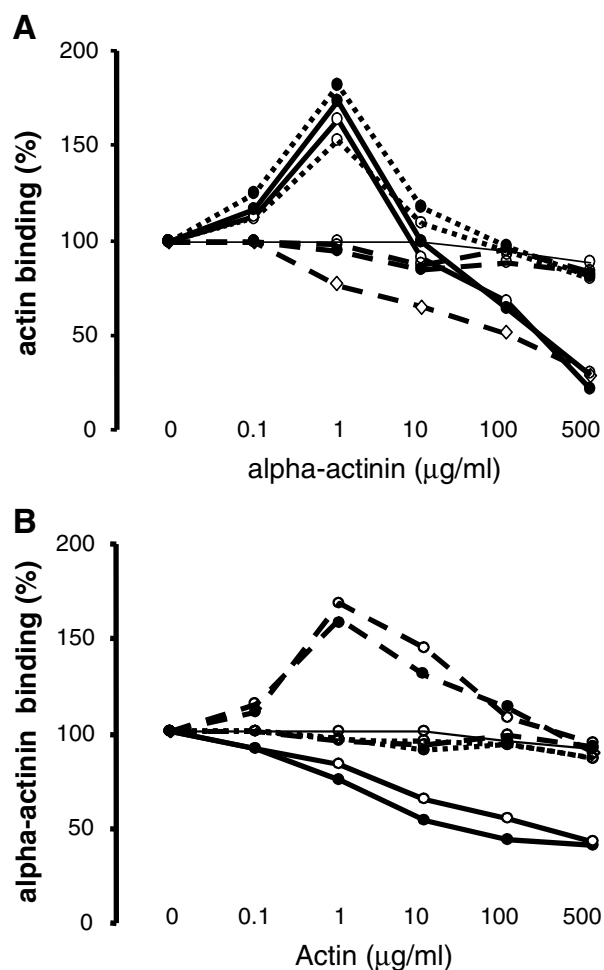
Fig. 2. Competition experiments. Two anti-filamentous actin (AFA) + /anti-α actinin + sera (*bold lines*): serum #1 (*filled circles*) and serum #2 (*open circles*); three AFA + /anti-α-actinin − sera (*dashed lines*): serum #3 (*filled circles*), serum #4 (*open circles*), and serum #5 (*diamond*); two AFA − /anti-α-actinin + sera (*dotted lines*): serum #6 (*filled circles*) and serum #7 (*open circles*) and a healthy control #8 (*thin line*) were analyzed. They were mixed with α-actinin (A) or actin (B) and assayed in the AFA (A) and the anti-α-actinin (B) enzyme-linked immunosorbent assays, respectively. The percentage of reduction was calculated as described in the Methods section.

not cross-react with α-actinin, and anti-α-actinin Abs do not cross-react with FA.

The influence of the FA–α-actinin complex formation on the binding of AFA and anti-α-actinin Abs was, therefore, evaluated using the α-actinin-column effluent as the source of AFA Abs, and its eluate as the source of anti-α-actinin Abs. The binding of the effluent on actin from AFA + /anti-α-actinin + sera, from AFA + /anti-α-actinin − serum #5 but not from AFA + /anti-α-actinin − sera #3/4 was affected by the FA–α-actinin complex
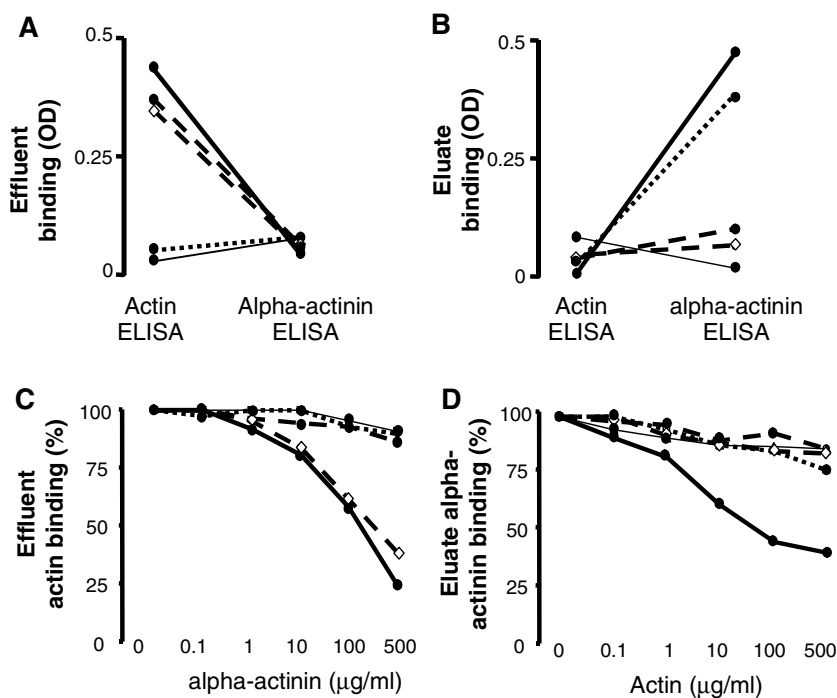
Fig. 3. Actin/α-actinin complex formation influences the binding of anti-filamentous actin (AFA) antibodies (Abs) to actin, and of anti-α-actinin Abs to actin. (A) and (B) Affinity purification of anti-α-actinin Abs on an α-actinin-column. Purified IgG from AFA $+$/anti-α-actinin $+$ sera (*bold line*), from AFA $+$/anti-α-actinin $-$ sera (serum #3: *dashed line*; serum #4: *filled circles*; and serum #5: *diamonds*), from AFA $-$/anti-α-actinin $+$ sera (*dotted line*) and a healthy control (serum #8: *thin line*) were applied to an α-actinin column. The effluents and the eluates were tested in the AFA (A) and the anti-α-actinin (B) enzyme-linked immunosorbent assays. (C) and (D) The effluent (C) and the eluates (D) were examined for their AFA (C) or anti-α-actinin (D) avidity in presence of α-actinin (C) or actin (D).

formation (Fig. 3C) suggesting that the binding epitope of those sera on FA is located on its binding site (Fig. 4A). The binding of the eluate on α-actinin from AFA $+$/α-actinin $+$ sera but not from AFA $-$/anti-α-actinin $+$ sera was reduced when FA was associated with α-actinin (Fig. 3D). The epitope of anti-α-actinin Ab of those sera on α-actinin is probably the FA binding site of α-actinin (Fig. 4B).

Characterization of Anti-α-Actinin Antibodies in AIH-1

Based on the frequency of anti-ssDNA IgG in AIH-1 and the known association between anti-dsDNA and anti-α-actinin Abs in SLE, the latter specificity was evaluated in combination with AFA and anti-α-actinin Abs. Anti-ssDNA was associated with 16/22 AFA positive sera ($P = 0.008$), with 16/21 anti-α-actinin $+$ sera ($P = 0.002$), and remarkably, with 12/14 AFA $+$/anti-α-actinin $+$ sera ($P = 0.004$).

To confirm the cross-reactivity of anti-ssDNA Abs with FA and α-actinin, competition experiments were conducted. Sera #1′ through #6′ were preincubated with increasing amount of FA, α-actinin or ssDNA prior to being retested in the three ELISAs. The binding of sera to FA was not modified by ssDNA (Fig. 5A), neither was their binding ssDNA by FA. This indicates that AFA and anti-ssDNA are independent in AIH-1. A cross-reactivity between anti-α-actinin and anti-ssDNA Abs was found for sera #1′ and 2″ (Fig. 5B and C). Such effect was specific for ssDNA, since no inhibition was observed when dsDNA was substituted to ssDNA in the α-actinin ELISA (Fig. 5D).

DISCUSSION

We started by confirming that AFA occur more commonly in AIH-1 (2, 6, 7) than in other autoimmune and non-autoimmune liver diseases. AIH-1 patients displayed
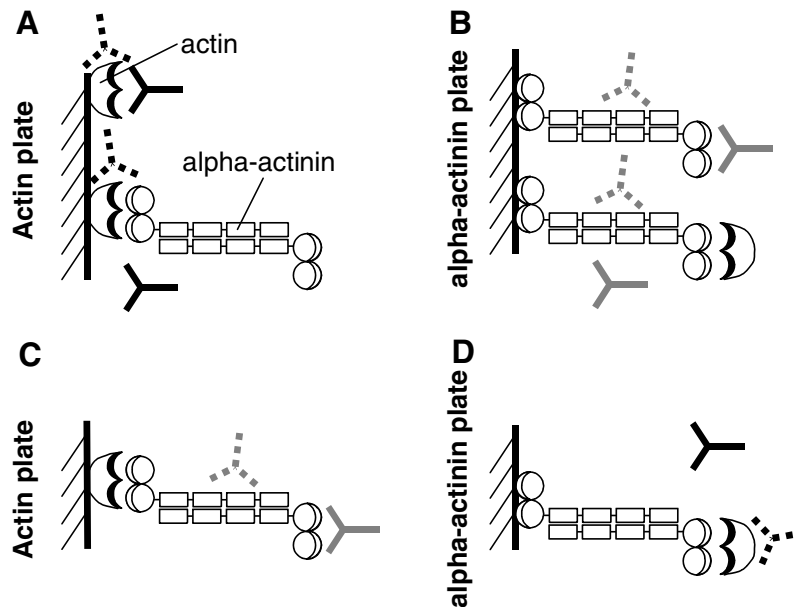
Fig. 4. (A) Because α-actinin masks the epitope for anti-filamentous actin (AFA) antibodies (Abs) and (B) actin masks the epitope for anti-α-actinin Abs, AFA from sera #1, 2, and 5 (*black bold* Abs), and anti-α-actinin from sera #1 and 2 (*grey bold* Abs) response is elicited by the actin/α-actinin complex formation. Binding of AFA (sera #3 and 4: *black dashed* Abs) and anti-α-actinin (sera #6 and 7: *grey dashed* Abs) is not modified. When using whole sera, anti-α-actinin (C) and AFA (D) could bind to the complex as observed in the range of 0.1–10 μg/mL, when the target is accessible.

significantly higher AFA titers, which are associated with SMA-G/T pattern as previously described (2, 6, 7, 21). However, AFA seropositivity may be found in a significant proportion of SMA-G/T seronegative patients (6), suggesting an increased sensitivity of the anti-FA ELISA probably due to the presentation of new immunodominant epitopes of F-actin coated onto the ELISA plates (2). On the other hand, in accordance with our recent publication (6) we observed that the increased sensitivity of the anti-FA ELISA comes to the cost of its lower specificity as it detects AFA in a considerable proportion of SMA seronegative liver disease controls, especially in PBC and other liver diseases (Table I). Activity status and HLA DR3, but not biological variables, were weakly associated with AFA (2, 7). It is interesting that AFA were strongly associated with anti-α-actinin Abs. That is, most of the AFA-positive AIH-1 sera were also positive in the anti-α-actinin test. A similar association has been noted in 11/13 AIH patients (11). Furthermore, AIH-1 patients with both AFA and anti-α-actinin Abs were those patients (all with SMA-G/T pattern on IIF) with active disease who were untreated at the time of sampling. This is in agreement with the observation that SMA and ANA disappearance was associated with improved laboratory and histological

features after treatment (26). There was no association between anti-α-actinin Abs and HLA DR3 or DR4, suggesting that the mechanisms for the production of AFA and for that of anti-α-actinin Abs may be different in susceptible individuals.

The coexistence of AFA + and anti-α-actinin + Abs tested in a large number of sera characterized AIH-1. Indeed, there was only 1 of 601 disease control and blood donors sera that displayed double reactivity. Anti-α-actinin Abs (44%) were more frequent than anti-SLA/LP (10%) in AIH-1. Thus, detection of anti-α-actinin Abs along with AFA could be used as an additional diagnostic marker for AIH-1, especially since these patients are active and have a short disease duration. However, the diagnostic relevance of anti-α-actinin as a predictive marker of AIH-1 flares or for treatment failure was out of the scope of the present study and was not formally tested.

Four groups of AIH-1 sera were identified. The first group (AFA + /anti-α-actinin +) consisted of non-cross-reactive Abs that were easily eluted from the α-actinin-column. The effluent and the eluate bound to actin and α-actinin, respectively. Each subset of Abs is affected by actin–α-actinin complex formation. Our localization of the AFA epitope in the α-actinin binding site of FA,
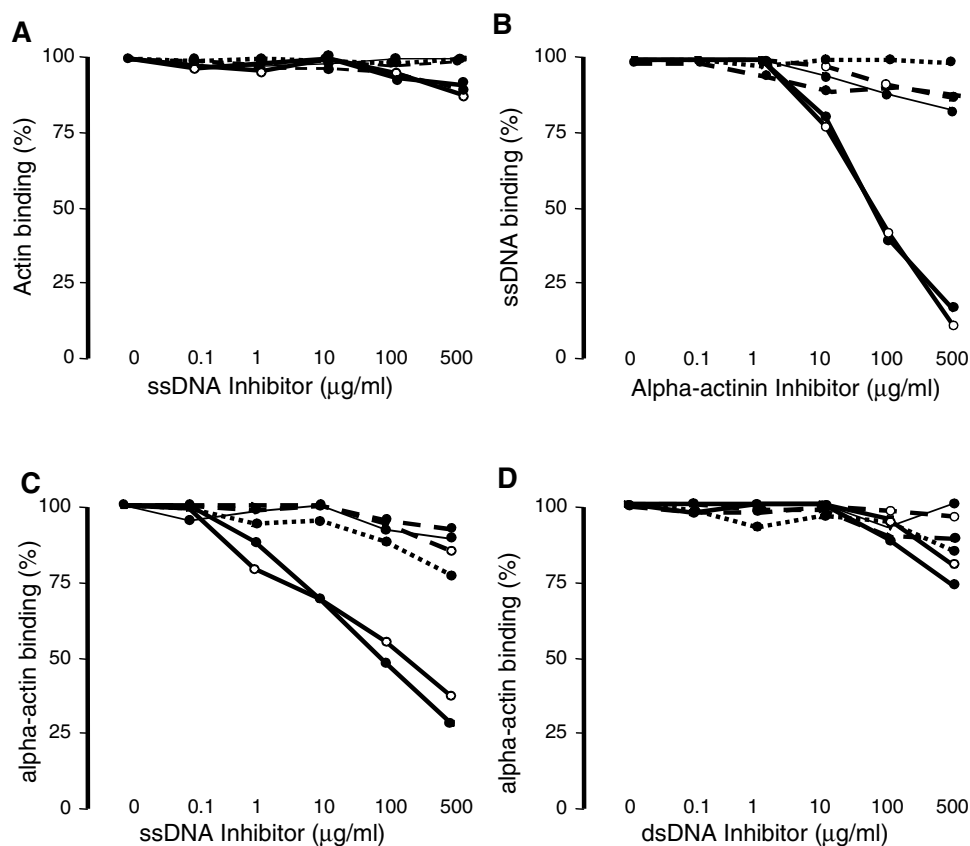
Fig. 5. Competition experiments: two anti-filamentous actin (AFA) + /anti-α-actinin + /anti-single stranded (ss)DNA + sera (*bold lines*), two AFA + /anti-α-actinin − /anti-ssDNA + sera (*dashed lines*), one AFA-/anti-α-actinin + /anti-ssDNA + (*dotted line*) and one healthy subject (*thin line*) were analyzed. Sera were mixed with inhibitor (0.1–500 μg/mL) and tested in the AFA (A), anti-ssDNA (B), and anti-α-actinin (C and D) enzyme-linked immunosorbent assays.

using two distinct technologies to analyze AFA from AIH-1 patients (8, 9), is consistent with one of the two α-actinin binding sites on FA (10). It is not surprising that anti-α-actinin Abs directed to the actin BD on α-actinin were detected. This is in agreement with anti-α-actinin Abs from SLE patients, which are also sensitive to the addition of FA in the anti-α-actinin ELISA (16). These were detected in 10 of 10 SLE patients who had developed glomerulonephritis, and disappeared after the onset of immunotherapy. Nevertheless, SLE anti-α-actinin Abs differ from AIH-1 anti-α-actinin Abs, since the former cross-react with dsDNA, but not with ssDNA, whereas the latter cross-react with ssDNA but not with dsDNA. Our second and third group of AFA + /anti-α-actinin − Abs differ in that one binds to FA in presence of α-actinin, whereas the other does not. The AFA − /anti-α-actinin + Ab fourth group consists of Abs that bind to α-actinin independent of the presence of actin, and do not cross-

react with ssDNA. This latest group of anti-α-actinin Abs is also detected in the sera of other liver diseases indicating that single reactivity against α-actinin is not specific for AIH-1.

The mechanism of liver damages in AIH-1 is not understood. Hepatocyte-specific autoAbs have been incriminated in AIH-1 through Ab-dependent cell-mediated cytotoxicity (27–32). Several target Ags have been shown to range from 49 to 136 kDa (30, 32). Only two have been identified: the 40-kDa cell surface asialoglycoprotein receptor (29) and the 52-kDa cytoplasmic UGA serine tRNA-associated protein (target of anti-SLA/LP) (30–32). Based on our results, we can further support that AFA + /anti-α-actinin + Abs which recognize specific disease epitopes could potentially contribute to the pathogenesis of AIH-1. These epitopes occur at the docking site between the two proteins (Fig. 4). Thus, Abs binding implies probably an effective disruption between F-actin

and α-actinin, a hypothesis, which needs to be tested. In SLE, nephritogenic anti-α-actinin Abs bind to α-actinin expressed on the surface of mesangial cells from lupus-prone mice and modify gene expression (33, 34). Taking into account the latter study along with our results in the present study, it seems rational to suggest the need of future investigation using similar experiments with hepatocytes, in an attempt to address whether anti-α-actinin could potentially contribute to the pathogenesis of AIH-1.

In conclusion, we show that almost one-third of AIH-1 sera reacted with FA and α-actinin. This subgroup of AIH-1 is characterized by active disease clinically and histologically, and is present in patients who are untreated. The combination of AFA $+$/anti-α-actinin $+$ Abs may, therefore, be used to differentiate those SMA-G/T positive patients with a diagnosis of AIH-1 as double reactivity was detected specifically, except one case, in AIH-1.

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