Journal of Clinical Laboratory Analysis 16:194–201 (2002)

Anti-Smooth Muscle Antibodies (ASMAs) and Anti-Cytoskeleton Antibodies (ACTAs) in Liver Diseases: A Comparison of Classical Indirect Immunofluorescence With ELISA

A. Zamanou,[1] A. Tsirogianni,[2] C. Terzoglou,[3] A. Balafas,[1] I. Economidou,[2] and P. Lymberi[1]*

[1]*Immunology Laboratory, Biochemistry Department, Hellenic Pasteur Institute, Athens, Greece*
[2]*Immunology-Histocompatibility Department, Evangelismos Hospital, Athens, Greece*
[3]*Immunology Department, Koryialenio-Benakio Hospital, Athens, Greece*

In the diagnosis of autoimmune hepatitis type I (AIH-I), the routine assay of indirect immunofluorescence (IFL), used for the detection of anti-smooth muscle antibodies (ASMAs), has a low predictive value. On the other hand, the enzyme-linked immunosorbent assay (ELISA), which detects anti-cytoskeleton antibodies (ACTAs), presents contradictory results concerning their specific antigenic target. In this study, we first looked for the immunological properties (isotypes and antigenic targets) of autoantibodies in AIH-I and two other control liver diseases: primary biliary cirrhosis (PBC) and viral hepatitis (VH), using ELISA based on cytoskeleton proteins: F-actin, G-actin, myosin, tropomyosin, troponin, desmin, vimentin, keratin, and an extract of HEp-2 carcinoma cells. We also compared the diagnostic value of IFL and ELISA. In contrast to previous studies, we found that actin was not specific for AIH-I. No autoantigen and no antibody class or subclass discriminated AIH-I from the control diseases. IFL is more suitable for AIH-I diagnosis, as 97% of AIH-I sera but only 22% of PBC sera were ASMA-positive. Additionally, 96% of ASMA-positive, and all ASMA-negative sera from all three liver diseases were ACTA-positive. ASMA were mainly IgG, while >50% of ACTA also contained IgA and IgM. These data suggest that ACTAs recognize additional epitopes as compared to ASMAs, and they frequently occur in all liver diseases. J. Clin. Lab. Anal. 16:194–201, 2002.

Key words: cytoskeleton proteins; primary biliary cirrhosis; autoimmune hepatitis type I; Ig classes and subclasses; HEp-2 cells; indirect immunofluorescence; ELISA; immunodiagnostics

INTRODUCTION

Anti-smooth muscle antibodies (ASMAs) are routinely detected by indirect immunofluorescence (IFL) on mouse stomach kidney sections. They are used for the diagnosis of autoimmune hepatitis type I (AIH-I), detected in titers exceeding 1-80 (1–3). However, ASMAs have a low predictive value for AIH-I (only 30%) since they are also detected in other liver diseases, such as primary biliary cirrhosis (PBC) and viral hepatitis (VH), as well as in healthy individuals (2–7). In order to improve the diagnostic value of IFL for AIH-I, the IFL patterns observed with ASMAs on tissue sections have been further studied (3,8). These studies revealed multiple ASMA patterns, suggesting that several antigens are involved in these reactions. The antigenic targets of ASMA were primarily found among cytoskeleton proteins, and several previous studies correlated ASMA with anti-cytoskeleton antibodies (ACTAs). These early studies were mainly based on 1) absorption experiments of the ASMA tissue patterns by purified cytoskeleton proteins (8–10); and 2) IFL using HEp-2 cells, fibroblasts, or other similar cell lines as a substrate (3,11–13). HEp-2 is a carcinoma cell line of human larynx epithelium with a well developed cytoskeleton, which gives characteristic and easily identified fluorescent ACTA profiles (e.g., actin cables pattern and

*Correspondence to: Dr. P. Lymberi, Immunology Laboratory, Biochemistry Dept., Hellenic Pasteur Institute, 127 Vas. Sofias Ave, 11521, Athens, Greece. E-mail: plymberi@mail.pasteur.gr

Received 1 November 2001; Accepted 18 March 2002
DOI 10.1002/jcla.10040
Published online in Wiley InterScience (www.interscience.wiley.com).

perinuclear intermediate filaments pattern) (11–16). Nevertheless, IFL does not allow the clear discrimination between the diverse antigens responsible for the fluorescence patterns of ASMA on either tissue sections or HEp-2 cells. Additionally, IFL is a semiquantitative test which is based on subjective interpretation of the immunofluorescence staining patterns. Thus, there is a reported wide variation in ASMA prevalence among patient groups and healthy individuals (3,6).

Subsequently, some investigators developed the enzyme-linked immunosorbent assay (ELISA), based on purified cytoskeleton proteins, in order to correlate ASMA with well defined antigenic targets and avoid the use of the classical IFL on tissue sections.

Indeed, ELISA has clear advantages as compared to IFL: it can identify the exact antigenic targets, it allows antibody quantitation, and because it is a more sensitive assay it requires only minimal amounts of antigens and sera. Studies based on ELISA showed that a great part of ASMA activities can be accounted for by autoantibodies reacting with 1) microfilaments such as actin, myosin, tropomyosin, and troponin; 2) intermediate filaments such as desmin and vimentin; and 3) microtubules such as tululin (16). However, the data concerning the antigenic specificity of ASMA in the different liver disease groups (e.g., AIH-I, PBC, and VH) have been contradictory. Some studies showed that ASMAs in different liver diseases have the same antigenic targets (16,17), whereas others support the view that actin is the specific target of ASMAs only in AIH-I (18,19) and that an ELISA screening test based on actin could be used for its diagnosis (18,19). Furthermore, there are contradictory data concerning the form of actin recognized by ASMAs in AIH-I: filamentous-polymerized actin (F-actin) (20) or globular-monomer (G-actin) (16,18).

The aims of this study were: 1) to test for the existence of a specific target antigen in AIH-I, as compared to the control groups PBC and VH by using ELISA based on purified cytoskeleton antigens (i.e., microfilaments such as F-actin, G-actin, myosin, tropomyosin, and troponin, and intermediate filaments such as keratin, vimentin, and desmin); and 2) to examine whether an ELISA using purified cytoskeleton antigens or HEp-2 cytoplasmic extract (which is rich in cytoskeleton proteins) can replace the classical IFL assay used for the detection of ASMAs on tissue sections in AIH-I diagnosis.

MATERIALS AND METHODS

Sera

Four groups of sera were studied. Group I (AIH-I group) consisted of 59 sera from patients with AIH-I. Among them, 57 sera were ASMA-positive (IFL titer $>$ 1:80) and two ASMA-negative. Group II (PBC group) comprised 23 sera from patients with PBC: five were ASMA-positive (IFL titer $>$ 1:80) and 18 ASMA-negative. All PBC sera were anti-mitochondrial antibody (AMA)-positive (IFL titer $>$ 1:160). Group III (VH group) included 47 sera from patients with VH B (16 sera) or C (31 sera): 45 were ASMA-positive (IFL titer $>$ 1:80) and two ASMA-negative. Group IV consisted of 40 sera from healthy donors (HDs), all of whom were ASMA-negative.

Antigens

Human skeletal F (filamentous) actin, G (globular) actin, myosin, troponin, and tropomyosin were prepared according to described methods (21,22). Vimentin from bovine lens, and desmin from chicken stomach were provided by Dr. C. Vorgias (Department of Biochemistry and Molecular Biology, University of Athens). Bovine skeletal tropomyosin, and troponin and keratin from human epidermis were purchased from Sigma (St. Louis, MO). The purity of all antigens was verified by SDS-PAGE under reducing and nonreducing conditions.

HEp-2 Cytoplasmic Extract

Cultured HEp-2 cells were harvested from flasks and washed twice with cold (4°C) phosphate-buffered saline (PBS). The cells were then suspended, at 10^8 cells/mL, in Triton X-100 lysis buffer with protease inhibitors (300 mM NaCl, 50 mM Tris-HCl pH 7.6, 0.5% Triton X-100, PMSF 1 mM, aprotinin 10 μg/mL). After 30–45 min on ice, centrifugation was performed at 10,000 *g* for 15 min at 4°C, and the supernatant (the cytoplasmic extract) was stored in aliquots at −70°C. Protein concentration was measured by Bradford kit (BioRad, Richmond, CA).

Polyclonal and Monoclonal Antibodies

Control monoclonal antibodies specific for desmin and vimentin (Boehringer, Mannheim, Germany) or for tropomyosin and actin (Sigma, St. Louis, MO) were used in western blotting. Polyclonal antibodies against human myosin were produced in adult New Zealand white rabbits. Briefly, two animals were injected subcutaneously with 100 μg of myosin. The antigen was emulsified with complete Freund's adjuvant (CFA) for priming immunizations, and incomplete Freund's adjuvant (IFA) for secondary immunizations. Sera were collected after four boostings and stored at −30°C until use.

Enzyme and FITC Conjugates

Alkaline phosphatase-labeled goat anti-human IgG (γ-specific) (Sigma, St. Louis, MO), sheep anti-human IgA (α-specific) (Farmalex, Athens, Greece), sheep anti-human IgM (μ-specific) (Sigma, St. Louis, MO), mouse anti-human IgG1, mouse anti-human IgG2, mouse anti-human IgG3, mouse anti-human IgG4 (Calbiochem, San Diego, CA), sheep anti-rabbit Ig, and rabbit anti-mouse Ig (Farmalex, Athens, Greece) were used in ELISA and western blotting. FITC-conjugated antibodies specific for human IgG, human IgM, and human IgA (Kallestad, Chaska, MN) were used in the IFL.

Screening of ASMAs in Sera

ASMA-positive sera were primarily typed by IFL on commercial mouse stomach-kidney sections using an FITC-anti-human Ig conjugate (Kallestad, Chaska, MN). Sera were tested using sequential twofold dilutions starting from a 1:40 dilution until an end point was reached. The incubation period with sera and conjugate was 20 min at room temperature (RT) and under humidity to avoid the sections drying. Sera with a titer > 1:80 were considered to be ASMA-positive. For the determination of ASMA isotype, isotype specific FITC conjugates (20 μg/mL) were used. Screening dilution of sera for ASMA of IgG, IgM, and IgA classes was 1:20, 1:10, and 1:5, respectively. Results were read with an optical microscope.

ELISA for the Determination of ACTA Specificities, Classes, and Subclasses

ELISA using purified cytoskeleton antigens

Indirect, noncompetitive ELISA was performed as described elsewhere (23), with minor modifications. Myosin, tropomyosin, troponin, vimentin, and desmin were coated in polystyrene flat-bottomed plates (Nalge Nunc International, Rochester, NY), at a concentration of 5 μg/mL in 0.1 M carbonate-bicarbonate buffer (CBC), pH 9.5. G-actin was coated in CBC at 10 μg/mL, while F-actin was coated at a concentration of 5 μg/mL in PBS-$MgCl_2$ 2 mM-ATP 0.2 mM. Finally, keratin was coated at 5 μg/mL in Tris-HCl 0.1 M, pH 7.4, containing 8 M urea. For the detection of IgG autoantibodies, sera were diluted 1:500 in F-actin assay; 1:200 in G-actin; 1:100 in myosin, keratin, and desmin assays; and 1:50 in tropomyosin, troponin, and vimentin assays. For IgA and IgM antibodies, sera were tested at 1:50 in all assays. IgG subclasses were detected by incubating sera with the coated antigens, at a dilution of 1:50 in BSA-PBS-0.1% Tween. Sera reactivity was revealed by class- and subclass-specific alkaline phosphatase-labeled anti-human IgG, IgM, IgA, IgG1, IgG2, IgG3, and IgG4 conjugates (1 μg/mL). P-nitrophenyl phosphate (1 mg/mL) was used as a substrate, and optical density (OD) at 405 nm was measured using a microplate reader (Dynatech, Chantilly, VA). A reference serum with OD corresponding to that of the cut-off point (mean OD value of the control sera + 2.5 SD), was always included in each plate in order to directly define positive sera. A serum was regarded as positive if the obtained OD value was higher than that of the reference serum.

HEp-2 ELISA

The HEp-2 extract was used for coating at a concentration of 100 μg/mL in CBC 0.01 M, pH 9.5. Sera were diluted 1:25, when screening for IgA and IgM antibodies was performed, and 1:100 for IgG. Isotype-specific alkaline phosphatase-labeled anti-human antibodies were used at a concentration of 1 μg/mL, in all cases. All steps were similar to those described above for cytoskeleton ELISA.

SDS-PAGE and Western Blotting for Analysis of the HEp-2 Cytoplasmic Extract

The HEp-2 extract, prepared as described in Materials and Methods, was loaded on SDS polyacrylamide gel (4% stacking gel and 7–17% gradient separating gel). The electrophoresis was performed under denaturing and reducing conditions, diluting 800 μg of the extract in 1 × sample buffer containing 10 mM Tris, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.002 M bromophenol blue. Protein bands were transferred elecrophoretically from the gel to nitrocellulose (NC) sheets (Hybond-C 45 Micron, Amersham, UK) at 125 mA, overnight at 4°C. Transferred bands were revealed using Ponceau S. For antibody detection, NC strips (individually vertically cut from an NC sheet) were incubated with Tris-buffered saline (TBS) containing 5% nonfat milk and 0.2% Tween-20, for 2 hr at RT, in order to block the remaining binding sites. After three washings with TBS containing 0.5% Tween-20, and one with TBS, the strips were incubated with monoclonal antibodies at 2 μg/mL concentration or with antisera at 1:100 dilution in TBS containing 1% milk and 0.2% Tween-20, for 2 hr at RT. Strips were washed as described above and then incubated with alkaline phosphatase-conjugated anti-human IgG (2 μg/mL), anti-human IgM (1 μg/mL), or anti-human IgA (2 μg/mL) for 90 min at RT. The staining reaction was performed with NBT-BCIP substrate buffer and was stopped with TBS containing 20 mM EDTA.

Statistical Analysis

The proportions of positive sera per antigen and antibody isotype were compared between the AIH-I,

PBC, VH, and HD groups by a χ^2-test. The distribution of antibody activity values (OD values) of sera in each patient group (AIH-I, PBC, and VH) was compared to that of sera in the HD group using the nonparametric Mann-Whitney test statistic. The comparison of antibody activity between patient groups was also performed with the same test.

RESULTS

IgG Serum Autoantibodies to Cytoskeleton Proteins

Sera from patients with AIH-I, PBC, and VH B and C, as well as sera from HDs, were tested by ELISA for the presence of IgG antibodies against eight panel antigens. The proportions of positive sera per disease group and antigen are shown in Table 1. Analysis of the results by the χ^2-test showed that in comparison to sera from HDs, a significantly higher proportion of sera from each disease group exhibited increased IgG antibody reactivity to most of the panel antigens. There was no significant difference in the prevalence of anti-G-actin (60% in AIH-I vs. 83% in PBC and 51% in VH B and C), anti-keratin (21% in AIH-I vs. 30% in PBC and 23% in VH B and C), anti-vimentin (25% in AIH-I vs. 37% in PBC and 18% in VH B and C), and anti-desmin (40% in AIH-I vs. 32% in PBC and 52% in VH B and C) IgG antibodies among the three disease groups, suggesting that anti-actin autoantibodies are not characteristic for AIH-I. On the contrary, a significantly higher incidence of IgG anti-F-actin, anti-tropomyosin, and anti-myosin antibodies ($P \leq 0.05$) was observed in PBC (61%, 81%, and 52%, respectively) as compared to AIH-I (24%, 35%, and 17%, respectively) and VH (19%, 30%, and 23%, respectively). Overall, the main antigenic targets for IgG autoantibodies in AIH-I (incidence $\geq 35\%$) are G-actin, tropomyosin, and desmin.

Subsequently, we compared the IgG antibody reactivities against these antigens among the three disease groups (Fig. 1). Mann-Whitney statistical analysis showed that no significant difference of IgG antibody reactivities (expressed as OD values) against G-actin, desmin, and tropomyosin was observed among the three disease groups.

IgA and IgM Serum Antibodies to Cytoskeleton Proteins

As shown in Table 1, a significantly higher proportion of sera exhibiting increased IgA antibody activity, as compared to HDs, was observed mainly in PBC against all the panel antigens and in AIH-I and VH B and C only against tropomyosin. A significantly higher prevalence of IgA anti-desmin ($P \leq 0.01$), anti-vimentin, and anti-myosin antibodies ($P \leq 0.05$) was found in PBC, as compared to the other two disease groups (53%, 37%, and 44%, respectively, in PBC vs. 6%, 10%, and 16% in AIH-I, and 3%, 3%, and 13% in VH B and C).

TABLE 1. Proportion of positive sera with IgG, IgA, and IgM autoantibodies to the panel of cytoskeleton antigens in the three disease groups and the group of healthy donors.

		Cytoskeleton antigens							
Disease group	Antibody isotypes	F-actin	G-actin	Myosin	Tropomyosin	Troponin	Keratin	Vimentin	Desmin
	IgG	24% (*)	**60% (**)**	17% (NS)	**35% (**)**	7% (NS)	21% (*)	25% (NS)	**40% (*)**
AIH-I[a]	IgA	14% (NS)	15% (NS)	16% (NS)	25% (*)	12% (NS)	19% (NS)	10% (NS)	6% (NS)
(n = 59)	IgM	**40% (**)**	19% (NS)	17% (NS)	23% (**)	26% (*)	30% (*)	18% (*)	27% (NS)
	IgG	**61% (**)**	**83% (**)**	**52% (**)**	**81% (**)**	19% (NS)	30% (*)	37% (*)	32% (NS)
PBC[b]	IgA	**48% (**)**	40% (*)	**44% (**)**	**52% (**)**	24% (*)	**48% (**)**	**37% (**)**	**53% (**)**
(n = 23)	IgM	**35% (**)**	26% (NS)	**44% (**)**	**43% (**)**	14% (NS)	**43% (**)**	1% (NS)	32% (NS)
	IgG	19% (NS)	**51% (**)**	23% (*)	30% (*)	15% (NS)	23% (*)	18% (NS)	**52% (**)**
VH[c]	IgA	21% (NS)	21% (NS)	13% (NS)	24% (*)	15% (NS)	17% (NS)	3% (NS)	3% (NS)
(n = 47)	IgM	23% (*)	32% (*)	17% (NS)	19% (**)	13% (NS)	26% (*)	21% (*)	18% (NS)
	IgG	2,5%	2.5%	2.5%	2.5%	7.5%	0	8%	10%
HD[d]	IgA	2,5%	5%	2.5%	0	0	2.5%	0%	8%
(n = 40)	IgM	2,5%	5%	2.5%	0	0	0	0%	8%

[a]Autoimmune hepatitis I.
[b]Primary biliary cirrhosis.
[c]Viral hepatitis B and C.
[d]Healthy donors.
*$P \leq 0.05$, by the χ^2-test, when compared to the control group; **$P \leq 0.01$, by the χ^2-test, when compared to the control group.
NS, statistically nonsignificant, by the χ^2-test, when compared to the control group.
Numbers in bold indicate high percentages ($\geq 35\%$).

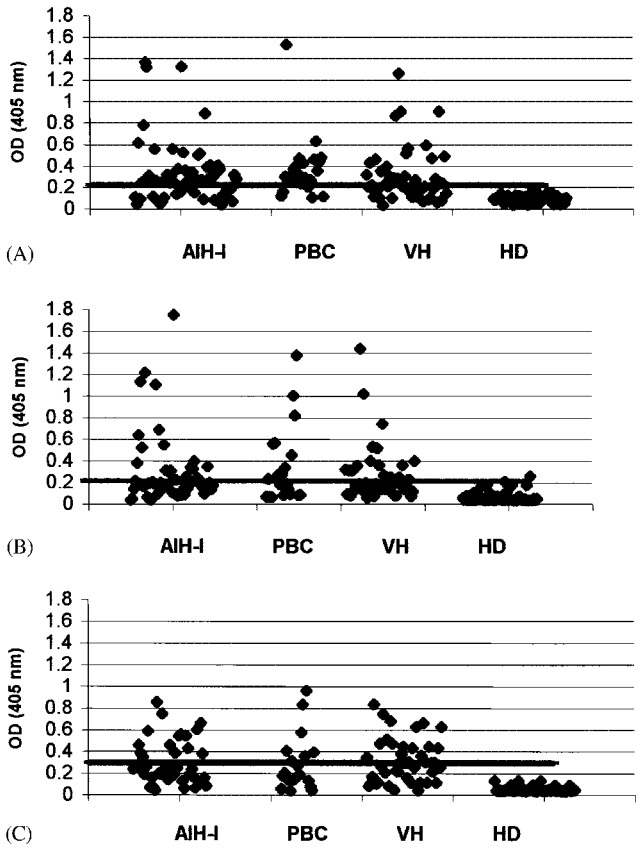

Fig. 1. Distribution of IgG reactivities against (**A**) G-actin, (**B**) tropomyosin, and (**C**) desmin in sera from AIH-I, PBC, VH, and HD groups. ELISA results are expressed as OD values of each serum at 405 nm. The cut-off values, defined by the mean OD value of HDs + 2.5 SD, are 0.222 for G-actin, 0.214 for tropomyosin, and 0.300 for desmin.

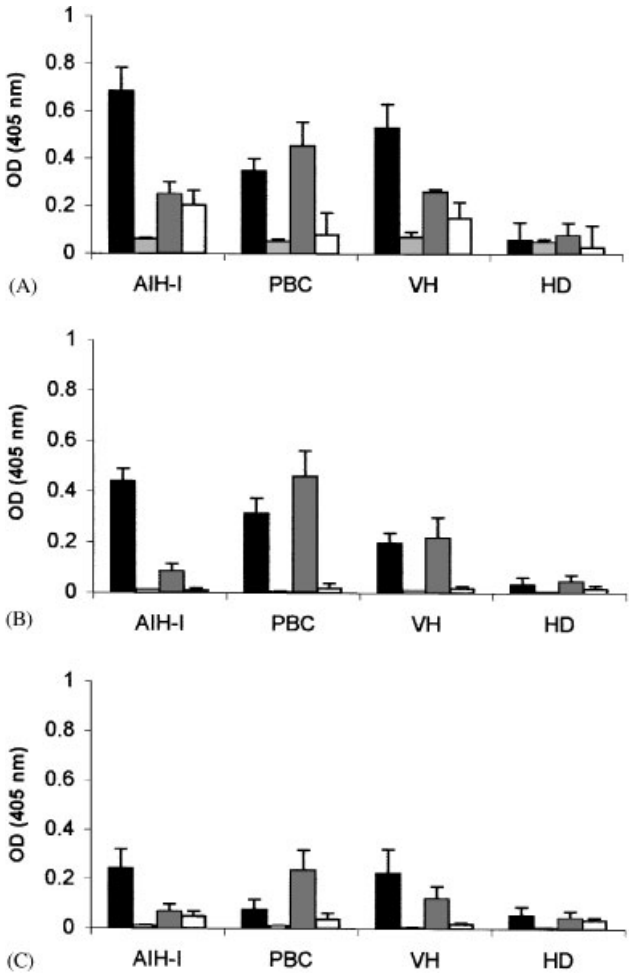

Fig. 2. IgG subclass distribution among autoantibodies reacting with (**A**) G-actin, (**B**) tropomyosin (TM), and (**C**) desmin in sera from AIH-I, PBC, VH, and HD groups. ELISA results are expressed in mean OD values + SD. Boxes follow the order IgG1, IgG2, IgG3, IgG4.

Finally, a significantly higher proportion of sera exhibiting increased IgM activity against most of the panel antigens was found in all disease groups as compared to HDs (Table 1).

IgG Subclasses of Serum Autoantibodies Reactive to Cytoskeleton Proteins

Sera from the AIH-I, PBC, VH B and C, and HD groups were tested by ELISA for the presence of IgG1, IgG2, IgG3, and IgG4 antibodies directed against the main antigenic targets of ACTA: G-actin, tropomyosin, and desmin. The results showed that in all disease groups, IgG autoantibodies against the three antigens were mainly of the IgG1 and IgG3 subclass (Fig. 2). IgG1 autoantibody activity to all three antigens was significantly higher, by Mann-Whitney statistical analysis ($P \leq 0.05$), in AIH-I sera (mean $OD_{value} = 0.684$, 0.439, and 0.241 for G-actin, tropomyosin, and desmin, respectively) as compared to PBC sera (mean $OD_{value} = 0.348$, 0.313, and 0.077 for G-actin, tropomyosin, and desmin, respectively), while the opposite occurred for IgG3 autoantibody activity (mean OD_{value} in AIH-I = 0.250, 0.083, and 0.066 for G-actin, tropomyosin, and desmin, respectively, vs. 0.455, 0.461, and 0.238, respectively, in PBC).

Anti-Cytoskeleton Activity and Isotypes of Serum Autoantibodies in ASMA-Positive and -Negative Sera: A Comparison of Classical IFL and ELISA

Sera from all disease groups were tested by IFL on mouse stomach-kidney sections for the presence of ASMAs of all three isotypes (IgG, IgA, and IgM). Sera were also tested by ELISA using purified cytoskeleton proteins (cytoskeleton ELISA) and HEp-2 cytoplasmic extract (HEp-2 ELISA). HEp-2 cells are used in this study as a source of cytoskeleton proteins. Analysis of a HEp-2 cytoplasmic extract by SDS-PAGE and western

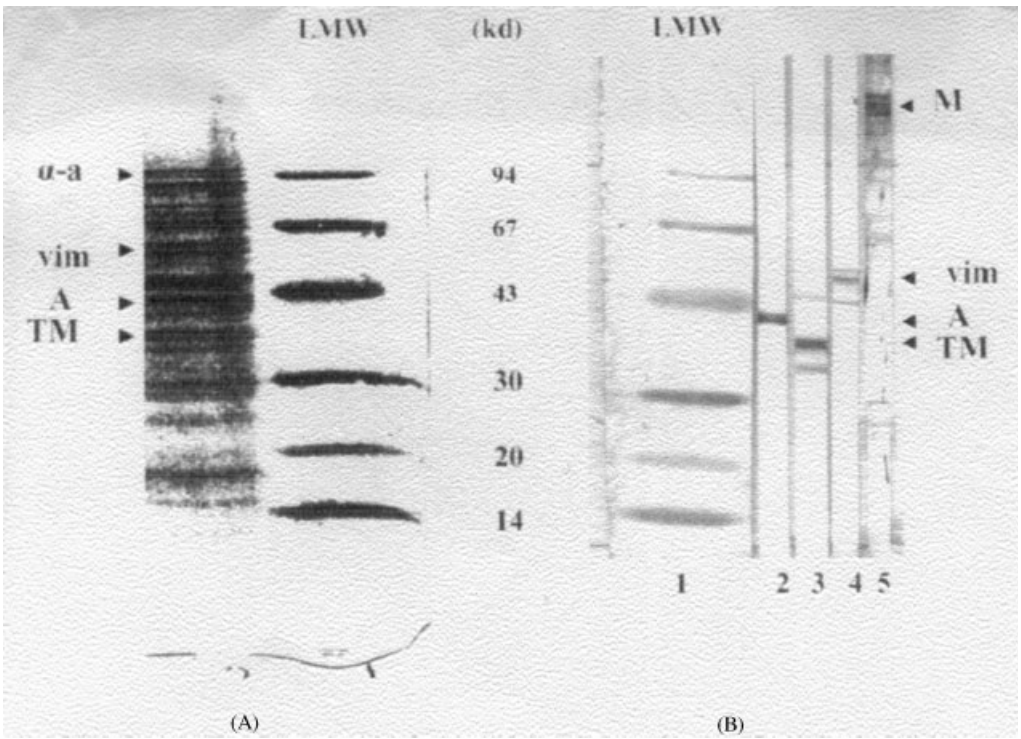

Fig. 3. Analysis of constituents of the HEp-2 cytoplasmic extract by (**A**) SDS-PAGE and (**B**) western blotting. A: The gel was stained with Coomassie blue. The antigens contained in the extract were identified by their molecular weights. LMW, low molecular weight; α-a, α-actinin (100 kd); Vim, vimentin (54 kd); A, actin (42 kd); TM, tropomyosin (35 kd). B: Proteins of the extract were separated by SDS-PAGE and then transferred to NC membranes. The NC was cut into strips. Lane 1: LMW markers after staining of the NC with Ponceau S. Lane 2: Incubation of the NC strip with a monoclonal anti-actin antibody. Lane 3: Incubation of the NC strip with a monoclonal anti-tropomyosin antibody. Lane 4: Incubation of the NC strip with a monoclonal anti-vimentin antibody. Lane 5: Incubation of the NC strip with a polyclonal anti-myosin antibody.

blotting showed that it contains a large amount of cytoskeleton proteins, including actin, myosin, tropomyosin, vimentin, and α-actinin (Fig. 3). Sera were considered to be positive by cytoskeleton ELISA if they reacted with at least one antigen. The comparative results are shown in Table 2.

Autoantibodies detected by IFL are almost exclusively of the IgG class: over 95% of positive sera in all tested groups contained IgG, while only 0–31% of positive sera contained IgA, and 10–59% IgM antibodies. On the contrary, using ELISA, IgM, and IgA, autoantibodies are also detected in a significant number of sera: over 50% of positive sera contained IgM and IgA antibodies, in almost all cases.

IFL and cytoskeleton ELISA revealed the same proportion of total positive sera (independently of the isotype), with the exception of PBC sera, which showed a low reactivity by IFL and a high reactivity by ELISA (22% of PBC sera reacted by IFL, while 100% ($P \leq 0.01$) and 85% ($P \leq 0.01$) reacted by cytoskeleton ELISA and HEp-2 ELISA, respectively). Sera from all disease groups reacted to a lesser extent by HEp-2 ELISA (100–92% of positive sera by cytoskeleton ELISA vs. 85–40% by HEp-2 ELISA). These results confirm that only IFL can discriminate between the two autoimmune liver diseases AIH-I and PBC, as the total proportion of sera reacting by IFL is significantly higher in AIH-I as compared to PBC (97% vs. 22% ($P \leq 0.01$)). On the contrary, there was no significant difference between the proportion of AIH-I, PBC, and VH sera reacting by cytoskeleton ELISA (93%, 100%, and 92%, respectively) and Hep-2 ELISA (65%, 85%, and 40%, respectively).

DISCUSSION

A serological profile of the different liver diseases (AIH-I, PBC, and VH) is important for their diagnosis. The immunological markers of PBC and VH are well defined as AMAs of M_2 specificity, and viral antigens or antiviral antibodies, respectively (2,15). On the other hand, ASMAs, which are routinely detected by IFL and used for the diagnosis of AIH-I, are autoantibodies that occur in a variety of diseases as well as in healthy individuals (3,6–8). Cytoskeleton proteins have been previously considered to be, at least in part, the

TABLE 2. Comparison of classical IFL and ELISA using cytoskeleton proteins or HEp-2 extract*

	Screening Methods											
	IFL[a]				Cytoskeleton ELISA				HEp-2 ELISA			
Disease group	Total positive[b]	IgG positive	IgM positive	IgA positive	Total positive	IgG positive	IgM positive	IgA positive	Total positive	IgG positive	IgM positive	IgA positive
AIH-I[c] (n = 59)	**97%**	90%	10%	30%	**93%**	90%	61%	34%	**65%**	37%	36%	13%
PBC[d] (n = 23)	**22%**	22%	13%	0	**100%**	100%	74%	78%	**85%**	80%	57%	38%
VH[e] (n = 47)	**96%**	93%	13%	6%	**92%**	83%	45%	49%	**40%**	33%	9%	11%

*Results are expressed as percentage of positive sera per disease group and antibody isotype (IgG, IgM, IgA).
[a]Indirect immunofluorescence.
[b]Positive sera for at least one isotype IgG, IgM or IgA.
[c]Autoimmune hepatitis type I.
[d]Primary biliary cirrhosis.
[e]Viral hepatitis B and C.

antigenic targets of ASMAs in liver diseases (9–16). ACTAs of distinct specificities have been correlated with different liver diseases (anti-actin antibodies with AIH-I, and anti-vimentin and anti-keratin antibodies with VH) (10,12,14,18,19). Previous studies by our group and other researchers (15–17) only partially confirmed the association of AIH-I with anti-actin antibodies. In the present study, we have shown that autoantibodies specific for either F- or G-actin are not associated exclusively with AIH-I. Therefore, an ELISA based on actin cannot be used for the diagnosis of AIH-I. This does not support the findings of previous investigators (18,19). In order to identify the form of actin recognized by autoantibodies, we developed an ELISA specific for each form. The coating conditions were appropriate for F- and G-actin to maintain their filamentous and globular conformation, respectively. The data showed that autoantibodies react mainly with G-actin rather than with F-actin, which was thought to be responsible for tissue and cellular fluorescent patterns (1,9). Our results suggest that these patterns result from the simultaneous reaction of autoantibodies with different microfilament antigens.

ACTAs from all disease groups were polyreactive and heterogeneous by ELISA. Most sera reacted with more than one antigen, and each serum exhibited its own reactivity profile, independently of the disease group. Apart from G-actin, the main antigenic targets of IgG autoantibodies in AIH-I are tropomyosin and desmin. The reactivity of AIH-I sera against these antigens was not significantly different from that of PBC and VH sera. Therefore, no serological discrimination of the liver diseases was evident using the eight panel antigens (F-actin, G-actin, tropomyosin, myosin, troponin, vimentin, desmin, and keratin). The same conclusion was derived from the Ig isotype and IgG subclass profiles. Only minor differences were found among the diseases, such as an increased IgA and IgG3 antibody reactivity in sera from PBC, and IgG1 antibody reactivity in sera from AIH-I and VH. However, these differences are not important as regards the diagnosis, since they cannot contribute to a clear differentiation of AIH-I from the other two disease groups. However, the predominance of IgG1 and IgG3 antibody subclasses, which are known to bind complement, suggests that ACTAs may contribute to the pathogenesis of liver diseases.

Our previous results (16) indicated that there is a significant correlation between ASMAs and ACTAs, even though 25% of ASMA-positive sera do not react with any of the cytoskeleton panel proteins. In the present study, by analyzing the Ig classes and subclasses, and using a similar panel of proteins, we found that only 4% of ASMA-positive sera (independently of the disease group) does not react with any of the cytoskeleton panel proteins. In addition, ASMA-negative sera (mainly from PBC patients), were all found to be ACTA-positive by ELISA. Another difference between IFL and ELISA arises from the analysis of the isotypes of serum autoantibodies, which showed that IFL detects autoantibodies almost exclusively of the IgG class, whereas autoantibodies of all classes (IgG, IgA, and IgM) are detected by ELISA in a significant number of sera.

Two explanations may account for the discrepancy between these two methods: 1) ASMAs (detected by IFL) may not be exclusively directed against cytoskeleton proteins, but may contain antibodies against other antigens or cytoskeleton-associated proteins. On the

other hand, ACTAs (detected by ELISA) may also be a distinct group of autoantibodies that presents additional reactivities not necessarily associated with those of ASMAs. 2) The epitopes accounting for the fluorescent patterns differ from those recognized in ELISA. This is possible because of the different conformational state of the antigens in the two assays: in IFL antigens conserve their native tertiary structure as they interact with each other and form complexes, while in ELISA the antigens used are purified. In addition, although cytoskeleton proteins are highly conserved molecules, antigens used in IFL and ELISA have different tissue origins and may have epitopic diversity.

In spite of the previously mentioned advantages of ELISA, as compared to IFL, it did not reveal any specific antigenic target or isotype for autoantibodies in AIH-I. The use in ELISA of a HEp-2 extract, which contains several cytoskeleton antigens in addition to those of the panel, only confirmed the finding that ACTAs are a group of autoantibodies that occur frequently in all liver diseases. Consequently, ELISA cannot replace IFL, which is the only method that discriminates between AIH-I and other liver diseases. Therefore, IFL remains the only suitable routine test for the diagnosis of AIH-I. We cannot exclude the possibility that a more detailed analysis of new antigenic targets of ASMAs and ACTAs, as well as the fine epitope mapping of the main antigenic targets (e.g., actin), may contribute to the identification of disease-related subspecificities and explain the role of these antibodies in AIH-I and other liver diseases. However, it appears that ASMAs and ACTAs are an epiphenomenon of AIH-I, and that the specific pathogenic antigen may be a liver-specific antigen.

ACKNOWLEDGMENTS

The authors thank Dr. G. Carayanniotis for useful discussions, and Dr. C. Vorgias for providing us with the desmin and vimentin.

REFERENCES

1. Whittingham S, Mackay IR. Smooth muscle autoantibodies. In: Peter JB, Shoenfeld Y, editors. Autoantibodies. Amsterdam: Elsevier Science BV; 1996. p 767–773.
2. Abuaf N, Johanet C, Homberg JC. Autoantibodies in autoimmune chronic active hepatitis. In: Krawitt E, Wiesner R, editors. Autoimmune liver diseases. New York: Raven Press, Ltd; 1991. p 93–109.
3. Bylund D, McHutchison J. Autoimmune liver diseases. Clin Lab Med 1997;17:483–497.
4. Toh BH. Smooth muscle autoantibodies and autoantigens. Clin Exp Immunol 1979;38:621–628.
5. Czaja AJ, Carpenter HA, Santrach PJ, Moore SB, Taswell HF, Homburger HA. Evidence against viruses as important causes of severe autoimmune hepatitis in the United States. J Hepatol 1993;18:342–352.
6. Kurki P. Cytoskeleton antibodies. In: Nishioka M, Toda G, Zeniya M, editors. Autoimmune hepatitis. Elsevier Science BV; 1994. p 185–198.
7. Nakamura RM, Bylund DJ. Diagnostic significance of autoantibodies in autoimmune chronic hepatitis. Clin Immunol Newsl 1991;11:161–167.
8. Bottazzo GF, Florin-Christensen A, Fairfax A, Swana G, Donicah D, Groschel-Stewart U. Classification of smooth muscle autoantibodies detected by immunofluorescence. J Clin Pathol 1976;29:403–410.
9. Gabbiani G, Ryan GB, Lamelin JP. Human smooth muscle autoantibody, its identification as antiactin antibody and a study of its binding to "nonmuscular" cells. Am J Pathol 1973;72: 473–484.
10. Lidman K, Biderfeld G, Fagraeus A, Norberg R, Torstensson R, Utter G. Anti-actin specificity of human smooth muscle antibodies in chronic active hepatitis. Clin Exp Immunol 1976;24: 266–272.
11. Zauli D, Crespi C, Bianchi FB, Pisi E. Immunofluorescent detection of anti-cytoskeleton antibodies using vinblastine-treated mononuclear cells. J Immunol Methods 1985;85:77–82.
12. Kurki P, Linder E, Miettinen A, Alfthan O. Smooth muscle antibodies of actin and non actin specificity. Clin Immunol Immunopathol 1978;9:443–453.
13. Kurki P, Virtanen I. The detection of human antibodies against cytoskeletal components. J Immunol Methods 1984;67:209–223.
14. Pedersen JS, Toh BH, Locarnini SA, Gust ID, Shyamala GNS. Autoantibody to intermediate filaments in viral hepatitis. Clin Immunol Immunopathol 1981;21:154–161.
15. Takaki A, Sakaguchi K, Ogawa S, Kawamoto H, Tsuji T. Specificities and clinical significance of anti-cytoskeleton antibodies in anti-smooth muscle antibody positive patients with chronic liver disease C. Acta Med Okayama 1994;48: 143–149.
16. Dighiero G, Lymberi P, Monot C, Abuaf N. Sera with high levels of anti-smooth muscle and anti-mitochondrial antibodies frequently bind to cytoskeleton proteins. Clin Exp Immunol 1990;82:52–56.
17. Kurki P, Miettinen A, Linder E, Pikkarainen P, Vuoristo M, Salaspuro MP. Different types of smooth muscle antibodies in chronic active hepatitis and primary biliary cirrhosis: their diagnostic and prognostic significance. Gut 1980;21:878–884.
18. Bretherton E, Brown C, Pedersen JS, et al. ELISA assay for IgG autoantibody to G-actin: comparison of chronic active hepatitis and acute viral hepatitis. Clin Exp Immunol 1983;51:611–616.
19. Leibovitch L, George J, Levi Y, Bakimer R, Shoenfeld Y. Anti-actin antibodies in sera from patients with autoimmune liver diseases and patients with carcinomas by ELISA. Immunol Lett 1995;48:129–132.
20. Andersen P, Small JV, Andersen HK, Sobieszek A. Reactivity of smooth muscle antibodies with F- and G-actin. Immunology 1979;37:705–709.
21. Spudish JA, Watt S. The regulation of rabbit skeletal muscle contraction. J Biol Chem 1971;246:4866–4871.
22. Whalen RG, Butler-Browne S, Gros F. Identification of a novel form of myosin light chain present in embryonic muscle tissue and cultured muscle cells. J Mol Biol 1978;126:415–431.
23. Mappouras D, Philippou G, Haralambous S, Tzartos S, Balafas A. Antibodies to acetylcholinesterase cross-reacting with thyroglobulin in myasthenia gravis and Graves' disease. Clin Exp Immunol 1995;100:336–343.