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Non-muscle myosin as target antigen for human autoantibodies in patients with hepatitis C virus-associated chronic liver diseases

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SUMMARY

Three patients with hepatitis C virus (HCV)-related chronic liver disease were shown to have autoantibodies strongly reacting with cytoskeletal fibres of non-muscle cells. The heavy chain of non-muscle myosin microfilament was the main target for those autoantibodies, as determined by (i) cell and tissue immunofluorescence studies showing colocalization with an anti-myosin antibody prototype; (ii) primary reactivity in immunoblotting with a 200-kD protein, using either MOLT-4 cells, human platelets, or affinity-purified non-muscle myosin as antigen extract; and (iii) immunoblotting of similar immunoreactive fragments in papain-digested MOLT-4 cell extracts, by using those human sera and antibody prototype. Autoantibodies to non-muscle myosin heavy chain were not previously reported in patients with chronic liver diseases, especially in those associated with HCV infection.

Keywords myosin autoimmunity chronic liver diseases hepatitis C virus

INTRODUCTION

More than half of all patients developing acute hepatitis C virus (HCV)-related post-transfusion hepatitis progress to chronic liver disease [1,2], either in the form of chronic active hepatitis or liver cirrhosis, with a substantial number progressing to hepatocellular carcinoma [3,4]. In cases of community-acquired infection, 62% develop chronic hepatitis in the USA [5]. Recent data show evidence for the presence of several autoimmune phenomena in patients with HCV infection, such as oligo- or polyarthritis [6,7], primary Sjögren's syndrome [8,9], membranoproliferative glomerulonephritis [10], cryoglobulinaemia [11–14], antinuclear, anti-mitochondrial, and anti-smooth muscle antibodies [11], rheumatoid factors [11,13], autoantibodies targeting a host protein named GOR, cloned from a chimpanzee infected with non-A, non-B hepatitis [15,16], and LKM-1 antibodies in adult patients with concomitant type 2 autoimmune hepatitis [17–21]. Some of the events were related to interferon-alpha (IFN-α) treatment [22] or to concomitant HCV infection due to exposure to human blood products [23].

Autoantibodies targeting cytoplasmic structures are commonly seen in autoimmune diseases [24–34] or in patients with active bacterial or viral infections [30,35,36], in most circumstances with lack of specificity for any particular disease entity.

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Cytoskeletal proteins like actin or myosin were shown to be targets of autoantibodies in autoimmune diseases [30–33,37–41], after cardiac surgery or heart diseases in humans and experimental animals [42–51], and accompanying some infections in humans or animals [30,40,52,53].

We report studies on three patients with chronic liver disease related to HCV infection in whom high titres of autoantibodies recognizing the heavy chain of non-muscle myosin were demonstrated.

PATIENTS AND METHODS

Patients and sera

Rabbit non-muscle myosin specific antibody was kindly provided by Velia M. Fowler (The Scripps Research Institute). This antibody preparation was isolated by affinity chromatography with native platelet myosin, and exclusively recognizes the myosin heavy chain [54]. Another myosin-specific antibody, a human monoclonal IgM to myosin heavy chain (SA-2), from a patient with myasthenia gravis and hyperplastic thymus [37,38], was a kind gift of Vanda A. Lennon (Mayo Clinic and Mayo Medical School, Rochester, MI). Normal human sera (NHS) were obtained from healthy donors in our laboratory.

Human sera from three patients (coded 225, 283, and 357) were selected on the basis of their common immunofluorescence (IF) pattern from 654 patients with chronic liver diseases who were being seen at the Shinshu University

Table 1. Clinical and laboratory data from three patients with anti-myosin antibodies

Patient	Age	Sex	Diagnosis	Antibody titre (IF)	Comments
225	74	F	Liver cirrhosis, HCV$^+$	1:1280	1985: abnormal liver tests; no previous blood transfusion or alcohol abuse; recent laboratory data: normal liver echographic examination; negative HBV serology, slight elevation of liver enzymes and total serum IgG, normal alpha-fetoprotein levels
283	66	F	Chronic hepatitis in transition to liver cirrhosis, HCV$^+$	1:2560	1987: liver dysfunction and biopsy compatible with early liver cirrhosis; no family history of liver disease; no history of hepatitis, alcohol or drug abuse, no surgery and no acupuncture treatment; recent laboratory data: normal liver echographic examination; liver tests compatible with mild chronic inflammation, negative HBV serology, normal alpha-fetoprotein levels
357	64	M	Chronic hepatitis, HCV$^+$	1:1280	1979: abnormal liver tests; father had liver cirrhosis; no history of alcohol or drug abuse, no previous surgery; recent laboratory data: mild hepatitis in liver biopsy; hepatic cyst and gallbladder polyp by echography; negative HBV serology, normal alpha-fetoprotein, slight elevation of liver enzymes and total IgG

IF, Immunofluorescence; HCV, hepatitis C virus; HBV, hepatitis B virus.

Hospital in Matsumoto, Japan. Clinical and laboratory data are presented in Table 1.

HCV serology

HCV serology was done in the laboratories of the Shinshu University School of Medicine by second generation ELISA, using the HCV non-structural protein C33c and the core protein C22 as antigens. Positive results in the three patients were confirmed by a standard polymerase chain reaction (PCR) assay that used nested primers from the highly conserved 5′ non-coding region of HCV. Among our patients, 396 (66·7%) were considered HCV-positive by the ELISA just described.

Indirect immunofluorescence

The standard indirect immunofluorescence (IIF) assay used commercial HEp-2 cells from Bion Enterprises Ltd. (Park Ridge, IL). Slides were incubated 60 min with first antibody in different dilutions, washed twice in PBS for 10 min, incubated with second antibody for 30 min, washed again, covered with glass slips using a 2:1 solution of glycerol: glycine buffer (3·5 g glycine, 0·18 g NaOH, 4·25 g NaCl, dH_2O 250 ml, pH 8·6), and read with a Olympus BH2-RFCA microscope.

The second antibodies were either FITC-labelled burro anti-human IgG, IgA, or IgM (Kallestad/Sanofi, Chaska, MN), rhodamine-labelled goat anti-human IgG (Caltag, San Francisco, CA), or FITC-labelled goat anti-rabbit IgG heavy and light chains, human adsorbed (Kirkegaard & Perry, Gaithersburg, MD), all at 1:100 dilution. For double-labelling studies, a mixture of rabbit and human antibodies was used in the first incubation, and a mixture of FITC-/rhodamine-labelled antibodies in the second incubation step, all on a 1:1 volume basis. Anti-mitochondrial and anti-smooth muscle antibodies were tested using mouse kidney/stomach slides (Kallestad/Sanofi, Chaska, MN) with the same incubation steps and FITC-labelled second antibodies as above. For IIF using liver sections, a rat was killed, its liver immediately embedded in OCT and frozen in dry ice/ethanol. Cryostat sections of 6 μm thickness were obtained, fixed with a 3:1 solution of acethone/methanol for 5 min at −20°C, washed in PBS, air-dried, and stored at −70°C until further processing. Before the incubation with first antibody, sections were blocked with undiluted normal goat serum for 20 min at room temperature. Double-staining studies in liver sections used the same second antibodies as above.

Immunoblotting

The standard procedure for immunoblotting has been described [55]. Briefly, MOLT-4 cells (CRL 1582; American Type Culture Collection, Rockville, MD), which is a T lymphocyte cell line, were grown to a concentration of $1{\cdot}5 \times 10^6$ cells/ml in 750 ml Falcon 3045 flasks (Becton Dickinson, Oxnard, CA) with RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) complemented by fetal bovine serum (FBS), L-glutamine, and gentamicin before use. Cells were collected by centrifugation, 5 min at 794 ***g***, resuspended in ice cold PBS, pelleted by centrifugation for 7 min at 794 ***g***, and treated with two volumes of buffer A (150 mM NaCl, 10 mM $MgCl_2.6H_2O$, 10 mM Tris pH 7·4, 0·5% NP-40 in dH_2O) for 5 min in ice. After another centrifugation step of 10 min at 8000 ***g***, the supernatant was collected, with protein concentration around 20 mg/ml. An equal volume of 2× Laemmli's sample buffer was mixed with the supernatant and boiled for 3 min before loading into a 7·5% slab gel. Transfer from gel to nitrocellulose (NC) membranes was performed at constant voltage of 50 V for 4 h. NC strips were blocked with 3% milk in PBS for 20 min and incubated 1 h with sera. After rinsing and washing in PBS–Tween, ^{125}I-labelled protein A (ICN Biomedicals, Inc., Costa Mesa, CA), 200 000–400 000 ct/min, was used as detecting reagent in a 30-min incubation, followed by new rinsing and washing procedure, and autoradiography on XAR-5 film in the presence of enhancing screen, at −70°C, for 12–48 h. Membranes were probed with NHS at 1:100 dilution, rabbit anti-non-muscle

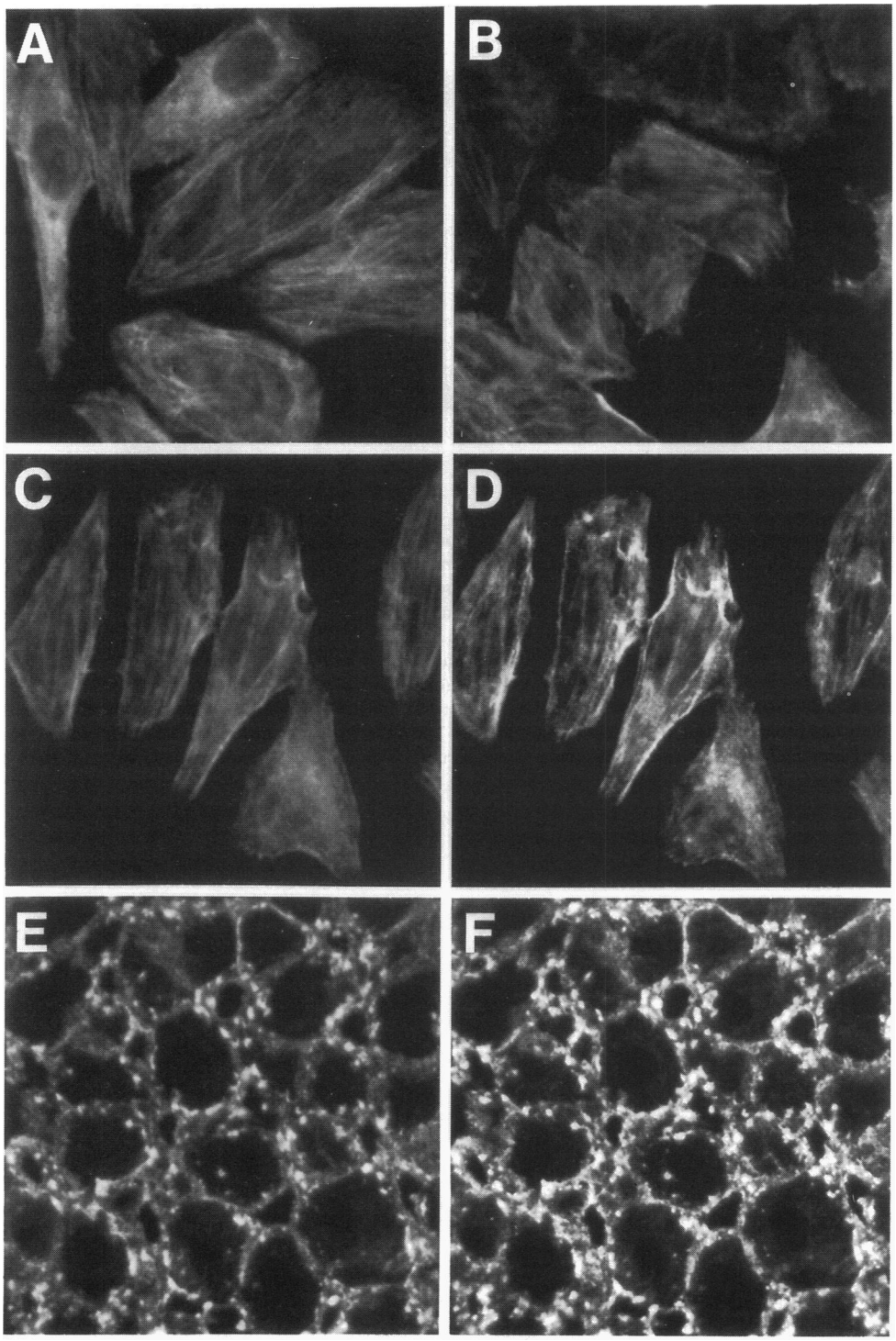

Fig. 1. Indirect immunofluorescence of anti-non-muscle myosin antibodies on HEp-2 cells and rat liver sections. (A,B) HEp-2 cells grown in monolayer were first incubated with sera 225 and 283, respectively, washed with PBS, and stained with rhodamine-labelled goat anti-human immunoglobulins as described in Patients and Methods. Note the typical radiating cable pattern of stress fibres as well as the absence of nucleoplasmic fluorescence. (C,D) Rhodamine-labelled staining of human serum 357 (C) co-localizing with the FITC staining obtained with the rabbit anti-non-muscle myosin prototype (D). The same cytoskeletal staining pattern is evident with both antibodies. (E,F) Fluorescence observed on a rat liver section using the same first and second antibodies as in C and D. Both antibodies display a polygonal pattern characteristic of non-muscle myosin in liver cells. (×500).

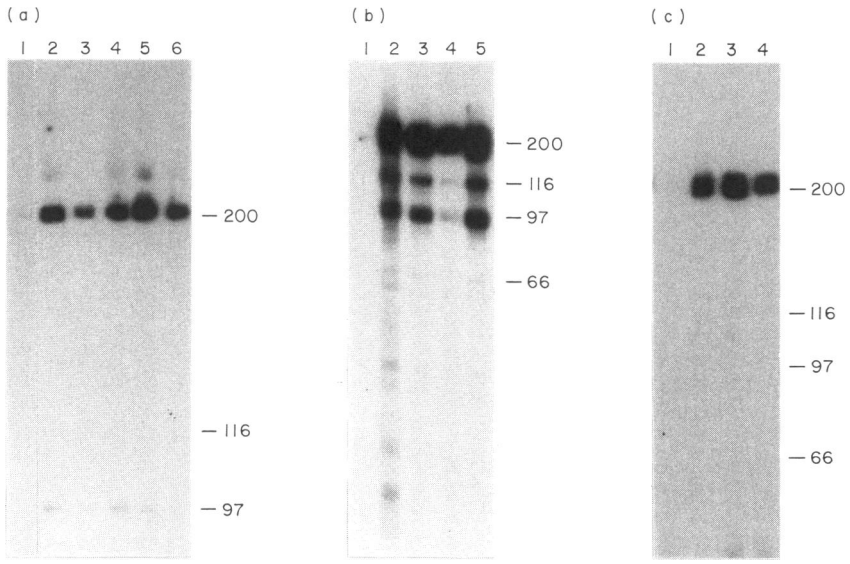

Fig. 2. Immunoblotting analysis using human and rabbit anti-non-muscle myosin antibodies. (a) Results of immunoblotting of cytoplasmic extract of MOLT-4 cells using normal human serum (lane 1, NHS), the rabbit anti-non-muscle myosin prototype antiserum (lane 2, 2 μg/ml; lane 3, 1 μg/ml), and human sera (lane 4, serum 225; lane 5, serum 283; lane 6, serum 357). Different antisera recognized a common band of 200 kD and also a degradation product with faint reactivity around 95 kD. (b) Human platelet extract was used as antigen source. Lane 1, NHS; lane 2, serum 225; lane 3, serum 283; lane 4, serum 357; lane 5, rabbit anti-non-muscle myosin prototype serum. Degradation products seen around 116 and 95 kD probably correspond to the myosin tail (fragments light meromyosin (LMM) + S2) and head (fragment S1), respectively. (c) Results of a two-part experiment, immunoprecipitation (IP) followed by immunoblotting. First, MOLT-4 cytoplasmic extract was immunoprecipitated with rabbit anti-non-muscle myosin prototype antibody and protein A-Sepharose beads (details in text). Then, the myosin-coated beads were analysed in immunoblotting using NHS (lane 1), serum 225 (lane 2), serum 283 (lane 3), and serum 357 (lane 4). These experiments used 7·5% SDS–PAGE gels.

myosin prototype in a concentration of 1–2 μg/ml, or patient's sera at 1 : 200 dilution in 3% milk–PBS–Tween. Alternatively, the procedure was conducted using human platelet extract obtained from normal blood donors, separated by centrifugation.

Affinity purification of myosin

MOLT-4 extract prepared as above was reacted with 0·1 volume of a 10% suspension of 1·5 g protein A-Sepharose (Pharmacia, Piscataway, NJ) containing 0·1% bovine serum albumin (BSA) in NET-2 buffer (50 mM Tris–HCl, pH 7·4, 150 mM NaCl, 0·1% NP-40, 0·1% deoxycholic acid, 0·1% SDS) for 15 min in a preclearing step. After centrifugation, the supernatant was mixed with rabbit anti-non-muscle myosin antibody, protein A-sepharose and NET-2 buffer, and incubated with end-over-end rotation for 60 min. All steps were done at 4°C. The Sepharose beads were washed three times in NET-2 buffer, resuspended in Laemmli's sample buffer, and boiled for 3 min before loading the supernatant into a 7·5% slab gel. SDS–PAGE was followed by standard immunoblotting with NHS or sera from chronic liver disease patients.

Partial peptide mapping

Papain (Sigma Chemical Co., St Louis, MO) in concentrations ranging from 12·5 μg/ml to 100 μg/ml in buffer E (200 mM Tris-HCl pH 6·9, 2 mM EDTA) was added in equal volumes to the MOLT-4 cell extract as described above and incubated for 30 min at 37°C [56]. One volume of 2× Laemmli's sample buffer was immediately added and samples were boiled for 3 min before being loaded on to a 7·5% SDS–PAGE gel, followed by immunoblotting with rabbit anti-non-muscle myosin prototype or human sera.

RESULTS

Radiating stress fibres corresponding to microfilaments were brightly decorated with the three human sera, and stained structures colocalized with the rabbit anti-non-muscle myosin heavy chain prototype (Fig. 1). High titres of autoantibodies were present, with patients 225 and 357 having positive IIF at 1 : 1280, and patient 283 at 1 : 2560. Anti-mitochondrial and anti-LKM antibodies were not detected. Anti-smooth muscle antibodies were positive at 1 : 640, 1 : 160, and 1 : 40 dilution with sera 225, 283, and 357, respectively. The three human sera reacted strongly with the brush-border region in renal tubules and in stomach epithelia, all positive at 1 : 640 dilution (data not shown). Experiments using different FITC-labelled conjugates demonstrated that the human antibodies were of the IgG isotype exclusively. The characteristic polygonal staining of myosin in rat liver cells can be seen in Fig. 1e,f, where

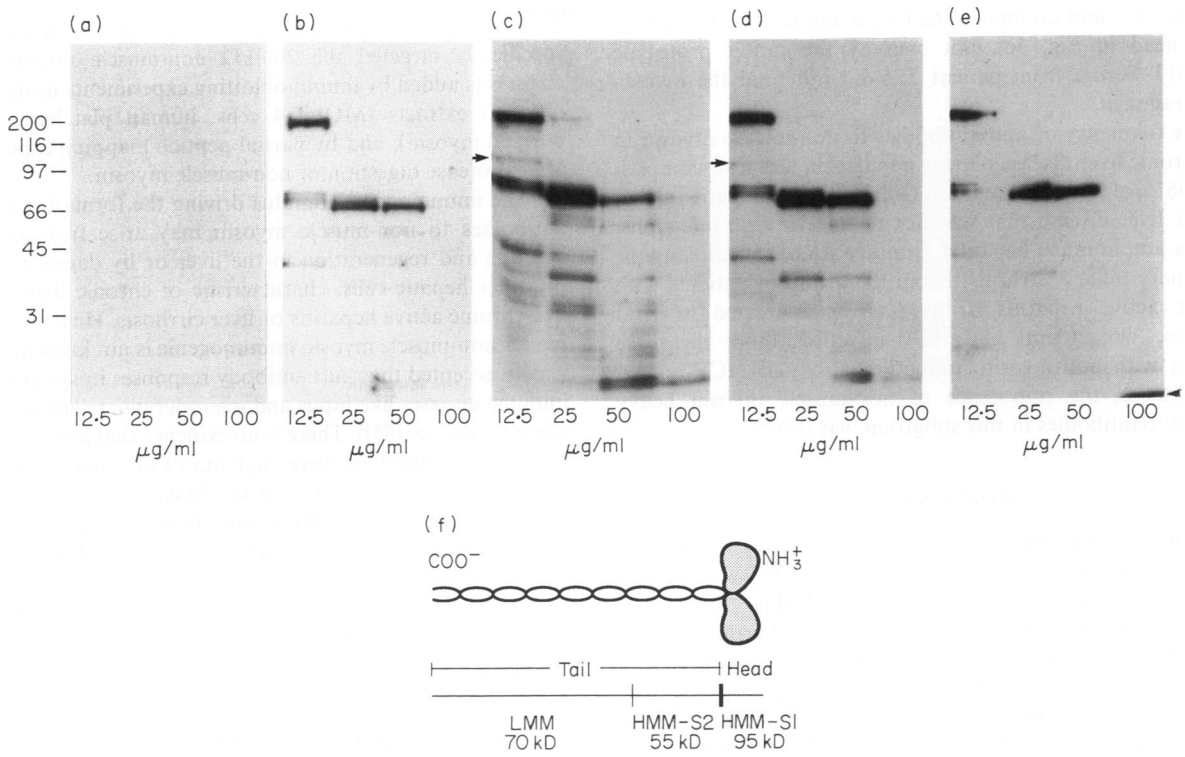

Fig. 3. Reactivity with peptide fragments of non-muscle myosin. Cytoplasmic extract from MOLT-4 cells was incubated with different concentrations of papain ranging from 12·5 μg/ml to 100 μg/ml, followed by SDS–PAGE in a 7·5% gel and immunoblotting. Panels a–e represent normal human serum (NHS), rabbit anti-non-muscle myosin prototype, and sera 225, 283, and 357, respectively. (f) Myosin heavy chain and its subdomains light meromyosin (LMM), heavy meromyosin S2 (HMM-S2), and HMM-S1, which are the major fragments commonly generated after protease digestion [58,86]. The pattern of bands obtained with human antisera was virtually identical to that seen with the rabbit prototype, with main reactivities seen at 200 kD (complete myosin heavy chain) and 70 kD (probably the LMM fragment) using low concentrations of papain (12·5 μg/ml). Sera 225 and 283 recognized additional fragments of lower molecular mass not reactive with the rabbit antiserum, although reactivities around 70 kD were predominant at papain concentration range of 25–50 μg/ml. At 100 μg/ml enzyme concentration, the human antisera still reacted faintly with peptides of 20 kD or less. Serum 357 had an additional strong band corresponding to a fragment with molecular mass around 10 kD (arrowhead). Only sera 225 and 283 recognized the HMM-S1 95-kD fragment when 12·5 μg/ml papain was used (arrows).

structures colocalize using either the rabbit prototype anti-myosin or human sera.

Figure 2 shows the results from immunoblotting analyses. Using either MOLT-4 cells or human platelet extracts, a major band of 200 kD, corresponding to the expected molecular mass of myosin heavy chain, was seen. There was co-migration of bands recognized by the three patients' sera and by the rabbit antibody prototype (Fig. 2a). In addition, the same patterns of reactivity were seen with possible heavy chain degradation products tentatively identified as the 125-kD tail fragment, and the 95-kD S1 fragment corresponding to the myosin head (Fig. 2b). The appearance of degradation products in the platelet extract was probably due to the absence of protease inhibitors in its preparation. The human MoAb (SA-2) to myosin also recognized a 200-kD band comigrating with the rabbit prototype and with the patients' sera in cell extract preparations (data not shown). Analyses of sera from the three patients over 1·5 years, with four samples collected at different time points for each individual, demonstrated no change over time. No reactivities in the lower molecular weight regions of the gels were seen that could be attributed to antibodies binding myosin light chains or actin. The confirmation that the human autoantibodies were reactive with myosin heavy chain was further demonstrated by immunoblotting of immunoprecipitates obtained from reaction of cytoplasmic extract with the prototype rabbit anti-myosin antibody (Fig. 2c).

Myosin is a well characterized protein and the major protease-generated fragments can be easily identified, so that this feature could be used to confirm further the specificity of human autoantibodies (Fig. 3). Extracts of MOLT-4 cells were digested with papain at different concentrations. The same major patterns of immunoreactive fragment recognition were observed between rabbit and human sera, with strong bands in the 200-kD and 70-kD regions, corresponding to the whole myosin molecule and to light meromyosin (LMM) respectively. Additional reactivities were seen with human sera. The latter bound several immunoreactive fragments of lower molecular weight (Fig. 3c,d), and also recognized distinct fragments like the 95-kD S1 head (arrows in Fig. 3c,d, sera

225 and 283) and an unidentified fragment of less than 10 kD (arrowhead in Fig. 3e), not seen by the rabbit prototype antibody. Serum from patient 357 did not bind the myosin head fragment.

The frequency of autoantibodies to non-muscle myosin in our chronic liver disease patient population was low, comprising 0·46% of 654 sera studied. Only 3% of our patients had chronic liver disease that was not related to viral infections, such as autoimmune hepatitis, primary sclerosing cholangitis, or primary biliary cirrhosis. From the group of patients with chronic active hepatitis or liver cirrhosis related to viral hepatitis, 396 patients had HCV infection. Since the three patients with anti-myosin antibodies also had HCV-related liver disease, the prevalence of non-muscle myosin heavy chain autoantibodies in this subgroup was 0·76%.

DISCUSSION

The tissue distribution and structure of myosin has been known for many years. Kühne, in the early 1860s [57,58], was the first to isolate a muscle protein called myosin. Today, three major classes of molecular motors are recognized, myosin, dynein, and kinesin, of which the most abundant in muscle cells is myosin. In non-muscle cells myosin has a low copy number [59], and is not organized in a sarcomere-like structure with actin as seen in muscle cells. Its function in those cells is mainly related to cytokinesis, organelle motility, and cell locomotion ('crawling') [58,60–64]. Contrary to the highly evolutionarily conserved actin, sarcomeric and non-muscle myosins have several different isoforms, determined by products of different genes or by post-translational modification, in accordance with their commitment to distinct cellular contractile functions [65–72].

In contrast to many reports of human autoantibodies to muscle myosin [37–40,42,43,46,49,73–82], autoantibodies targeting non-muscle myosin have been reported in only two patients. The first was a 68-year-old male with bronchiectasis [42], whose anti-myosin IgG antibodies reacted with liver, kidney and thyroid cells. The other was a 22-year-old male with myasthenia gravis and thymoma, from whom human IgM MoAbs were generated [37,38]. Serum and MoAbs reacted with thymic contractile proteins.

Our IIF studies in HEp-2 cells showed the characteristic decoration of stress fibres co-localized to structures also reacting with a rabbit antibody prototype specifically recognizing non-muscle myosin heavy chain. Using tissue substrates, we detected a honeycomb or polygonal pattern of IIF staining in liver cells, as well as staining of the brush border in kidney and stomach tissue (data not shown). Yasuura *et al.* [83], using monospecific antibody raised to rat hepatocyte myosin, were able to confirm the polygonal staining pattern of myosin in normal rat liver sections. Intestinal brush-border localization of myosin has been previously shown in chicken tissue [84]. Weak IIF staining at the luminal edge of kidney tubules, as well as polygonal pattern of rat liver staining, was a feature also described by Fairfax & Gröschel-Stewart with myosin antibodies [42]. Such findings suggest that cytoplasmic myosin tends to be localized immediately beneath the plasma membrane, co-localizing with actin. IIF experiments with our three human sera are in agreement with the characteristic staining of non-muscle myosin on cell or tissue substrates.

Further proof that autoantibodies in the human sera specifically targeted the 200-kD non-muscle myosin heavy chain was added by immunoblotting experiments using several different extracts (MOLT-4 cells, human platelets, affinity-purified myosin), and by partial peptide mapping experiments after protease digestion of non-muscle myosin.

The immunogenic stimulus driving the formation of autoantibodies to non-muscle myosin may arise from cycles of necrosis and regeneration in the liver or by damage inflicted on other hepatic cells, characteristic of chronic liver diseases like chronic active hepatitis or liver cirrhosis. How such events render non-muscle myosin immunogenic is not known, but it is widely accepted that autoantibody responses in systemic autoimmune diseases like lupus and scleroderma are antigen-driven (for a review see [85]). There is no evidence that any of the three patients reported here have stigmata of systemic autoimmune diseases, and there are no special clinical features of the liver disease which would set them apart from other patients in this category. It is also not clear what role chronic HCV infection could have played in eliciting such autoimmune phenomena in our patients. It is possible that immunohistochemical and molecular studies of liver tissue might provide some clues to the unique immune response to non-muscle myosin.

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