Clinical Chemistry 43:7
1188–1195 (1997)

Enzymes and Protein Markers

Serum concentrations of free ubiquitin and multiubiquitin chains

Koji Takada,[1*] Hidekazu Nasu,[4] Nozomu Hibi,[4] Yutaka Tsukada,[4] Toshiaki Shibasaki,[2] Kiyotaka Fujise,[3] Masahiro Fujimuro,[5] Hitoshi Sawada,[5] Hideyoshi Yokosawa,[5] and Kiyoshi Ohkawa[1]

Ubiquitin, which can conjugate with cellular proteins, is classified into two forms: free ubiquitin and multiubiquitin chains. The latter is active as a signal for degradation of the targeted proteins. We found both forms in human serum and, using two immunoassays, quantitated them in sera from healthy subjects and patients with some diseases. Because of putative leakage of erythrocyte ubiquitin, hemolytic serum and serum obtained after long incubation (>1–2 h) of blood at room temperature were excluded. Serum concentrations of multiubiquitin chains and free ubiquitin were substantially higher in rheumatoid arthritis and hemodialysis patients, respectively, than healthy subjects. Additionally, in acute viral hepatitis, serum multiubiquitin chain concentrations were increased in the acute phase, decreased in the recovery phase, and correlated with alanine and aspartate aminotransferase activities (r = 0.676 and 0.610, P <0.0001 and <0.001, respectively). Therefore, serum ubiquitin may have prognostic value.

INDEXING TERMS: radioimmunoassay • enzyme-linked immunosorbent assay • hemolysis • hemodialysis • rheumatoid arthritis • acute viral hepatitis

Ubiquitin, a 76-amino-acid protein, is present in the cytoplasm and nucleus of eukaryotic cells and can be covalently conjugated to cellular proteins by the enzymes of the ubiquitin conjugating system *[1, 2]*. In this process, several ubiquitin monomers are usually ligated sequentially to another ubiquitin moiety already linked to the protein, forming multiubiquitin chains *[3]*. The multiubiquitin chain acts as a signal to induce degradation of the target proteins by 26S proteasome *[2, 3]*. The ubiquitin-mediated proteolysis, a major pathway for selective protein degradation, plays a variety of regulatory roles in cellular processes, including stress response *[4-6]*, cell cycle *[7-9]*, gene expression *[10]* and apoptosis *[11]*. Ubiquitin may additionally be involved in the pathogenesis of various diseases. Intracellular accumulation of ubiquitin or ubiquitin conjugates has been detected in patients with neurodegenerative diseases *[12, 13]*, muscular diseases *[14]*, brain ischemia *[15, 16]*, and cancers *[17]*. Extracellular ubiquitin has been suggested to be involved in amyloid formation *[18]* and growth of hematopoietic cells *[19]*.

Cellular concentrations of the two forms of ubiquitin, free ubiquitin and multiubiquitin chains, are closely linked and change with various cellular events; e.g., heat stress increases multiubiquitin chains with the consumption of free ubiquitin *[5, 6, 20, 21]*. Thus, quantitation of both ubiquitin forms is valuable in investigations of ubiquitin-mediated phenomena in vivo. Several studies have revealed that ubiquitin concentrations in body fluids are increased in patients with various diseases: serum ubiquitin in parasitic and allergic disease *[22]*, plasma ubiquitin in chronic renal failure, especially in cases undergoing hemodialysis treatment *[23]*, and cerebrospinal fluid ubiquitin in Creutzfeldt–Jakob disease *[24]* and Alzheimer disease *[25, 26]*. However, these studies have not been entirely convincing because they have not determined which form of ubiquitin is preferentially being detected. Origin and metabolism of ubiquitin in the body fluid also have not been clarified.

We have recently developed two kinds of immunoassay: an RIA that preferentially detects free ubiquitin *[21]*, and an ELISA specific to the multiubiquitin chains *[20]*. In the present study, by using these assays, we have quantitated both ubiquitin forms in human serum from healthy

[1] Department of Biochemistry (I), [2] Department of Internal Medicine (II), and [3] Department of Internal Medicine (Kashiwa Hospital), Jikei University School of Medicine, Minato-ku, Tokyo 105, Japan.
[4] Department of Research Laboratory, SRL, Inc., Komiya-cho, Hachioji-shi, Tokyo 192, Japan.
[5] Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan.
[*] Author for correspondence. Fax 81-3-3435-1922; e-mail MHA03643@niftyserve.or.jp.
Received January 2, 1997; revised March 17, 1997; accepted March 18, 1997.

Downloaded from https://academic.oup.com/clinchem/article/43/7/1188/5640949 by U.S. Department of Justice user on 17 August 2022

subjects and patients with various diseases to find possible clinical application of the assays.

Materials and Methods

BLOOD COLLECTION AND SERUM PREPARATION

Blood was obtained from three healthy men (ages 28, 32, and 35 years) for assessment of serum processing conditions in the ubiquitin quantitation. Ten milliliters of blood was drawn from the antecubital vein and immediately aliquoted to check effects of the following conditions: (*a*) time from blood drawing to separation of the serum by centrifugation (0.5–6 h); (*b*) temperature of blood during the above conditions (room temperature or 4 °C); (*c*) time of storage of serum at 4 °C before freezing (0.5–10 h); (*d*) repeated freezing and thawing (to room temperature for 30 min/thaw) of serum.

We also collected blood from two groups of healthy volunteers (60 for group 1 and 96 for group 2), patients with systemic lupus erythematosus (SLE),[6] rheumatoid arthritis (RA), motor neuron disease (MND), and multiple sclerosis (MS) and patients on hemodialysis for chronic renal failure. Patients with SLE and RA were diagnosed according to the American Rheumatism Association criteria *[27, 28]*. Thirty-eight SLE patients (18 with clinically active disease, 20 with inactive disease) and 24 RA patients (all seropositive) were analyzed. Patients with MND and MS were diagnosed on the basis of their clinical symptoms, confirmatory paraclinical and imaging findings, and the exclusion of other neurological disease processes *[29, 30]*. The hemodialysis patients had been treated with cuprophane membrane dialyzers for 0.5–19.5 years (mean ± SD, 7.1 ± 5.4). In addition, seven patients (three men and four women, ages 25–52 years) with acute viral hepatitis (AVH) type A, diagnosed by means of a serological marker of IgM antibody to Hepatitis Virus A, were investigated. A total of 30 blood samples were obtained serially from all AVH patients (4–6 samples/patient) during the acute and recovery phases of AVH at 1–14-day intervals for ~1 month. Unless otherwise stated, blood was kept at room temperature and the resulting serum was separated by centrifugation within 2 h after the blood collection, then stored at −75 °C. All hemolytic sera (as determined macroscopically) were excluded.

REAGENTS AND CALIBRATION MATERIALS

Most of the reagents used were described previously *[20, 21]*. A reagent kit of Interference Check A (International Reagents Corp., Kobe, Japan) was used to check the interference of hyperbilirubinemia, hyperlipemia, and hemolysis in the immunoassays.

Bovine ubiquitin (Sigma, St. Louis, MO) was applied to a Mono Q column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mmol/L ethanolamine, pH 9.0, and highly purified monoubiquitin was eluted from the column with a linear gradient of sodium chloride. The monoubiquitin, dialyzed against phosphate-buffered saline, was quantified by absorbance at 280 nm *[31]* and used as a calibrator for free ubiquitin RIA.

Multiubiquitin chains were prepared as described previously *[20]*. Briefly, monoubiquitin (10 mg) was incubated overnight at 37 °C in 100 mmol/L Tris-HCl, pH 9.0, containing 15 ng of ^{125}I-labeled ubiquitin (4×10^6 cpm), 200 μg of lysozyme, ubiquitin-ligating enzyme mixtures (100 μg of E1/E2 and 300 μg of E3) *[32]*, 2 U of inorganic pyrophosphatase, 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, and 4 mmol/L ATP in a total volume of 1 mL. The reaction mixture was applied to a Superdex 200 HR 10/30 gel-filtration column (Pharmacia Biotech) and eluted with 50 mmol/L phosphate buffer, pH 7.2, containing 0.15 mol/L NaCl and 1 g/L 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate at a flow rate of 0.5 mL/min. The M_r of fractionated proteins was estimated by comparing their elution volumes with those of calibrator proteins *[20]*, and fractions with M_r >100 000 were selected. A mixture of the selected fractions, designated as the multiubiquitin chain reference preparation 1 (MUCRP1) *[20]*, was used to calibrate the multiubiquitin chain ELISA. The concentration of MUCRP1 estimated from the recovered ^{125}I-labeled ubiquitin radioactivity was 19 mg of ubiquitin equivalent per liter.

FREE UBIQUITIN RIA AND MULTIUBIQUITIN CHAIN ELISA

RIA and ELISA procedures were described previously *[20, 21]*. Briefly, in the RIA, 100 μL of each dilution of the calibrator or the twofold-diluted serum samples was transferred to respective tubes, and the rabbit antiserum to ubiquitin (US-1) and ^{125}I-labeled monoubiquitin were added to the tube (final volume 500 μL) and mixed. After a 20-h incubation at room temperature, diluted goat antiserum to rabbit γ-globulin containing normal rabbit serum and polyethylene glycol was added and incubated for an additional 1.5 h. Immune complex was obtained by centrifugation, and the radioactivity was counted.

In the ELISA, each dilution of the calibrator (MUCRP1) and the 10-fold-diluted samples (100 μL) was transferred to the respective wells of the microtiter plates, which had been coated with monoclonal antibody FK2 specific for conjugated ubiquitin *[32]*. The wells were incubated for 3 h at room temperature and then washed. One hundred microliters of biotinylated FK2 (4 mg/L) was added to each well and incubated for 2 h. After washing, 100 μL of a solution of peroxidase-conjugated streptavidin was

[6] Nonstandard abbreviations: AVH, acute viral hepatitis; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; MND, motor neuron disease; MS, multiple sclerosis; MUCRP1, multiubiquitin chain reference preparation 1; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; LAP, leucine aminopeptidase; γ-GTP, γ-glutamyltranspeptidase; CRP, C-reactive protein; DBi, direct bilirubin.

Downloaded from https://academic.oup.com/clinchem/article/43/7/1188/5640949 by U.S. Department of Justice user on 17 August 2022

added to each well and incubated for an additional 30 min. After washing, color was developed with the substrate 3,3′,5,5′-tetramethylbenzidine solution (Pierce, Rockford, IL) and measured at 450 nm. Calibrated curves were fitted by nonlinear regression analysis by using the computer software GraphPad PRISM (GraphPad Software, San Diego, CA), and sample concentrations were determined by interpolation.

We have already described the ranges of the calibration curves, the minimum detection limits, precision, and reproducibility of both assays *[20, 21]*. We have also certified that the RIA is an accurate method for measuring free ubiquitin in serum *[21]*. Accuracy of the ELISA in measurements of serum multiubiquitin chains was analyzed by a recovery test and a dilution test of human serum as described previously *[21]*.

SERUM FRACTIONATION BY GEL FILTRATION

Proteins in human serum from a healthy man, age 35 years, were fractionated by gel-filtration chromatography on a Superdex 200 HR 10/30 column. Before the chromatography, lipoproteins that could reduce the column performance were removed from the serum by dextran sulfate precipitation *[33]*. The lipoprotein-free serum derived from 110 μL of the serum was applied to the column and eluted with the same buffer used for the separation of MUCRP1. The M_r of the fractionated proteins was estimated as described above.

PREPARATION OF BLOOD CELL EXTRACTS

Erythrocytes, mononuclear cells, neutrophils, and platelets were separated from human peripheral blood by the modified method of Ficoll-Conray density centrifugation *[34]*. We prepared water-soluble extracts of these cells and estimated their protein concentration, using the previously described procedures *[20]*.

LABORATORY MEASUREMENTS

A sequential multiple automated analyzer (TBA-80 M; Toshiba, Tokyo, Japan) determined the following analytes in the serum specimens from AVH patients: aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), cholinesterase, alkaline phosphatase (ALP), leucine aminopeptidase (LAP), γ-glutamyltranspeptidase (γ-GTP), C-reactive protein (CRP), and direct bilirubin (DBi).

STATISTICAL ANALYSIS

Data are expressed as means ± SD. Means were compared by unpaired *t*-test unless stated otherwise, and test comparisons were analyzed by linear regression analysis. These statistical analyses were performed with GraphPad PRISM computer software. The diagnostic accuracy was evaluated by ROC curve analysis with the software GraphROC *[35, 36]*. Significance was set at $P < 0.05$.

Results

ASSAY VALIDATION

We carried out tests of recovery and dilution to assess the ELISA accuracy in measurements of serum multiubiquitin chains. Between 86% and 117% (96.1% ± 8.84%) of the added multiubiquitin chains were recovered in the recovery test. In the dilution test, the estimated multiubiquitin chain values of human sera at different dilutions were close to the calculated theoretical values (Fig. 1). These results indicate that the ELISA can be adopted for the quantitation of serum multiubiquitin chains.

FRACTIONATION OF HUMAN SERUM AND THEIR REACTIVITIES IN THE ASSAYS

The lipoprotein-free human serum was fractionated on a gel-filtration column, and the fractions were analyzed by both immunoassays (Fig. 2). The RIA-reactive response showed only one peak whose elution volume (18.6 mL) was coincident with that of monoubiquitin, a major form of free ubiquitin. However, the ELISA-reactive fractions were eluted from 8 to 14 mL and gave several distinct peaks whose M_r were estimated to be 50 000–1 000 000. This result is compatible with multiubiquitinated proteins showing a broad size spectrum *[37]*.

INTERFERENCE STUDIES

The addition of hemolysate (containing >0.5 g/L hemoglobin) greatly increased the estimated concentration of each ubiquitin in the samples, although highly purified hemoglobin (2 g/L), bilirubin (20–200 mg/L), and a lipid mixture [of egg yolk lecithin (91–910 mg/L) and triolein (85–850 mg/L)] showed no substantial interference in the assays (data not shown). Because erythrocytes contain large amounts of both ubiquitin forms (Table 1), the interference of the hemolysate may be a result of ubiquitin derived from erythrocytes.

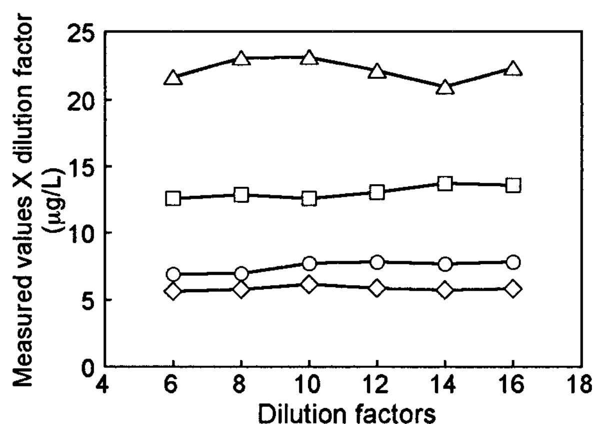

Fig. 1. Dilution tests of the ELISA for multiubiquitin chains.

Four human sera (○, □, ◇, and △) were serially diluted and used as the samples for the ELISA. Measured values (in terms of MUCRP1) obtained for each dilution, multiplied by the corresponding dilution factor, were plotted against the dilution factors.

Downloaded from https://academic.oup.com/clinchem/article/43/7/1188/5640949 by U.S. Department of Justice user on 17 August 2022

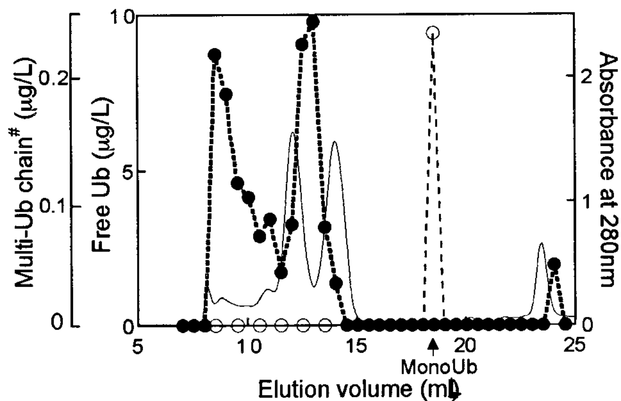

Fig. 2. Superdex 200 gel-filtration of human serum.

Lipoprotein-free human serum was fractionated by gel-filtration on Superdex 200. The concentrations of free ubiquitin (*Free Ub*, ○) and multiubiquitin chains (*Multi-Ub chain*, ●; # in terms of MUCRP1) in each fraction were estimated by the RIA and the ELISA, respectively, and absorbance at 280 nm of the eluate (——) was monitored. The location of eluted monoubiquitin in the separate experiment with the same column is indicated as *MonoUb* (- - - -).

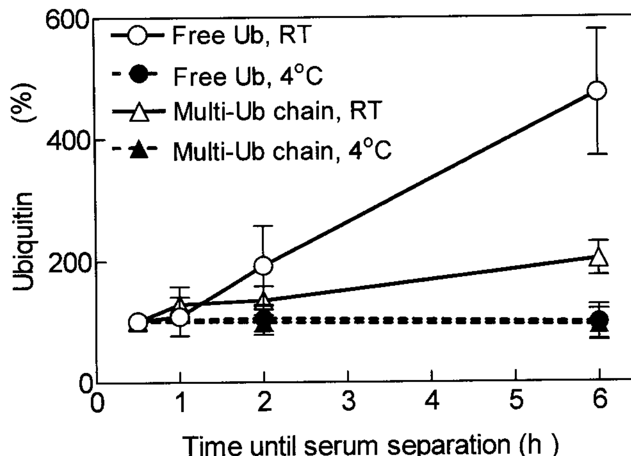

Fig. 3. Percentage of serum concentrations of free ubiquitin (*Free Ub*, ○ and ●) and multiubiquitin chains (*Multi-Ub chain*, △ and ▲) related to time until serum separation from the blood drawing.

The blood was kept at room temperature (*RT*) (○ and △) or 4 °C (● and ▲) until the serum separation. Each ubiquitin concentration was estimated by the immunoassays and calculated as the average (mean ± SD, n = 3), with estimates at 0.5 h being set to 100%.

EFFECTS OF SERUM PROCESSING CONDITIONS

Sera were separated from blood specimens kept for 1–6 h at room temperature or 4 °C, and their ubiquitin concentrations were estimated (Fig. 3). In the sera prepared at room temperature, both ubiquitin concentrations progressively increased with the time from blood drawing to serum separation. The rate of increase of free ubiquitin over time from blood drawing to serum separation was severalfold greater than that of multiubiquitin chains. However, in the sera prepared at 4 °C, the concentrations of free ubiquitin and multiubiquitin chains were not affected by the time for serum separation. Serum specimens showed no substantial change in free ubiquitin and multiubiquitin concentrations with storage for 0.5–10 h at 4 °C and with up to six freeze–thaws (data not shown).

SERUM UBIQUITIN CONCENTRATIONS IN HUMAN SUBJECTS

To establish the reference range, we analyzed the serum concentrations of both ubiquitin forms in two groups of healthy volunteers (Table 2 and Fig. 4). One was designated "control group 1," in which we kept the collected blood at 4 °C and separated the serum within 3 h after the blood collection. The other was designated "control group 2," in which blood was kept at room temperature and the resulting serum was separated at 5–6 h after the blood collection. Mean free ubiquitin concentration in control group 2 was significantly higher than in control group 1 ($P < 0.001$). Mean multiubiquitin chain concentration was also significantly, but only slightly, higher in control group 2 than in control group 1 ($P < 0.05$). There was no significant correlation between age and the ubiquitin concentrations (data not shown).

Because the patient sera were collected as part of a routine clinical evaluation and may be affected by incubation (up to 2 h) with the clots at room temperature, it is difficult to compare ubiquitin concentrations in the patient sera with those in the controls (Fig. 4). However, from the results shown in Fig. 3, we assume that the increase in ubiquitin induced by the sample processing in the patient sera was greater than in control group 1 but less than in control group 2. Thus the following observations suggest that serum ubiquitin concentrations are changed by several pathological conditions: (*a*) mean free ubiquitin concentration in hemodialysis patients was higher than in control group 2 ($P < 0.001$); (*b*) mean multiubiquitin chain concentration in patients with RA was higher than in control group 2 ($P < 0.001$ by Mann–

Table 1. Water-soluble ubiquitin concentrations in human blood cells.

	No. of experiments	Free ubiquitin	Multiubiquitin chains[a]
		Mean ± SD, μg/cells from 1 L of blood	
Erythrocytes	4	50 900 ± 9200	3930 ± 274
Mononuclear cells	3[b]	50.4 ± 3.61	0.836 ± 0.520
Neutrophils	3	60.4 ± 13.0	0.311 ± 0.050
Platelets	2	1.63 ± 0.405	0.050 ± 0.014

[a] Expressed in terms of MUCRP1.
[b] n = 2 for determination of multiubiquitin chains.

Downloaded from https://academic.oup.com/clinchem/article/43/7/1188/5640949 by U.S. Department of Justice user on 17 August 2022

Table 2. Serum ubiquitin concentrations in control subjects and patients with some diseases.

	Male cases	Female cases	Age, yr	Free ubiquitin, μg/L	Multiubiquitin chains[a], μg/L
			Mean ± SD		
Control group 1	32	28	31 ± 6	32.7 ± 13.8	3.40 ± 1.19
Control group 2	45	51	32 ± 8	126 ± 24.4	3.86 ± 1.55
SLE	4	34	36 ± 10	63.9 ± 24.7	3.88 ± 1.32
RA	4	19	37 ± 9	88.3 ± 43.5	5.47 ± 2.12
MND	13	16	56 ± 10	76.6 ± 38.4	2.76 ± 0.975
MS	2	8	47 ± 12	86.6 ± 36.8	2.57 ± 0.740
Hemodialysis	77	23	56 ± 12	307 ± 85.2	3.50 ± 2.23

[a] Expressed in terms of the standard MUCRP1.

Serum was prepared at 4 °C within 3 h (control group 1) or at room temperature within 6 h (control group 2) after the blood collection.

Whitney *U*-test); (*c*, *d*) mean multiubiquitin chain concentrations in patients with MND (*c*) or MS (*d*) were lower than in control group 1 ($P < 0.05$). ROC curve analysis was used to determine cutoff values for observations *a*, *b*, *c*, and *d*, estimated to be 171, 4.32, 2.66, and 2.36 μg/L, respectively (Fig. 5). These cutoff values were chosen to give the greatest diagnostic detection limit (0.97, 0.74, 0.59, and 0.50, respectively) and specificity (0.99, 0.73, 0.80, and 0.88, respectively).

Concentrations of multiubiquitin chains and free ubiquitin in a total of 30 serum samples from seven AVH patients were 9.62 ± 4.58 μg/L (in terms of MUCRP1; range 3.51–19.2) and 133 ± 57.2 μg/L (range 52.2–261), respectively. If the upper limit of the normal range is defined as mean ± 2 SD of control group 1 or 2 (Table 2), 77% or 63% of the sera were positive by the multiubiquitin chain ELISAs, which are comparable with the percentages from the liver function and enzyme tests (Table 3). In addition, serum multiubiquitin chain concentrations were increased in the acute phase and decreased in the recovery phase (a typical result is shown in Fig. 6), and correlated significantly with ALT, AST, and LDH activities (r = 0.676, 0.610, and 0.587; $P < 0.0001$, < 0.001, and < 0.01, respectively) (Table 3 and Fig. 7). Serum concentrations of free ubiquitin correlated weakly only with concentrations of multiubiquitin chains and CRP (r = 0.464 and 0.431; $P < 0.01$ and < 0.05, respectively).

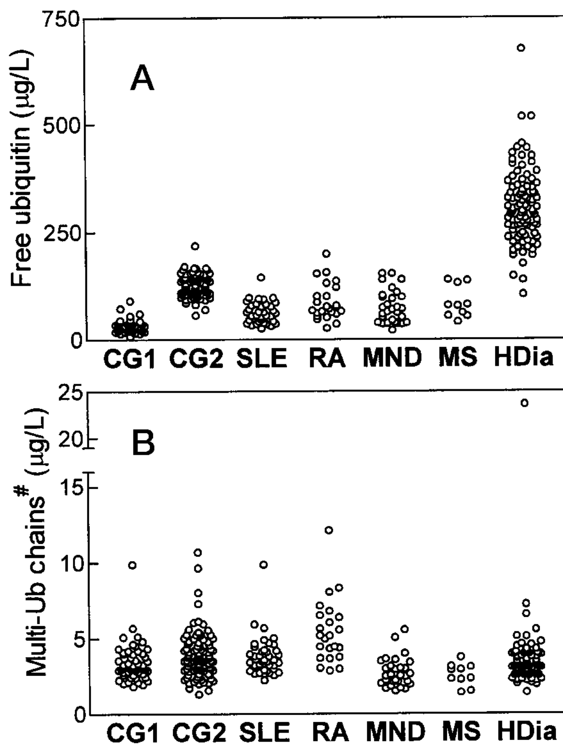

Fig. 4. Serum concentrations of free ubiquitin (*A*) and multiubiquitin chains (*Multi-Ub chains*, # in terms of MUCRP1) (*B*) in healthy subjects and patients with various diseases.

Each ubiquitin value was estimated by the immunoassays. Details of the samples are described in Table 2 and in *Materials and Methods*. *CG1*, control group 1; *CG2*, control group 2; *HDia*, hemodialysis.

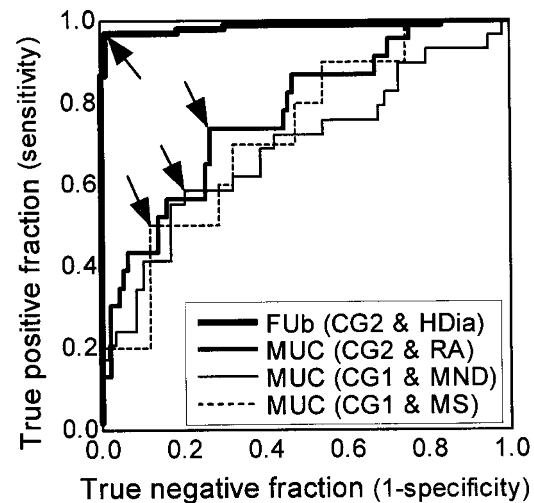

Fig. 5. ROC curves for serum ubiquitin (*FUb*, free ubiquitin; *MUC*, multiubiquitin chains).

Each curve was constructed with combined data from a selected control group and a selected patient group, as shown in Fig. 4. The selected groups are indicated in *parentheses*. Areas under the curves (and 95% confidence intervals) were 0.986 (0.968–1.00) for FUb in CG2 and HDia, 0.773 (0.666–0.880) for MUC in CG2 and RA, 0.689 (0.563–0.815) for MUC in CG1 and MND, and 0.738 (0.593–0.883) for MUC in CG1 and MS. Points derived from the cutoff values are indicated by *arrows*.

Downloaded from https://academic.oup.com/clinchem/article/43/7/1188/5640949 by U.S. Department of Justice user on 17 August 2022

Table 3. Positive rates of free ubiquitin, multiubiquitin chains, and laboratory tests, and correlation between each ubiquitin and the test data, in sera from patients with acute viral hepatitis.

	% positive[a,b]	Reference upper limit[b]	*r* with multiubiquitin chains[c]	*r* with free ubiquitin[c]
Multiubiquitin chains, μg/L	77, 63	5.78, 6.96		0.464**
Free ubiquitin, μg/L	90, 20	60.3, 175	0.464**	
AST, U/L	100	33	0.610***	0.209
ALT, U/L	100	35	0.676****	0.126
LDH, U/L	81	235	0.587**	0.159
ChE, U/L	17	700	−0.207	−0.214
ALP, U/L	57	300	−0.156	−0.246
LAP, U/L	80	80	0.312	0.280
γ-GTP, U/L	71	65 M, 27 F	0.175	−0.158
CRP, mg/L	55	3	0.075	0.431*
DBi, mg/L	97	3	0.151	0.286

[a] Ratio of serum specimens that gave higher estimates than reference upper limit to the total for 30 serum samples from the patients.
[b] For paired values, the first value is compared with the mean ± 2SD upper limit value calculated in control group 1, the second with that calculated in control group 2.
[c] Correlation coefficient calculated from all 30 serum samples from the patients. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Discussion

We demonstrated that free ubiquitin RIA and multiubiquitin chain ELISA are applicable to the estimation of each form of serum ubiquitin. The presence of both ubiquitin forms in human serum is also supported by the results of gel-filtration analysis (Fig. 2). In the ubiquitin-mediated proteolytic pathway, free ubiquitin and multiubiquitin chains have distinct cellular roles: the former is a pool for future conjugation to cellular proteins and the latter is an active form that induces their degradation *[3]*. Thus, the measurements of both ubiquitin forms are informative for deducing the cellular state of the ubiquitin system. We have stated previously that our immunoassays may be the only authentic and practical methods of quantifying both ubiquitin forms in the cells *[20, 21]*. Therefore, the present study is the first attempt to infer the systemic (or organic) states of the ubiquitin system from quantitation of both ubiquitin forms in human serum.

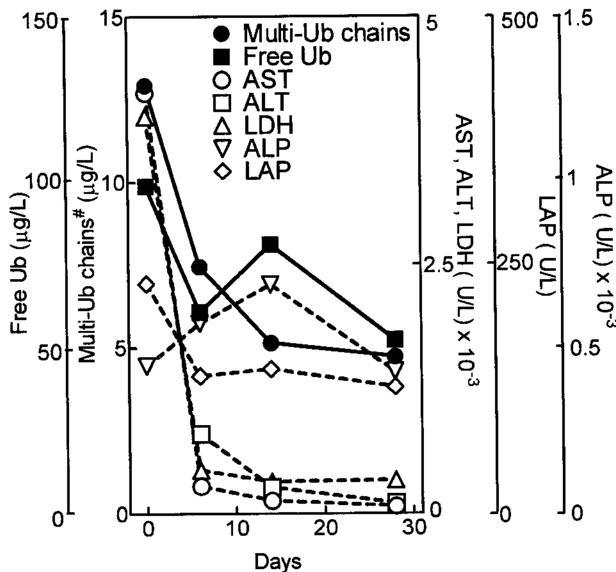

Fig. 6. Serial changes of serum concentrations of free ubiquitin (*Free Ub*), multiubiquitin chains (*Multi-Ub chains*, # in terms of MUCRP1), and liver function and enzyme tests in the patient with AVH (female, age 25 years).

The quality of the serum sample affected the ubiquitin quantitation (Figs. 3 and 4). Thus, we concluded that hemolytic serum must be avoided and serum must be separated from the clots quickly (within 1 h from blood collection at room temperature) or under chilled conditions (within 6 h from blood collection at 4 °C). Because the free ubiquitin concentrations of slightly reddened serum were within the range of control group 1, selection with the naked eye appears sufficient to eliminate the effect of hemolysis. Overestimation of serum ubiquitin by hemolysis is easy to understand because of the presence of ubiquitin in the erythrocytes (Table 1). However, it is difficult to explain why the longer (>1 h) incubation of blood at room temperature without hemolysis caused the

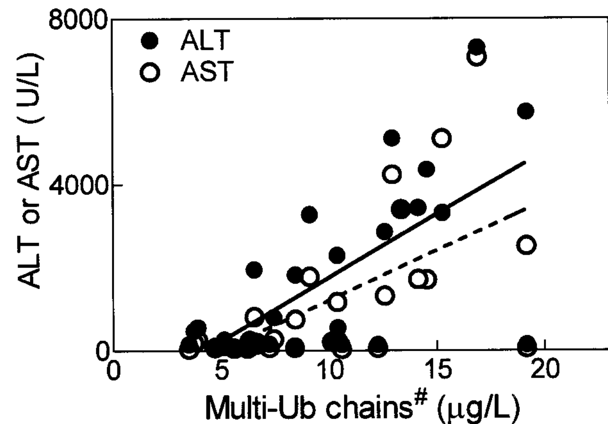

Fig. 7. Correlation between multiubiquitin chain concentration (*Multi-Ub chains*, # in terms of MUCRP1) and ALT (●) or AST (○) activity in sera from patients with AVH.

The regression lines with ALT (*solid line*) ($r = 0.676$, $P < 0.0001$, $y = 304x - 1280$) and with AST (*dotted line*) ($r = 0.610$, $P < 0.001$, $y = 237x - 1160$) are indicated.

Downloaded from https://academic.oup.com/clinchem/article/43/7/1188/5640949 by U.S. Department of Justice user on 17 August 2022

increase of ubiquitin values. We propose that it was caused by a unknown mechanism involving ubiquitin release from erythrocytes. Clot formation seems to be involved in this process because the 6-h incubation of blood supplemented with anticoagulants (either EDTA or heparin) was less effective for free ubiquitin increase (unpublished observation).

To evaluate ubiquitin concentrations in the patient sera, we used two groups of sera from healthy volunteers, control groups 1 and 2 (Table 2 and Fig. 4). The latter sera, separated after a long incubation (5–6 h) with clots, showed a greater increase of free ubiquitin than the former because of the putative ubiquitin release from erythrocytes. On the other hand, the former sera were prepared at 4 °C and thus were expected to show accurate concentrations of ubiquitin with little interference. Unfortunately, as described in *Results*, the samples from patients were not prepared under such an ideal condition. Therefore, comparison of serum ubiquitin concentrations was limited, and only the four observations described in *Results* are established. If the condition of serum separation is known for each patient sample, then values might be normalized against a calibrated curve as indicated in Fig. 3. However, we could not determine the exact condition of each sample, and thus such a method is not applicable to this case.

Okada et al. *[23]* reported that plasma ubiquitin increased in hemodialysis patients. We found that hemodialysis increased only free ubiquitin but not multiubiquitin chains in the serum. Thus, metabolism of each serum ubiquitin is distinct. Further studies are required to clarify the relation between serum ubiquitin and diseases tested in the present study.

Another aim of this study was to find potential clinical applications of the ubiquitin measurements. Serum concentrations of multiubiquitin chains but not free ubiquitin in AVH patients changed in accordance with the clinical course (Fig. 6) and correlated with the biochemical determinants of liver injury (Table 3). Ubiquitin conjugation to cellular proteins is greatly enhanced by acute stress, e.g., heat shock *[5, 6]* and ischemia *[15, 16]*. Therefore, multiubiquitin chains may be inducible by acute hepatocellular damages in AVH, and they are probably released into the blood by hepatonecrotic injury. On the basis of this assumption, serum multiubiquitin chain concentration reflects not only hepatocellular damage but also cytoprotective response to cell injury. Some AVH sera showed high concentrations of multiubiquitin chain in spite of low concentrations of ALT and AST (Fig. 7), and might reflect the cytoprotective response rather than damage. In addition, we recently found that multiubiquitin chain concentrations in sera from patients with drug-induced hepatitis were increased and highly correlated with ALP and LAP activities but poorly correlated with AST and ALT activities (unpublished observation). These results strongly suggest that the serum multiubiquitin chain is a novel marker for monitoring the clinical course of acute hepatic damage.

In the present study, AVH is proposed as a possible target for clinical application of multiubiquitin chain ELISA. However, ubiquitin is present in all cells in the human body; thus other diseases accompanied by acute cellular damage, e.g., acute renal failure, might also be candidates for the target. In addition, several studies have revealed the great involvement of ubiquitin-dependent proteolysis in cell proliferation via cell cycle control *[7-9]*. Consequently, malignant neoplasms seem to be another candidate. Further studies are in progress in our laboratory to assess serum ubiquitin in these diseases.

We are grateful to K. Shima (Department of Neurology, National Sapporo Minami Hospital) for providing some patient sera and T. Inoue (Department of Internal Medicine I, Jikei University School of Medicine) for valuable discussion. We also thank Y. Negishi, T. Kubota, K. Muramatsu, and the members of Hachioji Laboratory of SRL, Inc., for their cooperation in blood sampling and T. Hirakawa for technical assistance. This study was supported in part by a Grant in Aid for Scientific Research on Priority Areas (Intracellular Proteolysis) from the Ministry of Education, Science, and Culture. Some of the authors have applied for a patent on the ELISA.

References

1. Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J Biol Chem 1983;258:8206–14.
2. Hershko A, Ciechanover A. The ubiquitin system for protein degradation. Annu Rev Biochem 1992;61:761–807.
3. Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK, et al. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science 1989;243:1576–83.
4. Finley D, Ozkaynak E, Varshavsky A. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. Cell 1987;48:1035–46.
5. Carlson N, Rogers S, Rechsteiner M. Microinjection of ubiquitin: changes in protein degradation in HeLa cells subjected to heat-shock. J Cell Biol 1987;104:547–55.
6. Parag HA, Raboy B, Kulka RG. Effect of heat shock on protein degradation in mammalian cells: involvement of the ubiquitin system. EMBO J 1987;6:55–61.
7. Glotzer M, Murray AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. Nature 1991;349:132–8.
8. Nishizawa M, Furuno N, Okazaki K, Tanaka H, Ogawa Y, Sagata N. Degradation of Mos by the *N*-terminal proline (Pro2)-dependent ubiquitin pathway on fertilization of *Xenopus* eggs: possible significance of natural selection for Pro2 in Mos. EMBO J 1993;12:4021–7.
9. Funabiki H, Yamano H, Kumada K, Nagao K, Hunt T, Yanagida M. Cut2 proteolysis required for sister-chromatid separation in fission yeast. Nature 1996;381:438–41.
10. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell 1994;78:773–85.

Downloaded from https://academic.oup.com/clinchem/article/43/7/1188/5640949 by U.S. Department of Justice user on 17 August 2022

11. Delic J, Morange M, Magdelenat H. Ubiquitin pathway involvement in human lymphocyte γ-irradiation-induced apoptosis. Mol Cell Biol 1993;13:4875–83.

12. Mori H, Kondo J, Ihara Y. Ubiquitin is a component of paired helical filaments in Alzheimer's disease. Science 1987;235:1641–4.

13. Lowe J, Mayer RJ, Landon M. Ubiquitin in neurodegenerative diseases. Brain Pathol 1993;3:55–65.

14. Askanas V, Serdaroglu P, Engel WK, Alvarez RB. Immunolocalization of ubiquitin in muscle biopsies of patients with inclusion body myositis and oculopharyngeal muscular dystrophy. Neurosci Lett 1991;130:73–6.

15. Hayashi T, Takada K, Matsuda M. Changes in ubiquitin and ubiquitin–protein conjugates in the CA1 neurons after transient sublethal ischemia. Mol Chem Neuropathol 1991;15:75–82.

16. Hayashi T, Tanaka J, Kamikubo T, Takada K, Matsuda M. Increase in ubiquitin conjugates dependent on ischemic damage. Brain Res 1993;620:171–3.

17. Ishibashi Y, Takada K, Joh K, Ohkawa K, Aoki T, Matsuda M. Ubiquitin immunoreactivity in human malignant tumours. Br J Cancer 1991;63:320–2.

18. Alizadeh KK, Normand J, Chronopoulos S, Ali A, Ali KZ. Amyloid enhancing factor activity is associated with ubiquitin. Virchows Arch A Pathol Anat Histopathol 1992;420:139–48.

19. Daino H, Shibayama H, Machii T, Kitani T. Extracellular ubiquitin regulates the growth of human hematopoietic cells. Biochem Biophys Res Commun 1996;223:226–8.

20. Takada K, Nasu H, Hibi N, Tsukada Y, Ohkawa K, Fujimuro M, et al. Immunoassay for the quantification of intracellular multi-ubiquitin chains. Eur J Biochem 1995;233:42–7.

21. Takada K, Hibi N, Tsukada Y, Shibasaki T, Ohkawa K. Ability of ubiquitin radioimmunoassay to discriminate between monoubiquitin and multi-ubiquitin chains. Biochim Biophys Acta 1996;1290:282–8.

22. Asseman C, Pancre V, Delanoye A, Capron A, Auriault C. A radioimmunoassay for the quantification of human ubiquitin in biological fluids: application to parasitic and allergic diseases. J Immunol Methods 1994;173:93–101.

23. Okada M, Miyazaki S, Hirasawa Y. Increase in plasma concentration of ubiquitin in dialysis patients: possible involvement in β_2-microglobulin amyloidosis. Clin Chim Acta 1993;220:135–44.

24. Manaka H, Kato T, Kurita K, Katagiri T, Shikama Y, Kujirai K, et al. Marked increase in cerebrospinal fluid ubiquitin in Creutzfeldt–Jakob disease. Neurosci Lett 1992;139:47–9.

25. Wang GP, Iqbal K, Bucht G, Winblad B, Wisniewski HM, Grundke-Iqbal I. Alzheimer's disease. Paired helical filament immunoreactivity in cerebrospinal fluid. Acta Neuropathol 1991;82:6–12.

26. Kudo T, Iqbal K, Ravid R, Swaab DF, Grundke-Iqbal I. Alzheimer's disease. Correlation of cerebro-spinal fluid and brain ubiquitin levels. Brain Res 1994;639:1–7.

27. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.

28. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.

29. Li TM, Day SJ, Alberman E, Swash M. Differential diagnosis of motorneuron disease from other neurological conditions. Lancet 1986;27:31–3.

30. Nesbit GM, Forbes G, Scheithauer BW, Okazaki H, Rodriguez M. Multiple sclerosis: histopathologic and MR and/or CT correlation in 37 cases at biopsy and three cases at autopsy. Radiology 1991;180:467–74.

31. Haas AL, Wilkinson KD. The large scale purification of ubiquitin from human erythrocytes. Prep Biochem 1985;15:49–60.

32. Fujimuro M, Sawada H, Yokosawa H. Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. FEBS Lett 1994;349:173–80.

33. Frisch-Niggemeyer W. Rapid separation of immunoglobulins M from immunoglobulins G antibodies for reliable diagnosis of recent rubella infections. J Clin Microbiol 1975;2:377–81.

34. Wakeyama H, Takeshige K, Takayanagi R, Minakami S. Superoxide-forming NADPH oxidase preparation of pig polymorphonuclear leucocyte. Biochem J 1982;205:593–601.

35. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine [Review]. Clin Chem 1993;39:561–77.

36. Kairisto V. Optimal bin widths for frequency histograms and ROC curves. Clin Chem 1995;41:766–7.

37. Hershko A, Heller H. Occurrence of a polyubiquitin structure in ubiquitin–protein conjugates. Biochem Biophys Res Commun 1985;128:1079–86.

Downloaded from https://academic.oup.com/clinchem/article/43/7/1188/5640949 by U.S. Department of Justice user on 17 August 2022