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Abundance of Immunologically Active Alanine Aminotransferase in Sera of Liver Cirrhosis and Hepatocellular Carcinoma Patients

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BACKGROUND: Although alanine aminotransferase (ALT) is a widely used indicator of liver function, ALT enzymatic activity may not always reflect the degree of liver damage. Improved methods or approaches would be useful.

METHODS: Monoclonal antibodies (mAbs) to ALT were generated to develop a sandwich enzyme immunoassay system. We used an immunoassay to measure ALT mass concentration and a common biochemical analyzer to assay ALT enzymatic activity in serum samples from patients with liver diseases and healthy individuals. The results from the 2 methods were compared and analyzed by ROC curve analysis.

RESULTS: The ALT sandwich enzyme immunoassay system demonstrated reliable performance in linearity, recovery, and imprecision studies. The ALT activity assay exhibited a higher diagnostic accuracy in acute hepatitis (AH) patients, but the ALT immunoassay exhibited higher sensitivity and specificity in patients with chronic liver diseases. The areas under the ROC curve for ALT mass and enzymatic activity were 0.82 and 0.98, respectively, in AH, 0.99 and 0.52 in hepatocellular carcinoma (HCC), and 0.94 and 0.45 in liver cirrhosis (LC). Serum samples from HCC and LC patients had higher amounts of ALT–immunoglobulin complexes [median A_{450}, 1.7 (interquartile range, 1.4–1.9)] than the other groups [1.3 (interquartile range, 0.9–1.6)].

CONCLUSIONS: Our analysis of sera from the HCC and LC patient groups revealed considerable amounts of immunologically active but catalytically inactive ALT. The amount of the ALT–immunoglobulin complex increased with the severity of the liver disease. The 2-site immunoassay method may be useful in the differential diagnosis of some causes of liver disease.

Alanine aminotransferase (ALT)[4] participates in intermediary metabolism and in liver gluconeogenesis *(1)*. ALT is released from liver tissue into the circulation in proportion to the degree of hepatocellular damage due to toxic substances, viral infections, or other causes of liver damage *(2)*. In most types of liver disease, serum ALT exhibits greater enzymatic activity than serum aspartate aminotransferase *(3)*, and its activity has been considered one of the more sensitive markers of hepatocellular injury and liver disease progression *(4)*. The development of an accurate diagnostic method is critical for early detection and proper treatment of liver disease, because patients are often asymptomatic until their livers have deteriorated severely *(5)*. In clinical laboratories, serum ALT activity is commonly measured by a spectrophotometric assay in which the transamination reaction is coupled to a second reaction that reduces pyruvate to lactate via lactate dehydrogenase and NADH.

In this study, we developed a sandwich immunoassay that uses murine monoclonal antibodies (mAbs) generated against human recombinant ALT1 protein (hrALT) to quantify serum ALT mass concentration. We measured ALT enzymatic activity and mass concentration in the sera of liver disease patients and evaluated the results with ROC curve analysis to assess and compare the diagnostic accuracies of the immunoassay and the enzymatic activity assay *(6)*.

Serum samples were obtained from patients and healthy volunteers who enrolled at Hallym University Medical Center (ChunCheon, Korea). The institutional review board approved the current study plan, and informed consent was obtained from all participants. ALT enzymatic activity (L-type GPT J2 kit; Wako) and other biochemical tests were performed with a Hitachi 747 chemical analyzer. The categorization of patients to acute hepatitis (AH), chronic hepatitis, hepatocellular carcinoma (HCC), and liver cirrhosis (LC) was determined via physician review of the biochemical and clinical test results. The hrALT enzyme was expressed in bacteria, purified, and used as an immunogen for the production of the mouse mAbs *(7)*. The specific activity of the purified hrALT was assayed at 1099 IU/mg. Immunizations, cell fusion, screening of hybridoma cells, and production of mAbs

[4] Nonstandard abbreviations: ALT, alanine aminotransferase; mAb, monoclonal antibody; hrALT, human recombinant ALT1 protein; AH, acute hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

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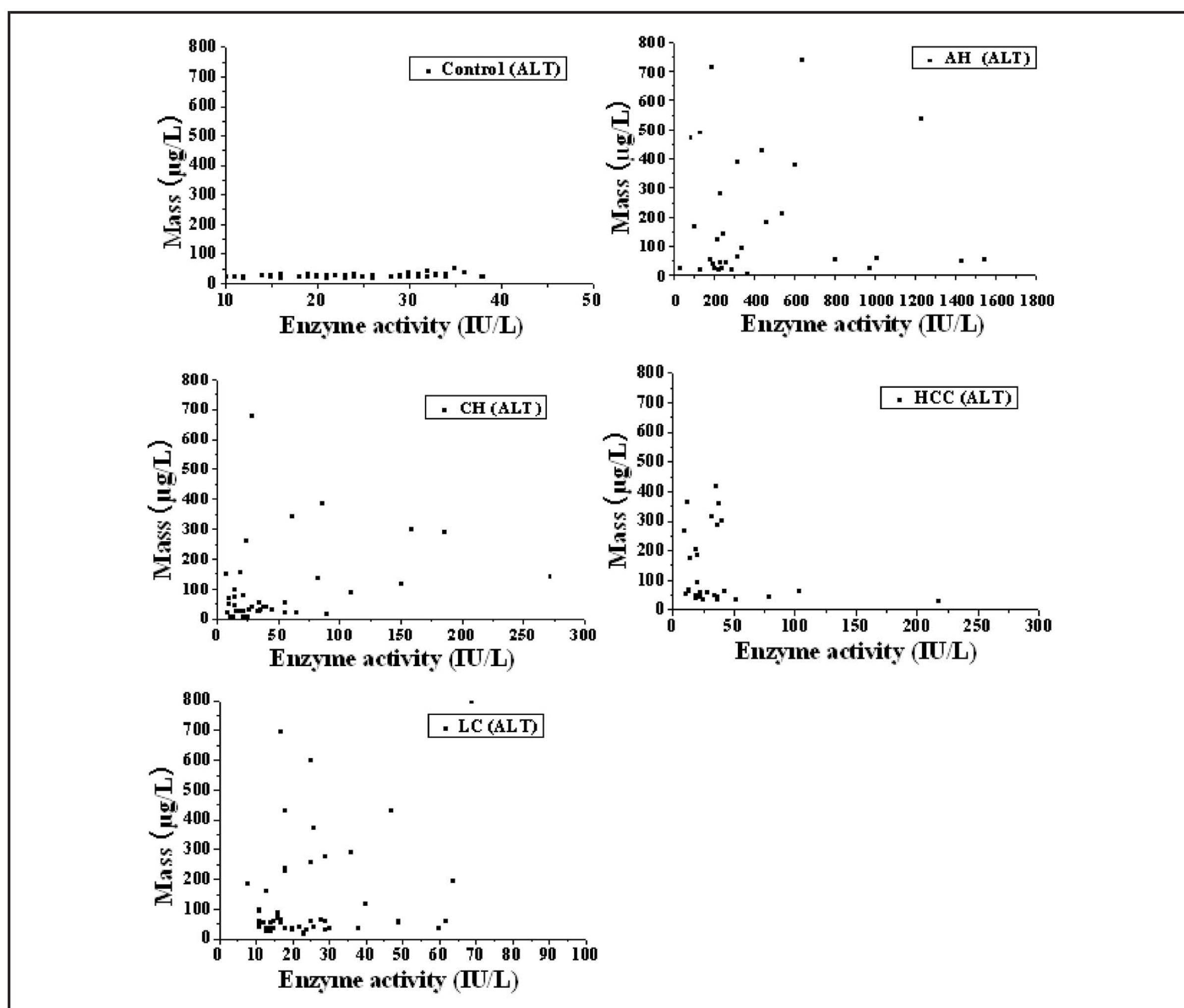

Fig. 1. Distribution of serum ALT mass concentration and enzymatic activity in healthy control and patients with different liver diseases.

Serum samples were measured side by side for ALT mass with an immunoassay and for enzymatic activity with a biochemical analyzer. AH patients had the highest median value for both ALT mass and enzymatic activity. As liver disease progressed, the ALT enzymatic activity decreased, but the ALT mass concentration had a tendency to increase. No significant correlation between serum ALT mass concentration and serum ALT enzymatic activity was observed. Note the different scales of enzyme activity on the *x* axes for the different groups. CH, chronic hepatitis.

were conducted with standard methods. The best analytical sensitivity was obtained when anti-ALT64C4 mAb and anti-ALT90C1 mAb were used as the capture antibody and the detection antibody, respectively; this combination was used in a sandwich enzyme immunoassay system for measuring the serum ALT mass concentration (data not shown). Differences between medians or means were evaluated for statistical significance with the Student *t*-test and ANOVA with Bonferroni adjustment. Pearson correlation coefficients and least-squares linear regression were used to evaluate correlations between the patient and control groups. P values <0.05 were considered statistically significant.

A calibration curve was prepared for the sandwich enzyme immunoassay system with different amounts of hrALT (0–500 μg/L). For calibration, we diluted control serum from a healthy individual (enzymatic activity, 9 IU/L) 5-fold with PBS (136 mmol/L NaCl, 2 mmol/L KCI, 8 mmol/L Na_2HPO_4, 1.4 mmol/L KH_2PO_4) and spiked the serum with known hrALT concentrations. Linear regression of A_{450} values on hrALT concentration in the calibrator solutions had an r value of 0.998 ($y = 0.0013x +$

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Table 1. Analysis of ROC curves.[a]

ALT	All patients		AH		CH		HCC		LC	
	Mass	Activity	Mass	Activity	Mass	Activity	Mass	Activity	Mass	Activity
AUC	0.87	0.66	0.82	0.98	0.74	0.81	0.99	0.52	0.94	0.45
95% CI	0.82–0.91	0.60–0.73	0.73–0.89	0.93–1.00	0.65–0.82	0.72–0.87	0.94–1.00	0.42–0.62	0.88–0.97	0.36–0.54
Cutoff	31.2 μg/L	30.0 IU/L	39.8 μg/L	44.0 IU/L	32.3 μg/L	31.0 IU/L	30.3 μg/L	8.0 IU/L	31.2 μg/L	31.0 IU/L
Sensitivity, %	77.2	48.8	68.6	94.3	53.5	53.5	100	28.1	86.5	25.0
Specificity, %	92.9	90.9	98.6	100.0	95.7	91.4	90.0	95.7	92.9	91.4

[a] P values for the differences in ALT mass and activity between the control group and the various groups of patients with liver diseases were all statistically significant ($P < 0.05$) except for ALT mass for the AH group ($P = 0.51$) and for ALT enzyme activity for the HCC group ($P = 0.40$) and the LC group ($P = 0.27$). CH, chronic hepatitis; AUC, area under the ROC curve.

0.0632, where y is the A_{450} value and x is the hrALT concentration). Imprecision and recovery studies were conducted to evaluate the analytical performance of the ALT immunoassay system. Within- and between-run assay CVs were 0.8%–6.9% and 4.9%–9.4%, respectively; recoveries were within 8% at all concentrations tested (see Table 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol55/issue5).

Fig. 1 shows the distribution of serum ALT mass concentrations and enzymatic activities in the healthy control group and the 4 groups with liver disease. No significant correlation between serum ALT enzymatic activity and serum ALT mass concentration was observed (all r values <0.58). The distribution pattern for AH patients was different from those of the other patients. AH patients showed a mixed pattern of high mass/low activity and low mass/high activity. The LC and HCC groups, however, displayed a pattern of high mass/low activity, suggesting that a considerable amount of immunologically active but catalytically inactive ALT enzyme exists in the sera of HCC and LC patients. As expected, median enzymatic activities varied widely among patients with different forms of liver disease, with the highest values found in AH patients (333.0 IU/L; interquartile range, 129.5–761.0 IU/L). The chronic hepatitis, HCC, and LC patients showed activities of 32.0 IU/L (interquartile range, 24.0–80.0 IU/L), 18.0 IU/L (interquartile range, 8.0–31.0 IU/L), and 15.0 IU/L (interquartile range, 10.0–30.0 IU/L), respectively (see Table 2 in the online Data Supplement). These data indicated that serum ALT enzymatic activity decreases as liver tissue damage progresses. In contrast, the ALT mass concentration tended to increase as liver disease progressed. Because the ALT activity assay we used was not supplemented with pyridoxal phosphate, we used 35 samples selected from the healthy control group and the groups of patients with different liver diseases to check whether the activity assay was affected by the presence of pyridoxal phosphate. We found no meaningful differences in ALT activity between the methods with and without pyridoxal phosphate; we thus concluded that the decreased activities in the HCC and LC patient groups were not due to vitamin B_6 deficiency (data not shown).

We used ROC curve analysis to evaluate ALT mass and ALT activity values for the groups of patients with liver diseases (Table 1; area under the ROC curve, 0.87 vs 0.66, respectively). This analysis indicated that the immunoassay method had a higher accuracy in HCC and LC patients than the activity method. The P values for both HCC and LC were <0.0001 vs the control group. At the obtained optimal cutoff values, the diagnostic sensitivity and specificity of the ALT immunoassay method were 100% and 90.0%, respectively, for the HCC group, and 86.5% and 92.9% for the LC group; however, the P values for the ALT enzyme activities in the HCC and LC groups were 0.54 and 0.20, respectively, suggesting no ability of the enzymatic activity assay to discriminate patients with these chronic liver diseases from the control group. This result is consistent with the results of previously reported studies *(8)*.

In the chronic liver disease groups, ALT mass concentration had a tendency to increase as liver disease progressed, whereas ALT activity decreased. These observations can be explained by proteolysis or some other alteration of the ALT protein after its release into the serum. Posttranslational regulation is common for serum and tissue proteins, and several factors involved in protein modifications are well known to reduce enzymatic activity *(9, 10)*. One possibility is the formation of a complex between the ALT enzyme and an antibody, as has been found in the inhibition of lactate dehydrogenase *(11)*. An antibody against ALT might bind to the enzyme and thus block enzymatic activity,

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probably by interfering with the access of substrate or cofactor. Antibody-bound ALT with reduced catalytic activity has been documented in the sera of patients with liver disease *(10)*.

We examined whether the healthy control group and the patient groups differed in the amounts of ALT–IgA complex and found that the HCC and LC groups had higher amounts of the ALT–IgA complex than the other groups [median A_{450}, 1.7 (interquartile range, 1.4–1.9) vs 1.3 (interquartile range, 0.9–1.6)] (see figure in the online Data Supplement); however, the concentrations of the ALT–IgA complex were significantly higher in all of the patient groups compared with the control group (all $P < 0.05$). Recently, several reports described high IgA concentrations in serum and a high incidence of IgA nephropathy in LC patients and indicated that the amount of aspartate aminotransferase–IgA complexes increased as liver disease progressed *(12, 13)*. Other modifications of serum ALT, such as glycation or fragmentation by proteolysis, could inhibit the interaction of ALT with a cofactor or substrate or could convert the functional dimeric protein to a monomeric form *(8, 14)*. Sera from healthy individuals and patients have been reported to contain considerably more immunologically active aspartate aminotransferase than the catalytically active protein *(15)*. Although the authors of the latter report did not consider the ALT protein or pursue whether the relationship between mass concentration and enzymatic activity changes with the progression of liver disease, their result seems very relevant to the key point of the current study.

In conclusion, our analysis of sera revealed that HCC and LC patients have considerable amounts of immunologically active but catalytically inactive ALT and higher amounts of the ALT–IgA complex. Our sandwich enzyme immunoassay method for determining serum ALT mass concentration performed well and exhibited good sensitivity and specificity. The immunoassay method we have described may be useful as a screening tool and may be useful in the differential diagnosis of liver disease.

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