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Augmenter of liver regeneration (ALR) may promote liver regeneration by reducing natural killer (NK) cell activity in human liver diseases

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Abstract: Cytotoxicity of liver natural killer cells against regenerating hepatocytes has been reported as a possible mechanism of regeneration failure in fulminant hepatitis. An augmenter of liver regeneration (ALR) inhibits liver natural killer cell activity in rats. In this study, we measured hepatic expression of ALR mRNA, blood levels of ALR, and peripheral blood natural killer cell activity in patients with various types of acute liver disease to investigate the relationship between failure of liver regeneration and hepatic natural killer cells. Hepatic ALR mRNA expression was higher in liver disease patients than in non-liver disease controls, and a correlation was found between serum ALR values and hepatic levels of ALR mRNA. In acute liver injury, the serum ALR level also showed a negative correlation with NK activity. ALR was produced by and released from the liver at the time of hepatic injury. Our findings suggest that ALR may protect against failure of regeneration by inhibition of hepatic natural killer cell activity in acute liver injury.

Key words: augmenter of liver regeneration (ALR), liver regeneration, NK cell activity

Introduction

Fulminant hepatitis has a poor prognosis although various methods of treatment have been developed. At present, liver transplantation has become a practical form of therapy for fulminant hepatitis, but there are problems related to insufficient donors and the need for long-term administration of immunosuppressants. Therefore, it is necessary to improve the results of conservative therapy, which are unsatisfactory at present.

The liver has remarkable regenerative power,[1] and adequate regeneration usually occurs in patients with acute hepatitis, despite extensive hepatocellular necrosis. However, in fatal fulminant hepatitis, the number of regenerating hepatocytes is significantly lower than that in surviving patients,[2] and it appears that regeneration failure causes death in such patients.

Thus, promotion of regeneration is important in patients with acute hepatic failure, such as those with fulminant hepatitis. Various treatments have been proposed to promote hepatic regeneration, e.g., glucagon—insulin therapy,[3] but the results are still not adequate. In addition, administration of growth factors is currently being studied. However, high blood levels of hepatocyte growth factor (HGF), a typical growth factor, are already observed in fulminant hepatitis, and the prognosis of patients with persistently high levels is poor.[4] Accordingly, there are doubts as to whether exogenous administration of this factor can be effective. There is also a dispute about the role of HGF in carcinogenesis.[5–7] Transforming growth factor alpha (TGF-α) is also involved in hepatic regeneration,[8,9] but exogenous administration to promote hepatocyte growth is considered difficult because of the risk of carcinogenesis,[10–12] and it has not been used clinically. Therefore, a method for promoting hepatic regeneration without using growth factors is desirable.

Hepatic stimulator substance was discovered in the cytosol of neonatal rat liver and after partial hepatectomy in rats by LaBrecque and Pesch.[13] It has since been detected in many animals and also in human fetal liver.[14–16] In experiments on rabbits, the expression of hepatic stimulator substance protein reached a peak 24 h after partial hepatectomy, and it was produced for only 18 to 36 h.[17] This substance promoted liver regeneration when administered to mice after partial hepa-

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tectomy or after they were treated with carbon tetrachloride,[18] and its growth-promoting action was specific for hepatocytes.[16,19] Hagiya et al.[20] cloned the cDNA of hepatic stimulator substance, clarified its primary structure and called it augmenter of liver regeneration (ALR). Natural killer (NK) cells in the liver[21] and spleen[22] show specific cytotoxicity against regenerating hepatocytes, but Francavilla et al.[23] found that ALR inhibited hepatic NK cell activity. The number of liver NK cells reaches a peak 2 weeks after partial hepatectomy and then gradually decreases and returns to the baseline level after about 1 month.[24] It appears that NK cells in the liver and spleen are involved in suppressing liver regeneration in patients with fulminant hepatitis and that specific cytotoxicity against regenerating hepatocytes is one mechanism of regeneration failure. ALR acts specifically on the liver and appears to promote liver regeneration by inhibiting hepatic NK activity, but the expression of ALR and its relationship with NK activity have not been investigated in human liver.

In this study, we measured ALR mRNA expression in liver tissue, serum ALR concentration, and peripheral blood NK activity in patients with various types of acute liver disease to clarify the relationship between ALR and liver regeneration in human acute hepatic failure.

Patients and methods

Patients

All patients studied were admitted to the First Department of Internal Medicine of Yamaguchi University between September 1993 and October 1997. Informed consent of the patients was obtained following the guidelines established by the Yamaguchi University Human Research Committee. Liver disease was classified as follows: Acute hepatitis (AH) was diagnosed when viral markers were positive in patients who had had normal liver function test results in the past and no previous history of liver disease. Fulminant hepatitis (FH) was diagnosed in acute hepatitis patients whose prothrombin time was ≤40% and who developed at least grade 2 hepatic coma within 8 weeks of onset. Chronic hepatitis (CH) and liver cirrhosis (LC) were diagnosed by liver function tests and histological examination of biopsy specimens. Acute exacerbation of chronic hepatitis (EX) was diagnosed in patients who showed a sharp increase in alanine aminotransferase (ALT) levels during the course of chronic hepatitis. Patients were classified as being in the recovery stages of acute hepatitis (AHR) and acute exacerbation of chronic hepatitis (EXR) when the ALT level returned to low and stable levels.

Sample collection and preparation

Blood samples, collected early in the morning after the patients had fasted overnight, were let stand for 30 min at room temperature. Serum was then separated by centrifugation and kept frozen at −80°C until determination of ALR. Liver biopsy was performed at laparoscopy. The larger portion of each biopsy specimen was fixed in formalin, embedded in paraffin, and examined histologically. About 3 to 5 mm of each specimen was immediately placed in guanidine isothiocyanate buffer at the time of biopsy, frozen in liquid nitrogen, and stored at −80°C for isolation of RNA.

Determination of serum ALR levels

Fifty-five patients with liver disease (Table 1) and 58 controls were studied. The patients with liver disease included 10 with acute hepatitis, 4 recovering from acute hepatitis, 7 with fulminant hepatitis, 6 with exacerbation of chronic hepatitis, 6 recovering from exacerbation, 17 with chronic hepatitis, and 5 with cirrhosis. Control serum ALR levels were determined in healthy individuals with no biochemical abnormalities. The serum ALR level was determined by an enzyme-linked immunosorbent assay (ELISA) method using standard human ALR, an anti-human ALR antibody, and a biotinylated anti-human ALR antibody (Toyobo, Ohtsu, Japan).

For this ELISA, ALR antibody was incubated overnight at 4°C. After blocking with Block Ace (Snow Brand, Sapporo, Japan), 20 to 100 pg/ml of diluted standard human ALR and serum samples were incubated at 37°C for 1.5 h. Biotinylated anti-human ALR antibody was incubated at 37°C for 1.5 h. Streptavidin horseradish peroxidase conjugate (Amersham, Buckinghamshire, UK) was incubated at 37°C for 1 h. The ELAST ELISA Amplification System (Du Pont, Wilmington, DE, USA) was used according to the manufacturer's

Table 1. Liver disease patients in whom serum ALR level was determined

	A	B	C	nAnBnC	Total
AH	7	3			10
AHR	1	3			4
FH		5		2	7
EX		4	2		6
EXR		5	1		6
CH		3	14		17
LC		3	2		5

ALR, augmenter of liver regeneration; A, hepatitis A virus (HAV); B, HBV; C, HCV; nAnBnC, liver disease of unknown etiology excluding HAV, HBV, and HCV; AH, acute hepatitis; AHR, recovery stage of AH; FH, fulminant hepatitis; EX, acute exacerbation of chromic hepatitis; EXR, recovery stage of EX; CH, chronic hepatitis; LC, liver cirrhosis

protocol. O-phenylenediamine dihydrochloride solution was incubated at room temperature until the color had clearly developed (about 15 min). Color development was stopped with 2N H_2SO_4 and the absorbance was determined at 490 nm using an automatic plate reader.

Investigation of ALR mRNA expression

Liver biopsy tissue was obtained from 22 patients with liver disease (Table 2) and two controls without liver disease. The patients with liver disease consisted of 4 recovering from acute hepatitis, 4 recovering from exacerbation of chronic hepatitis, 9 with chronic hepatitis, and 5 with cirrhosis.

The control liver tissues were obtained from a patient with cholecystitis at cholecystectomy and from a patient who died after a traffic accident.

Competitive reverse-transcriptase polymerase chain reaction (RT-PCR)

Because the liver tissue specimens were very small, competitive RT-PCR was used,[25,26] according to the method of Masuhara et al.[8] In the PCR, a cDNA with a known concentration (Mimic; Clontech Laboratories, Palo Alto, CA) was used as the competitor.

Synthesis of Mimic competitor

The Mimic competitor (ALR MIMIC) with the ALR base sequence[27] was prepared using the PCR Mimic TM construction kit (Clontech Laboratories). Composite primers (ALR MF and ALR MR for ALR M) were designed to hybridize the target gene primers (ALR F and ALR R) (Table 3).

Table 2. Liver disease patients in whom ALR mRNA level was determined

	A	B	C	Total
AHR	1	3		4
EXR		4		4
CH		2	7	9
LC		3	2	5

A, HAV; B, HBV; C, HCV; AHR, EXR, CH, LC, as in Table 1 footnote

Isolation of total RNA

Liver tissue was homogenized, using a Polytron homogenizer (PTA7; Kinematica, Lucerne, Switzerland), and total RNA was extracted by the method of Chomczynski and Sacchi.[28]

Synthesis of cDNA

First-strand cDNA was prepared from 1 μg of total RNA with oligo (dT) as the primer, using a First-strand cDNA Synthesis Kit (Clontech Laboratories).

Dilution of Mimic

Ten-fold serial dilutions of 1×10^{26} attmoles/ml of ALR M were prepared from 1×100 attmoles/ml. cDNA synthesized from total RNA obtained from 25 ng of liver tissue was placed in a tube containing serially diluted Mimic and amplified according to the manufacturer's protocol. Based on the results of a preliminary study using ten-fold serial dilutions, twofold serial dilutions (2M1–2M9) were prepared, ranging from 10×10^{23} attmoles/ml to 0.039×10^{-3} attmoles/ml.

PCR

A stock solution was prepared according to the manufacturer's protocol. Specific primers (ALR F and ALR R) were used in a total volume of 25 μl. cDNA (25 ng) prepared from liver tissue total RNA and twofold serial dilutions of Mimic (25 μl) were added to a tube and the mixture was amplified by 33 PCR cycles of 45 s at 94°C, 45 s at 57°C, and 120 s at 72°C.

Determination of NK activity

Twelve patients with liver disease (Table 4) were studied; of 7 with acute hepatitis and 5 with fulminant hepatitis.

Table 3. Primers for PCR

ALR F	**GTC GAC TTC AAG ACG TGG AT**
ALR R	**TAG TCA CAG GAG CCA TCC TT**
ALR MF	**GTC GAC TTC AAG ACG TGG AT**C GCA AGT GAA ATC TCC TCC G
ALR MR	**TAG TCA CAG GAG CCA TCC TT**T TGA GTC CAT GGG GAG CTT T

Bold face type indicates gene-specific primer sequence; underlining indicates sequence that hybridizes to the neutral DNA fragment
PCR, polymerase chain reaction

NK activity

Peripheral blood was collected in a syringe containing acid-citrate-dextrose (ACD) solution and cooled immediately. NK activity was measured by the ^{51}Cr release method. Peripheral blood was obtained by centrifugation on Ficoll-Hypaque gradients (density, 1.077) at 400 *g* for 25 min at 25°C. The mononuclear cells were collected from the gradient interface. After a washing with phosphate-buffered saline, RPMI 1640 medium, containing 10% fetal bovine serum, was added and the cell count was adjusted to 1×10^6/ml. Next, cultured K-562 cells were harvested by centrifugation, 50 to 100 µl of ^{51}C was added, and the mixture was incubated at 37°C for 1 h. After the cells were washed with phosphate-buffered saline, RPMI 1640 medium, containing 10% fetal bovine serum, was added and the cell count was adjusted to 1×10^6/ml. Then the target cells were placed in each well of a microplate. 1N HCl was added to obtain maximum release and RPMI 1640 medium containing 10% fetal bovine serum was added to obtain spontaneous release (controls). Peripheral blood mononuclear cells were added to the other wells at an effector/target ratio of 20. Centrifugation was performed for 5 min at 800 rpm, using a plate centrifuge, followed by incubation for 3.5 h in 5% CO_2. The culture supernatant was collected from each well and radioactivity was measured with a g-scintillation counter. NK activity was calculated as follows:

$$\text{NK activity (\%)} = \frac{\text{Experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{Maximum release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Statistical analysis

Measured values were analyzed statistically by the Mann-Whitney *U*-test and Spearman's rank correlation analysis. A difference of $P < 0.05$ was considered statistically significant.

Table 4. Liver disease patients in whom natural killer cell activity level was determined

	A	B	nAnBnC	Total
AH	3	4		7
FH		3	2	5

A, HAV; B, HBV; nAnBnC, liver disease of unknown etiology excluding HAV, HBV, and HCV; AH and FH, as in Table 1 footnote

Results

Serum ALR level

Figure 1 shows the ALR levels in the different groups of subjects. The level was 3.0 ± 7.1 pg/ml (mean [SD]) for the controls ("normal" in Fig. 1), 823.3 ± 1340.9 for fulminant hepatitis, 104.6 ± 114.5 for acute hepatitis,

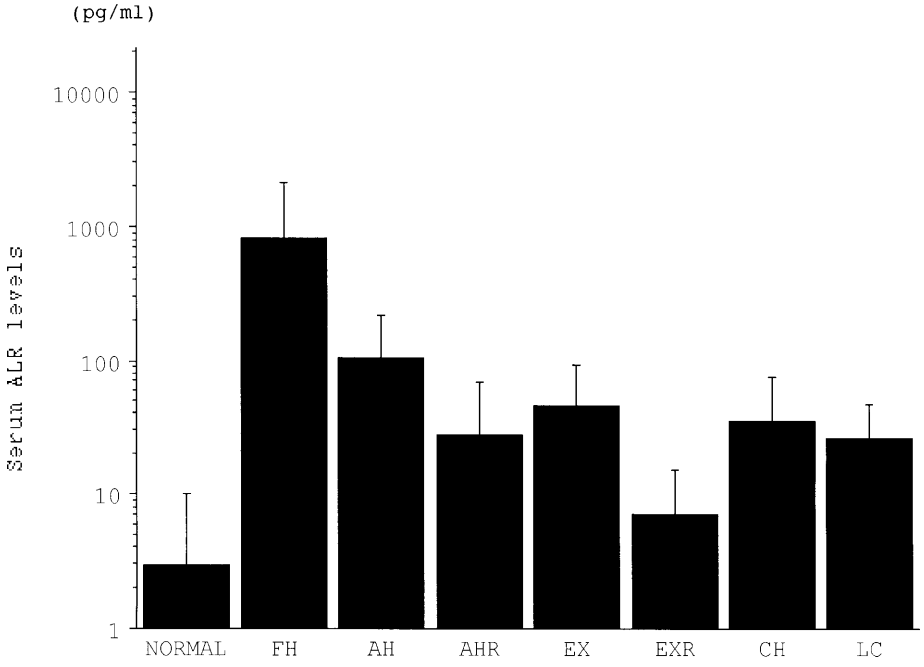


Fig. 1. Serum augmenter of liver regeneration (*ALR*) levels in various types of liver disease. Serum ALR levels were significantly higher in all types of liver disease than in the controls (normal; $P < 0.05$) and were also significantly higher in fulminant hepatitis (*FH*) than in the other liver diseases ($P < 0.05$). Values are means ± SD. *AH*, acute hepatitis; *AHR*, recovery stage of acute hepatitis; *EX*, acute exacerbation of chronic hepatitis; *EXR*, recovery stage of chronic hepatitis; *CH*, chronic hepatitis; *LC*, liver cirrhosis

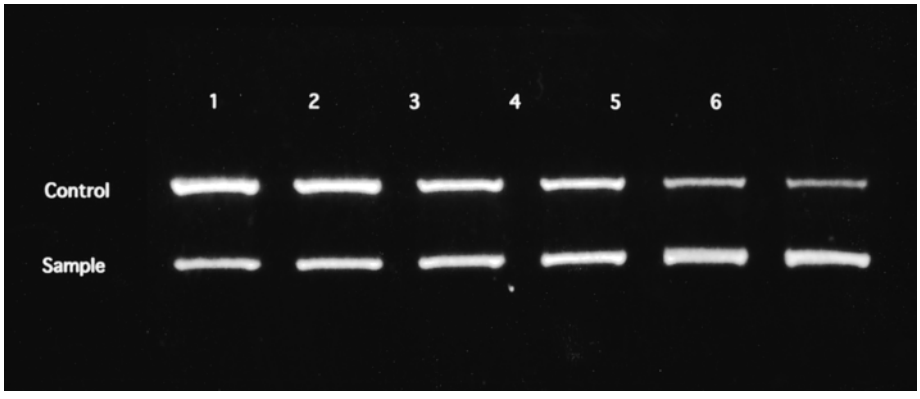
28.0 ± 40.9 for patients recovering from acute hepatitis, 45.8 ± 47.4 for patients with exacerbation of chronic hepatitis, 7.2 ± 8.2 for patients recovering from exacerbation of chronic hepatitis, 35.1 ± 41.7 for chronic hepatitis, and 26.8 ± 21.1 for cirrhosis. The serum ALR level was higher in patients with AH and FH, although the difference between acute and chronic was not significant. The values in patients with each type of liver disease were significantly higher than that in the controls. ALR levels were also significantly higher in fulminant hepatitis than in any other liver disease.

Hepatic ALR mRNA expression

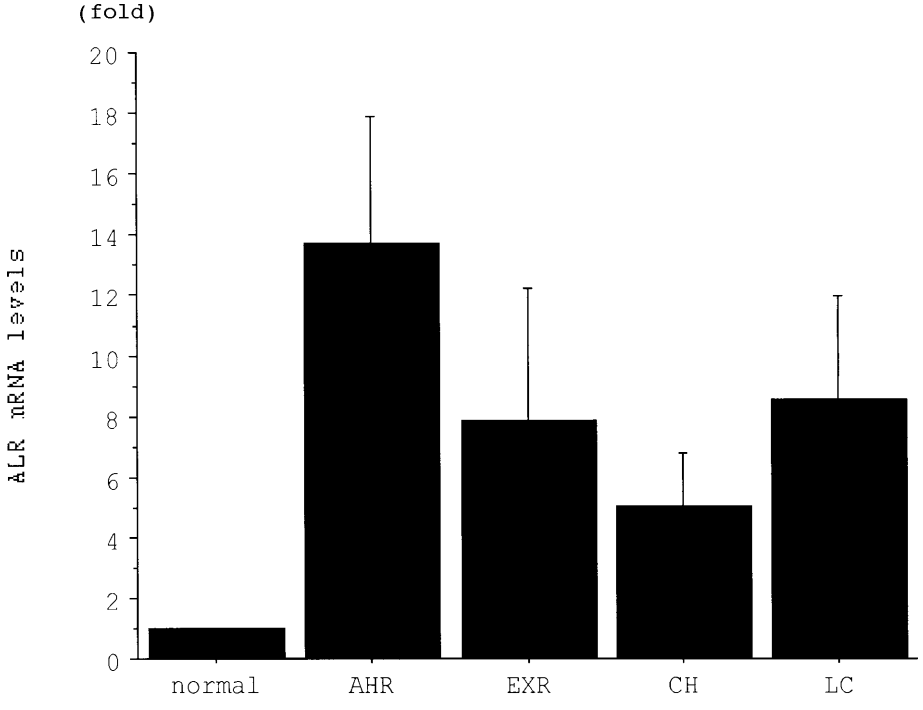
ALR mRNA levels measured in liver tissue samples by the competitive RT-PCR method are shown in Fig. 2a as a ratio of the control ALR mRNA level. The mRNA level was 13.7 ± 8.3 × 10^{-5} attmoles/ml (mean [SD]) for patients recovering from acute hepatitis, 7.8 ± 8.3 for patients recovering from exacerbation of chronic hepatitis, 5.1 ± 5.2 for chronic hepatitis, and 8.6 ± 7.6 for cirrhosis (Fig. 2b). The mRNA level was higher in each liver disease than in the controls without liver disease. In addition, expression of ALR mRNA was highest in patients recovering from acute hepatitis. ALR mRNA levels showed no correlation with ALT or aspartate aminotransferase (AST) levels (data not shown).

NK activity

Figure 3 shows peripheral blood NK activity. The value was 45.9 ± 15.4% (mean [SD]) for patients with acute hepatitis, and 14.8 ± 8.6% for patients with fulminant hepatitis. NK activity was significantly lower in fulminant hepatitis than in acute hepatitis ($P < 0.01$).



a



b

Fig. 2. **a** Detection of ALR mRNA in liver samples reverse transcriptase-polymerase chain reaction by RT-PCR. The higher molecular weight bands are the PCR products of ALR Mimic (Clontech Laboratories), whereas the lower molecular weight bands are the ALR PCR products. cDNA synthesized from 25 ng of total RNA from each liver sample and two-fold serially diluted ALR MIMIC (2M3–2M8) were added to each tube, and PCR was performed as described in "Patients and methods". *Lane 1*, ALR Mimic (2M3) and ALR PCR product; *lane 2*, 2M4 and ALR PCR product; *lane 3*, 2M5 and ALR PCR product; *lane 4*, 2M6 and ALR PCR product; *lane 5*, 2M7 and ALR PCR product; *lane 6*, 2M8 and ALR PCR product. **b** ALR mRNA levels in patients with liver disease. ALR mRNA levels are shown relative to the control (*normal*) ALR mRNA level

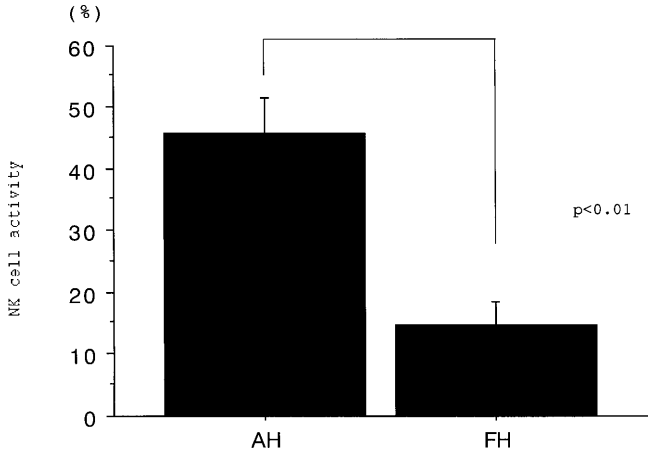


Fig. 3. Natural killer cell (*NK*) activity in patients with fulminant hepatitis (*FH*) and acute hepatitis (*AH*). NK activity was significantly lower in FH than in AH ($P < 0.01$)

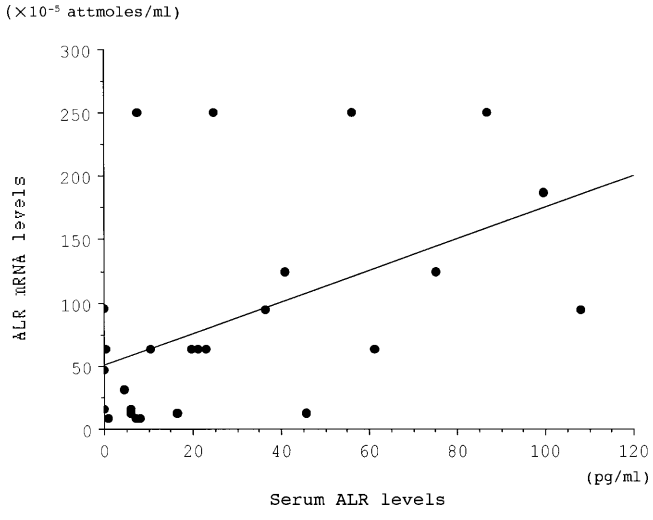


Fig. 4. Correlation between hepatic ALR mRNA expression and serum ALR level. A positive correlation was found between ALR mRNA expression in liver tissue and serum ALR level ($\rho = 0.53$; $P < 0.01$; $y = 1.2x + 50.6$

Correlation between peripheral blood ALR levels or NK activity and hepatic ALR mRNA expression

When the correlation between peripheral blood ALR levels and liver tissue ALR mRNA levels was investigated, the two parameters showed a positive correlation ($\rho = 0.53$; $P < 0.001$) (Fig. 4). When the correlation between peripheral blood NK activity and the peripheral blood ALR level was investigated, serum ALR values showed a negative correlation with NK activity ($\rho = -0.80$; $P < 0.001$) (Fig. 5).

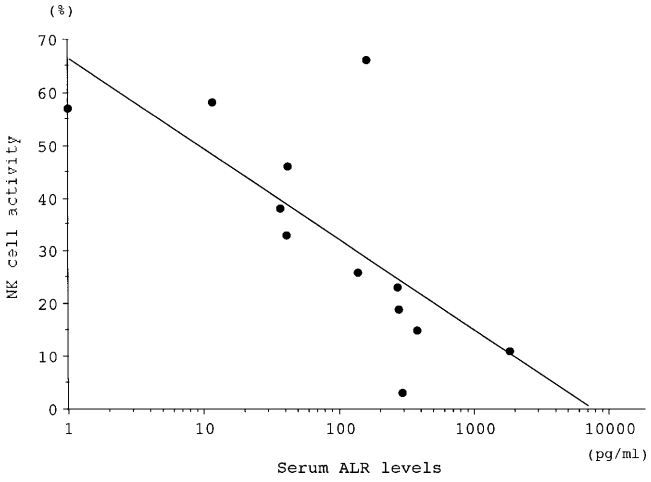


Fig. 5. Correlation between NK cell activity and serum ALR level. The serum ALR level showed a negative correlation with NK activity ($\rho = -0.80$; $P < 0.001$; $y = -22.31 \log x + 78.1$

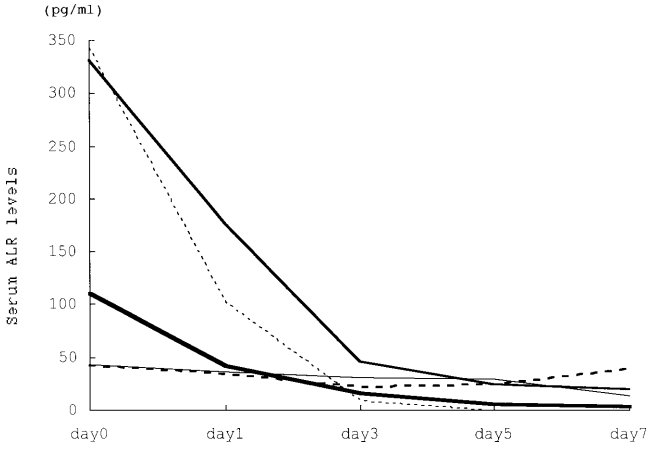


Fig. 6. Profile of serum ALR levels in five patients after admission for acute hepatitis. Serum ALR levels were determined on admission and on the first, third, fifth, and seventh days after admission. The highest values in each patient were found at the time of admission

Changes in peripheral blood ALR levels in acute liver disease

Changes in the peripheral blood ALR level over time were examined in five patients with acute hepatitis (Fig. 6). When the level on admission and on the first, third, fifth, and seventh days after admission was compared, the highest value in each patient was at the time of admission, and ALR levels decreased subsequently.

Discussion

Various mechanisms are involved in liver regeneration in acute liver disease. It is known that the immunosup-

pressants cyclosporin[29] and FK506[30] promote liver regeneration, and we previously found that the immune system was involved in suppressing liver regeneration.[31] Therefore, we have attempted to use immunosuppressants to treat fulminant hepatitis.[32] However, treatment was difficult because of the high risk of complications such as infections. Since NK cells in the liver and spleen are known to show specific cytotoxicity against regenerating hepatocytes, liver regeneration could be promoted if agents were administered which only caused immunosuppression localized to the liver. Francavilla et al.[23] found that ALR inhibited hepatic NK activity in rats and that ALR had a high liver specificity, suggesting it might be such an agent.

In the present study, hepatic ALR mRNA expression was higher in patients with liver disease than in non-liver disease controls, and a correlation was found between serum ALR values and hepatic levels of ALR mRNA. Therefore, it seems that ALR is produced by the liver during liver disease and this increases the ALR concentration in the peripheral blood. ALR was liver-specific, but variable RNA expression in nonhepatic tissues, particularly testis,[20] has been shown. Our data suggested that ALR may play a role in liver regeneration because hepatic ALR mRNA levels were higher in acute liver disease than in chronic liver disease. In addition, investigation of sequential changes in serum ALR levels in acute hepatitis revealed peak values on admission, with a gradual decrease thereafter. This result corresponded with data obtained in rats showing that the production of ALR first occurs in the initial stage of liver disease.

A negative correlation was observed between NK activity in peripheral blood and serum ALR level. To our knowledge, there have been no reports on the relationship between NK activity in the liver and in the peripheral blood during liver disease. However, NK activity in the peripheral blood appears to reflect NK activity in the liver to some extent, because ALR has been reported to inhibit NK activity only in the liver and not in the peripheral blood.[23] As we have not measured purified NK cell activity or the population of NK cells in the peripheral blood, further examinations are necessary. Promotion of liver regeneration by ALR in humans may involve suppression of damage to regenerating hepatocytes by the inhibition of hepatic NK activity.

In conclusion, this study showed that ALR production in the liver was enhanced during acute liver disease, and the findings suggest that ALR plays a role in liver regeneration by inhibiting hepatic NK activity. In the future, it would be interesting to clarify the direct action of ALR on liver regeneration.

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