Table 4 the comparison of liver function tests pre- and post-splenectomy

	Pre-splenectomy	24 weeks post-splenectomy	*P*
Prothrombin time (%)	87.9	94.7	0.03
Albumin (g/dL)	2.9 ± 0.4	3.1 ± 0.4	NS
Median total bilirubin (mg/dL)	1.05 (0.50–2.07)	0.90 (0.46–2.24)	NS

NS, not significance.

splenectomy was performed. However, open surgery is a highly invasive method, and thus partial splenic arterial embolization (PSE) has been performed in previous patients with HCC to reduce spleen volume and control hypersplenism.[19,20] PSE, via interventional radiology, is less invasive than open splenectomy but platelet counts post-PSE are less than that after splenectomy. As it is necessary that platelet counts be more than about $10 \times 10^4/mm^3$ for treatment with a full-dose chemotherapy regimen, some PSE patients will not meet this criteria. In such a case, a control trial is necessary.

The WBC and platelet counts of all patients in the splenectomy group increased following surgery, and thus (excepting two cases) were able to be treated with the full-dose chemotherapy regimen. Most patients in the non-splenectomy group (8/12, 66.6%) were unable to receive a full therapeutic dose of chemotherapy. Consequently, the response rate in the splenectomy group was significantly better than that the non-splenectomy group. The response rate in the splenectomy group was very high (68.1%). In this study, patients without portal thrombus were included, achieving a response rate of 61.1%.

The 2-year survival rate was also significantly better in the splenectomy group, suggesting that splenectomy should be performed in order to improve the prognosis of HCC patients with leucopenia and thrombocytopenia. A prospective study will be necessary in the future.

Splenectomy has been reported to be associated with a high risk of overwhelming post-splenectomy syndrome, bleeding, and portal thrombosis. In the present study, the only complications were two cases of portal thrombosis (9.0%), indicating that splenectomy is a safe and feasible approach in this clinical situation. Recently, patients with hypersplenism, due to liver cirrhosis, have undergone interferon therapy for the eradication of the HCV and reduction of occurrence of HCC. Splenectomy may also be useful for these patients.

Splenectomy might be not only be useful for increasing platelet counts, but also in improving liver function. There are several reports indicating that splenectomy could improve liver function for patients with liver cirrhosis. In our patients, only the prothrombin time was significantly increased after splenectomy. The progression of cancer and degree of nutrition might have precluded improvements in other liver function parameters (Table 4).

In conclusion, splenectomy allowed an increased proportion of the patients studied to complete full-dose chemotherapy, increased the proportion of patients whose tumor(s) responded to chemotherapy and prolonged survival.

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肝動注癌化学療法における脾摘術とPSEの功罪

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索引用語：脾臓摘出術，PSE，肝動注癌化学療法，肝細胞癌

1 はじめに

肝細胞癌は慢性肝障害を背景に発症する．慢性肝障害の多くは門脈圧亢進症を合併しており，その結果種々の臨床症状を現す．具体的な症状として食道・胃静脈瘤，門脈圧亢進症性胃腸症，腹水，肝性脳症，脾機能亢進症などがある．

近年，肝動脈塞栓療法により治療効果が得られない症例や脈管浸潤をきたした進行肝癌症例に対し肝動注化学療法が普及しその有用性が報告されている(表1)．肝細胞癌患者の多くに血球減少があり，抗癌剤を連日使用することやインターフェロン(IFN)の使用によりさらに血球減少をきたし，しばしば治療を中断せざるをえない．門脈圧亢進症において，血球減少などの合併症を改善する目的に脾臓摘出術，部分的脾動脈塞栓術(partial splenic embolization; PSE)が用いられ始めている．

本稿では肝動注化学療法患者における脾臓摘出術とPSEの適応，治療効果，また両治療法の功罪について述べる．

2 肝動注化学療法施行患者における血球減少

進行肝癌に対する肝動注化学療法は2つに大別される．1つはリザーバーカテーテルと皮下留置型ポートを使用し持続的に抗癌剤を動注するもの[1~6]，もう1つはシスプラチンなどを使用し単回の抗癌剤動注を繰り返す方法である[7]．特にリザーバーシステムによる肝持続動注療法では複数の抗癌剤の連日使用を行ったり，IFNを使用したりすることにより血球減少をきたしやすい．当科におけるリザーバーシステムによる肝持続動注療法を施行した際，治療開始前より血球減少を呈していることも重なり，特に血小板において半数を超える症例に高度の減少がみられた．術前の血小板が10万/μlの症例であってもgrade 3以上の血小板低下がみられた．これらの患者は治療の中断を余儀なくされる．実際に抗癌剤による十分な効果もみられなかった．以上より抗癌剤投与中の血球減少，特に

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表1　進行肝細胞癌に対する肝動注化学療法

投与薬剤	報告者(年)	奏功率(%)	1年生存率/2年生存率(%)
IFN α, 5FU	Ota[1] (2002)	13/29 (45)	49 / 29
IFN α, 5FU	Obi[2] (2006)	61/116 (53)	34 / 18
CDDP, 5FU	Ando[3] (2002)	23/48 (48)	45 / 31
CDDP, 5FU Leucovorin	Yamasaki[4] (2005)	14/29 (48)	48 / 24
CDDP, エトポシド	Sangro[5] (2002)	10/26 (38)	33 / 24
MTX, CDDP 5FU, IFN α	Kaneko[6] (2002)	13/29 (45)	− / 15
CDDP	吉川[7] (2007)	27/80 (33.8)	67.5 / 50.8

表2　脾臓摘出術，PSEの適応

汎血球減少
腹水
門脈圧亢進性胃腸症
食道胃静脈瘤
肝性脳症

血小板減少は抗癌剤の不十分な投与につながり，結果として奏功が得られない原因の1つとなっていると考えている．

3 肝細胞癌の肝動注化学療法施行患者における脾臓摘出術とPSEの適応

門脈圧亢進症患者に対する脾臓摘出術またはPSEの適応を示す(表2)．これらの中で化学療法を施行する際に問題となるのは血球減少である．当科では化学療法施行前の白血球2,000/μl未満または血小板が8万/μl以下の症例は，同意が得られた場合抗癌剤投与前に積極的に脾臓摘出術またはPSEを行うようにしている．肝動注療法施行中または休薬中にNCI-CTCAE分類にてgrade 3以上の血球減少をきたし治療の中断を余儀なくされる

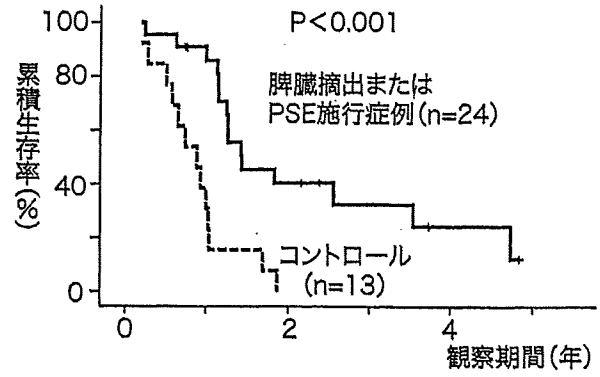

図1

症例についても脾臓摘出術またはPSEの適応としている．PSEの施行時期は可能であればカテーテル留置時に行っている．血管造影に要する回数が1回で終了することと化学療法を早く開始できるためである．PSE施行後血小板上昇に2〜3週を要するため化学療法の開始日はそれ以降になるためである．PSEは田尻らの方法に準じて塞栓物質のみで行っている[11]．塞栓物質によるPSEは簡便であり30分程度の時間で施行可能であるためカテーテル留置に手間がかからなければ同時に行ってもよいものと思われる．血行改変に時間を要する症例では患者の負担を考え，後日

表3　肝硬変症例に対する脾臓摘出術とPSE

治療	報告者（年）	術前血小板/白血球（/μL）	術後血小板/白血球（/μL）	主な合併症
脾臓摘出	緒方[8]（2005）	4.2±1.5万/2,712±1,354	15.3±4.8万/5,131±1,187	発熱22%，門脈血栓17%，腹水7%
脾臓摘出	Sugawara[9]（2000）	4.7±0.3万/記載なし	23.1±2.6万/記載なし	門脈血栓23%
脾臓摘出	Watanabe[10]（2007）	3.3±1.2万/2,500±1,100	22.0±15万/5,600±1,800	門脈血栓18.8%，腹水8%，胸水4%
脾臓摘出	自験例	5.1±3.8万/	22.1±6.5万/μl/	門脈血栓13.6%，腹水9.1%，胸水4.5%
PSE	Tajiri[11]（2007）	8.2±3.9万/記載なし	16.7±6.9万/記載なし	記載なし
PSE	渡辺[12]（2007）	3.8万/記載なし	8.6万/記載なし	脾膿瘍2例
PSE	Hayashi[13]（2007）	4.5±1.2万/記載なし	11.6±5.1万/記載なし	発熱・左側腹部痛（程度不明）100% 胸水7.1%，腹水2.4%，胃潰瘍2.4%
PSE	Sangro B[14]（1993）	5.5±2.0万/3,016±1,317	18.04±7.7万/9,901±3,007	15日以上の発熱7.5%，胸水10% 腹水30%
PSE	自験例	4.5±2.3万/2,510±950	14.2±5.5万/4,300±1,100	15日以上の発熱5.0%，腹水15%，胸水5%

PSEを施行するようにしている．

4 脾臓摘出術，PSE施行患者における肝動注化学療法の治療成績

2002年1月から2007年6月までに，肝動注化学療法患者に対し22例脾臓摘出術，2例PSEを施行した．同時期に脾臓摘出術またはPSEを施行せずに肝動注化学療法を施行した患者をコントロールとして完遂率，奏功率，生存率を比較した．脾臓摘出術またはPSEを施行した症例では，カテーテルトラブルによる2例を除く全症例で完遂が可能であった．十分な抗癌剤投与を行うことにより，奏功率は向上し，生存率は脾臓摘出術やPSEを行った症例が有意に良好であった（図1）．

5 肝動注化学療法における脾臓摘出術，PSEの功罪

表3に門脈圧亢進症患者における脾臓摘出術とPSEによる血球改善の報告を示す．両治療法ともに有意な血球改善がみられている．当科においてもIFN投与や肝癌局所療法施行前などにも脾臓摘出術やPSEを施行している．これらの患者を含めた当科で施行した脾臓摘出，PSEの血小板回復は両治療ともに血小板数は10万/μl以上に改善したが，脾臓摘出症例はPSEに比べ高い上昇をきたした（脾臓摘出22.1±6.5万/μl，14.2±5.5万/μl）．さらに術前血小板数により血小板の改善の程度を比較した場合，術前血小板数3.5万/μl未満の症例では脾臓摘出術では十分な血小板上昇が得られたのに対し（18.9±

表4　肝癌化学療法患者における脾臓摘出術とPSEの長所と短所

	長所	短所
脾臓摘出術	・血球数改善が確実 ・肝予備能の改善	・術後重篤な感染症，門脈血栓症発生の危険性がある ・PSEに比べ侵襲あり
PSE	・手技が簡便 ・術後感染症，門脈血栓は稀 ・肝予備能の改善	・血球改善が不十分な症例あり

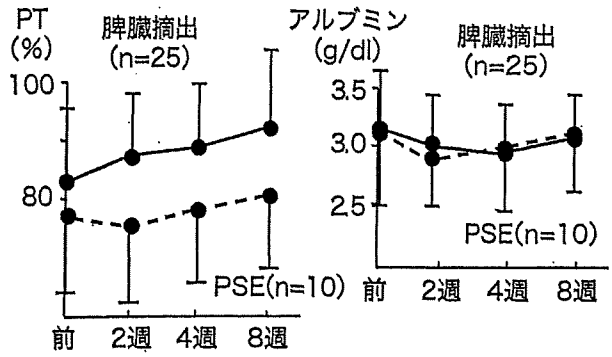

図2

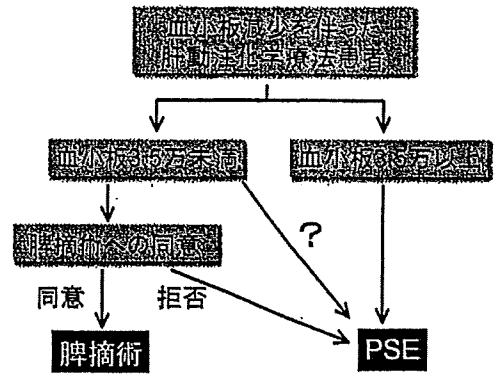

図3

5.0万/μl)，PSE後では10万前後への上昇しか得られなかった(10.1±3.4万/μl)．以前の報告も同様でありWatanabeらの報告[10]では，脾臓摘出により術前平均3.3万/μlの血小板が平均22万/μlに改善したのに対し，渡辺らの報告ではPSEは平均3.8万/μlの血小板は8.6万/μl程度にしか改善できていなかった[12]．術前血小板脾臓摘出血小板10万の症例までgrade 3以上の血小板減少がみられていることから肝持続動注を主眼とした場合，使用薬剤や投与方法によっては術前3.5万/μl未満の症例においてPSEでは血小板の上昇程度は不十分であることが示唆される．したがって術前血小板数が3.5万/μlを下回るような極めて血小板数が低い症例は脾臓摘出術が行われるべきである．一方，脾臓摘出術は肺炎球菌ワクチン投与や術後管理の工夫によりその頻度は減少しているものの，門脈血栓症や術後重症感染症の危険性は存在する．表3に示す通り脾臓摘出術では高率に門脈血栓を合併している．逆にPSEでは門脈血栓症は稀である．現在ステロイドやNSAIDs投与の工夫により以前に比べPSEは負担の少ない治療になっている．術前血小板数が3.5万/μl以上の症例では簡便で低侵襲なPSEの良い適応と考えられる．脾臓摘出術による術後門脈血栓症や重篤な感染症のリスクを考えた場合，術前血小板数が3.5万/μlを超えるようなある程度血小板数が保たれている症例ではPSEが選択されるべきである(図2)．3.5万/μlという数字が脾臓摘出術とPSEの選択基準として妥当か否かは，今後症例を集積しさらに検討が必要である．これらの処置は肝予備能を改善することが報告されている[8~14]．両治療法の間にPT，アルブミンで両治療法ともに改善はみられたが，その改善の程度に差はみられなかった(図3)．

6 おわりに

血球数減少を合併した肝動注化学療法患者では，脾臓摘出術やPSEを行うことにより予後の改善が期待できると考えられる．

血球消費を抑制する観点から脾臓に対する有用性を述べたが，その一方で血球産生を向上する治療も重要である．白血球についてはG-CSFが広く使用され，今後はPEG製剤が普及してくるものと思われる．血小板産生については，現在トロンボポエチンレセプター活性化作用を持つ第2世代の血小板増加薬が開発され臨床試験が順調に進んでいる．特に経口投与可能なEltrombopag (SB-497115)[15,16]は臨床試験が進行中である．これらの薬剤[17]が日本でも使用されるようになれば，今後脾臓摘出術やPSEを施行する意義が問われる可能性はある．数年後には脾臓に対する治療自体が再考されるものと考えられる．

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Long-term outcome of branched-chain amino acid treatment in patients with liver cirrhosis

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Clinical impact of protein-energy malnutrition (PEM) on the outcome of liver cirrhosis is well documented. As a candidate interventional modality to improve PEM in cirrhosis, effects of branched-chain amino acid (BCAA) supplementation on event-free survival and quality of life (QOL) was first reported by Yoshida *et al.* in 1989. Although critical arguments still continue regarding the effects of BCAA, several randomized trials in the last 5 years have brought positive results, and seem to have settled the discussion in a favorable direction for the efficacy of BCAA in liver cirrhosis. Actually, The European Society for Clinical Nutrition and Metabolism (ESPEN) upgraded the recommendation of BCAA supplementation in decompensated liver cirrhosis in the latest revision of its guidelines in 2006, by referring to the literatures from Italy and Japan. Particularly in these two long-term randomized studies with 1–2 years-supplementation, event-free survival was estimated by employing composite endpoints such as aggravation of hepatic failure (ascites, peripheral edema, hepatic encephalopathy, and jaundice), rupture of esophageal or gastric varices, development of liver cancer, and death from any cause. Both trials agreed on the effect of BCAA to reduce the incidence of hepatic failure, thus contributing to the rise in the event-free survival. Quality of life is another essential marker of outcome survey. Marchesini, Muto, and Nakaya reported the improved QOL in cirrhotics with BCAA supplementation. In particular, quantitative analysis of QOL measured by Short Form 36 (SF-36) questionnaire demonstrated a significant recovery of general heath perception score in BCAA supplemented patients in a randomized trial. In this article, the long-term outcome of BCAA treatment in liver cirrhosis will be reviewed with its action mechanisms. In addition, the effects of BCAA treatment on the incidence of liver cancer in obese patients with type C liver cirrhosis, significance of obesity as a risk factor for type C liver cancer, and a possible role of Body Mass Index to estimate the histological grade of fat deposition in the liver will be briefly discussed.

Key words: branched-chain amino acids (BCAA), liver cancer, liver cirrhosis, liver failure, obesity, survival

INTRODUCTION

IT IS WELL known that the liver plays a central role in the nutrient metabolism. After intestinal absorption, the majority of both macronutrients, consisting of carbohydrate, lipid and protein, and micronutrients, such as vitamins and trace minerals, are taken up by the liver, stored there, and transported to peripheral tissues depending on its demand. In addition, carrier proteins of these nutrients are also produced by the liver. Hence, nutrient metabolism is often impaired in liver diseases, leading to protein and energy malnutrition (PEM). The incidence of PEM varies among liver diseases, but reaches up to 85% particularly in advanced cirrhosis.[1,2]

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CLINICAL SIGNIFICANCE OF PROTEIN-ENERGY MALNUTRITION (PEM) IN THE OUTCOME OF LIVER CIRRHOSIS

PROTEIN NUTRITIONAL STATE is commonly estimated anthropometrically by arm muscle circumference (AMC)[1] or blood biochemically by serum albumin level.[2] Similarly, energy nutritional state is measured by triceps skinfold thickness (TSF)[1] or by indirect calorimetry.[2] The grade of PEM progresses in parallel with the increasing severity of liver cirrhosis,[2] and is regarded as a most significant factor that determines the survival rate of the patients with liver cirrhosis.[1,2] As possible interventions for PEM of cirrhotics, current guidelines recommend branched-chain amino acid

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(BCAA) supplementation for protein malnutrition[3] and divided meals including a late evening snack (LES) for energy malnutrition.[3,4] However, effects of such nutritional support on the survival rate itself have long been controversial until recently.

EFFECTS OF BRANCHED-CHAIN AMINO ACID TREATMENT ON EVENT-FREE SURVIVAL AND QUALITY OF LIFE IN LIVER CIRRHOSIS

BENEFICIAL EFFECTS OF BCAA supplementation on the survival rate of cirrhotic patients was first reported by Yoshida *et al.* in 1989.[5] Although critical arguments still continue regarding such effects of BCAA,[6] several randomized trials in the last 5 years have brought positive results,[7–10] and seem to have settled the discussion in a favorable direction toward the efficacy of BCAA in liver cirrhosis. Actually, the European Society for Clinical Nutrition and Metabolism (ESPEN) upgraded the recommendation of BCAA supplementation in decompensated liver cirrhosis in the latest revision of its guidelines in 2006,[3] by referring to the literature, references 7 and 9 in this paper. Particularly in these two long-term randomized studies with 1–2 years-supplementation,[7,9] event-free survival was estimated by employing composite endpoints including aggravation of hepatic failure (ascites, peripheral edema, hepatic encephalopathy, and jaundice), rupture of esophageal or gastric varices, development of liver cancer, and death from any cause. Both trials agreed on the effect of BCAA in reducing the incidence of hepatic failure, thus contributing to the rise in the event-free survival.[7,9]

Quality of life (QOL) is another essential marker of outcome survey.[11] Marchesini,[7] Muto,[9] and Nakaya[10] reported the improved QOL in cirrhotics with BCAA supplementation. In particular, quantitative analysis of QOL measured by the Short Form 36 (SF-36) questionnaire demonstrated a significant recovery of general health perception scores in BCAA-supplemented patients in a randomized trial.[9]

EFFECTS OF BRANCHED CHAIN AMINO ACID TREATMENT ON INCIDENCE OF LIVER CANCER IN TYPE C LIVER CIRRHOSIS

AMONG COMPOSITE ENDPOINTS as described before, development of liver cancer was significantly inhibited by BCAA supplementation in type C cirrhotic patients with body mass index above 25.[12] In contrast, BCAA showed no inhibitory effect on the incidence of liver cancer in lean cirrhotics with a BMI below 25.[12]

RISK FACTORS FOR TYPE C LIVER CANCER: SIGNIFICANCE OF OBESITY

CONCURRENT PRESENCE OF diabetes mellitus,[13–19] hyperinsulinemia, or obesity[1,7,13,20–24] is regarded as a significant risk factor for the development of liver cancer. This concern has not been appreciated sufficiently in Japan, since the proportion of obese patients among all cirrhotics was low until the year 1995[2] (see Fig. 1). However, the cirrhotic cohort registered in 2000 showed the proportion as high as that in the age- and sex-matched general population[12] (Fig. 1). These data suggest that obesity prevailed in such a short period in patients with liver cirrhosis.

In such a patient cohort, obesity was a significant risk factor for liver carcinogenesis in addition to other risk factors including sex (male), lower serum albumin concentration, higher serum alpha-fetoprotein concentration and the presence of diabetes mellitus.[12]

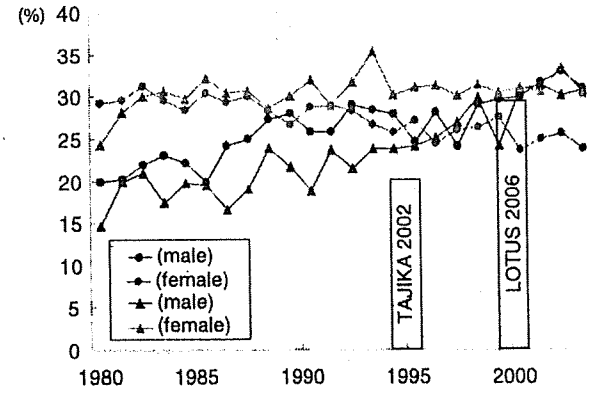

Figure 1 Proportion of overweight/obese subjects in patients with liver cirrhosis and in the general population in Japan. Overweight and obese were defined by body mass index above 25 and 30, respectively. Lines indicate the proportion of overweight/obese subjects in the general population according to National Health Survey conducted by the Ministry of Health, Labor, and Welfare of Japan. Bars indicate the proportion of overweight/obese patients in cirrhotics in the year 1995 (Tajika M, *et al.*[2]) and 2000 (LOTUS trial,[11]), respectively. (-●-) 50–59 years (male); (-●-) 50–59 years (female); (-▲-) 60–69 years (male); (-▲-) 60–69 years (female)

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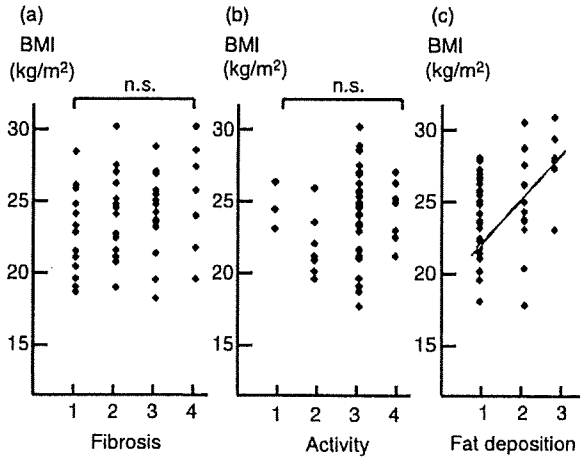

Figure 2 Correlations between body mass index (BMI) and the histological grades of fibrosis (a), inflammatory activity (b) and fat deposition (c) in the liver of type C chronic hepatitis and cirrhosis. The grade of hepatic fat deposition was defined as 1 with <10% of the total parenchymal area, two with 10–30%, and three with >30%. $P < 0.01$ for panel C by Spearman's rank correlation test. n.s., not significant.

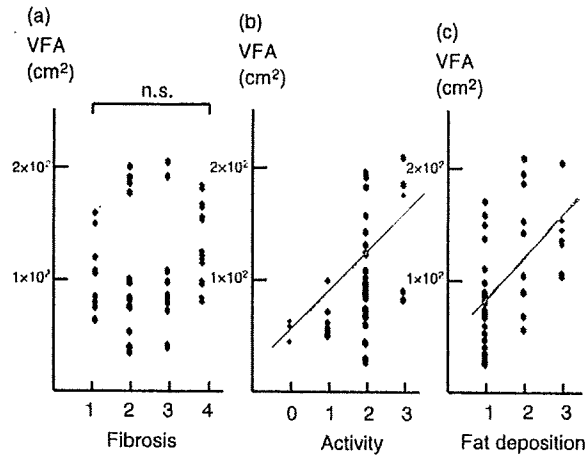

Figure 3 Correlations between visceral fat area (VFA) and the histological grades of fibrosis (a), inflammatory activity (b) and fat deposition (c) in the liver of type C chronic hepatitis and cirrhosis. $P < 0.01$ for panels B and C by Spearman's rank correlation test. n.s., not significant.

BODY MASS INDEX ESTIMATES THE HISTOLOGICAL GRADE OF FAT DEPOSITION IN THE LIVER

OBESITY IS USUALLY defined by body mass index above 25 in Japan and above 30 in western countries. The promotional role of obesity in liver carcinogensis can be hypothesized in two ways; (i) obesity induces fat deposition in the liver, leading to generation of lipid peroxiside and reactive oxygen species (ROS) and (ii) obesity induces general insulin resistance. Reactive oxygen species damage DNA and may act at the very early stage of carcinogenesis, while insulin resistance and resulting hyperinsulinemia promote the growth of cancer cells.

Figure 2 indicates correlations between body mass index (BMI) and the histological grades of fibrosis (Fig. 2a), inflammatory activity (Fig. 2b) and fat deposition (Fig. 2c) in the liver of type C chronic hepatitis and cirrhosis. These data suggest the direct relation between obesity and fat deposition in the liver as described above.

Figure 3 indicates correlations between visceral fat area (VFA) and the histological grades of fibrosis (A), inflammatory activity (B) and fat deposition (C) in the liver of type C chronic hepatitis and cirrhosis. Visceral fat area is generally accepted as the most important factor that affects the development of metabolic syndrome. However, it is interesting that a more simple and easy-to-measure parameter, BMI, showed a higher correlation coefficient with hepatic fat deposition than the visceral fat area (Table 1).

Figure 4 indicates that hepatic fat deposition affects both the histological grades of liver fibrosis and inflammatory activity in type C chronic hepatitis and cirrhosis. Although these findings do not directly support the relation between obesity and liver carcinogenesis, hepatic fat deposition seems to determine the progression of the activity of liver disease.

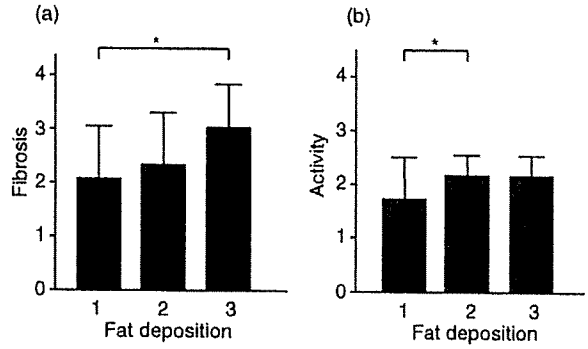

Figure 4 Hepatic fat deposition affects both the histological grades of liver fibrosis and inflammatory activity in type C chronic hepatitis and cirrhosis. Values are expressed as mean and standard deviation. *$P < 0.05$ by Kruskal-Wallis test.

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Table 1 Correlations between blood biochemical parameters and the histological grade of fat deposition in the liver

	Spearman correlation coefficient		Multiple regression coefficient	
	r	*P* value	*r*	*P* value
Triglyceride	0.386	<0.01		
FBS	0.348	<0.01	0.008	<0.0001
T-bil	0.103	n.s.		
AST	0.160	n.s.		
ALT	0.092	n.s.		
T-chol	0.047	n.s.		
HbA1c	0.217	n.s.		
Hyaluronic acid	0.284	n.s.		
P3NP	0.341	n.s.		
Type 4 collagen	0.081	n.s.		
HCV-RNA	−0.009	n.s.		
Serum-Fe	0.236	n.s.		
Ferritin	0.277	n.s.		
Platelet count	0.007	n.s.		
BMI	0.579	<0.01	0.100	<0.0001
Visceral fat	0.555	<0.01	0.084	<0.0001

FBS, fasting blood sugar; T-bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-chol, total cholesterol; HbA1c, hemoglobin A1c; P3NP, amino terminal propeptide of type 3 procollagen; HCV-RNA, hepatitis C virus-ribonucleic acid; BMI, body mass index.

CONCLUSION

LONG-TERM TREATMENT with BCAA improves the clinical outcome of cirrhotic patients by reducing the event of liver failure and, in obese patients, by inhibiting liver carcinogenesis. The mechanism of such action by BCAA and, furthermore, the role of obesity in liver carcinogenesis will be a very important and interesting target of future basic and clinical studies.

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Role of Vα14$^+$ NKT cells in the development of Hepatitis B virus-specific CTL: activation of Vα14$^+$ NKT cells promotes the breakage of CTL tolerance

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Keywords: CTL, hepatitis B virus, NKT cell

Abstract

CTLs are thought to be major effectors for clearing viruses in acute infections including hepatitis B virus (HBV). Persistent HBV infection is characterized by a lack of or a weak CTL response to HBV, which is thought to reflect tolerance to HBV antigens. In the present study, we found that alpha-galactosylceramide (α-GalCer), a ligand for Vα14-positive NKT cells, strongly enhanced the induction and proliferation of HBV-specific CTLs by HBsAg. In HBsAg transgenic mice, which are thought to be tolerant to HBV-encoded antigens, administration of HBsAg or α-GalCer alone failed to induce HBsAg-specific CTLs, but they were induced by co-administration of both compounds. Furthermore, by limiting dilution analysis, we confirmed the existence of HBsAg-specific CTL precursors in the HBsAg transgenic mice immunized with HBsAg and α-GalCer. A blocking experiment using antibodies to cytokines and CD40 ligand showed that IL-2 and CD40–CD40L interaction mediate the enhancement of CTL induction caused by α-GalCer through NKT cell activation. Our results may open up a new method for clearing the virus from patients with persistent HBV infection.

Introduction

Most perinatal hepatitis B virus (HBV) infections become persistent due to the failure to mount an effective immune response. Individuals with such persistent infection usually become asymptomatic carriers (ASCs) who are thought to be immunologically tolerant to HBV-encoded antigens. However, this tolerance is eventually broken and the development of chronic hepatitis (CH) is observed. Once CH has been developed, a weak CTL response to HBV becomes detectable and is thought to form an important part of the pathogenesis of CH, liver injury and down-regulation of the vial replication (1).

In many types of viral infections, CTLs have been shown to play critical roles on the clearance of the viruses (2–4). The same scenario is applicable in HBV infection. However, the clearance or continuous suppression of HBV is not observed in all the cases with CH possibly because of the

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weakness of CTL response. Therefore, for the establishment of the effective therapy of type B CH, it is necessary to elucidate the reason for the weakness of CTL response and to seek the method for boosting the response.

In addition, the mechanism for the development of CH from ASC state still remains unknown. Breaking tolerance in a CTL level should be one of the key mechanisms for this phenomenon. To confirm the hypothesis above and to establish therapeutic model, an appropriate animal model system is necessary because of the technical and ethical difficulties in human study.

Studies on HBV have progressed rapidly since the HBV transgenic mice system was established by Chisari *et al.* (5). With this system, it has been has proven that HBV-specific CTLs cause acute or fulminant hepatitis (6) and are directly cytopathic *in vivo* (7). In addition, with this system, it has been shown that HBV-specific CTLs have mechanism to suppress HBV gene expression and replication without killing hepatocytes (8, 9), although the destruction of both infected and bystander cells by CTLs are thought to be another important mechanism to terminate viral infection (10, 11). Since HBV transgenic mice used in our study is deeply tolerant against HBsAg in both cellular and humoral immune responses, it may be a suitable model to study the mechanism for breaking tolerance and the immunological treatment for chronic HBV infection.

Recently, a novel lymphoid lineage, $V\alpha14^+$ NKT cells, distinct from mainstream T cells, B cells and NK cells has been identified (12, 13). These cells are found in relative abundance in tissue such as spleen, bone marrow, thymus and liver and characterized by the co-expression of NK cell receptors and semi-invariant T cell receptors encoded by Vα14 and Jα18 gene segments. It is well known that activated $V\alpha14^+$ NKT cells strongly produce T_h1 and two cytokines, and we expected that this cell fraction can affect CTL induction.

We here report that NKT cell activation strongly helps to induce specific CTLs by the HBsAg immunization even in HBV transgenic mice, deeply tolerant to HBsAg in a CTL level. We think that our findings may help explaining the development of CH from ASC state and that they may suggest the novel immunological approach for the treatment of CH B.

Methods

Mice

Male BALB/c ($H\text{-}2^d$) and B10.D2 ($H\text{-}2^d$) mice (8–10 weeks, 25–30 g) were obtained from Japan SLC Inc. (Shizuoka, Japan). HBsAg transgenic mice lineage 107-5D [official designation Tg (Alb-1, HBV) Bri66; inbred B10.D2, $H\text{-}2^d$] in which the HBV envelope-coding region is under the control of the mouse albumin promoter was generously provided by Francis V. Chisari (Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA, USA). Mice deficient in Vα14 NKT cells [Jα281 knockout (KO)] were established by specifically deleting the Jα18 gene segment by homologous recombination (14) and backcrossed with BALB/c mice.

Cell lines and reagents

The $H\text{-}2^d$ mastcytoma cell line P815 was obtained from the American Type Culture Collection (Rockville, MD, USA). P815 cells expressing HBV-preS1, 2 and S (P815preS1) and the HBsAg-specific $CD8^+$ CTL clone 6C2 were generously provided by Francis V. Chisari (6, 7). mAbs specific to murine IL-2 (clone JES6-1A12), IL-4 (clone 30340.11), IFN-γ (clone H22), tumor necrosis factor (TNF)-α (TN3-19.12) and CD40L (clone 208109) were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant murine IL-2 and CD40L were purchased from R&D Systems. PE-conjugated anti-mouse CD40L (clone MR1) was purchased from eBioscience.

Establishment of HBsAg-specific CTLs

Immunization was performed using HBsAg (Advanced ImmunoChemical Inc., Long Beach, CA, USA) with or without alpha-galactosylceramide (α-GalCer) (generously provided by the Pharmaceutical Research Laboratory, Kirin Brewery, Gunma, Japan). HBsAg was intra-peritoneally (i.p.) administered at a dose of 10 μg per mouse in 0.2 ml of PBS (Invitrogen Corp. Carlsbad, CA, USA). α-GalCer was administered i.p. at a dose of 1 μg per mouse in 0.2 ml of PBS. In some experiments, IL-2 (4×10^4 U per mouse) and/or CD40L (100 μg per mouse) were administered i.p. instead of α-GalCer. Spleen cells were prepared from immunized mice 7 days after the immunization. The immunized spleen cells (4×10^6 per well) were cultured in 24-well plates with mitomycin C (MMC)-treated P815preS1 (2×10^5 per well) in complete RPMI 1640 (Sigma–Aldrich, St Louis, MO, USA) containing 10% heat-inactivated FCS (Invitrogen Corp.) and 5% EL-4 supernatant as a source of T cell growth factor. The immunized spleen cell lines and clones were re-stimulated with MMC-treated spleen cells (4×10^6 per well) and P815preS1 (2×10^5 per well) every 7 days. HBsAg-specific CTL clones were established by limiting dilution.

Blocking experiment using neutralizing antibodies to IL-2, IL-4, IFN-γ, TNF-α and CD40L

To evaluate the role of various cytokines and ligands in the induction of HBsAg-specific CTLs *in vivo*, mAbs to IL-2, IL-4, IFN-γ, TNF-α and CD40L (100 μg per mouse) were administered i.p. on days 0 and 3 of the immunization with HBsAg or HBsAg and α-GalCer. After re-stimulation *in vitro*, the proportion of HBsAg-specific cells was measured by flow cytometric analysis using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The mAbs to IL-2, IL-4, IFN-γ, TNF-α and CD40L were purchased from R&D Systems.

Cytotoxicity assay

The cytolytic activity of HBsAg-specific CTLs was assessed using a Europium release assay as described previously (15). Target cells (P815 or P815preS1) were labeled with Eu diethylenetriaminepentaacetate (80 mM; Wako Pure Chemical Industries Ltd, Osaka, Japan). Labeled targets (5×10^3 cells) and various number of effector cells were added in a final volume of 200 μl to each well of 96-well round-bottomed

plates and incubated for 4 h at 37°C. Next, 20 μl of the culture supernatant was mixed with 100 μl of enhancing solution (Wallac Oy, Tuerku, Finland), and the released Eu was measured using a time-resolved fluorometer (1230 Arcus, Wallac Oy). The percentage of Eu release was determined from the following equation: %Eu release = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. Maximal release was measured after lysis with Triton X-100. Spontaneous release was 10–20% of maximal release.

Detection of HBsAg-specific CTLs by flowcytometry

Immunodominant HBsAg-specific CTLs in mice are known to be restricted by H-2L^d of MHC class I, and the shortest peptide HBsAg peptide resulting in maximal activity is S28–39 (IPQSLDSWWTSL) (HBsAgS28–39) (7). Therefore, peptide-loaded recombinant soluble dimeric mouse H-2L^d:Ig (mouse IgG1; BD PharMingen, San Diego, CA, USA) was prepared by mixing soluble dimeric mouse H-2L^d:Ig for 48 h at 4°C with a 160-fold molar excess of HBsAgS28–39. The peptide-loaded soluble dimeric mouse H-2L^d:Ig (4 μg) was then added to CD8$^+$ cells prepared from immunized splenocytes or re-stimulated splenocytes. After incubation for 1 h at 4°C, the cells were stained with FITC-conjugated anti-mouse CD8α and PE-conjugated anti-mouse IgG1 (BD PharMingen). The proportion of HBsAg-specific cells was measured by flow cytometric analysis on a FACScan (Becton Dickinson Immunocytometry Systems).

Frequency analysis for HBsAg-specific CTLs by limiting dilution

We performed limiting dilution analysis as previously described (16). CD8-positive cells from the immunized mice were isolated by using MACS system. These CD8-positive cells (three cells per well) were cultured in 96-well plates with MMC-treated spleen cells (4×10^5 per well) and MMC-treated P815preS1 (2×10^4 per well) in complete RPMI 1640 and 5% EL-4 supernatant. The cells were re-stimulated with MMC-treated spleen cells and P815preS1 every 7 days. After 14 days, the number of wells in which the cells proliferated was counted and the proportion of HBsAg-specific CTLs was measured by flow cytometric analysis on a FACScan as described above.

Isolation of Vα14$^+$ NKT, CD4$^+$ T and CD8$^+$ T cells

Spleen cells were separated into NK marker-positive and -negative cells using anti-DX5-conjugated magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To label Vα14$^+$ NKT cells with recombinant soluble dimeric mouse CD1d:Ig (BD PharMingen), DX5-positive cells were mixed with recombinant soluble dimeric mouse CD1d:Ig that had been pre-incubated with a 40-fold molar excess of the Vα14$^+$ NKT cell-specific ligand α-GalCer. After thorough washing, the stained cells were incubated with rat anti-mouse IgG1-conjugated immunomagnetic beads (Dynal Biotech ASA, Oslo, Norway). Bead-bound cells were magnetically removed using magnetic particle concentrator-1 (Dynal Biotech ASA). To purify CD4$^+$ or CD8$^+$ T lymphocytes, NKT cells expressing CD4 or CD8 were removed. DX5-negative cells were removed using anti-CD4 or anti-CD8α magnetic microbeads (Miltenyi Biotec GmbH). The magnetically labeled cells were purified by using VarioMACS system (Miltenyi Biotec GmbH). The isolated cells were served for the analysis of cytokine mRNA expression by real-time PCR.

Real-time reverse transcription–PCR

Total RNA was isolated and transcribed into complementary DNA (cDNA) using an RNeasy Mini Kit and an Omniscript Reverse Transcriptase Kit (QIAGEN GmbH, Hilden, Germany). The resulting cDNA was used as a template for real-time PCR along with primer–probe sets for the IL-2, IL-4, IFN-β, IFN-γ, TNF-α and CD40L (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA, USA) and 2× Taqman universal PCR master mix (Applied Biosystems) according to the manufacturer's recommendations. IFN-α mRNAs were detected using SYBR Green reverse transcription (RT)–PCR. Primer–probe sets for 18S were used as an internal control in each reaction (Applied Biosystems). Real-time PCR data were analyzed using sequence detector software (Applied Biosystems).

Intracellular cytokine staining

For intracellular staining, the splenocytes from the mice administrated with HBsAg and α-GalCer were incubated for 4 h with brefeldin A (10 μg ml^{-1}). Then, cells were fixed and permeabilized with the Cytofix/Cytoperm buffer (BD PharMingen) and stained with PE-conjugated anti-mouse IFN-γ (clone XMG1.2), FITC-conjugated anti-mouse IL-4 (clone BVD6-24G2), PE-conjugated anti-mouse IL-2 (clone JES6-5H4) and PE-conjugated anti-mouse TNF-α (clone MP6-XT22) (eBioscience). Samples were acquired on a FSCStar flow cytometer and data analysis was conducted using CellQuest software (BD PharMingen).

Statistics

Values are expressed as means ± SEMs. Differences between experimental and control groups were analyzed by the Kruskal–Wallis test followed by Scheffe's *F*-test. Significance was established at $P < 0.05$.

Results

Effect of NKT cell activation on the efficiency of HBsAg-specific CTL induction

Since it is well known that activated Vα14$^+$ NKT cells strongly produce T$_h$1 and two cytokines, we expected that this cell fraction can affect CTL induction. To determine if Vα14$^+$ NKT cells contribute to the induction of HBsAg-specific CTLs, we immunized Jα18$^{+/+}$ [wild-type (WT)] mice and Jα18$^{-/-}$ (KO) mice with i.p. injection of HBsAg and/or α-GalCer. It is well known that the efficiency of HBsAg-specific CTL induction is higher in HBV DNA immunization than in the immunization with HBsAg protein (17). However, considering further clinical application in human, we decided to use HBsAg protein for the immunization to induce HBsAg-specific CTLs. We examined HBsAg-specific lysis of CTL lines from Jα18 WT mice (Fig. 1A). Injection of HBsAg alone induced 19.2 and 38.3% HBsAg-specific lysis at an effector to target cell ratio

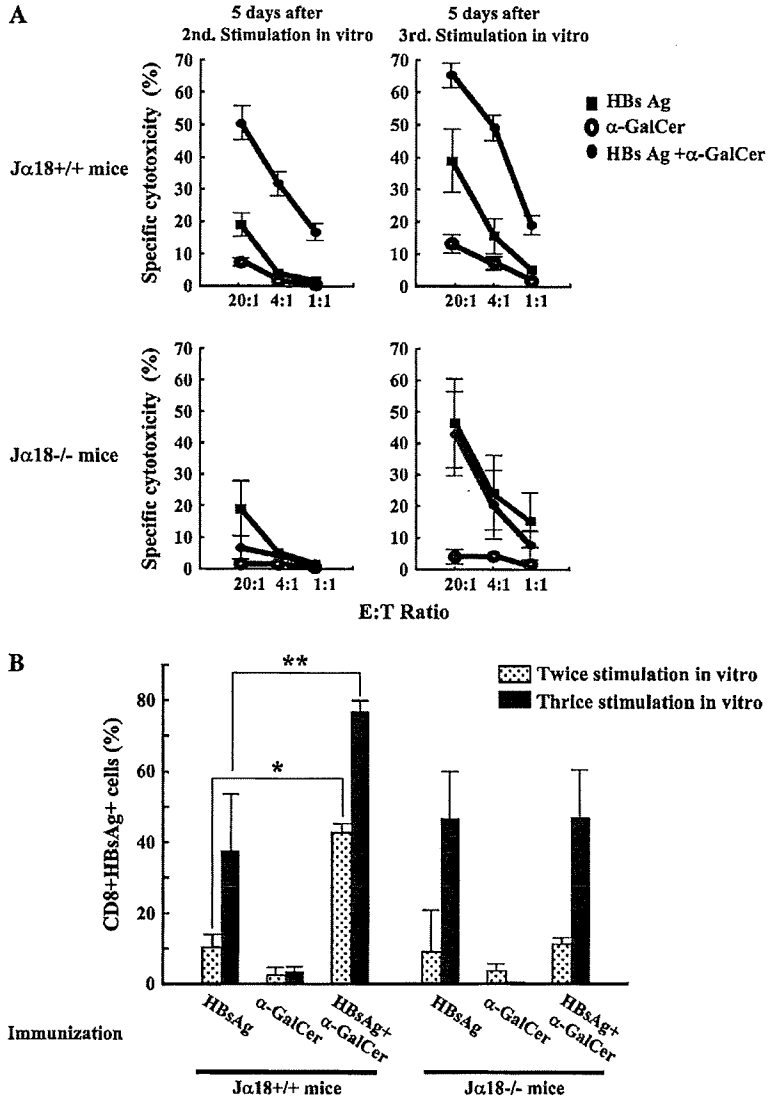

Fig. 1. HBsAg-specific cytolytic activity and HBsAgS28–39 specificity in the cell lines from immunized Jα18 WT and Jα18 KO mice. Jα18 WT or Jα18 KO mice were immunized with HBsAg (10 μg per mouse), α-GalCer (1 μg per mouse) or both. Splenocytes from these mice were prepared 7 days after immunization and were incubated with MMC-treated preS1-transfected P815 cells. (A) For the assay, effector cells (splenocytes) were incubated for 4 h with Eu-labeled target cells (P815 cells and preS1-transfected P815 cells) at an effector to target cell ratio of 20:1, 4:1 or 1:1. The percent specific cytotoxicity was calculated by subtracting the percent cytotoxicity of effector cells for P815 cells (HBsAg negative) from that for preS1-transfected P815 cells (HBsAg positive). Spontaneous release was always <20% of the total. Each data point and error bar represent the mean and the SEM, respectively, of results for triplicate samples. (B) Induction of HBsAg-specific $CD8^+$ cell responses was assessed by flow cytometric analysis using fluorescent dimeric H-$2L^d$–HBsAgS28–39 complexes. FACS profiles of Jα281 WT and Jα18 KO mice receiving HBsAg, α-GalCer or both are shown, with mean percentages of HBsAg $dimer^+$ $CD8^+$ cells plotted for each treatment. Each bar and error bar represent the mean and the SEM, respectively, of results for triplicate samples. Difference of percentages of HBsAg $dimer^+$ $CD8^+$ cells in the presence or absence of α-GalCer are compared. $^*P < 0.01$, $^{**}P < 0.05$.

of 20:1 following two or three *in vitro* stimulations, respectively. Injection of HBsAg with α-GalCer markedly enhanced specific lysis (50.2 and 65.3% after two and three stimulations, respectively) against HBsAg, but α-GalCer alone did not induce HBsAg-specific CTL responses. This enhancement of the HBsAg-induced CTL response by α-GalCer was not observed in Jα18 KO mice (Fig. 1A).

Next, we examined CTL frequencies using recombinant soluble dimeric H-$2L^d$ Ig, which can be used to stain $CD8^+$ T cells that are reactive for the H-$2L^d$-binding peptide HBsAgS28–39 (Fig. 1B). After two or three *in vitro* stimulations, the HBsAg-specific $CD8^+$ T cell counts in CTL lines from Jα18 WT mice immunized with a combination of HBsAg and α-GalCer were higher than those from WT mice

immunized with HBsAg alone. Also, there was no HBsAg-specific $CD8^+$ T cell from splenocytes of mice immunized with α-GalCer alone. The results of the cytotoxicity assays and the frequency analyses of HBsAg-specific CTLs indicate that α-GalCer strongly enhanced the induction and proliferation of HBsAg-specific CTLs in Jα18 WT mice.

Cytokine and CD40L production by spleen in response to i.p. HBsAg and α-GalCer in Jα18 WT and Jα18 KO

Previous studies demonstrated that several cytokines play important roles in the induction and proliferation of antigen-specific CTLs. Therefore, we measured the mRNA levels for IL-2, IL-4, IFN-γ, TNF-α and CD40L in whole spleens from Jα18 WT mice and Jα18 KO mice stimulated with HBsAg alone or a combination of HBsAg and α-GalCer (Fig. 2A). Using real-time RT–PCR, we found that the expression of IL-2, IL-4, IFN-γ and TNF-α mRNA in the splenocytes from Jα18 WT mice was enhanced by immunization with both HBsAg and α-GalCer but not with HBsAg alone. The enhancement of mRNA expression for these cytokines reached maximum levels ~7 h after immunization with HBsAg and α-GalCer. In contrast, the enhancement of cytokine mRNA expression was not observed in Jα18 KO mice. The mRNA expression of CD40L seemed to be constitutive both in Jα18 WT and KO mice. There was no significant change in the expression of CD40L in Jα18 WT and KO mice by the addition of α-GalCer during the observation period. Next, we measured protein production levels of cytokines in splenocytes after injection of HBsAg and α-GalCer by intracellular cytokine staining. The intracellular staining shown in Fig. 2(B) indicated that IL-2, IL-4, IFN-γ and TNF-α production of splenocyte increased after immunization of a combination

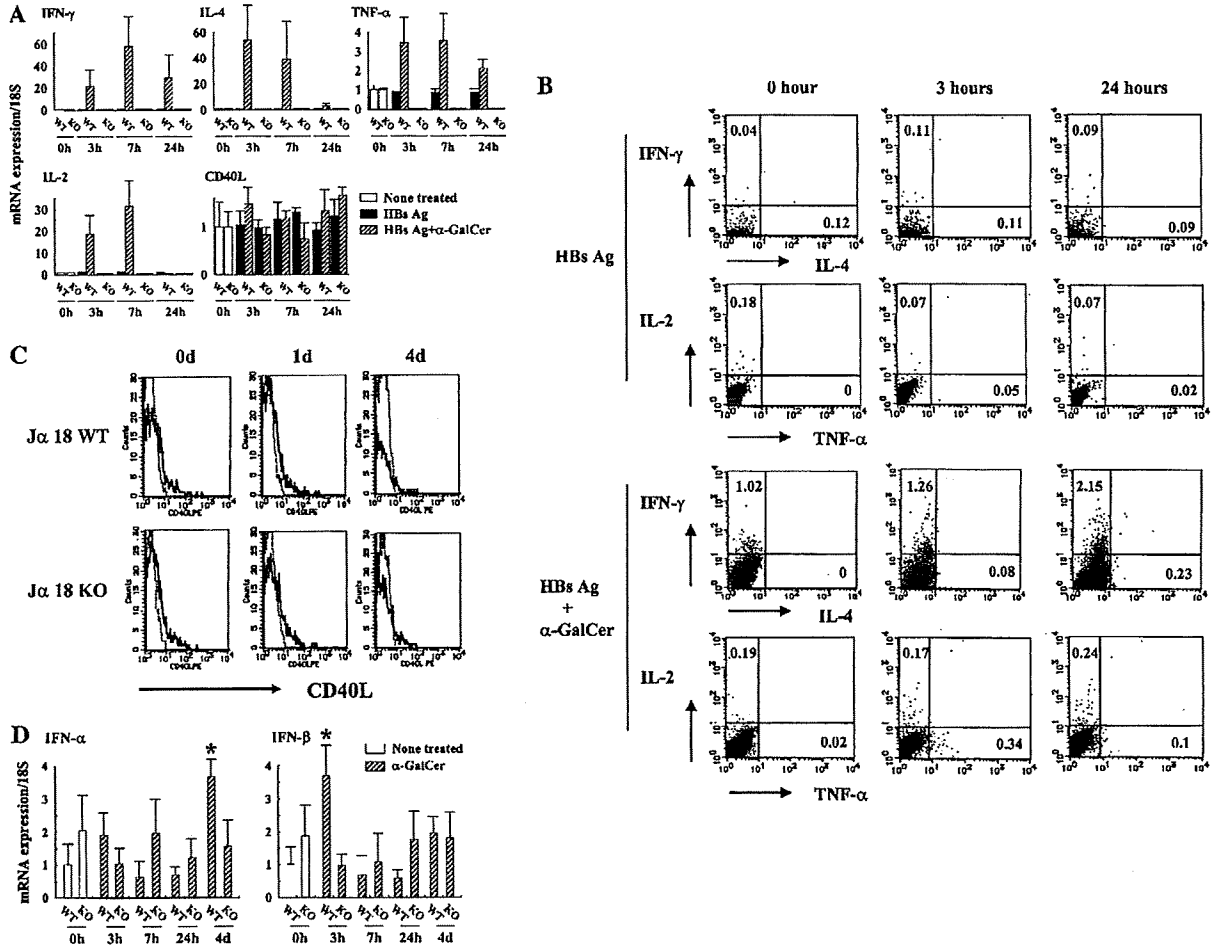

Fig. 2. Cytokine and CD40L production by spleen in response to i.p. HBsAg and α-GalCer. (A) Total spleen mRNA was prepared from Jα18 WT and KO mice immunized with HBsAg (10 μg per mouse) in the presence or absence of α-GalCer (1 μg per mouse) 3, 7 and 24 h after treatment. The expression of IL-2, IL-4, IFN-γ, TNF-α and CD40L mRNA was analyzed by real-time RT–PCR. (B) Flow cytometric analysis for intracellular cytokine produced by splenocytes taken from mice 0, 3 and 24 h after injection of HBsAg and α-GalCer, or HBsAg alone, and cultured for 4 h in brefeldin A. (C) CD40L expression on splenic CD3-positive cells 0 day, 1 day and 4 days after injection of HBsAg and α-GalCer. (D) The expression of IFN-α and IFN-β mRNA was analyzed by real-time RT–PCR. Results were normalized by the expression of 18S mRNA. Each bar and error bar represent the mean and the SEM, respectively, of results for triplicate samples. $*P < 0.05$ compared with non-treated WT mice.

of HBsAg and α-GalCer. On the other hand, HBsAg-alone injection did not enhance the cytokine production in splenocytes. Furthermore, we examined the surface CD40L expression of splenocytes by flow cytometer. CD40L expression was not affected by the injection of α-GalCer as a result of real-time PCR (Fig. 2C).

Previous study demonstrated that α-GalCer activated intrahepatic NKT cells to secrete antiviral cytokines (IFN-γ and IFN-α/β) in the liver and had the potential to control viral replication during natural HBV infection (18). Therefore, we evaluated mRNA levels for IFN-α and IFN-β in whole spleens from Jα18 WT mice and Jα18 KO mice stimulated with α-GalCer (Fig. 2D). IFN-α mRNA expression level significantly was elevated 4 days after the immunization of α-GalCer. On the other hand, IFN-β mRNA expression level was rapidly increased after α-GalCer injection.

Roles of IL-2, IL-4, IFN-γ, TNF-α and CD40L on the induction of CTLs

We tested the effect of neutralizing antibodies to IL-2, IL-4, IFN-γ, TNF-α and CD40L on the induction of HBsAg-specific CTLs from Jα18 WT mice immunized with both HBsAg and α-GalCer. As shown in Fig. 3, the frequency of HBsAg-specific CTLs from mice immunized with HBsAg and α-GalCer was reduced by the administration of antibodies to IL-2 and CD40L. Although antibodies to IL-4 and TNF-α reduced the frequency of HBsAg-specific CTLs from mice immunized with HBsAg alone, these antibodies did not affect the frequency from mice immunized with both HBsAg and α-GalCer.

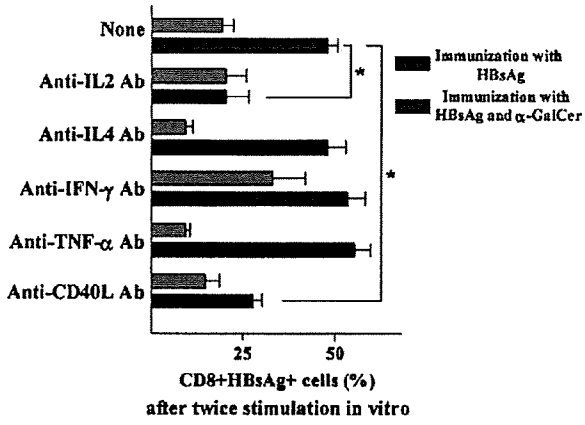

Fig. 3. Effect of IL-2, IL-4, IFN-γ, TNF-α and CD40L on the induction of HBsAg-specific CTLs. Jα18 WT mice were injected with neutralizing antibodies to IL-2, IL-4, IFN-γ, TNF-α or CD40L and then immunized with HBsAg (10 μg per mouse) in the presence or absence of α-GalCer (1 μg per mouse). Splenocytes prepared 7 days after immunization were incubated with MMC-treated P815/preS1 transfectants. Induction of HBsAg-specific $CD8^+$ cell responses was assessed by flow cytometric analysis using fluorescent dimeric $H\text{-}2L^d$–HBsAgS28–39 complexes. FACS profiles of mice receiving HBsAg or both HBsAg and α-GalCer are shown, with mean percentages of HBsAg $dimer^+$ $CD8^+$ cells plotted for each treatment group. Each bar and error bar represent the mean and the SEM, respectively, of results for triplicate samples. Difference of percentages of HBsAg $dimer^+$ $CD8^+$ cells in the presence or absence of neutralizing antibodies are compared. $^*P < 0.05$.

Next, we tested if co-administration of IL-2 and CD40L can substitute the enhancing effect of α-GalCer on CTL induction. Recombinant IL-2 and CD40L were co-administrated with HBsAg, and cytotoxicity of HBsAg-specific CTL lines and the frequency of HBsAg-specific CTLs were analyzed. After single stimulation *in vitro*, HBsAg-specific cytotoxicity of CTL lines from the mice immunized with HBsAg and α-GalCer was equal to that from mice immunized with IL-2, CD40L and HBsAg. The frequency of HBsAg-specific $CD8^+$ T cells in CTL lines from the mice immunized with IL-2, CD40L and HBsAg was rather higher than that from the mice immunized by the different protocols (Fig. 4).

It was recently reported that repeated stimulation of Vα14 NKT cells with α-GalCer treatments led to a change in their cytokine profile (19). We evaluated the effect of repeated stimulation of VαNKT cells with α-GalCer on the induction of HBsAg-specific CTLs and IL-2 production in Jα18 WT mice. Jα18 WT mice were immunized with HBsAg, HBsAg and once injection of α-GalCer or HBsAg and three times injection of α-GalCer. Induction of HBsAg-specific $CD8^+$ T cell responses was assessed by flow cytometric analysis. The frequency of HBsAg-specific $CD8^+$ T cells in CTL lines from the mice immunized with HBsAg and once α-GalCer was higher than that from the mice immunized by the other protocols (Fig. 5A). Furthermore, we found that the expression of IL-2 mRNA in the splenocytes from Jα18 WT mice was enhanced by immunization with once α-GalCer injection but not three times α-GalCer injection *in vivo* (Fig. 5B).

Cytokine mRNA expression of splenic $V\alpha14^+$ NKT, $CD4^+$ T cells and $CD8^+$ T cells from Jα18 WT mice immunized with HBsAg and α-GalCer

Next, we isolated $V\alpha14^+$ NKT, $CD4^+$ $DX5^-$ and $CD8^+$ $DX5^-$ cells from whole splenocytes by immunomagnetic separation and then examined which cell fractions produced these cytokines in the mice immunized with HBsAg and α-GalCer (Fig. 6). The expression of IFN-γ mRNA was enhanced in all the cell fractions, especially in $V\alpha14^+$ NKT and in $CD8^+$ T cells. IL-2 mRNA expression was also enhanced in all the fractions; however, the highest enhancement was observed in $CD4^+$ T cells. The enhancement of IL-4 mRNA expression was seen in $V\alpha14^+$ NKT and $CD4^+$ T cells. TNF-α mRNA expression was enhanced in $CD8^+$ T cells but not in $V\alpha14^+$ NKT and $CD4^+$ T cells. CD40L mRNA was expressed constitutively in $CD4^+$ T cells but not in $V\alpha14^+$ NKT or $CD8^+$ T cells. The expression level of CD40L was not changed by the addition of α-GalCer.

Induction of HBsAg-specific CTLs in HBsAg transgenic mice

HBsAg transgenic mice, which are immunologically tolerant to HBV-encoded antigens, are a model of chronic HBV infection (20). In order to test the possibility for inducing HBsAg-specific CTLs even in conditions of chronic HBV infection, we evaluated the HBsAg-specific CTL activity of harvested spleen cells from transgenic mice immunized with HBsAg or both HBsAg and α-GalCer following two or three *in vitro* stimulations with the P815preS1 transfectants. As shown in Fig. 7(A), HBsAg-specific cytotoxicity was induced only in mice immunized with both HBsAg and α-GalCer; CTL activity was not observed in spleen cells from the mice immunized with either HBsAg or α-GalCer alone. Furthermore,

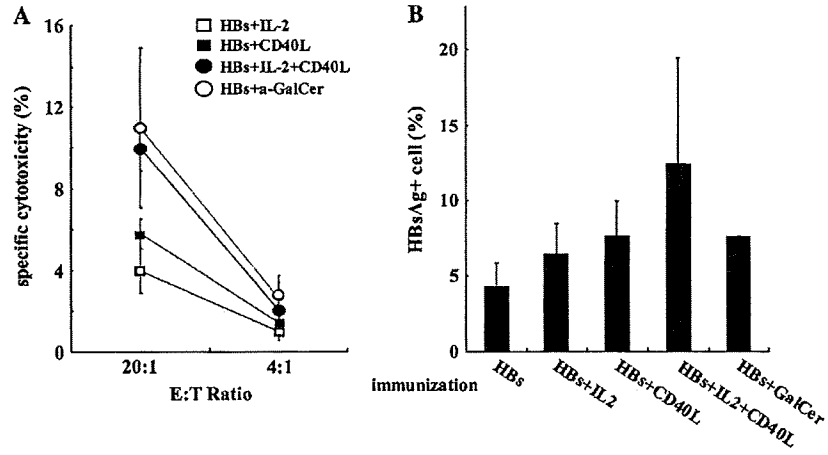

Fig. 4. Role of IL-2 and CD40L on the induction of HBsAg-specific CTLs. Jα18 WT mice were administrated (4×10^4 U per mouse) or CD40L (100 μg per mouse) or both of IL-2 (4×10^4 U per mouse) and CD40L (100 μg per mouse) or α-GalCer (1 μg per mouse) with HBsAg (10 μg per mouse). Splenocytes from these mice were prepared 7 days after immunization and were incubated with MMC-treated preS1-transfected P815 cells. (A) For the assay, effector cells were incubated for 4 h with Eu-labeled target cells (P815 cells and preS1-transfected P815 cells) at an effector to target cell ratio of 20:1 or 4:1. The percent specific cytotoxicity was calculated by subtracting the percent cytotoxicity of effector cells for P815 cells from that for preS1-transfected P815 cells. Each data point and error bar represent the mean and the SEM, respectively, of results for triplicate samples. (B) Induction of HBsAg-specific $CD8^+$ cell responses was assessed by flow cytometric analysis using fluorescent dimeric H-$2L^d$–HBsAgS28–39 complexes. Each bar and error bar represent the mean and the SEM, respectively, of results for triplicate samples.

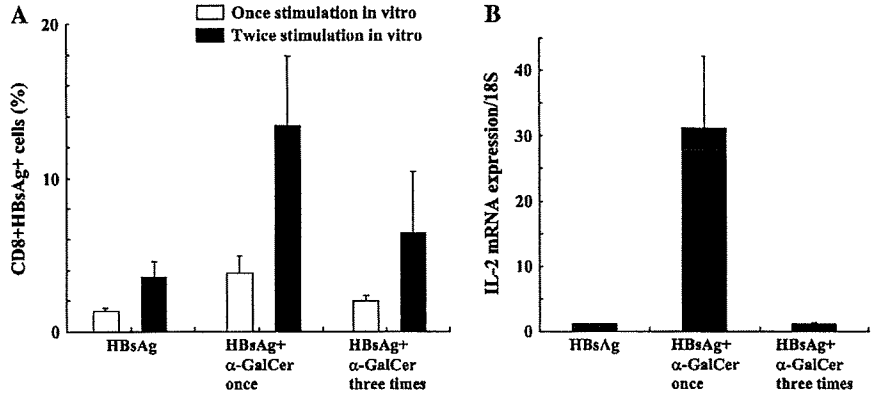

Fig. 5. Effect of repeated stimulation of VαNKT cells with α-GalCer on the induction of HBsAg-specific CTLs in Jα18 WT mice. Jα18 WT mice were immunized with HBsAg (10 μg per mouse), HBsAg and once injection of α-GalCer (1 μg per mouse) or HBsAg and three times injection of α-GalCer at interval 3 days. (A) Splenocytes from these mice were prepared 10 days after immunization and were incubated with MMC-treated preS1-transfected P815 cells. Induction of HBsAg-specific $CD8^+$ cell responses were assessed by flow cytometric analysis using fluorescent dimeric H-$2L^d$–HBsAgS28–39 complexes. Each bar and error bar represent the mean and the SEM, respectively, of results for triplicate samples. (B) Total spleen mRNA was prepared from Jα18 WT mice immunized 7 h after last α-GalCer injection. The expression of IL-2 mRNA was analyzed by real-time RT-PCR. Each bar and error bar represent the mean and the SEM, respectively, of results for triplicate samples.

quantitative flow cytometric analysis revealed that HBsAg-specific $CD8^+$ T cells appeared in the HBsAg transgenic mice only when they were immunized with HBsAg and α-GalCer (Fig. 7B).

Next, we tried to induce HBsAg-specific CTL clones in HBsAg transgenic mice after immunization with a combination of HBsAg and α-GalCer. By limiting dilution, we established CTL clones with high cytolytic activity from HBsAg transgenic mice (Table 1).

Analysis of HBsAg-specific CTL precursor frequency in HBsAg transgenic mice immunized with HBsAg and α-GalCer

To quantitate the number of HBsAg-specific CTL precursors in the HBsAg transgenic mice immunized with HBsAg and α-GalCer *ex vivo*, we used a limiting dilution technique. The $CD8^+$ T cells isolated by immunomagnetic beads from immunized mice were seeded at three cells per well and stimulated weekly with irradiated autologous splenocytes and P815preS1 *in vitro*. After 2 weeks stimulation, the number of

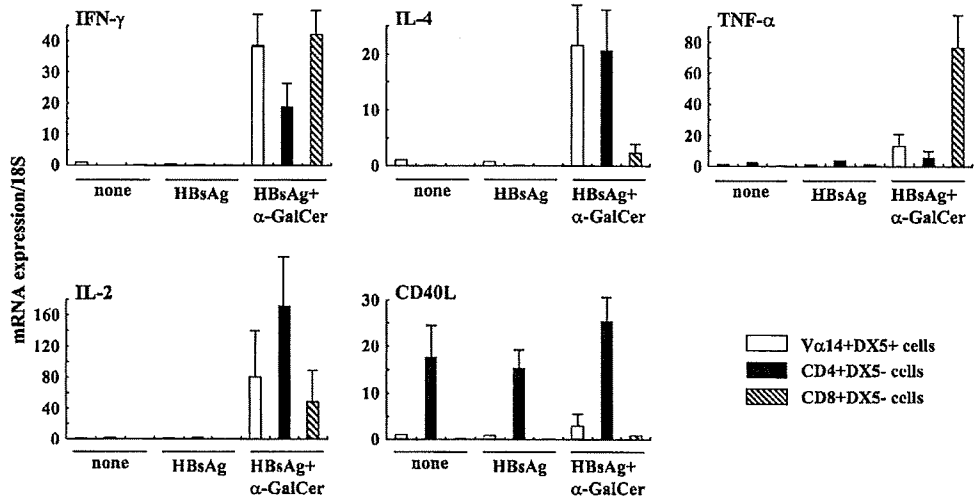

Fig. 6. Expression of IL-2, IL-4, IFN-γ, TNF-α and CD40L mRNA by splenic Vα14$^+$ NKT, CD4$^+$ or CD8$^+$ cells from Jα18 WT mice immunized with HBsAg and α-GalCer. Jα18 WT mice were immunized with HBsAg (10 μg per mouse), α-GalCer (1 μg per mouse) or both. Splenic Vα14$^+$ NKT, CD4$^+$ and CD8$^+$ cells were purified using immunomagnetic separation 7 h after treatment and used to prepare total mRNA. The expression of IL-2, IL-4, IFN-γ, TNF-α and CD40L mRNA was analyzed by real-time RT-PCR. Results were normalized by the expression of 18S mRNA.

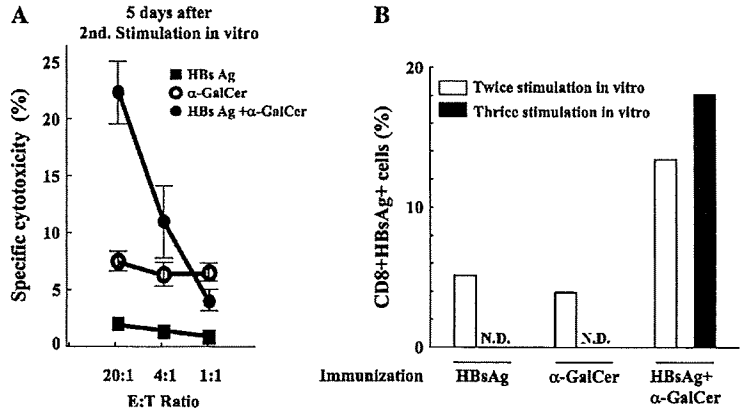

Fig. 7. Induction of HBsAg-specific CTLs in HBsAg transgenic mice immunized with HBsAg and α-GalCer. Cytotoxic activity of HBsAg-specific CTLs induced in HBsAg transgenic mice (A) and flow cytometric analysis of the frequency of HBsAg-specific CD8$^+$ cells in HBsAg transgenic mice (B). HBsAg transgenic mice were immunized with HBsAg (10 μg per mouse), α-GalCer (1 μg per mouse) or both. Splenocytes prepared 7 days after inoculation were incubated with MMC-treated P815/preS1-transfected cells. Induction of HBsAg-specific CD8$^+$ cell responses were assessed by a cytotoxic assay and flow cytometric analysis using dimeric H-2L^d–HBsAgS28–39 complexes. Each data point and error bar represent the mean and the SEM, respectively, of results for triplicate samples (A).

the wells with proliferating cells was counted and those cells were tested for HBsAg specificity. With the splenocytes from HBsAg transgenic mice immunized with HBsAg alone, the proliferation of the cells was observed only in four wells out of 960 wells, none of those cells showed specificity to HBsAgS28–39. In contrast, the proliferation was observed in 99 wells out of 960 wells, and 89% (88/99) of them showed HBsAgS28–39 specificity in the case of co-immunization with HBsAg α-GalCer (Table 2).

Discussion

It is well known that CTLs play a key role not only in hepatocellular injury (6, 7) but also in viral clearance (8, 9). On the other hand, a lack of or a weak CTL response is observed in individuals with chronic HBV infection, and it is considered to be one of the main reasons for the persistence of HBV infection (1). Recent studies revealed that DNA vaccination and dendritic cell (DC) immunization overcome the tolerance of CTLs to autoantigens (17, 21) and tumors (22, 23). In HBsAg transgenic mice, repetitive immunization with a recombinant vaccinia virus encoding HBsAg breaks the tolerance at the B cell level but not the T cell level (20). Furthermore, an artificially synthesized peptide that includes specific epitopes for helper T lymphocytes and CTLs has been shown to break CTL tolerance in HBV transgenic mice (24). To attempt breaking CTL tolerance by modifying