

よる IgE 抗体産生の選択的抑制効果とは独立していることが示唆された。さらに、蛍光標識した OVA、あるいは、消化を受けると蛍光を発する OVA (DQ-OVA) をリポソームと結合させたものをマクロファージ培養中に添加して共焦点蛍光顕微鏡を用いた解析を行ったところ、オレイン酸からなるリポソームに結合した OVA はステアリン酸からなるリポソームに結合した OVA と比べて同一時間内により多くマクロファージに貪食され (図 4a)、かつ消化を受ける (図 4b) ことが示された。このことから、いわゆるアジュバント効果は抗原提供細胞による認識され易さと相関することが示唆された⁴⁾。

リポソーム表面結合抗原によって誘導される IgE 選択的無反応は T 細胞に非依存的である

OVA-リポソームおよび OVA-alum 免疫マウス由来の CD4 陽性 T 細胞について、T 細胞欠損マウス (ヌードマウス) において抗 OVA 抗体産生を誘導す

る能力を比較検討した⁵⁾。表 2 に示すように、OVA-リポソーム免疫マウス由来 CD4 陽性 T 細胞をヌードマウスに移入し、OVA-alum を免疫すると、抗 OVA IgE 産生が誘導された。これに対して、OVA-alum 免疫マウス由来 CD4 陽性 T 細胞をヌードマウスに移入し、OVA-リポソームを免疫すると、抗 OVA IgE 産生は誘導されなかった。このように、IgE 産生の選択的抑制はヌードマウスを OVA-リポソームで免疫したときに観察され、T 細胞ドナーの免疫方法にはよらなかった。IL-10 および CD8 陽性 T 細胞が IgE 産生の調節に関与することを示唆する報告がされているが、IL-10 の活性を中和するモノクローナル抗体、および CD8 陽性 T 細胞を除去するモノクローナル抗体を *in vivo* 投与しても OVA-リポソームによって誘導される IgE 抗体産生の選択的抑制に影響は見られなかったことから、OVA-リポソームによって誘導される IgE 抗体産生の選択的抑制に IL-10 あるいは CD8 陽性 T 細胞は関与しないと考えられた。



図 4a 2種類の異なる脂質組成のリポソームに結合した OVA のマクロファージによる貪食
蛍光標識した OVA をステアリン酸 (Stearoyl)、オレイン酸 (Plepyl) からなるリポソームと結合させ、マクロファージ培養中に添加した。培養開始後 60 分における共焦点蛍光顕微鏡像を示す。



図 4b 2種類の異なる脂質組成のリポソームに結合した OVA のマクロファージによる消化
消化を受けると蛍光を発する OVA (DQ-OVA) をステアリン酸 (Stearoyl)、オレイン酸 (Plepyl) からなるリポソームと結合させ、マクロファージ培養中に添加した。培養開始後 60 分における共焦点蛍光顕微鏡像を示す。

表2 OVA 免疫マウス由来脾臓 CD4 陽性 T 細胞を移入した T 細胞欠損マウスにおける抗 OVA 抗体産生

immunization of T-cell donor	immunization of recipient mice	Anti-OVA antibodies	
		IgG ($\mu\text{g/ml}$)	IgE ELISA titer
no	OVA-liposome	12.3 \pm 8.7	N.D.
	OVA-alum	17.7 \pm 5.2	N.D.
OVA-liposome	OVA-liposome	144.7 \pm 24.3	N.D.
	OVA-alum	124.3 \pm 12.8	105.6 \pm 10.7
OVA-alum	OVA-liposome	178.0 \pm 28.3	N.D.
	OVA-alum	246.8 \pm 29.4	139.3 \pm 12.3

OVA-リポソームあるいは OVA-alum で免疫したマウスの脾臓由来 CD4 陽性 T 細胞を T 細胞欠損マウスに移入した後、OVA-リポソームあるいは OVA-alum で免疫を行い、細胞移入 4 週間後に採血を行って血清中の抗 OVA 抗体を測定した。

T 細胞欠損マウスへの CD4 陽性 T 細胞移入実験によって OVA-リポソーム免疫マウス由来 CD4 陽性 T 細胞は抗 OVA IgE 抗体産生を支持することが示され、OVA-alum 免疫マウス由来 CD4 陽性 T 細胞を移入したヌードマウスにおいても OVA-リポソームで免疫すると抗 OVA IgE 産生が誘導されないことが示されたことから、OVA-リポソームによる IgE 産生の抑制機構に CD4 陽性 T 細胞は関与していないことが明らかになった。CD4 陽性 T 細胞のかわりに脾臓細胞から B 細胞を除去したものをを用いた時にも同様の結果が得られたことから、OVA-リポソームによって誘導される IgE 産生の選択的抑制においては B 細胞が重要な役割を担っていることが示唆された。

これらの結果は、同一の抗原に対して異なるアジュバントを使用することにより異なるパターンの免疫応答が誘導されることを示している。OVA は元来アレルギー性を有しているが、リポソームあるいは CFA を使用することにより IgE 産生が選択的に抑制される。OVA-CFA による IgE 産生の抑制には Th1 が関与しているが OVA-リポソームによる IgE 産生の抑制には Th1 は関与していない。このことから、Th1 および Th2 のバランスによらない IgE 産生調節機構が存在することが示唆された。

不飽和脂肪酸からなるリポソームを用いて
作製されたリポソーム表面結合抗原は
細胞傷害性 T 細胞 (CTL) を誘導し、
腫瘍拒絶を誘導する

上述のように、リポソームの脂質組成を変えることによりアジュバント効果に変化することが示されたが、このような量的な変化だけでなく、リポソームの

脂質組成を変えることによってリポソーム表面結合抗原が液性免疫 (抗体産生)、あるいは細胞性免疫 (CTL 活性化) を誘導する、という質的变化がもたらされること近年の検討の結果、明らかになった⁹⁾。マクロファージのクラス II compartment を赤色に蛍光標識しておき、リポソームに結合させる OVA を緑色に蛍光標識したものをマクロファージ培養中に添加すると、図 5a に示すように、飽和脂肪酸からなるリポソームに結合した OVA は貪食を受けた後すべてマクロファージのクラス II compartment に集結するが、不飽和脂肪酸からなるリポソームに結合した OVA の一部は細胞質に留まることがわかった。さらに、消化を受けて蛍光を発する OVA (DQ-OVA) をリポソームに結合させて同様の検討を行ったところ、不飽和脂肪酸からなるリポソームに結合した OVA の一部はクラス II compartment の外で消化を受けることがわかった (図 5b)。この結果は試験管内での CD4 および CD8 陽性 T 細胞への抗原提供を検討した結果ともよく一致した。すなわち、飽和脂肪酸からなるリポソームに結合した OVA を OVA 免疫マウス由来の T 細胞および抗原提供細胞の培養中に添加すると CD4 陽性 T 細胞だけが活性化したが、不飽和脂肪酸からなるリポソームに結合した OVA は CD4 陽性 T 細胞だけでなく CD8 陽性 T 細胞も活性化した (表 3)。

これらの結果から、不飽和脂肪酸からなるリポソームに結合した抗原は、外来性抗原であるにもかかわらず抗原提供細胞において MHC クラス I を介して CD8 陽性 T 細胞に cross-present されることが明らかになった。そこで、OVA のペプチドを発現した腫瘍細胞株 E.G7 をマウスに移植して担癌マウスを作製し、不飽和脂肪酸からなるリポソームに結合した OVA の



図 5a リポソームに結合した OVA のマクロファージ細胞中における局在
クラス II を赤色の蛍光で標識したマクロファージの培養中に、緑色の蛍光で標識した OVA と飽和脂肪酸 (Saturated) あるいは不飽和脂肪酸 (Unsaturated) からなるリポソームとの結合物を添加した。培養開始後 60 分における共焦点蛍光顕微鏡像を示す。

図 5b リポソームに結合した OVA のマクロファージ細胞中における消化
クラス II を赤色の蛍光で標識したマクロファージの培養中に、消化を受けると蛍光を発する OVA (DQ-OVA) と飽和脂肪酸 (Saturated) あるいは不飽和脂肪酸 (Unsaturated) からなるリポソームとの結合物を添加した。培養開始後 60 分における共焦点蛍光顕微鏡像を示す。

表 3 OVA をパルスしたマクロファージによる CD4/CD8 陽性 T 細胞の活性化
マウス脾臓より得られた抗原提供細胞を OVA あるいはリポソーム結合 OVA でパルスした後、OVA 免疫マウス脾臓由来 CD4/CD8 陽性 T 細胞と共培養し、培養上清中のサイトカインを測定した。

<i>in vitro</i> Ag	Liposomes	CD4 T-cells		CD8 T-cells	
		IL-5	IFN- γ	IL-5	IFN- γ
none		ND	ND	ND	ND
OVA solution		96.2 \pm 12.5	ND	ND	ND
OVA-liposome	Saturated	910.2 \pm 23.0	88.7 \pm 45.0	ND	ND
OVA-liposome	Unsaturated	1,065.5 \pm 31.9	115.1 \pm 28.6	163.3 \pm 99.1	149.9 \pm 83.8

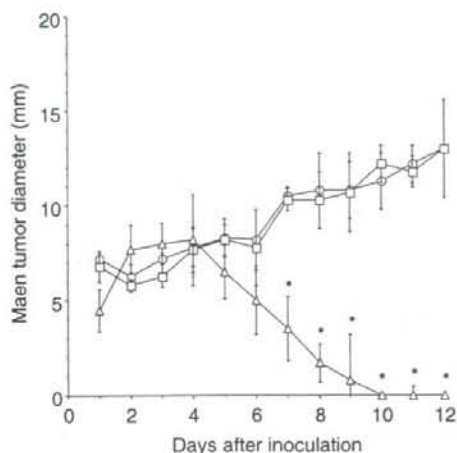


図6 腫瘍抗原ペプチドを投与したマウスにおける腫瘍拒絶
C57BL/6 マウスに E.G7 を移植し、腫瘍径が約 5 mm となった時に腫瘍の周囲に OVA ペプチド結合リポソームを CpG、抗 IL-10 モノクローナル抗体と共に投与して腫瘍径の変化を観察した。○：CpG、抗 IL-10 モノクローナル抗体のみ投与。□：ペプチド溶液を CpG、抗 IL-10 モノクローナル抗体とともに投与。△：ペプチド結合リポソームを CpG、抗 IL-10 モノクローナル抗体とともに投与。

ペプチドを CpG、抗 IL-10 抗体とともにこのマウスに投与したところ、図 6 に示すように、投与後 10 日以内に腫瘍が消滅した。一方、CpG、抗 IL-10 抗体だけを投与したマウス、およびペプチドを溶液状にて投与した群では腫瘍拒絶は観察されなかった。図 7 に示すように、腫瘍拒絶が観察されたマウス由来の脾臓 CD8 陽性 T 細胞中では OVA ペプチドに特異的な細胞群が有意に増加していることが観察されたことから、不飽和脂肪酸からなるリポソームに結合したペプチドは CD8 陽性 T 細胞を活性化し、細胞性免疫を誘導することが示された。

おわりに

現行のワクチンにアジュバントとして用いられている水酸化アルミニウムは、取扱の簡便さ、低コスト、高いアジュバント効果、等の利点を有するが、一方で、ワクチン接種後のアレルギー反応に関与する IgE 抗体産生を誘導するほか、液性免疫は誘導するが細胞性免疫は誘導しにくく、代謝を受けることなく体内に蓄積する、等の欠点がある。本研究は現行のワクチンアジュバントが持つこれらの問題を解決することを目的として開始された。リポソーム表面結合抗原は IgE 抗体産生を選択的に抑制し、IgG 抗体産生を増強す

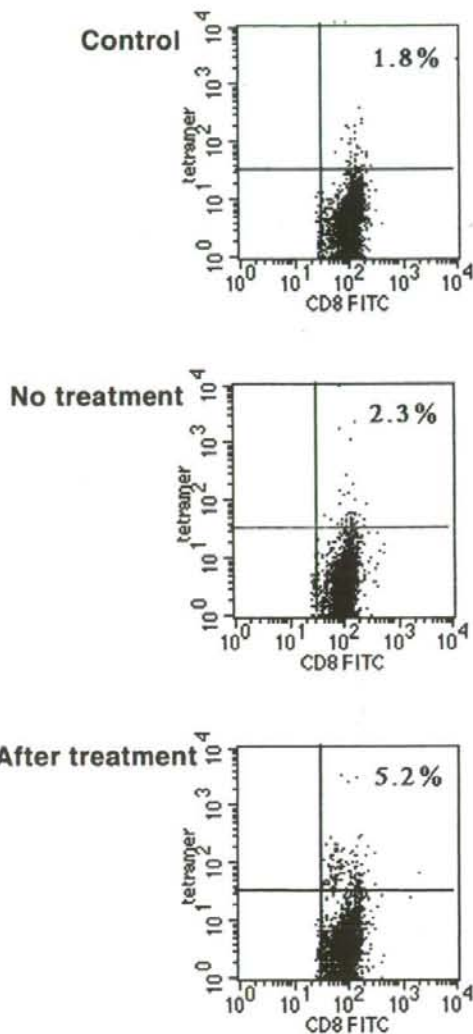


図7 腫瘍を拒絶したマウスにおける CTL 誘導
マウス脾臓由来 CD8 陽性細胞の OVA ペプチド-MHC クラス I (H2-K^b) 複合体を FACS を用いて解析した。Control：正常マウス。No treatment：腫瘍拒絶の観察されなかった担癌マウス。After treatment：図 6 の実験において腫瘍拒絶の観察されたマウス。

る。さらに、リポソームは生体の細胞膜構成成分からなるため、きわめて毒性が低く、代謝を受けて生体内に残留しないことから、リポソーム表面結合抗原が現行のアルミニウムアジュバントに代わるワクチンの処方として期待された。これに加えて、近年の検討の結果、リポソームの脂質組成を選択することによって細胞性免疫をよく誘導することのできる処方が開発されたことから、リポソーム表面結合抗原をアレルギー反応を誘導しにくいワクチンのほか、細胞性免疫

の誘導を目標とするウイルスワクチン、腫瘍治療薬の開発に応用することが期待される。

これまでに得られた知見をもとに、我々は現在、リポソーム表面結合抗原を高病原性鳥インフルエンザ、Severe acute respiratory syndrome (SARS) といった新興感染症に対するワクチン、および、腫瘍治療薬の創製に応用するべく検討を行っている。

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Identification of HLA-A2- or HLA-A24-Restricted CTL Epitopes Possibly Useful for Glypican-3-Specific Immunotherapy of Hepatocellular Carcinoma

Hirofumi Komori,^{1,2} Tetsuya Nakatsura,¹ Satoru Senju,¹ Yoshihiro Yoshitake,¹ Yutaka Motomura,^{1,2} Yoshiaki Ikuta,^{1,2} Daiki Fukuma,¹ Kazunori Yokomine,¹ Michiko Harao,^{1,2} Toru Beppu,² Masanori Matsui,³ Toshihiko Torigoe,⁴ Noriyuki Sato,⁴ Hideo Baba,² and Yasuharu Nishimura¹

Abstract Purpose and Experimental Design: We previously reported that glypican-3 (GPC3) was overexpressed, specifically in hepatocellular carcinoma (HCC) and melanoma in humans, and it was useful as a novel tumor marker. We also reported that the preimmunization of BALB/c mice with dendritic cells pulsed with the H-2K^d-restricted mouse GPC3₂₉₈₋₃₀₆ (EYLISLEEL) peptide prevented the growth of tumor-expressing mouse GPC3. Because of similarities in the peptide binding motifs between H-2K^d and HLA-A24 (A*2402), the GPC3₂₉₈₋₃₀₆ peptide therefore seemed to be useful for the immunotherapy of HLA-A24⁺ patients with HCC and melanoma. In this report, we investigated whether the GPC3₂₉₈₋₃₀₆ peptide could induce GPC3-reactive CTLs from the peripheral blood mononuclear cells (PBMC) of HLA-A24 (A*2402)⁺ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice to identify the HLA-A2 (A*0201)-restricted GPC3 epitopes to expand the applications of GPC3-based immunotherapy to the HLA-A2⁺ HCC patients.

Results: We found that the GPC3₁₄₄₋₁₅₂ (FVGEFFTDV) peptide could induce peptide-reactive CTLs in HLA-A2.1 (HHD) transgenic mice without inducing autoimmunity. In five out of eight HLA-A2⁺ GPC3⁺ HCC patients, the GPC3₁₄₄₋₁₅₂ peptide-reactive CTLs were generated from PBMCs by *in vitro* stimulation with the peptide and the GPC3₂₉₈₋₃₀₆ peptide-reactive CTLs were also generated from PBMCs in four of six HLA-A24⁺ GPC3⁺ HCC patients. The inoculation of these CTLs reduced the human HCC tumor mass implanted into nonobese diabetic/severe combined immunodeficiency mice.

Conclusion: Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of HCC patients.

Hepatocellular carcinoma (HCC) is now spreading rapidly, especially in Asian and Western countries. It is clear that patients with hepatitis B or C-based liver cirrhosis are at high risk for developing HCC (1), and patients with hepatitis

treated surgically or by other therapies are also at high risk for recurrence (2). Furthermore, the liver function of these patients is often very poor, so further treatment for recurrence is often restricted. As a result, the prognosis of HCC remains poor, and new therapies for the prevention of cancer development and recurrence, i.e., adjuvant therapy, is urgently needed. As for melanoma, the age-adjusted incidence rates have been increasing in most fair-skinned populations in recent decades (3). In 2005, it is estimated that 59,580 Americans will be diagnosed to have melanoma, and 7,770 will die from the disease (4).

We and others previously reported that glypican-3 (GPC3) was overexpressed in most types of HCC (5-9) and melanoma in humans (8), and we also previously reported that an H-2K^d-restricted antigenic peptide, the mouse GPC3₂₉₈₋₃₀₆ (EYLISLEEL) peptide, could be recognized by mouse CD8⁺ CTLs. In addition, these CTLs rejected tumor expressing mouse GPC3 both *in vitro* and *in vivo* (10). Because the structural motifs of peptides bound to HLA-A24 (A*2402) and mouse H-2K^d are similar, we investigated whether the GPC3 peptide was also useful as a cancer immunotherapy modality for HLA-A24⁺ HCC patients. The gene frequency of HLA-A24 (A*2402) is relatively high in Asian populations, especially in the Japanese, whereas it is low in Caucasians. On the other hand, The gene frequency of

Authors' Affiliations: Departments of ¹Immunogenetics and ²Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University; ³Department of Microbiology, Saitama Medical School, Saitama; and ⁴Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan
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Note: T. Nakatsura is currently in the Immunotherapy Section, Investigative Treatment Division, Center for Innovative Medicine, National Cancer Center East, Kashiwa, Japan.

Requests for reprints: Yasuharu Nishimura, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. Phone: 81-96-373-5310, Fax: 81-96-373-5314; E-mail: mxnshim@gpo.kumamoto-u.ac.jp.

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HLA-A2 (A*0201) is high among various ethnic groups, including both Asians and Caucasians (11). Therefore, it is suggested that the HLA-A2-restricted and GPC3-derived CTL epitopes might be very useful for the immunotherapy of many patients with HCC and melanoma all over the world. In the present study, we identified human GPC3-derived CTL epitopes restricted by HLA-A2 using HLA-A2.1 (HHD) transgenic mice (Tgm) and examined whether these HLA-A2 or HLA-A24-restricted epitope peptides could induce GPC3-reactive CTLs from peripheral blood mononuclear cells (PBMC) of patients with HCC.

Materials and Methods

Mouse. HLA-A2.1 (HHD) Tgm; H-2D^b-β2m^{-/-} double knockout mice introduced with human β2m-HLA-A2.1 (α1 α2)-H-2D^b (α3 transmembrane cytoplasmic) (HHD) monochain construct gene were generated in the Department SIDA-Retrovirus, Unité d'Immunité Cellulaire Antivirale, Institut Pasteur, France (12, 13) and kindly provided by Dr. F.A. Lemonnier. Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) female mice at 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan).

Patients, blood samples, and cell lines. Blood samples from patients with HCC were obtained during routine diagnostic procedures after obtaining a formal agreement signed by the patients in Kumamoto University Hospital from April to September 2005. Human liver cancer cell lines, SK-Hep-1 and T2-A0201 (a TAP-deficient and HLA-A*0201-positive cell line; refs. 14, 15), were provided by Kyogo Ito of Kurume University. Human liver cancer cell lines HepG2 and HuH-7 endogenously expressing GPC3, and GPC3⁻ colon cancer cell line SW620, were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). C1R-A*2402 (an HLA-A*2402 transfectant of C1R cells expressing a trace amount of HLA class I molecule; ref. 15) were generous gifts from Dr. Masafumi Takiguchi. The expression of HLA-A2 and HLA-A24 in these cell lines were examined using flow cytometry with an anti-HLA-A2 monoclonal antibody (mAb), BB7.2 and anti-HLA-A24 mAb (One Lambda, Inc., Canoga Park, CA), respectively, in order to select target cell lines for CTL assays. The origins and HLA genotypes of these cell lines have been described elsewhere (16, 17). These cells were maintained *in vitro* in RPMI 1640 or DMEM supplemented with 10% FCS.

Induction of GPC3-reactive mouse CTLs and IFN-γ enzyme-linked immunospot assay. Human GPC3-derived peptides (purity >90%) sharing the amino acid sequences with mouse GPC3 and carrying

binding motifs for HLA-A*0201-encoded molecules, were identified using BIMAS software (Bioinformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) and we purchased a total of nine peptides carrying HLA-A2 (A*0201) binding motifs (Table 1) from Biologica (Tokyo, Japan). The immunizations of mice with peptides were done as previously described (7). In brief, bone marrow (BM) cells (2×10^6) from HLA-A2.1 (HHD) Tgm were cultured in RPMI 1640 supplemented with 10% FCS, together with granulocyte macrophage colony-stimulating factor (5 ng/mL) and 2ME (0.8 ng/mL) for 7 days in 10-cm plastic dishes, and these BM-dendritic cells (DC) were pulsed with the mixture of GPC3 peptides carrying HLA-A2 binding motifs (1 μmol/L for each peptide) at 37°C for 2 hours. We primed the HLA-A2.1 (HHD) Tgm with this syngeneic BM-DC vaccine (5×10^5 /mouse) into the peritoneal cavity once a week for two weeks. Seven days after the last immunization, the spleens were collected and CD4⁺ spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production by CD4⁺ spleen cells cocultured with the BM-DC. The CD4⁺ spleen cells (2×10^6 /well) were stimulated with syngeneic BM-DC (2×10^5 /well) pulsed with each peptide *in vitro*. Then, 6 days later, the frequency of cells producing IFN-γ/ 2×10^4 CD4⁺ spleen cells upon stimulation with syngeneic BM-DC (1×10^4 /well), pulsed with or without each peptide, was assayed in an enzyme-linked immunospot (ELISPOT) assay as previously described (18).

Induction of GPC3-reactive human CTLs. We isolated PBMCs from the heparinized blood of HLA-A24⁺ and/or HLA-A2⁺ Japanese patients with HCC or healthy donors by means of Ficoll-Conray density gradient centrifugation, and peripheral monocyte-derived DCs were generated as described previously (19, 20). CD8⁺ T cells were isolated using CD8 microbeads (Miltenyi Biotec) from the PBMC of the same donors, and thereafter, peptide-reactive CD8⁺ CTLs were generated (19, 20). Five days after the last stimulation, the cytotoxic activities of the CTLs were measured by a ⁵¹Cr release assay.

CTL responses against cancer cell lines. CTLs were cocultured with each cancer cell line as a target cell (5×10^3 /well) at the indicated effector/target ratio and ⁵¹Cr release assay was done as described (21). The blocking of HLA-class I or HLA-DR, was done as follows. Before the coculture of CTLs with a cancer cell line in a ⁵¹Cr release assay or ELISPOT assay, target cancer cells were incubated for 1 hour with 10 μg/mL anti-class I mAb W6/32 or 10 μg/mL anti-HLA-DR mAb, H-DR-1, and then the effects of mAbs on either the cytotoxic activity or production of IFN-γ by CTLs were examined as reported previously (22).

Histologic and immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the GPC3₁₄₄₋₁₅₂ peptides and the staining of

Table 1. GPC3-derived peptides conserved between human and mouse GPC3 and predicted to be bound to HLA-A2 (A*0201)

A2-binding peptide	Position	Subsequence residue listing	HLA-A2 binding score*
GPC3A2-1	44-52	RLQPGLKVV	879
GPC3A2-2	102-110	FLIQNAAV	319
GPC3A2-3	144-152	FVGEFFTDV	828
GPC3A2-4	155-163	YILGSDINV	162
GPC3A2-5	169-177	ELFDSLFPV	1055
GPC3A2-6	254-268	RMLTRMWYC	1259
GPC3A2-7	281-289	VMQGCMAGV	196
GPC3A2-8	326-334	THDSIQYV	496
GPC3A2-9	522-560	FLAELAYDL	402

*Binding scores were estimated by using BIMAS software (<http://bimas.dart.nih.gov/cgi-bin/molbio/ken.parker.comboform>).

apoptotic cells with terminal deoxynucleotidyl transferase-mediated nick end labeling methods (ApopTag fluorescein *in situ* apoptosis detection kits; Serologicals Corporation, Norcross, GA) in tumor specimens of patients with HCC were done as described previously (23, 24). In addition, immunohistochemical staining of HLA-class I in HCC tumor tissue specimens were done by using anti-HLA-class I mAb, EMR 8-5.⁵

Detection by ELISA of the serum-soluble GPC3 protein. Detection of the serum-soluble GPC3 protein was done by an indirect ELISA using the rabbit anti-GPC3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (7). We used recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) as a standard, and the presence of >106 ng/mL of serum GPC3 protein was considered to be positive.

Transfer of CTLs to the NOD/SCID mice implanted with a human HCC cell line. The transfer of GPC3-reactive CTLs to the immunodeficient mice implanted with a human HCC cell line was done as described previously (7). Briefly, we *s.c.* inoculated SK-Hep1/GPC3 cells (1×10^7) positive for both HLA-A2 and HLA-A24 at the right flank of NOD/SCID mice. When the diameter of these tumors reached 5×5 mm on day 9 after tumor inoculation into mice, we intravenously injected the mixture of GPC3 peptide-reactive CTL lines or irrelevant HIV peptides; HLA-A2-restricted SLYNTYATL peptide and HLA-A24-restricted RYLRDQQLL peptide, stimulated CD8⁺ T cells (3×10^6) established from four HLA-A24-positive or two HLA-A2-positive HCC patients, or saline alone. T cells were *i.v.* injected one more times on day 14. The CD8⁺ T cells stimulated with HLA-A24-restricted GPC3₂₉₈₋₃₀₆ peptide or HIV (RYLRDQQLL) peptide and derived from two independent HLA-A24⁺ HCC patients were mixed, and injected into three NOD/SCID mice on day 9, and the mixture of peptide-stimulated CD8⁺ T cells from two other HLA-A24⁺ HCC patients distinct from the T cell donors at the first injection, were injected into the mice on day 14. The HLA-A2-restricted peptide-stimulated CD8⁺ T cells from one HLA-A2⁺ HCC patient were also injected into a NOD/SCID mouse on day 9, followed by the injection on day 14 with the peptide-stimulated CD8⁺ T cells derived from another HLA-A2⁺ HCC patient.

Statistical analysis. The two-tailed Student's *t* test was used to evaluate the statistical significance of differences in the data obtained by ELISPOT assay. The statistical significance of the differences in several factors between patients showing a successful CTL induction and other patients was assessed by a χ^2 test. $P < 0.05$ was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Identification of HLA-A2-restricted CTL epitopes by using HLA-A2.1 (HHD) Tgm. To identify HLA-A2-restricted epitopes by using HLA-A2.1 (HHD) Tgm, we selected nine kinds of peptides having amino acid sequences conserved between human and mouse GPC3 and having high predicted binding scores to HLA-A2 (A*0201; Table 1). CD4⁺ spleen cells from HLA-A2.1 (HHD) Tgm immunized *i.p.* twice with BM-DCs pulsed with the mixture of these nine peptides were again stimulated *in vitro* with BM-DCs pulsed with each peptide, and we found that CD4⁺ spleen cells stimulated *in vitro* with the GPC3₁₄₄₋₁₅₂ peptide produced the largest amount of IFN- γ in a peptide-specific manner in ELISPOT assays. These CD4⁺ spleen cells (2×10^4 /well), showed 36 ± 2.85 spot counts/well, in response to the BM-DCs pulsed with the GPC3₁₄₄₋₁₅₂ peptide,

whereas they showed 23 ± 1.84 spot counts/well in the presence of BM-DCs without peptide loading ($P < 0.005$) indicating that about $(36-23) / 2 \times 10^4 = 0.065\%$ of CD4⁺ spleen cells were reactive to the GPC3 peptide. When we used syngeneic BM-DCs pulsed with a HLA-A2-binding HIV-derived peptide; SLYNTYATL as a control, no significant response (8.84 ± 1.73) was observed. The summation of the diameter of the IFN- γ ELISPOT observed in CD4⁺ spleen cells stimulated with the GPC3₁₄₄₋₁₅₂ peptide pulsed BM-DCs was $1,878 \pm 131 \mu\text{m}$, that stimulated with the HIV-derived SLYNTYATL peptide pulsed BM-DCs was $437 \pm 77 \mu\text{m}$, and that observed in the presence of BM-DC without peptide loading was $762 \pm 131 \mu\text{m}$ ($P < 0.001$). These assays were done thrice with similar results. As shown in Fig. 1B, the differences in the spot counts (left) or spot diameters (right) between stimulations with peptide pulsed BM-DC and BM-DC without peptide loading clearly revealed the GPC3₁₄₄₋₁₅₂ peptide-specific response of CD4⁺ spleen cells. As for other peptides, no significant peptide-specific response was observed. These results suggest that the GPC3₁₄₄₋₁₅₂ peptide could be a CTL epitope peptide in HLA-A2.1 (HHD) Tgm, and we also expected this GPC3₁₄₄₋₁₅₂ peptide to be an epitope for human CTLs.

The immunization of the HLA-A2-restricted peptide, GPC3₁₄₄₋₁₅₂, did not induce autoimmunity in HLA-A2.1 (HHD) Tgm. It is well known that melanocyte-differentiation antigens such as MART-1 or gp100 are very useful for immunotherapy of melanoma patients, but they sometimes cause autoimmunity, such as vitiligo or uveitis, following vaccination. We previously reported that the immunization of the GPC3₂₉₈₋₃₀₆ peptide did not cause autoimmunity in BALB/c mouse (9). To investigate whether the immunization of mice with HLA-A2-restricted GPC3-derived peptides causes autoimmunity, the immunohistochemical staining of several organs with anti-CD4 and anti-CD8 mAb was done in HLA-A2.1 (HHD) Tgm immunized with a mixture of nine GPC3 peptides 7 days before the analysis. As shown in Fig. 2, we could not find any pathologic changes, such as lymphocyte infiltration or tissue destruction and repair in skin, lung, brain, heart, liver, and kidney of HLA-A2.1 (HHD) Tgm. The same result was also observed when mice were vaccinated with the GPC3₁₄₄₋₁₅₂ peptide alone ($n = 3$; data not shown). These results indicate that the GPC3₁₄₄₋₁₅₂ peptide-reactive CD8⁺ CTLs do not attack the normal tissue specimens that we investigated.

Induction of GPC3-reactive CTLs from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. We evaluated the cytotoxic activity of CTLs that were induced with the GPC3₂₉₈₋₃₀₆ or GPC3₁₄₄₋₁₅₂ peptide from PBMCs isolated from HCC patients. PBMCs were isolated from HCC patients positive for HLA-A24 and/or HLA-A2, and CD8⁺ T cells sorted from the PBMCs were cocultured with autologous monocyte-derived DCs pulsed with each peptide as described in Materials and Methods. CTLs from PBMCs of HLA-A2⁺ HCC patients stimulated with the GPC3₁₄₄₋₁₅₂ peptide or CTLs from PBMCs of HLA-A24⁺ HCC patients stimulated with the GPC3₂₉₈₋₃₀₆ peptide exhibited cytotoxicity against peptide-pulsed target cells. The representative data of CTLs restricted by HLA-A2 or HLA-A24 were shown in Fig. 3A. The CTLs induced from PBMCs of patient A2-8 showed cytotoxic activity to T2-A0201 cells (HLA-A2+) pulsed with the GPC3₁₄₄₋₁₅₂ peptide, but not to T2-A0201 cells without peptide loading by ⁵¹Cr release assay. The CTLs induced from PBMCs of patient A24-12 exhibited cytotoxic

⁵ T. Torigoe, et al. Immunohistochemical analysis of HLA class I expression in tumor tissues revealed unusually high frequency of down-regulation in breast cancer tissues submitted.

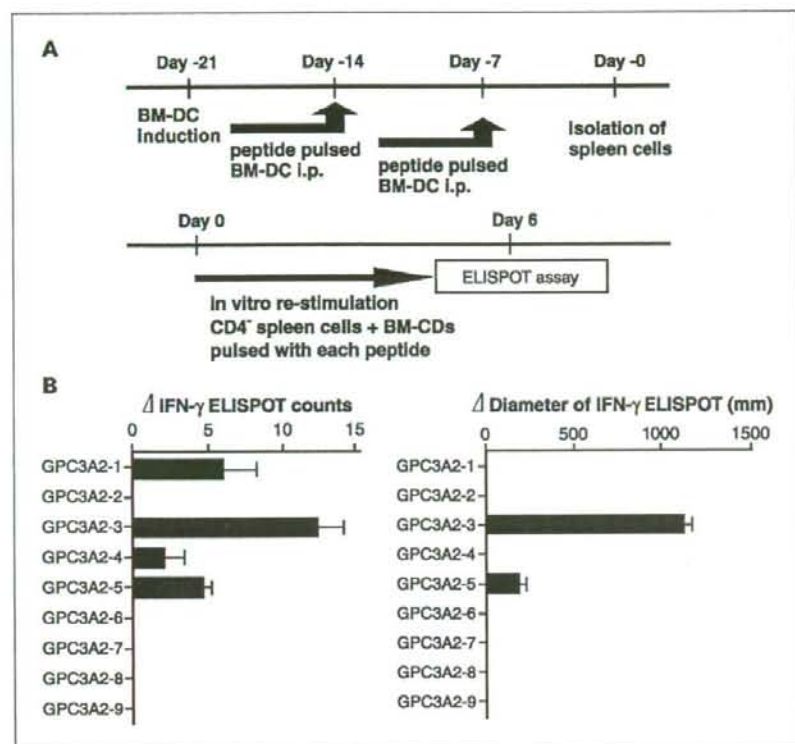


Fig. 1. Identification of HLA-A2-restricted CTL epitopes of GPC3 by using HLA-A2.1 (HHD) Tgm. **A**, protocol for identification of GPC3-derived and HLA-A2-restricted CTL epitopes. We primed the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5) pulsed with the mixture of GPC3-derived peptides carrying HLA-A2 (A*0201) binding motif into the peritoneal cavity once a week for two weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^+$ spleen cells (2×10^6 /well) were stimulated with syngeneic BM-DCs (2×10^5 /well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^+$ spleen cells as responder cells in ELISPOT assay to evaluate GPC3-specific response of CTLs. **B**, bar graph. IFN- γ ELISPOT counts/ 2×10^6 $CD4^+$ spleen cells cocultured with peptide pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*left*). Bar graph, summation of IFN- γ ELISPOT diameters/ 2×10^6 $CD4^+$ spleen cells cocultured with peptide-pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*right*). Columns, mean of triplicate assays; bars, SE. All assays were done thrice with similar results.

activity to the C1R-A*2402 cells (HLA-A24+) pulsed with the GPC3₂₉₈₋₃₀₆ peptide, but not to C1R-A*2402 cells without peptide loading. These results indicate that these CTLs had peptide-specific cytotoxicity. Other CTLs induced from the nine patients A2-1, A2-2, A2-3, A2-4, A24-1, A24-3, A24-4, A24-6, and A24-7 similarly exhibited peptide-specific cytotoxicity against peptide-pulsed target cells (data not shown).

Furthermore, we used GPC3 transfectants, SK-Hep1/GPC3 (GPC3+, HLA-A2+, HLA-A24+) or SW620/GPC3 (GPC3+, HLA-A2+, HLA-A24+) as target cells and examined whether we could find GPC3-specific cytotoxic activity of CTLs. As shown in Fig. 3B, the CTLs induced from PBMCs of patient A2-3 by stimulation with the GPC3₁₄₄₋₁₅₂ peptide showed specific cytotoxicity against SK-Hep1/GPC3, but not against GPC3-negative SK-Hep1. Similarly, the GPC3₂₉₈₋₃₀₆ peptide-induced CTLs showed specific cytotoxicity against SW620/GPC3 in

patient A24-7 or against SK-Hep1/GPC3 in patient A24-12, but not against SK-Hep1 or SW620, respectively, which did not endogenously express GPC3. These findings indicate that these peptides can be processed naturally in cancer cells, and the peptides in the context of HLA-A2 or HLA-A24 can be expressed on the cell surface of cancer cells to be recognized by the CTLs.

When we think about the application of GPC3 to cancer immunotherapy, the most important point is that these GPC3-reactive CTLs can exhibit specific cytotoxicity to the tumors endogenously expressing GPC3. We thus investigated whether these CTLs could kill human HCC cell lines expressing both endogenous GPC3 and the restriction HLA class I molecules. As shown in Fig. 3C, we could generate GPC3-reactive CTLs by stimulation with the GPC3₁₄₄₋₁₅₂ peptide and these CTLs exhibited cytotoxic activity to HepG2 (GPC3+, HLA-A2+, and

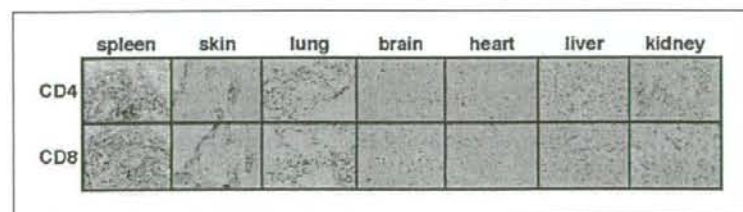


Fig. 2. Immunohistochemical staining with anti-CD4 or anti-CD8 mAb in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the GPC3₁₄₄₋₁₅₂ peptides. These tissue specimens were removed and analyzed 7 days after the second DC vaccination (original magnification, $\times 200$).

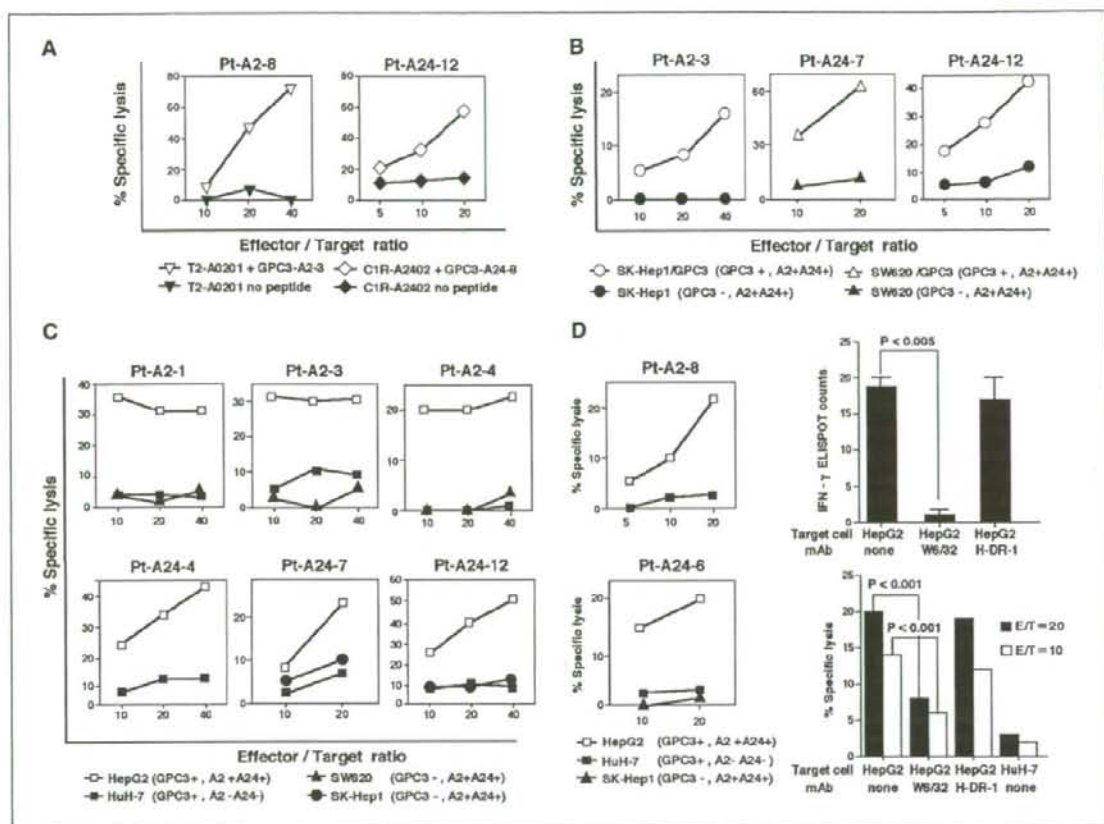


Fig. 3. CTL induction from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. **A** and **B**, GPC3 peptide-reactive CTLs were generated from CD8⁺ T cells of HLA-A2⁺ and/or HLA-A24⁺ HCC patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the GPC3₁₄₄₋₁₅₂ or GPC3₂₉₈₋₃₀₆ peptide, the CTLs were subjected to a standard ⁵¹Cr release assay at the indicated effector/target ratio. Their cytotoxicity against the GPC3₂₉₈₋₃₀₆ peptide pulsed C1R-A2402 cells or T2-A0201 cells, and each unpulsed cells (**A**), or GPC3⁻ HLA-A2⁺, HLA-A24⁺ HCC cell line SK-Hep-1, GPC3⁻ HLA-A2⁺, HLA-A24⁺ colon cancer cell line SW620, and those cell lines transfected with the human GPC3 gene; SK-Hep-1/GPC3 or SW620/GPC3 (**B**) were examined by a ⁵¹Cr release assay. **C** and **D**, GPC3⁺ HLA-A2⁺, HLA-A24⁺ HCC cell line HepG2, GPC3⁺ HLA-A2⁺, HLA-A24⁺ HCC cell line HuH-7, and GPC3⁻ tumor cell lines SW620 and SK-Hep1 were used as target cells (**left**). Points, percentage of specific lysis calculated based on the mean values of a triplicate assay. **D**, Inhibition of cytotoxicity by anti-HLA class I mAb (**right**). After the target HepG2 cells were incubated with anti-HLA class I mAb (W6/32, IgG_{2a}) or anti-HLA DR mAb (H-DR-1, IgG_{2a}), respectively, for 1 hour, the CTLs generated from PBMCs of patient A2-8 by stimulation with GPC3₁₄₄₋₁₅₂ peptide (**top**) or CTLs generated from patient A24-6 using the GPC3₂₉₈₋₃₀₆ peptide (**bottom**) were added. IFN- γ production (**top**; IFN- γ ELISPOT assay) and cytotoxicity (**bottom**; ⁵¹Cr release assay) were markedly inhibited by W6/32, but not by H-DR-1.

HLA-A24+), but not to HuH-7 (GPC3+, HLA-A2-, and HLA-A24-) or SW620 (GPC3-, HLA-A2+, and HLA-A24+) in patients A2-1, A2-3, and A2-4. Similarly, we could generate GPC3-reactive CTLs by stimulation of PBMCs with the GPC3₂₉₈₋₃₀₆ peptide and these CTLs exhibited cytotoxic activity to HepG2, but not to HuH-7 or SK-Hep-1 (GPC3-, HLA-A2+, HLA-A24+) in patients A24-4, A24-7, and A24-12.

In an HLA-class I blocking experiment, anti-HLA class I mAb W6/32 markedly inhibited the IFN- γ production stimulated with HepG2 cells in ELISPOT assay of CTLs generated from patient A2-8 by stimulation with the GPC3₁₄₄₋₁₅₂ peptide (Fig. 3D, top), and inhibited cytotoxic activity against HepG2 cells in ⁵¹Cr release assay of CTLs generated from patient A24-6 by stimulation with the GPC3₂₉₈₋₃₀₆ peptide (Fig. 3D, bottom), but anti-HLA-DR mAb, H-DR-1 did not inhibit the response of CTLs. These results clearly indicate that these CTLs recognized HepG2 in a HLA-class I-restricted manner.

As shown in Table 2, we could induce GPC3-reactive CTLs from PBMCs in ~50% of either the HLA-A2- or HLA-A24-positive HCC patients. In patients A2-6, A24-5, A24-9, and A24-11 who did not express GPC3 in tumor tissues, GPC3-reactive CTLs could not be induced from their PBMCs. Among eight HLA-A2-positive HCC patients who expressed GPC3 in HCC tissue or produced soluble GPC3 in sera, patients A2-1, A2-2, A2-3, A2-4, A2-6, A2-7, A2-9, and A2-10, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (50%). In patient A2-6, GPC3 was detected only in the serum but not in HCC tumor tissue. It was thought to be possible that the majority of GPC3 protein was secreted away in this type of HCC cell as described previously (7). Among six HLA-A24-positive patients who expressed GPC3 in tumor tissue, patients A24-1, A24-2, A24-3, A24-6, A24-10, and A24-12, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (67%). We also examined whether it was possible to

induce GPC3-specific CTLs from PBMCs isolated from healthy donors (each HLA type, $n = 3$), but we failed to generate GPC3-specific and HLA-A2- or HLA-A24-restricted CTLs even though PBMCs were stimulated with the peptides thrice *in vitro* (data not shown). These results suggest that GPC3-reactive CTLs could only be induced in patients who expressed GPC3 in tumor tissue, thus, indicating the existence of GPC3-reactive CTL precursors in patients with GPC3⁺ HCC. We also examined whether GPC3-reactive CTLs could be generated more frequently from PBMCs isolated from HCC patients positive for serum-soluble GPC3. As shown in Table 2, the presence of serum-soluble GPC3 did not correlate statistically with the successful induction of GPC3-reactive CTLs. As a result, we could not observe the enhancement of CTL induction efficiency via possible antigen presentation of soluble serum GPC3 through HLA-class II pathways to CD4⁺ T cells or cross-presentation through the HLA class I pathway to CD8⁺ T cells (25, 26) in patients positive for serum GPC3.

Inoculation of the GPC3 peptide-induced CTLs reduced growth of a GPC3⁺ human HCC tumor cell line implanted into NOD/SCID mouse. To investigate the effects of GPC3 peptide-reactive CTL inoculation into the mice implanted with the GPC3⁺ human HCC cell line, we s.c. inoculated SK-Hep1/GPC3

cell lines positive for both HLA-A2 and HLA-A24 into NOD/SCID mice, and i.v. injected the mixture of CTLs generated from several HCC patients positive for HLA-A2 or HLA-A24 into mice implanted with SK-Hep1/GPC3 when the diameter of these tumors reached 5 × 5 mm in size as described in Materials and Methods. The CTLs injected into mice were prepared by stimulating peripheral blood CD8⁺ T cells with HLA-A2- or HLA-A24-restricted GPC3-epitope peptides or control-irrelevant HIV peptides as described in Materials and Methods. The tumor sizes of four individual mice in each group (Fig. 4A) and mean ± SD of tumor sizes in each group (Fig. 4B) were evaluated. After 5 days from the second inoculation of GPC3 peptide-reactive CTLs, the tumor size of SK-Hep1/GPC3 was apparently reduced in comparison to the size of tumor mass implanted into NOD/SCID mice injected with control T cells or saline alone ($P < 0.01$). These results clearly indicate the efficacy of adoptive GPC3 peptide-reactive CTL transfer therapy for GPC3⁺ tumor in mice.

Discussion

In this article, we identified HLA-A24-restricted or HLA-A2-restricted GPC3 CTL epitope peptides, and found that

Table 2. Expression of GPC3 in HCC tissue, quantification of serum-soluble GPC3, and GPC3-specific CTL induction in HCC patients

	Age	Gender	State of tumor*	GPC3 expression [†]	Serum GPC3 [‡]	HLA expression [§]	CTL induction
HLA-A2 (A*0207)-positive patients							
Pt-A2-1	80	F	IIIa	+	+	+	+
Pt-A2-2	72	M	II	+	+	+	+
Pt-A2-3	67	F	II	ND	+	ND	+
Pt-A2-4	54	M	I	+	-	+	+
Pt-A2-5	57	M	I	ND	-	ND	-
Pt-A2-6	66	M	I	-	+	-	-
Pt-A2-7	54	M	IIIa	+	-	+	-
Pt-A2-8	73	M	II	ND	-	ND	+
Pt-A2-9	68	F	IIIa	+	-	+	-
Pt-A2-10	54	M	II	+	+	+	-
HLA-A24 (A*2402)-positive patients							
Pt-A24-1	60	M	IVa	+	+	+	+
Pt-A24-2	57	M	IVa	+	+	+	-
Pt-A24-3	75	F	IIIa	+	+	+	+
Pt-A24-4	59	M	IIIa	ND	-	ND	+
Pt-A24-5	52	M	IVb	-	-	+	-
Pt-A24-6	65	M	I	ND	+	ND	+
Pt-A24-7	61	M	I	ND	-	ND	+
Pt-A24-8	74	M	II	ND	-	ND	-
Pt-A24-9	59	M	IVb	-	-	-	-
Pt-A24-10	69	M	IVa	+	+	+	-
Pt-A24-11	72	M	II	-	-	+	-
Pt-A24-12	61	M	IIIa	+	+	+	+

Abbreviations: F, female; M, male; ND, not determined.

*Tumor-node-metastasis classification.

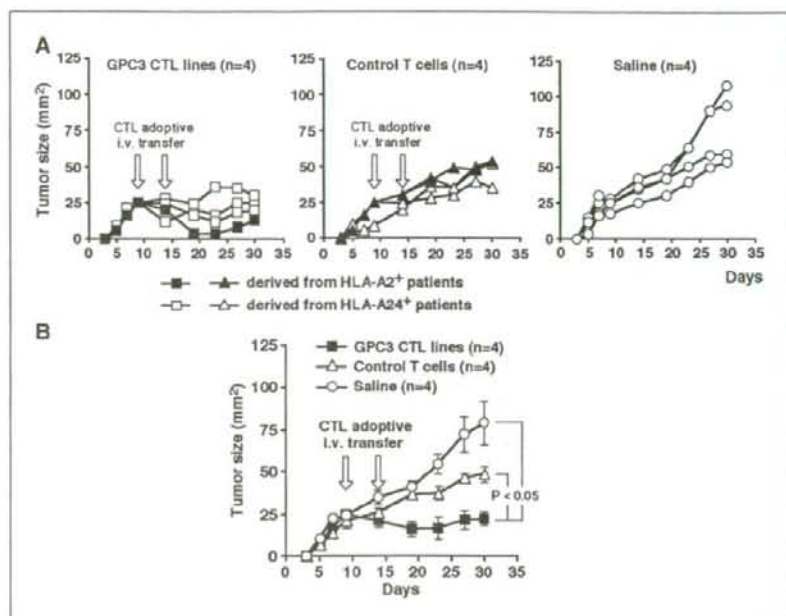
[†]Positive (+) or negative (-) staining of tumor cells in contrast with peritumor normal tissue as background staining.

[‡]Serum levels >106 ng/mL were evaluated as positive.

[§]Immunohistochemical staining of the membrane of tumor cells was evaluated as positive.

^{||}Specific lytic activity (≥20%) at E:Ratio = 20 against HepG2 target cells was evaluated as positive by ⁵¹Cr release assay.

Fig. 4. Marked inhibition of growth of a GPC3-transfected human HCC cancer cell line, SK-Hep1/GPC3, engrafted into NOD/SCID mice after adoptive transfer of human CTLs induced by the GPC3 peptides. **A**, when tumor size reached 25 mm² on day 9 after s.c. tumor implantation, human CTLs (3×10^6) reactive to HLA-A2-restricted (■) GPC3 peptide and generated from one HLA-A2⁺ donor, or those reactive to HLA-A24-restricted (□) GPC3 peptide and pooled from two HLA-A24⁺ donors were i.v. inoculated. On day 14, the inoculation of CTLs generated from the donors distinct from those at the first injection was repeated. The control CD8⁺ T cells stimulated with irrelevant HLA-A2-restricted (▲) or HLA-A24-restricted (△) HIV peptides were also injected into mice as a control. Tumor volumes in NOD/SCID mice given twice on days 9 and 14 with GPC3 epitope peptide-induced CTL lines ($n = 4$), control CD8⁺ T cells ($n = 4$), or saline alone ($n = 4$). Tumor size was expressed in square millimeters. **B**, points, mean tumor sizes in each group of mice; bars, \pm SD ($n = 4$). Statistical significance was evaluated using *t* test.



GPC3-reactive CTLs could be generated from PBMCs stimulated with these peptides in ~50% of HCC patients. Vaccination based on these peptides did not induce autoimmunity in HLA-A2.1 (HHD) Tgm of a B57Bl/6 background. We previously identified the GPC3₂₉₈₋₃₀₆ peptide to be a CTL epitope in BALB/c mouse, and we expected that this GPC3 peptide might also be present in human CTL in a HLA-A24-restricted manner. As expected, we could generate HLA-A24-restricted and the GPC3₂₉₈₋₃₀₆ peptide-reactive human CTLs in this study. As a result, BALB/c mice may be useful for identifying HLA-A24-restricted CTL epitopes. HLA-A2.1 (HHD) Tgm was reported to be a versatile animal model for the preclinical evaluation of peptide-based immunotherapy (12, 13). We could also find its usefulness for the identification of HLA-A2-restricted antigenic epitope in this study.

In this study, we wanted to identify the most effective major CTL epitopes derived from GPC3. As a result, we used BM-DCs derived from HLA-A2.1 (HHD) Tgm and pulsed BM-DCs with the mixture of GPC3 peptides for the vaccination of mice. Some of the peptides tested stimulated the weak response of CTLs in an ELISPOT assay, and these peptides might also be useful for future analysis. It was recently reported that peptides having a weak affinity to MHC, which could not be predicted by a BIMAS system, could induce peptide-reactive CTLs with a cytotoxic activity (27). To search for more peptides that can be applicable for immunotherapy, it may be necessary to check these minor CTL epitopes in the future. In this study, the GPC3-derived peptides predicted to have high binding affinity to HLA-A2 molecules and having the amino acid sequences conserved between human and mouse GPCs were selected for the analysis. When we analyzed the amino acid sequence of human GPC3 protein, all of the top 28 human GPC3 peptides having high binding scores (>100) to HLA-A2 molecules shared

the same amino acid sequences with mouse GPC3. Therefore, it is unlikely that we excluded many candidates of human GPC3-derived and HLA-A2-restricted CTL epitopes from the analysis by selecting the peptides having amino acid sequences shared between human and mouse GPC3. Furthermore, we have to consider the differences in the T cell repertoire in mice and humans. Thereby, we may miss GPC3 peptides recognized by human CTLs but not by mouse CTLs.

Considering ideal immunogenic target molecules for cancer immunotherapy, it is important to select a tumor antigen that could not be lost by tumor cells through immunoeediting (28, 29). Recently, Capurro et al. reported that GPC3 is involved in the carcinogenesis and proliferation of HCC via regulation of noncanonical Wnt signals (30). Therefore, it may be possible that tumor cells cannot lose the GPC3 expression in order to continue to grow. Furthermore, according to an immunohistochemical analysis of the expression of HLA-class I molecules using newly developed specific mAb, EMR 8-5,⁵ we found that almost all HCC cells expressed HLA-class I as far as we could examine (Table 2). For these reasons, we think that GPC3 is a very useful candidate as a target tumor antigen for the immunotherapy of HCC. We and others previously reported that the expression of GPC3 in HCC was detected from an early stage and the quantification of the soluble GPC3 protein in sera was useful for a diagnosis of HCC at an early stage (5, 7). As a result, GPC3-based immunotherapy might be able to prevent the appearance of HCC in patients with hepatitis B or C-based liver cirrhosis.

In this study, we found that it is possible to induce GPC3-reactive CTLs by the stimulation of PBMCs with the two major GPC3 epitopes *in vitro* in 50% of the HCC patients having an appropriate HLA-class I allele. However, it is necessary to investigate more patients to estimate the probability of a

successful induction of GPC3-reactive CTLs in HCC patients. We intended to know whether there was any correlation between successful induction of GPC3 peptide-reactive CTLs and prognosis or CTL infiltration into tumor tissue of these patients, therefore, we investigated the seven index cases; patients A2-10, A24-1, A24-2, A24-4, A24-9, A24-11, and A24-12, to see whether there was any correlation between successful induction of GPC3 peptide-reactive CTLs and prognosis or CTL infiltration into the tumor tissue of these patients. In three patients, A24-1, A24-4, and A24-12, who could generate GPC3 peptide-reactive CTLs, patient A24-12 recurred at 6 months after operation. In four patients, A2-10, A24-2, A24-9, and A24-11, who failed to induce GPC3-peptide-reactive CTLs, patient A24-9, whose HCC did not express GPC3, recurred at 6 months after operation, and patient A24-2 recurred at 3 months after operation and died 3 months after recurrence. These three recurred patients had extremely strong tumor invasion to the vasculature. Therefore, it was difficult to evaluate the correlation between the positive CTL response and clinical improvement at the present stage, and we have to increase the number of patients investigated and to do further statistical analyses on these relationships. In patients who could be examined for the infiltration of CD8-positive cells into their tumor specimens and for the existence of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells in tumor tissue, patients A2-10, A24-1, A24-2, and A24-9, there was no strong correlation between the positive GPC3 peptide-reactive CTL response and for the existence of CD8-positive or terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells in the tumor tissues (data not shown). As shown in Fig. 4, we observed a regression of the tumor masses in NOD/SCID mice implanted with SK-Hep1/GPC3 and transferred *i.v.* with the GPC3 peptide-reactive CTLs in comparison to the mice injected with control CD8⁺ T cells or saline alone. Although the regression of tumor growth was

observed for 2 weeks after the second transfer of CTLs, the tumors began to enlarge again after that period. We thought it was important to continue the transfer of CTLs again and again to obtain continuous regression of the GPC3-expressing tumor. These data suggest that the adoptive *i.v.* transfer of GPC3-reactive CTLs into mice bearing GPC3⁺ tumors was useful to inhibit tumor growth in the mouse tumor model.

In addition, it is most important to confirm the usefulness of GPC3-specific *in vivo* cancer immunotherapy in patients with HCC. Investigation of the presence of GPC3-specific CTLs in patients with melanoma are also eagerly awaited. We previously reported that DC differentiated *in vitro* from mouse embryonic stem cells transfected with the mouse GPC3 gene (24, 31) induced protective immunity against mouse melanoma cell line B16 F10 (32). We are now preparing a translational study of GPC3-based immunotherapy to reduce the risk of recurrence in HCC patients treated surgically. We will try to use the GPC3 epitope peptides identified in this study first, whereas in the second phase, we will make a trial of the peptide-pulsed DC vaccine. We expect that GPC3-based immunotherapy may be a novel treatment strategy that could potentially help to prevent the appearance, advance, and/or recurrence of HCC and melanoma.

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Immunogenic Variation between Multiple HLA-A*0201-Restricted, Hepatitis C Virus-Derived Epitopes for Cytotoxic T Lymphocytes

SATOSHI OHNO,^{1,2} OSAMU MORIYA,¹ TAKAYUKI YOSHIMOTO,³
HIDENORI HAYASHI,² TOSHITAKA AKATSUKA,¹ and MASANORI MATSUI¹

ABSTRACT

CD8⁺ cytotoxic T lymphocytes (CTLs) play a critical role in the immune control of Hepatitis C Virus (HCV) infection. In the current study, a number of HLA-A*0201-restricted CTL epitopes derived from HCV were evaluated by examining the peptide-binding affinity for major histocompatibility complex (MHC) class I molecules, the stability of peptide-MHC complexes, killing activities of peptide-induced CTLs, and frequencies of intracellular interferon (IFN)- γ -positive CD8⁺ T cells. Among 24 peptides tested, 15 peptides induced high or medium killing activities of peptide-specific CTLs. Thirteen of the 15 peptides exhibited high or medium binding affinities for HLA-A*0201 molecules, indicating that the high binding affinity for MHC class I molecules is an important factor for immunogenicity. In contrast, the stability of peptide-MHC class I complexes was not correlated with killing activities of peptide-induced CTLs. Furthermore, only a limited number of peptides could induce high or medium frequencies of IFN- γ -producing CD8⁺ T cells, which were generally considered to play a crucial role for the clearance of HCV. Analyses of the immunogenicity of CTL epitopes such as in the current study should provide important information about the design of an efficient HCV vaccine that induces vigorous, sustained, and broad HCV-specific CTL responses.

INTRODUCTION

CHRONIC INFECTION with Hepatitis C Virus (HCV) is a serious issue because this infection often leads to the development of cirrhosis and hepatocellular carcinoma (25). However, any treatment of HCV infection is not successful in most cases, and an effective HCV vaccine is not available so far. Therefore, it is urgent to develop an efficient and safe HCV vaccine.

It is well documented that major histocompatibility complex (MHC) class I-restricted, CD8⁺ cytotoxic T

lymphocytes (CTLs) play a major role in the immune control of various virus infections. In the case of HCV infection, spontaneous resolution of acute HCV infection was associated with vigorous HCV-specific CTL responses in chimpanzees (5) and humans (10,14,30). Hence, HCV-specific CTLs are likely to be crucial to eradicate HCV. However, in most cases, the cellular immune response fails to clear HCV and, eventually, more than 60% of infected individuals develop chronic hepatitis. This viral persistence might be explained by impaired functions of cellular immunity, including T cells

¹Department of Microbiology, Saitama Medical School, Saitama, Japan.

²Department of Pathological Biochemistry, Faculty of Pharmaceutical Sciences, Josai University, Saitama, Japan.

³Intractable Immune System Disease Research Center, Tokyo Medical University, Tokyo, Japan.

and dendritic cells (1,13,30,33), and viral escape arising from mutation of key epitopes recognized by T cells (6,7,22,29,31). Furthermore, in chronic HCV infection, the precursor frequency of HCV-specific CTLs is extremely low although HCV-specific CTLs are detectable in both peripheral blood and liver (14,23,24). Therefore, one may support an idea that an HCV vaccine should efficiently elicit HCV-specific CTL responses that are qualitatively and/or quantitatively sufficient for viral clearance.

To date, a number of CTL epitopes derived from HCV have been identified (2,4,10,27,28,32,34). However, for most of them their immunogenicity has not been well characterized. Only one previous study provided extensive analyses of the immunogenicity of HCV-derived CTL epitopes (11). In the current study, 24 HLA-A*0201-restricted, HCV-derived epitopes were evaluated by examining peptide-binding affinity for HLA-A*0201 molecules, the stability of peptide-HLA-A*0201 complexes, killing activities of peptide-induced CTLs, and frequencies of intracellular interferon (IFN)- γ -positive CD8⁺ T cells. Identification of highly immunogenic, immunodominant epitopes should be useful for the design of an efficient HCV vaccine that induces vigorous, sustained, and broad HCV-specific CTL responses.

MATERIALS AND METHODS

Mice

HHD mice express a transgenic HLA-A*0201 monochain, designated HHD, in which human β_2 -microglobulin (β_2m) is covalently linked to a chimeric heavy chain composed of HLA-A*0201 (α_1 and α_2 domains) and H-2D^b (α_3 , transmembrane, and cytoplasmic domains) (17,20). Because the innate H-2D^b and mouse β_2m genes have been disrupted by homologous recombination, the only MHC class I molecule on the cell surface, HHD, is efficiently used by HLA-A*0201-restricted CTLs. Eight- to 12-week old mice were used for all experiments. Mice were housed in appropriate animal care facilities at Saitama Medical School (Saitama, Japan) and handled according to international guidelines for experiments with animals.

Synthetic peptides

T1

As shown in Table 1, 24 HLA-A*0201-restricted peptides derived from HCV Core, E1, E2, NS3, NS4, or NS5 were synthesized by Qiagen (Tokyo, Japan). All the peptides had previously been defined as epitopes for HCV-specific CTLs (Table 1) (2,4,10,27,28,32,34).

Cell lines

The HHD gene-transfected mouse lymphoma cell line RMA-HHD (H-2^b) was previously described (20). The HLA-A*0201⁺ human lymphoblastoid cell line T2 (26), in which the transporter associated with antigen processing (TAP)-1 and TAP-2 genes are deficient, and the human kidney cell line 293 (8) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). T2 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (R-10). 293 and RMA-HHD cells were cultured in Dulbecco's modified Eagle's medium with 10% FCS (D-10) and D-10 containing G418 (500 μ g/ml) (Sigma, St. Louis, MO), respectively.

Plasmid and adenoviruses

The interleukin (IL)-12 expression plasmid, designated p3XFLAG-IL-12, was previously described (17). Plasmid DNA was purified by ultracentrifugation to equilibrium in cesium chloride-ethidium bromide gradients. Replication-defective recombinant adenoviruses expressing HCV structural proteins including Core, E1, and E2 (Adex1SR3ST) and HCV nonstructural proteins including NS3, NS4, and NS5A (Adex1CA3269) were described previously (15,17,32). Wild-type adenovirus (Adex1w) was used as a control. Virus was amplified in 293 cells and titered in standard plaque assays.

Peptide-binding assay

The peptide-binding assay was performed as described (21). Briefly, T2 cells were suspended in AIM-V serum-free medium (Invitrogen Life Technologies, Rockville, MD) supplemented with 100 nM human β_2m (Sigma) and were incubated with synthetic peptide at various concentrations overnight at 37°C. Cells were stained with the conformationally sensitive, anti-HLA-A*0201 monoclonal antibody (mAb) BB7.2 (19), followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody (Sigma). Mean fluorescence intensity (MFI) was measured by flow cytometry (FACScan; BD Biosciences Immunocytometry Systems, Mountain View, CA). The concentration of each peptide that yields the half-maximal MFI of T2 cells pulsed with a control peptide, NS3-1585 (Table 1), was calculated as the half-maximal binding level (BL₅₀) (12). For most peptides, experiments were performed three times, and data are given as mean values \pm standard error of the mean (SEM).

Complex stability assay

Peptides that showed a BL₅₀ of less than 100 μ M in the peptide-binding assay were evaluated for their half-lives as peptide-MHC class I complexes at 37°C by com-

TABLE 1. BINDING OF HCV-DERIVED PEPTIDES TO HLA-A*0201 MOLECULES AND STABILITY OF PEPTIDE-HLA-A*0201 COMPLEXES

Name	Residues	Sequence (Ref.)	BL ₅₀ (μM) ^a	Stability (h) ^b
NS5-2252	2252-2260	ILDSFDPLV (4)	19.8 ± 6.9	11.9 ± 3.6
Core-178	178-187	LLALLSCLTV (2)	23.0 ± 2.3	17.7 ± 2.7
NS4-1666	1666-1674	VLVGGVLAA (27)	27.0 ± 7.3	2.9 ± 0.3
Core-132	132-140	DLMGYIPLV (2)	27.5 ± 3.6	3.6 ± 0.4
NS4-1769	1769-1777	HMWNFISGI (27)	28.3 ± 6.9	13.4 ± 1.7
NS5-1992	1992-2000	VLSDFKTWL (32)	37.8 ± 2.5	10.2 ± 0.9
Core-35	35-44	YLLPRRGPRLL (2)	42.5 ± 3.8	24<
NS4-1789	1789-1797	SLMAFTAAV (4)	45.3 ± 2.5	24<
NS3-1585	1585-1593	YLVA YQATV (34)	49.6 ± 0.9	24<
NS5-2145	2145-2154	LLREEVSFRV (32)	51.2 ± 14.3	2.5 ± 0.1
NS4-1920	1920-1928	WMNRLIAFA (27)	57.1 ± 9.1	2.7 ± 0.2
NS3-1169	1169-1177	LLCPAGHAV (4)	60.0	9.7 ± 1.8
NS3-1131	1131-1139	YLVTRHADV (27)	60.3 ± 6.3	5.5 ± 0.5
NS3-1073	1073-1081	CINGVCWTV (4)	66.9 ± 17.0	8.1 ± 0.5
NS4-1851	1851-1859	ILAGYGAGV (2)	68.8 ± 23.8	4.8 ± 1.1
NS3-1406	1406-1415	KLVALGINAV (4)	76.7 ± 15.4	24<
NS4-1671	1671-1680	VLAALAAAYCL (34)	104.3	NT
E1-363	363-371	SMVGNWAKV (10)	130.7 ± 26.1	NT
E2-686	686-694	ALSTGLIHL (2)	156.9 ± 7.8	NT
E1-220	220-227	ILHTPGCV (10)	166.6 ± 34.0	NT
NS4-1807	1807-1816	LLFNILGGWV (2)	172.5	NT
E2-728	728-736	FLLLDARAV (27)	204.5	NT
E1-257	257-266	QLRRHIDLLV (28)	416.4	NT
E2-726	726-734	LLFLLLADA (27)	607.7	NT

Abbreviations: 24<, more than 24; NT, not tested.

^aData of peptide-binding assays are shown as BL₅₀, indicating a concentration of each peptide that yields the half-maximal MFI of T2 cells pulsed with NS3-1585. For most peptides, data are given as mean values ± SEM of three independent experiments. For several peptides, data are shown as mean values of two independent experiments.

^bPeptides that showed a BL₅₀ less than 100 μM in the peptide-binding assay were evaluated for their half-lives (hours) as peptide-class I complexes at 37°C by complex stability assay. For each peptide, experiments were performed three times, and data are given as mean values ± SEM.

plex stability assay as described previously (21). In brief, T2 cells were incubated with 100 μM peptide and 100 nM human β2m (Sigma) overnight at 37°C, and were then incubated for 1 h at 37°C in R-10 containing brefeldin A (Sigma) at 10 μg/ml to block the egress of new MHC class I molecules. At the indicated time points, an aliquot was stained with BB7.2 (19), followed by FITC-labeled anti-mouse IgG antibody. For each peptide, experiments were performed three times, and data are given as mean values ± SEM.

Immunization

Mice were immunized as described previously (16,17). Briefly, mice were intramuscularly injected twice via the tibialis muscles with 30 μg of p3XFLAG-IL-12, and then immunized intraperitoneally with 5 × 10⁷ plaque-form-

ing units (PFU) of either Adex1SR3ST, Adex1CA3269, or Adex1w. The interval between immunizations was 2 weeks.

Cytotoxic assay

CTL assays were carried out as described previously (17). In brief, 2 to 3 weeks after immunization, mice were killed. Spleen cells were then cultured for 1 week with irradiated (30 Gy), syngeneic naive spleen cells prepulsed with synthetic peptide at 10 μM, and employed as effector cells in standard ⁵¹Cr release assays. RMA-HH8 cells were pulsed with or without an appropriate peptide at 10 μM for 1 h, labeled with 100 μCi of Na₂⁵¹CrO₄, and used as target cells. After a 4-h incubation of effector cells together with target cells, the supernatant of each well was harvested and radioactivity was counted. Re-

sults were calculated as the mean of a triplicate assay. Percent specific lysis was calculated according to the formula: % specific lysis = $[(\text{cpm}_{\text{sample}} - \text{cpm}_{\text{spontaneous}}) / (\text{cpm}_{\text{maximum}} - \text{cpm}_{\text{spontaneous}})] \times 100$. Spontaneous release represents the radioactivity released by target cells in the absence of effectors, and maximum release represents the radioactivity released by target cells lysed with 5% Triton X-100. At least three mice per group were used in each experiment. Each experiment was repeated three times. Statistical analyses were performed by Student *t* test. $p < 0.05$ was considered statistically significant.

Intracellular IFN- γ staining

Intracellular cytokine staining (ICS) was performed as described (17,18). Briefly, spleen cells of three to five immunized mice per group were pooled and resuspended in R-10. In each well of a 96-well round-bottom plate, 2×10^6 spleen cells were incubated with

brefeldin A (GolgiPlug, $0.2 \mu\text{L}/\text{well}$; BD Biosciences Pharmingen, San Diego, CA) for 5 h at 37°C in the presence or absence of a peptide at a final concentration of $10 \mu\text{M}$. Cells were then washed once with ice-cold phosphate-buffered saline (PBS) containing 1% FCS and 15 mM sodium azide (fluorescence-activated cell-sorting [FACS] buffer), and incubated for 10 min at 4°C with rat anti-mouse CD16/CD32 mAb (Fc Block; BD Biosciences Pharmingen). After incubation, the cell surface was stained with FITC-conjugated rat anti-mouse CD8 α mAb (clone 53-6.7; BD Biosciences Pharmingen) for 30 min at 4°C . After washing twice with FACS buffer, cells were fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences Pharmingen), and stained with phycoerythrin (PE)-conjugated rat anti-mouse IFN- γ mAb (clone XMG1.2; BD Biosciences Pharmingen). After washing with $1 \times$ Perm/Wash solution (provided with the kit), flow cytometric analyses were performed.

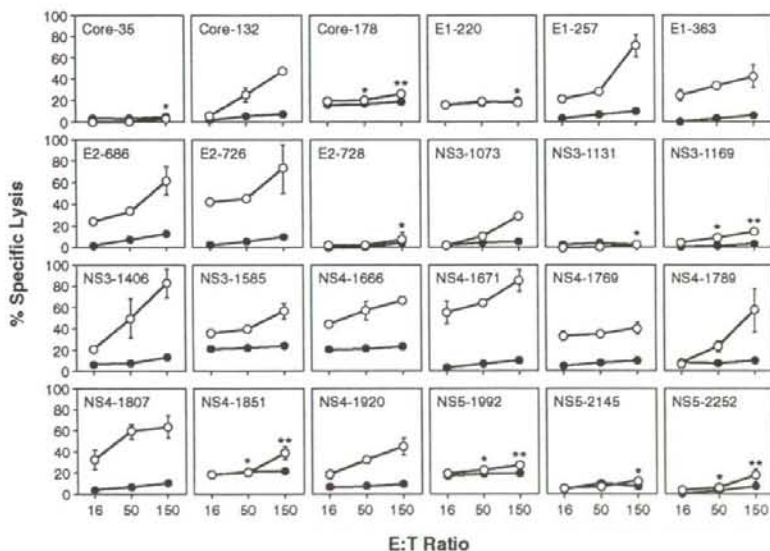


FIG. 1. CTL activities specific for 24 HCV-derived peptides. Mice were injected with p3XFLAG-IL-12, and then immunized intraperitoneally with 5×10^7 PFU of either Adex1SR3ST or Adex1CA3269. Two to 3 weeks after immunization, spleen cells of Adex1SR3ST-immunized mice and Adex1CA3269-immunized mice were prepared and stimulated *in vitro* for 1 week with each peptide derived from the HCV structural proteins (Core, E1, and E2), and nonstructural proteins (NS3, NS4, and NS5), respectively. After 1 week, ^{51}Cr release assays were performed at various E:T ratios with RMA-HHD cells pulsed with (open symbols) or without (solid symbols) a relevant peptide as target. Data are representative of one of three independent experiments and are shown as the means \pm SEM of triplicate wells. At least three mice per group were used in each experiment. Percentages of specific lysis of target cells pulsed with each peptide were compared with percentages of lysis of target cells pulsed with no peptide by Student *t* test (* $p > 0.05$; ** $p < 0.05$).

RESULTS

*Binding of HCV-derived peptides to HLA-A*0201 molecules*

Twenty-four HCV-derived peptides (Table 1) that have previously been defined as CTL epitopes (2,4, 10,27,28,32,34) were analyzed for their ability to bind to HLA-A*0201 molecules. The peptide-binding assay shows the binding affinity of a peptide for HLA-A*0201 molecules by measuring the stabilization of HLA-A*0201 on the TAP-deficient cell line T2 (26) pulsed with each peptide (21). According to the BL₅₀ values shown in Table 1, the peptides examined were classified into three categories: 16 high binders displaying BL₅₀ values less than 100 μM; 5 medium binders displaying BL₅₀ values ranging from 100 to 200 μM; and 3 low binders displaying BL₅₀ values of more than 200 μM. These data indicated that most of the epitopes were high binders. In particular, Core-132, Core-178, NS4-1666, NS4-1769, and NS5-2252 showed extremely high binding affinities for HLA-A*0201 molecules. On the other hand, E1-257 and E2-726 showed quite low binding capacities for HLA-A*0201 molecules.

Half-lives of peptide-class I complexes

Sixteen high binders were further evaluated by complex stability assay (Table 1). It might be expected that

the long half-life of the peptide-MHC class I complexes should increase the cell surface concentration of the peptide, thereby increasing the avidity of the cell-cell interaction. An epitope present at high cell surface concentration might be able to better stimulate epitope-specific T cells. Interestingly, half-lives of the peptide-MHC class I complexes at 37°C were found to vary among the 16 high binders (Table 1). In the cases of Core-35, NS3-1406, NS3-1585, and NS4-1789, the half-life of the complexes was more than 24 h. In contrast, the half-lives of complexes associated with NS4-1666, NS4-1920, and NS5-2145 were only 2.9, 2.7, and 2.5 h, respectively. These data indicate that the stability of the peptide-MHC class I complexes is not always correlated with the binding affinity of a peptide for MHC class I molecules.

Induction of CTLs specific for HCV-derived epitopes

We next investigated the induction of CTLs specific for each epitope, using HHD mice. Mice were immunized with either Adex1SR3ST or Adex1CA3269 via the intraperitoneal route of inoculation. Adex1SR3ST and Adex1CA3269 are replication-defective recombinant adenoviruses that express the HCV structural proteins (Core, E1, and E2) and the HCV nonstructural proteins (NS3, NS4, and NS5A) under the control of the CAG promoter, respectively (15,17,32). After 2 weeks, spleen cells were harvested and stimulated *in vitro* with syn-

TABLE 2. DETECTION OF CYTOLYTIC ACTIVITIES AND QUANTITATION OF IFN- γ -SECRETING CD8⁺ T CELLS IN RESPONSE TO EACH PEPTIDE DERIVED FROM HCV

Peptide	Lysis (%) ^a		ICS ^b		Peptide	Lysis (%)		ICS	
	(+)	(-)	Mi	Mc		(+)	(-)	Mi	Mc
Core-35	3.4	5.5	0.22	0.00	Core-132	47.7	7.0	6.83	0.02
Core-178	12.5	5.0	0.00	0.01	E1-220	3.9	5.1	0.04	0.02
E1-257	72.1	10.0	0.08	0.03	E1-363	42.9	6.3	0.08	0.02
E2-686	62.0	11.9	2.36	0.01	E2-726	73.2	9.9	0.04	0.05
E2-728	7.5	4.6	0.00	0.03	NS3-1073	29.1	5.5	0.11	0.01
NS3-1131	2.1	2.5	0.05	0.03	NS3-1169	15.2	4.0	0.00	0.05
NS3-1406	82.8	12.2	0.02	0.02	NS3-1585	48.4	9.8	0.02	0.00
NS4-1665	59.4	8.2	0.05	0.00	NS4-1671	85.3	9.7	0.17	0.01
NS4-1769	40.2	9.5	0.07	0.04	NS4-1789	57.4	9.8	0.02	0.04
NS4-1807	63.6	10.0	0.08	0.02	NS4-1851	27.6	7.1	0.00	0.02
NS4-1920	44.7	9.4	0.31	0.02	NS5-1992	13.4	4.5	0.74	0.03
NS5-2145	11.6	7.6	0.05	0.09	NS5-2252	17.7	7.4	0.08	0.02

^aSpleen cells of immunized mice were stimulated *in vitro* with each peptide, and CTL assays were performed. Data are shown as percent specific lysis of target cells pulsed with (+) or without (-) each peptide at an E:T ratio of 150.

^bICS, intracellular cytokine staining; spleen cells of mice immunized with Adex1SR3ST or Adex1CA3269 (Mi), or control mice injected with Adex1w (Mc), were stimulated *in vitro* with each peptide for 5 h. ICS was then performed by staining for cell surface CD8 and antigen-induced intracellular IFN- γ in spleen cells. Data indicate percentages of intracellular IFN- γ -positive cells in CD8⁺ cells.

genic spleen cells pulsed with each peptide. Spleen cells of mice immunized with the wild-type adenovirus Adex1w were stimulated *in vitro* with peptide-pulsed cells as well, and were used as negative controls. One week later, CTL assays were performed at various effector:target (*E:T*) ratios. As shown in Fig. 1 and Table 2, 13 peptides including Core-132, E1-257, E1-363, E2-686, E2-726, NS3-1406, NS3-1585, NS4-1666, NS4-1671, NS4-1769, NS4-1789, NS4-1807, and NS4-1920 elicited strong peptide-specific CTL responses. Eleven of them were either high binders or medium binders of HLA-A*0201 (Tables 1 and 3). However, E1-257 and E2-726 exhibited low binding affinities for HLA-A*0201 molecules (Tables 1 and 3). On the other hand, five peptides, Core-35, E1-220, E2-728, NS3-1131, and NS5-2145, failed to induce peptide-specific CTLs (Fig. 1 and Table 2). Interestingly, all these peptides were high or medium binders with only one exception, E2-728, which was a low binder (Tables 1 and 3). These data strongly suggest that the high binding affinity for MHC class I molecules may be necessary but not sufficient for the im-

munogenicity of CTL epitopes. Any significant CTL response was not observed in spleen cells of mice injected with Adex1w after *in vitro* stimulation with each peptide (data not shown).

Quantitation of IFN- γ -producing CD8⁺ T cells in response to HCV-derived peptides

To further examine the immunogenicity of HCV-derived peptides, frequencies of HCV epitope-specific CD8⁺ T cells were examined by staining for the cell surface CD8 molecule and antigen-induced intracellular IFN- γ in the spleen cells of immunized mice. Because the cells were stimulated *in vitro* with an appropriate peptide for only 5 h, the possibility of substantial *in vitro* expansion of responder cells was precluded (3). As shown in Table 2 and Fig. 2, high frequencies of IFN- γ -producing CD8⁺ T cells were detected for Core-132 (6.83%) and E2-686 (2.36%), which belong to the high binder and medium binder groups, respectively (Tables 1 and 3). As shown in Fig. 1 and Table 2, both peptides induced high

TABLE 3. CLASSIFICATION OF TWENTY-FOUR HCV-DERIVED CTL EPITOPES ON THE BASIS OF CURRENT DATA

Type I Antigens ^a				
Peptide	Affinity ^b (high/medium)	Lysis ^c (high/medium)	ICS ^d (high/medium)	Stability ^e
Core-132	27.5	40.7	6.81	3.6
NS4-1920	57.1	35.3	0.29	2.7
NS3-1073	66.9	23.6	0.10	8.1
NS4-1671	104.3	75.6	0.16	NT
E2-686	156.9	50.1	2.35	NT
Type II Antigens				
Peptide	Affinity (high/medium)	Lysis (high/medium)	ICS (low/ND)	Stability
NS4-1665	27.0	51.2	0.05	2.9
NS4-1769	28.3	30.7	0.03	13.4
NS4-1789	45.3	47.6	ND	24<
NS3-1585	49.6	38.6	0.02	24<
NS4-1851	68.8	20.5	ND	4.8
NS3-1406	76.7	70.6	ND	24<
E1-363	130.7	36.6	0.06	NT
NS4-1807	172.5	53.6	0.06	NT
Type III Antigens				
Peptide	Affinity (low)	Lysis (high)	ICS (medium)	Stability
E1-257	416.4	62.1	0.05	NT
E2-726	607.7	63.3	ND	NT

(continued)