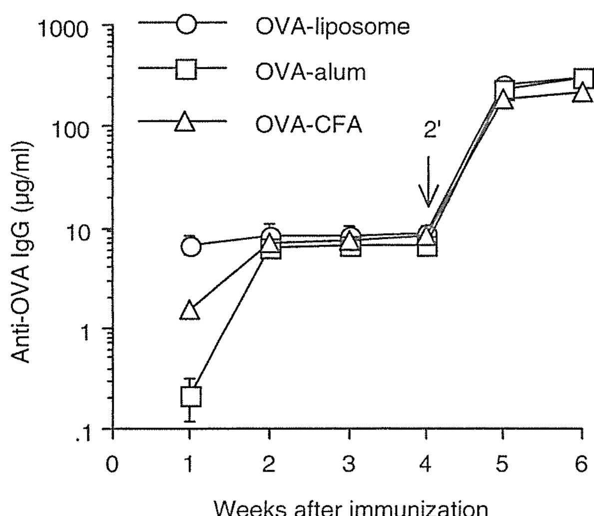


図 1a　OVA 免疫マウスにおける抗 OVA IgG 抗体産生
BALB/c マウスに3種類の異なるアジュバントを用いて OVA を0週、4週の2回免疫し、血清中の抗 OVA IgG 抗体を酵素抗体法 (ELISA) を用いて測定した。

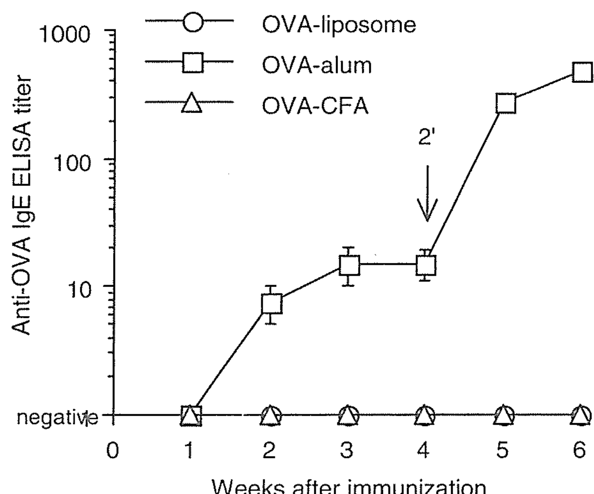


図 1b　OVA 免疫 BALB/c マウスにおける抗 OVA IgE 抗体産生
図 1a の血清について抗 OVA IgE 抗体を ELISA を用いて測定した。

表 1　OVA 免疫マウスにおける抗 OVA IgG1/IgG2a 抗体産生

immunization	anti-OVA antibodies (μg/ml)	
	IgG1	IgG2a
OVA-liposome	260.2±72.7	239.4±80.8
OVA-alum	252.8±70.4	32.6±5.4*
OVA-CFA	29.2±11.5	132.7±21.7*

図1の実験における初回免疫6週後の血清について、抗 OVA 抗体サブクラスを測定した。

生を検討した結果、OVA-リポソーム免疫群、OVA-alum 免疫群においては検討したすべての Th1 (IL-2 および IFN-γ) および Th2 (IL-4 および IL-5) サイトカインの顕著な産生が認められた。抗 OVA IgE 産生を誘導しなかった OVA-リポソーム免疫群においても Th1 サイトカインだけでなく Th2 サイトカインの産生が観察され、IgE 抗体産生の抑制効果と T 細胞サイトカイン産生のプロファイルが相関しない結果となった。これに対して、OVA-CFA 免疫群では IL-4 および IL-5 の産生が見られず、典型的なタイプ I 免疫応答が誘導されていることが示唆された。

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が IL-12 に依存的であるか否かについて IL-12 ノックアウトマウスを用いて検討したところ、図 2a に示すように、OVA-リポソームおよび OVA-alum 免疫群では同等の抗 OVA IgG 抗体産生が誘導されたが OVA-CFA 免疫群では他の2群と比較して顕著に低値であったことから、OVA-CFA によって誘導される抗 OVA IgG 抗体産生は IL-12 に依存的であることが示唆された。抗 OVA IgE 抗体産生は OVA-alum 免疫群で顕著に観察されたが、OVA-リポソーム免疫群では観察されなかった (図 2b)。このことから、OVA-リポソームによる IgE 産生の選択的抑制効果は IL-12 に非依存的な機序によるものであることが示唆された。

OVA-リポソームと OVA-CFA はともに IgE 抗体抗体産生を選択的に抑制するという点で共通しているが、OVA-リポソーム免疫マウスの CD4 陽性 T 細胞は顕著に Th2 サイトカインを産生するのに対して OVA-CFA 免疫マウスの CD4 陽性 T 細胞は Th2 サイトカインを産生しなかったことから、OVA-CFA は典型的な Th1 タイプ免疫応答の誘導を介して IgE 抗体産生の選択的抑制を誘導するが OVA-リポソームは必ずしもそうではないことが示唆された。このことは、Ig 抗体サブクラスの検討と、IL-12 欠損マウスを用いた検討によって裏付けられた。IL-12 は Th1 の誘導において中心的役割を担うことが知られており、OVA-CFA によって誘導される抗 OVA IgG 抗体産生は IL-12 欠損マウスにおいて正常マウスと比較して低レベルであったが OVA-リポソームは正常マウスと同レベルの抗 OVA IgG 抗体産生を誘導した。

IgE 選択的無反応は抗原とリポソームとの結合方法によらず誘導される

当初、抗原とリポソームとの結合はグルタールアルデヒド (GA) を用いて行ったが[1]、この方法では GA によって重合した抗原がリポソームに結合する。抗

原-リポソーム結合物によって誘導されるIgE抗体産生の選択的抑制効果が、抗原とリポソームとの結合によるものであるか、またはGAによる抗原の重合によるものであるかを確認する目的で、GAの他にN-(6-maleimidocaproyloxy) succinimide (EMCS)、disuccinimidyl suberate (DSS)、N-succimidyl 3-(2-pyridyldithio) propionate (SPDP) の3種類の架橋試薬を使用してOVA結合リポソームを作製し、比較検討を行った[2]。その結果、いずれの結合方法でOVA結合リポソームを作製した場合においても抗OVA IgE抗体産生は誘導されず、抗OVA IgG抗体産生は同程度に誘導された。一方、OVAとリポソームとの混合溶液、あるいはラテックス粒子にOVAを物理吸着させたものは顕著な抗OVA IgE抗体産生を誘導したことから、IgE抗体産生の選択的抑制を誘導するためには抗原とリポソームとの化学結合が必須であるが、抗原とリポソームとの架橋方法にはよらないことが示唆された。

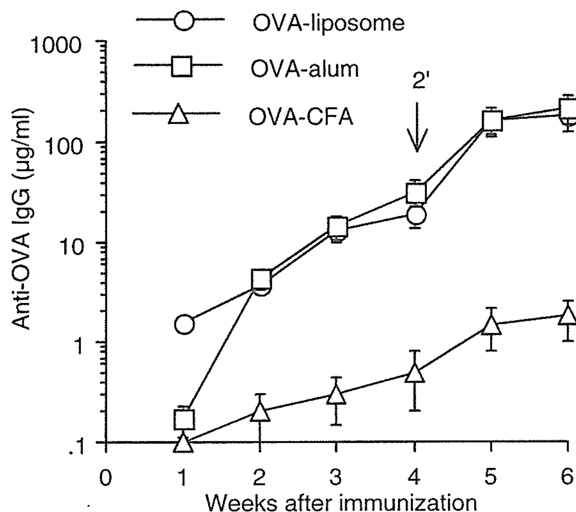


図2a　OVA免疫IL-12ノックアウトマウスにおける抗OVA IgG抗体産生
IL-12ノックアウトマウスに3種類の異なるアジュバントを用いてOVAを0週、4週の2回免疫し、血清中の抗OVA IgG抗体をELISAを用いて測定した。

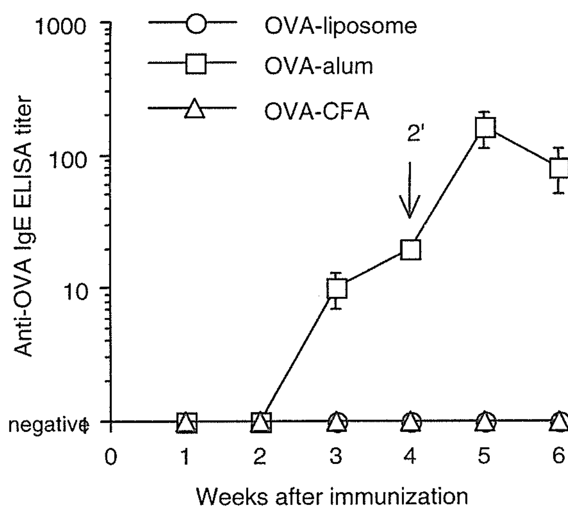


図2b　OVA免疫IL-12ノックアウトマウスにおける抗OVA IgE抗体産生
図2aの血清について抗OVA IgE抗体をELISAを用いて測定した。

IgG抗体産生の誘導におけるアジュバント効果はリポソームの脂質組成によって異なる

不飽和脂肪酸 (オレイン酸)、およびアシル鎖長の異なる飽和脂肪酸 (ミリスチン酸、パルミチン酸、ステアリン酸) を用いて脂質組成の異なる4種類のリポソームを作製し、それぞれについて表面にOVAを結合したものをマウスに投与して抗体産生誘導能の比較を行った[3]。その結果、図3に示すように、抗原特異的IgG抗体産生は不飽和脂肪酸からなるリポソームを使用した群において最も高値であり、飽和脂肪酸の中ではアシル鎖長 (ミリスチン酸＝14、パルミチン酸＝16、ステアリン酸＝18) の短いほど高値のIgG抗体産生が誘導された。IgG抗体産生の誘導能はリポソームの膜流動性の高さと相関したことから、リポソームの膜流動性とアジュバント効果との間に密接な関連があると考えられた。また、いずれの場合にもIgE抗体産生は誘導されなかったことから、リポソームのアジュバント効果とリポソーム表面結合抗原に

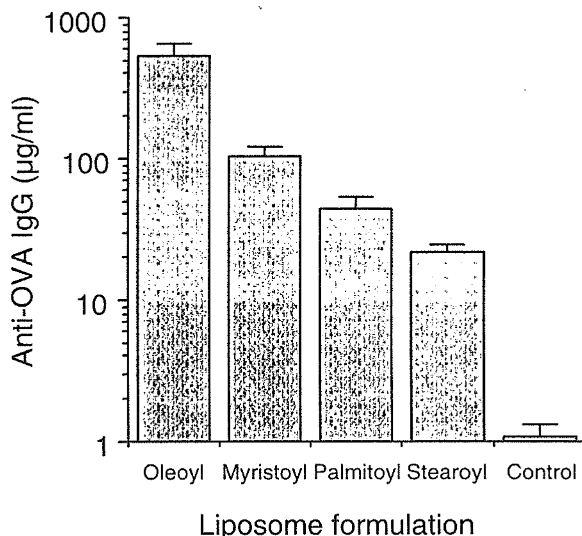


図3　脂質組成の異なるリポソームと結合したOVAによって誘導される抗OVA IgG抗体産生
オレイン酸、ミリスチン酸、パルミチン酸、ステアリン酸からなるリポソームにOVAを結合させ、BALB/cマウスに0週、4週の2回免疫した。初回免疫後6週における血清中抗OVA IgG抗体価を示す。

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。

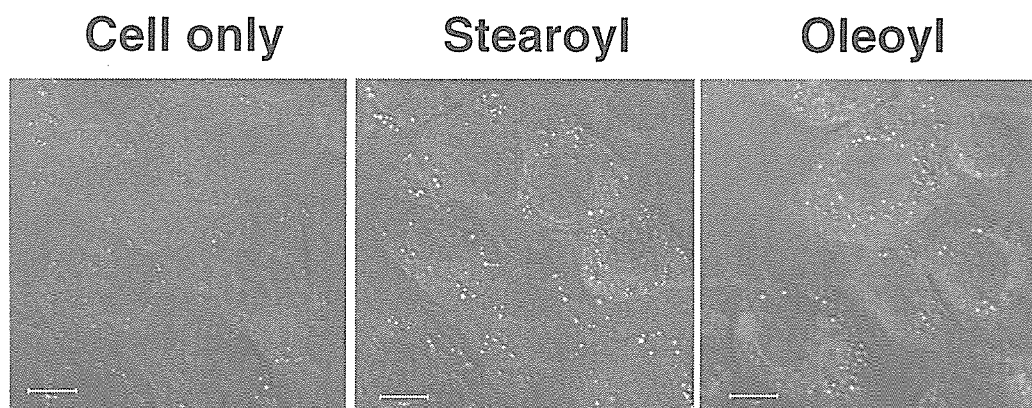


図4a　2種類の異なる脂質組成のリポソームに結合したOVAのマクロファージによる貪食
蛍光標識したOVAをステアリン酸 (Stearoyl)、オレイン酸 (Plepyl) からなるリポソームと結合させ、マクロファージ培養中に添加した。培養開始後60分における共焦点蛍光顕微鏡像を示す。

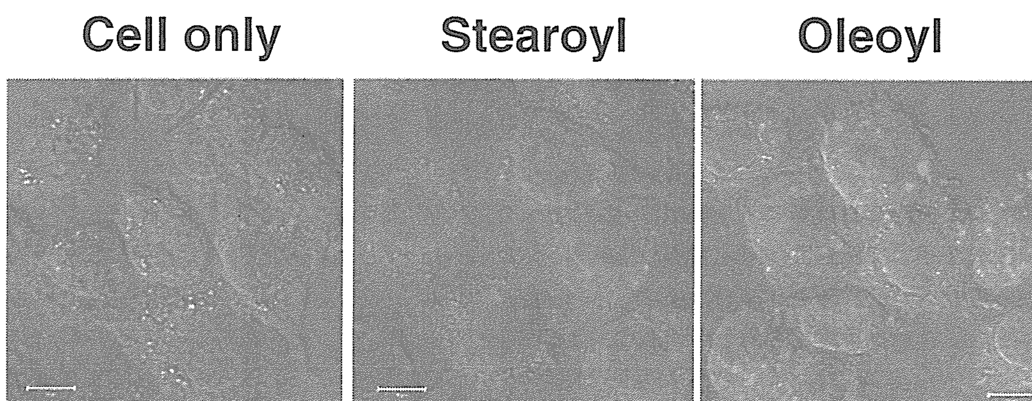


図4b　2種類の異なる脂質組成のリポソームに結合したOVAのマクロファージによる消化
消化を受けると蛍光を発するOVA (DQ-OVA) をステアリン酸 (Stearoyl)、オレイン酸 (Plepyl) からなるリポソームと結合させ、マクロファージ培養中に添加した。培養開始後60分における共焦点蛍光顕微鏡像を示す。

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 (μg/ml)	IgE ELISA titer
no	OVA-liposome	12.3±8.7	N.D.
	OVA-alum	17.7±5.2	N.D.
OVA-liposome	OVA-liposome	144.7±24.3	N.D.
	OVA-alum	124.3±12.8	105.6±10.7
OVA-alum	OVA-liposome	178.0±28.3	N.D.
	OVA-alum	246.8±29.4	139.3±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細胞のかわりに脾細胞からB細胞を除去したもの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は元来アレルゲン性を有しているが、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が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、という質的変化がもたらされることが近年の検討の結果、明らかになった[6]。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

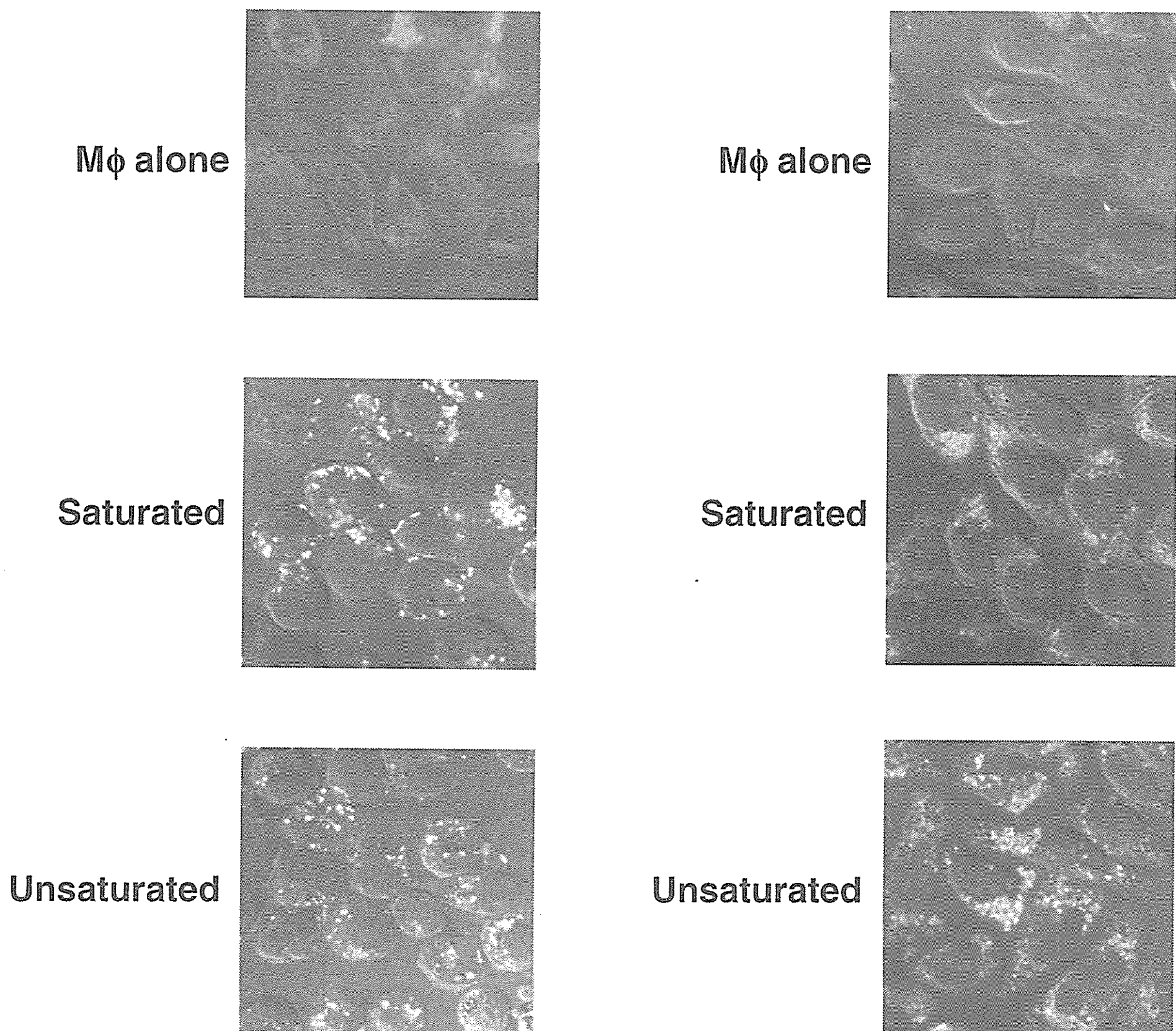


図 5a　リポソームに結合した OVA のマクロファージ細胞中における局在
クラス II を赤色の蛍光で標識したマクロファージの培養中に、緑色の蛍光で標識した OVA と飽和脂肪酸 (Saturated) あるいは不飽和脂肪酸 (Unsaturated) からなるリポソームとの結合物を添加した。培養開始後 60 分における共焦点蛍光顕微鏡像を示す。

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。

in vitro 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±12.5	ND	ND	ND
OVA-liposome	Saturated	910.2±23.0	88.7±45.0	ND	ND
OVA-liposome	Unsaturated	1,065.5±31.9	115.1±28.6	163.3±99.1	149.9±83.8

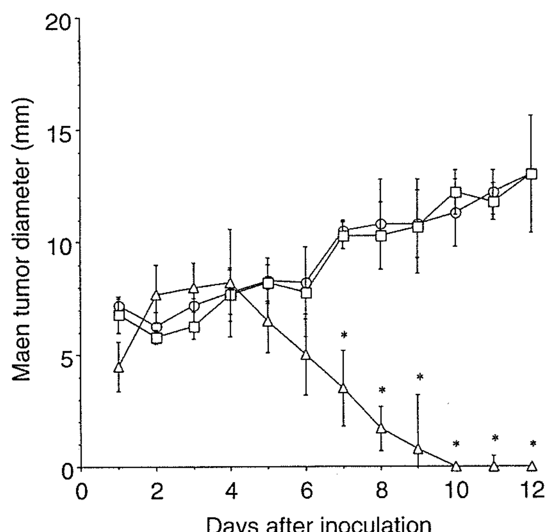
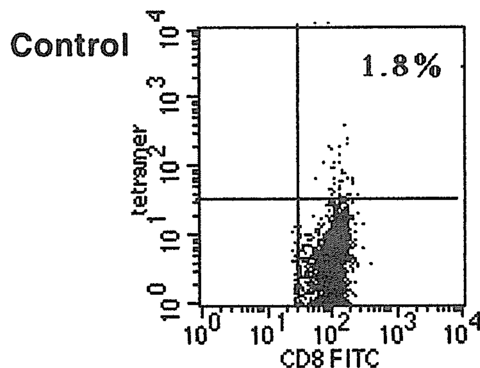
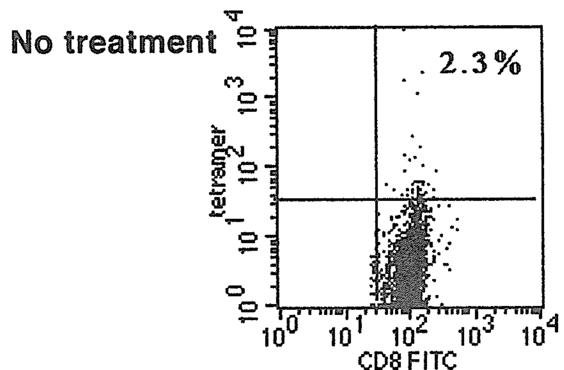


図6　腫瘍抗原ペプチドを投与したマウスにおける腫瘍拒絶
C57BL/6マウスにE.G7を移植し、腫瘍径が約5 mmとなった時に腫瘍の周囲にOVAペプチド結合リポソームをCpG、抗IL-10モノクローナル抗体と共に投与して腫瘍径の変化を観察した。○：CpG、抗IL-10モノクローナル抗体のみ投与。□：ペプチド溶液をCpG、抗IL-10モノクローナル抗体とともに投与。△：ペプチド結合リポソームをCpG、抗IL-10モノクローナル抗体とともに投与。





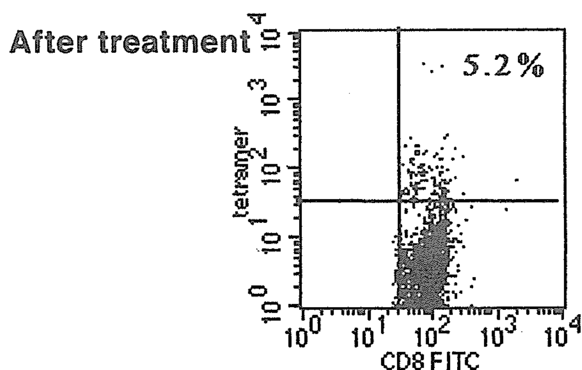


図7　腫瘍を拒絶したマウスにおけるCTL誘導
マウス脾臓由来CD8陽性細胞のOVAペプチド-MHCクラスI (H2-K^b) 複合体をFACSを用いて解析した。Control：正常マウス。No treatment：腫瘍拒絶の観察されなかった担癌マウス。After treatment：図6の実験において腫瘍拒絶の観察されたマウス。

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。

文　献

1) Naito S, Horino A, Nakayama M, Nakano Y, Nagai T, Mizuguchi J, Komuro K, Uchida T: Ovalbumin-liposome conjugate induced IgG but not IgE antibody production. Int Arch Allergy Immunol **109**: 223-228, 1996
2) Nakano Y, Mori M, Nishinohara S, Takita Y, Naito S, Horino A, Kato H, Taneichi M, Komuro K, Uchida T: Antigen-specific, IgE-selective unresponsiveness induced by antigen-liposome conjugates: Comparison of four different conjugation methods. Int Arch Allergy Immunol **120**: 199-208, 1999
3) Nakano Y, Mori M, Nishinohara S, Takita Y, Naito S, Kato H, Taneichi M, Komuro K, Uchida T: Surface-linked liposomal antigen induces IgE-selective unresponsiveness regardless of the lipid components of liposomes. Bioconj Chem **12**: 391-395, 2001
4) Tanaka Y, Kasai M, Taneichi M, Naito S, Kato H, Mori M, Nishida M, Maekawa N, Yamamura H, Komuro K, Uchida T: Liposomes with differential lipid components exert differential adjuvanticity in antigen-liposome conjugates via differential recognition by macrophages. Bioconj Chem **15**: 35-40, 2004
5) Taneichi M, Naito S, Kato H, Tanaka Y, Mori M, Nakano Y, Yamamura H, Ishida H, Komuro K, Uchida T: T cell-independent regulation of IgE antibody production induced by surface-linked liposomal antigen. J Immunol **169**: 4246-4252, 2002
6) Taneichi M, Ishida H, Kajino K, Ogasawara K, Tanaka Y, Kasai M, Mori M, Nishida M, Yamamura H, Mizuguchi J, Uchida T: Antigens chemically coupled to the surface of liposomes are cross-presented to $CD8^+$ T cells and induce potent antitumor immunity. J Immunol 2006 in press

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

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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 GPC3$_{298-306}$ (EYILSLEEL) 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 GPC3$_{298-306}$ peptide therefore seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC and melanoma. In this report, we investigated whether the GPC3$_{298-306}$ 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 GPC3$_{144-152}$ (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 GPC3$_{144-152}$ peptide-reactive CTLs were generated from PBMCs by *in vitro* stimulation with the peptide and the GPC3$_{298-306}$ 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 GPC3$_{298-306}$ (EYILSLEEL) 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

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HLA-A2 (*A*0201*) is high among various ethic 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-$2D^{b-/-}\beta 2m^{-/-}$ 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, Unite d' Immunite 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 $HLA\text{-}A24^+$ and/or $HLA\text{-}A2^+$ 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 ^{51}Cr 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 ^{51}Cr 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 ^{51}Cr 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 $GPC3_{144\text{-}152}$ 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	RLQPGLKWV	879
GPC3A2-2	102-110	FLIIQNAAV	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	TIHDSIQYV	496
GPC3A2-9	522-560	FLAELAYDL	402

*Binding scores were estimated by using BIMAS software (http://bimas.dcrt.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 $GPC3_{298-306}$ 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 $GPC3_{144-152}$ 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 $\pm$ 2.85 spot counts/well, in response to the BM-DCs pulsed with the $GPC3_{144-152}$ peptide, whereas they showed 23 $\pm$ 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 $\pm$ 1.73) was observed. The summation of the diameter of the IFN-γ ELISPOT observed in $CD4^-$ spleen cells stimulated with the $GPC3_{144-152}$ peptide pulsed BM-DCs was 1,878 $\pm$ 131 μm, that stimulated with the HIV-derived SLYNTYATL peptide pulsed BM-DCs was 437 $\pm$ 77 μm, and that observed in the presence of BM-DC without peptide loading was 762 $\pm$ 131 μm ($P < 0.001$). 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 $GPC3_{144-152}$ peptide-specific response of $CD4^-$ spleen cells. As for other peptides, no significant peptide-specific response was observed. These results suggest that the $GPC3_{144-152}$ peptide could be a CTL epitope peptide in HLA-A2.1 (HHD) Tgm, and we also expected this $GPC3_{144-152}$ peptide to be an epitope for human CTLs.

The immunization of the HLA-A2-restricted peptide, $GPC3_{144-152}$, 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 $GPC3_{298-306}$ 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 $GPC3_{144-152}$ peptide alone ($n = 3$; data not shown). These results indicate that the $GPC3_{144-152}$ 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 $GPC3_{298-306}$ or $GPC3_{144-152}$ 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 $GPC3_{144-152}$ peptide or CTLs from PBMCs of HLA-$A24^+$ HCC patients stimulated with the $GPC3_{298-306}$ 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 $GPC3_{144-152}$ peptide, but not to T2-A0201 cells without peptide loading by ^{51}Cr 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.

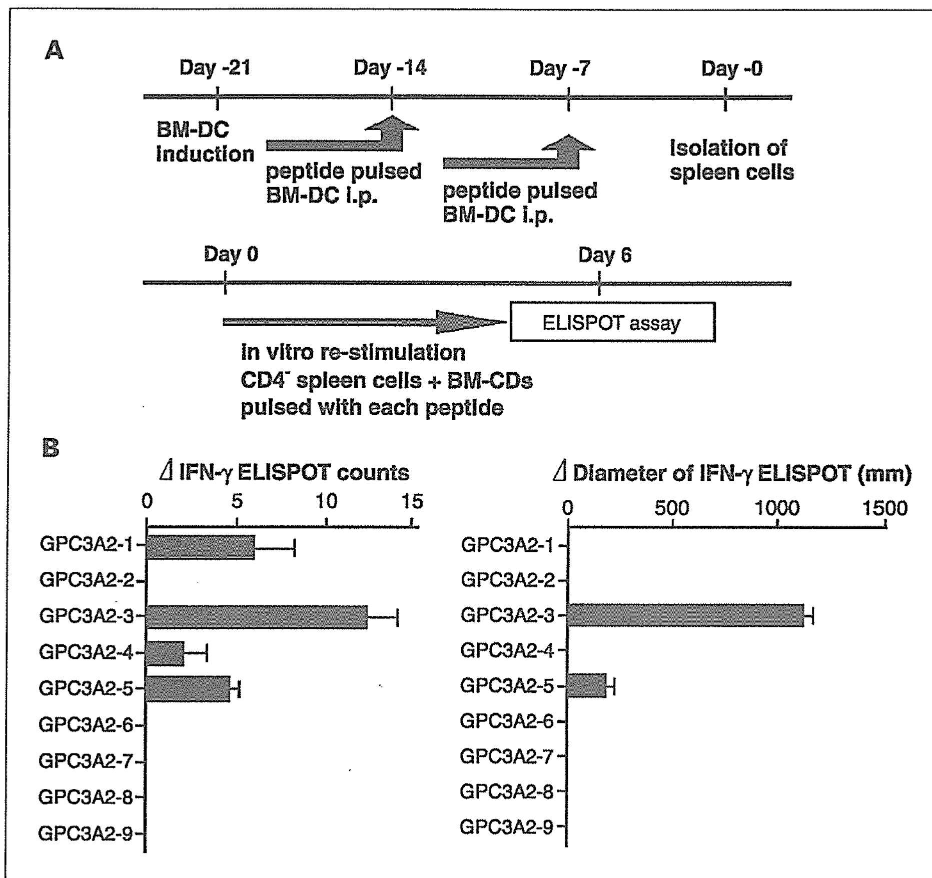

Fig. 1. Identification of HLA-A2-restricted CTL epitopes of GPC3 by using HLA-A2.1 (HHD) Tgm. *A,* protocol for identification of GPC3-derived and HLA-A2-restricted CTL epitopes. We primed the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5) pulsed with the mixture of GPC3-derived peptides carrying HLA-A2 (*A*0201*) 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 ELISPOTassay to evaluate GPC3-specific response of CTLs. *B, bar graph,* IFN-γ ELISPOTcounts/2×10^4 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/2×10^4 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 $GPC3_{298-306}$ 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 $GPC3_{144-152}$ peptide showed specific cytotoxicity against SK-Hep1/GPC3, but not against GPC3-negative SK-Hep1. Similarly, the $GPC3_{298-306}$ 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 $GPC3_{144-152}$ peptide and these CTLs exhibited cytotoxic activity to HepG2 (GPC3+, HLA-A2+, and

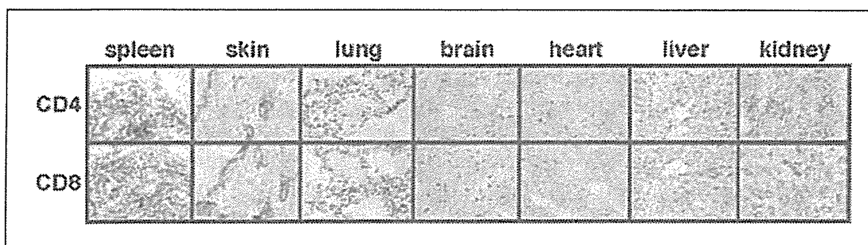

Fig. 2. Immunohistochemical staining with anti-CD4 or anti-CD8 mAb in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the GPC3 $_{144-152}$ peptides. These tissue specimens were removed and analyzed 7 days after the second DC vaccination (original magnification, ×200).

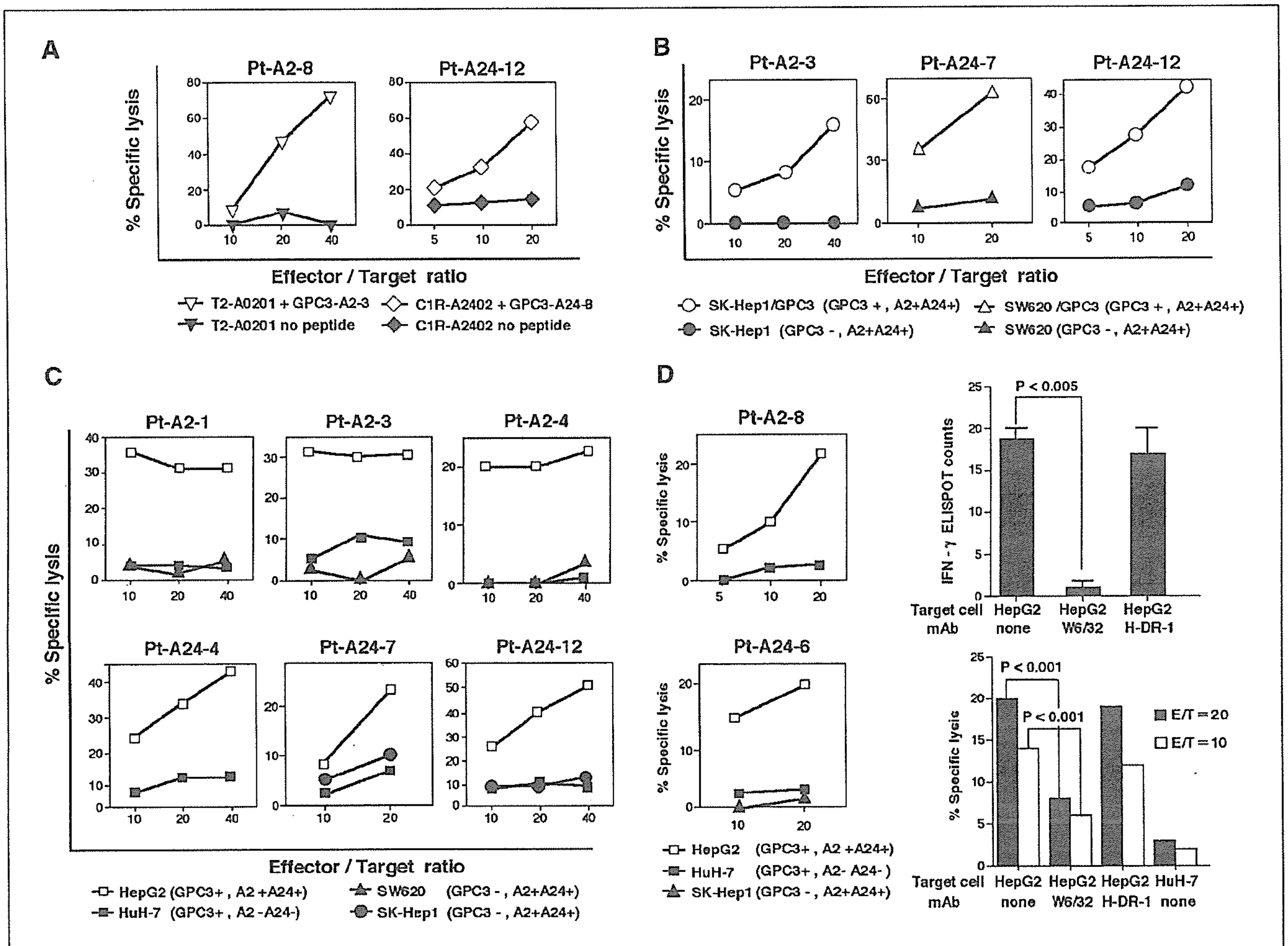

Fig. 3. CTL induction from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. *A* and *B*, GPC3 peptide-reactive CTLs were generated from $CD8^+$ T cells of HLA-$A2^+$ and/or HLA-$A24^+$ HCC patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the $GPC3_{144-152}$ or $GPC3_{298-306}$ peptide, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio. Their cytotoxicity against the $GPC3_{298-306}$ 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 ^{51}Cr 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 $GPC3_{144-152}$ peptide (*top*) or CTLs generated from patient A24-6 using the $GPC3_{298-306}$ peptide (*bottom*) were added. IFN-γ production (*top*; IFN-γ ELISPOT assay) and cytotoxicity (*bottom*; ^{51}Cr 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 $GPC3_{298-306}$ 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 $GPC3_{144-152}$ peptide (Fig. 3D, top), and inhibited cytotoxic activity against HepG2 cells in ^{51}Cr release assay of CTLs generated from patient A24-6 by stimulation with the $GPC3_{298-306}$ 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*0201*) – 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:Tratio = 20 against HepG2 target cells was evaluated as positive by ^{51}Cr release assay.

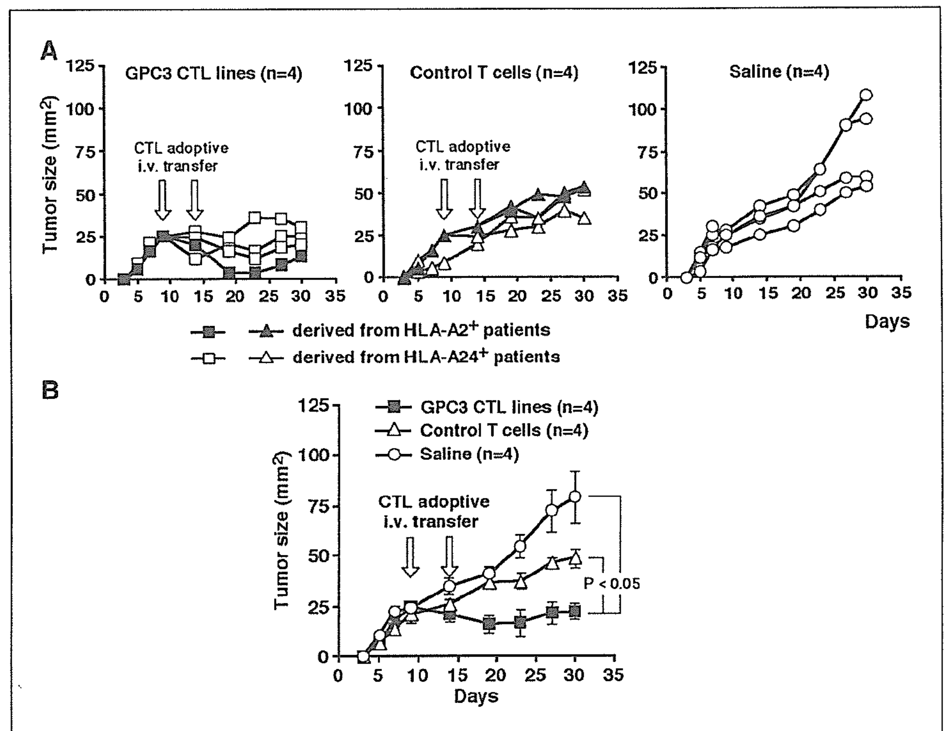

Fig. 4. Marked inhibition of growth of a GPC3-transfected human HCC cancer cell line, SK-Hep1/GPC3, engrafted into NOD/SCID mice after adoptive transfer of human CTLs induced by the GPC3 peptides. *A,* when tumor size reached 25 mm^2 on day 9 after s.c. tumor implantation, human CTLs (3×10^6) reactive 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,* ±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 $GPC3_{298-306}$ 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 $GPC3_{298-306}$ 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 immunoediting (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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References

1. Schafer DF, Sorrell MF. Hepatocellular carcinoma. Lancet 1999;353:1253-7.
2. Tung-Ping Poon R, Fan ST, Wong J. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. Ann Surg 2000;232:10-24.
3. Tsao H, Atkins MB, Sober AJ. Management of cutaneous melanoma. N Engl J Med 2004;351: 998-1012.
4. Jemal A, Murray T, Ward E, et al. Cancer statistics. CA Cancer J Clin 2005;55:10-30.
5. Hippo Y, Watanabe K, Watanabe A, et al. Identification of soluble NH_2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 2004;64:2418-23.
6. Capurro M, Wanless IR, Sherman M, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125:89-97.
7. Nakatsura T, Yoshitake Y, Senju S, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003;306:16-25.
8. Nakatsura T, Kageshita T, Ito S, et al. Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 2004;10:6612-21.
9. Zhu ZW, Friess H, Wang L, et al. Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut 2001;48:558-64.
10. Nakatsura T, Komori H, Kubo T, et al. Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 2004; 10:8630-40.
11. Browning M, Krausa P. Genetic diversity of HLA-A2: evolutionary and functional significance. Immunol Today 1996;17:165-70.
12. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Perarnau B. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from β2 microglobulin (β2m) HLA-A2.1 monochain transgenic H-2Db β2m double knockout mice. J Exp Med 1997; 185:2043-51.
13. Firat H, Garcia-Pons F, Tourdot S, et al. H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. Eur J Immunol 1999; 29:3112-21.
14. Henderson RA, Michel H, Sakaguchi K, et al. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. Science 1992;255:1264-6.
15. Karaki S, Kariyone A, Kato N, Kano K, Iwakura Y, Takiguchi M. HLA-B51 transgenic mice as recipients for production of polymorphic HLA-A, B-specific antibodies. Immunogenetics 1993;37:139-42.
16. Wadee AA, Paterson A, Coplan KA, Reddy SG. HLA expression in hepatocellular carcinoma cell lines. Clin Exp Immunol 1994;97:328-33.
17. Matsui M, Machida S, Itani-Yohda T, Akatsuka T. Downregulation of the proteasome subunits, transporter, and antigen presentation in hepatocellular carcinoma, and their restoration by interferon-γ. J Gastroenterol Hepatol 2002;17:897-907.
18. Bourgault Villada I, Moyal Barracco M, Ziol M, et al. Spontaneous regression of grade 3 vulvar intraepithelial neoplasia associated with human papillomavirus-16-specific CD4(+) and CD8(+) T-cell responses. Cancer Res 2004;64:8761-6.
19. Yoshitake Y, Nakatsura T, Monji M, et al. Proliferation potential-related protein, an ideal esophageal cancer antigen for immunotherapy, identified using complementary DNA microarray analysis. Clin Cancer Res 2004;10:6437-48.
20. Monji M, Nakatsura T, Senju S, et al. Identification of a novel human cancer/testis antigen, KM-HN-1, recognized by cellular and humoral immune responses. Clin Cancer Res 2004;10:6047-57.
21. Makita M, Hiraki A, Azuma T, et al. Antilung cancer effect of WT1-specific cytotoxic T lymphocytes. Clin Cancer Res 2002;8:2626-31.
22. Gomi S, Nakao M, Niiya F, et al. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-specific CTLs. J Immunol 1999;163:4994-5004.
23. Kai M, Nakatsura T, Egami H, Senju S, Nishimura Y, Ogawa M. Heat shock protein 105 is overexpressed in a variety of human tumors. Oncol Rep 2003;10: 1777-82.
24. Matsuyoshi H, Senju S, Hirata S, Yoshitake Y, Uemura Y, Nishimura Y. Enhanced priming of antigen-specific CTLs *in vivo* by embryonic stem cell-derived dendritic cells expressing chemokine along with antigenic

protein: application to antitumor vaccination. J Immunol 2004;172:776–86.

25. Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 2002; 20:621–67.

26. Thomas AM, Santarsiero LM, Lutz ER, et al. Mesothelin-specific CD8(+) T cell responses provide evidence of *in vivo* cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. J Exp Med 2004;200:297–306.

27. Bredenbeck A, Losch FO, Sharav T, et al. Identification of noncanonical melanoma-associated T cell epitopes for cancer immunotherapy. J Immunol 2005; 174:6716–24.

28. Kawakami Y, Rosenberg SA. Human tumor antigens recognized by T-cells. Immunol Res 1997;16:313–39.

29. Tsuboi A, Oka Y, Udaka K, et al. Enhanced induction of human WT1-specific cytotoxic T lymphocytes with a 9-mer WT1 peptide modified at HLA-A*2402-binding residues. Cancer Immunol Immunother 2002; 51:614–20.

30. Capurro MI, Xiang YY, Lobe C, Filmus J. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res 2005;65:6245–54.

31. Senju S, Hirata S, Matsuyoshi H, et al. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. Blood 2003; 101:3501–8.

32. Motomura Y, Senju S, Nakatsura T, et al. Embryonic stem cell-derived dendritic cells expressing Glypican-3, a recently identified oncofetal antigen, induce protective immunity against highly metastatic mouse melanoma, B16–10. Cancer Res 2006;66: 2414–22.

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

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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

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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 (β2m) 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 β2m 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

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 μg/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 β2m (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 (BL_{50}) (12). For most peptides, experiments were performed three times, and data are given as mean values ± standard error of the mean (SEM).

Complex stability assay

Peptides that showed a BL_{50} of less than 100 μM 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	YLLPRRGPRL (2)	42.5 ± 3.8	24<
NS4-1789	1789–1797	SLMAFTAAV (4)	45.3 ± 2.5	24<
NS3-1585	1585–1593	YLVAYQATV (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	VLAALAAYCL (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	FLLLADARAV (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.

[a]Data of peptide-binding assays are shown as BL_{50}, 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.

[b]Peptides that showed a BL_{50} 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 5×10^7 plaque-forming 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 ^{51}Cr release assays. RMA-HHD cells were pulsed with or without an appropriate peptide at 10 μM for 1 h, labeled with 100 μCi of $Na_2{}^{51}CrO_4$, 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 = $[(cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous})] \times 100$. 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 μL/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 μM. 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 CD8α 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× Perm/Wash solution (provided with the kit), flow cytometric analyses were performed.

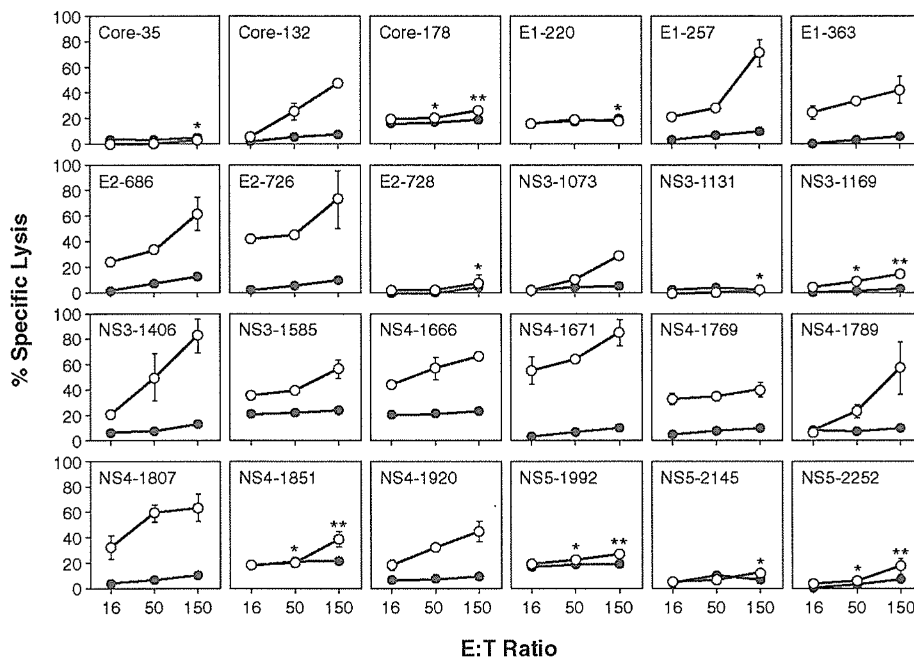

FIG. 1. CTL activities specific for 24 HCV-derived peptides. Mice were injected with p3XFLAG-IL-12, and then immunized intraperitoneally with 5×10^7 PFU of either Adex1SR3ST or Adex1CA3269. Two to 3 weeks after immunization, spleen cells of Adex1SR3ST-immunized mice and Adex1CA3269-immunized mice were 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 ± 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 (*$p > 0.05$; **$p < 0.05$).