Table 3. TRG-derived peptides used for this study

HLA-B52 binding peptide				
peptide name	a.a.position[a]	length	HLA-B52 affinity[b]	a.a.sequence
TRG1–9	9–18	10	72	HPLPQIQLFI
TRG1–20	20–28	9	220	YQLCLTNIF
TRG1–25	25–33	9	20	TNIFHILII
TRG2–23	23–32	10	75	VGVCVCVNII
TRG2–41	41–50	10	24	KQLEGRACLI
TRG3–9	9–17	9	30	VCVCVCSII
TRG3–8	8–17	10	24	CVCVCVCSII
TRG3–25	25–34	10	62	VGYTIGLCLI
TRG5–11	11–19	9	34	LGGQGGRIM
TRG7–46	46–54	9	54	IQIIFRPEI

[a] Amino acid positions in each open reading frame are shown.
[b] HLA-B52-binding score by BIMAS.

The cytotoxic activity of TRG peptide-stimulated PBMC was further examined by a standard 6-h ^{51}Cr-release assay after obtaining a sufficient number of cells by long-term culture with IL-2. Representative results are shown in Fig. 5B. The TRG peptide-stimulated PBMC were cytotoxic to HLA-B52$^+$TRG$^+$ lung cancer cells (11–18). In contrast, these cells were not cytotoxic to HLA-B52$^-$TRG$^+$ (QG56) and HLA-B52$^+$ TRG$^-$ (LC65A) lung cancer cells, or HLA-B52$^+$ TRG$^-$ PHA-blasts. Peptide specificity was confirmed by cytotoxicity to TRG1–20 peptide-loaded SS-EBB (Fig. 5B, right panel). PBMC stimulated with a control Flu-peptide did not show cytotoxicity against any target used in this experiment (data not shown). These CTL activities were inhibited by anti-HLA class I, anti-HLA-BC, and anti-HLA-Bw4 mAb, the last of which reacts to a subgroup of HLA-B containing HLA-B52 molecules, and by anti-CD8, but were not inhibited by anti-HLA-DR, anti-CD4, or anti-CD14 mAb. Representative results are shown in Fig. 5C.

2.4 Induction of HLA-B62-restricted CTL

Because the BIMAS search revealed that TRG1–20 had a relatively high affinity for HLA-B1501, which is identical to serologically determined HLA-B62, we attempted to determine whether TRG1–20 has the ability to induce HLA-B1501-restricted and tumor-reactive CTL. TRG1–20-stimulated PBMC of seven HLA-B62$^+$ cancer patients (two with non-small-cell lung cancer, four with prostate cancer, and one with melanoma) were tested for their reactivity to TRG1–20-loaded HLA-B62$^+$ AY-EBB cells and to HLA-B1501$^+$ (B62$^+$) TRG$^+$ tumor cells, respectively. The PBMC from three of seven patients produced significant levels of IFN-γ in response to both the peptide-loaded HLA-B62$^+$ AY-EBB cells (Fig. 6A, left panel) and the HLA-B1501$^+$TRG$^+$ tumor cells (Fig. 6A, right panel).

TRG1–20-stimlated PBMC (HLA-B62$^+$/HLA-B52$^-$) were further tested for their reactivity to COS7 cells that had been cotransfected with TRG and either HLA-B1501 or control HLA-B5201 cDNA. These PBMC produced significant levels of IFN-γ by recognition of these cells in a dose-dependent and HLA-B1501-restricted manner (Fig. 6B), suggesting that TRG1–20 peptide is able to induce CTL in PBMC from both HLA-B52$^+$ and HLA-B62$^+$ donors in a HLA-B-restricted manner. The cytotoxic activity of the TRG1–20-stimulated PBMC was further examined, and representative results are shown in Fig. 6C. The TRG1–20-stimulated PBMC were cytotoxic to HT1376 (HLA-B1501$^+$TRG$^+$) and PACA2 (HLA-B1501$^+$TRG$^+$), but failed to react with HLA-B1501$^-$TRG$^+$ QG56 cells, HLA-B1501$^+$ TRG$^-$ KIM-1 tumor cells (Fig. 6C), or autologous PHA-blasts (data not shown). Cytotoxicity was inhibited by anti-HLA class I, anti-CD8, anti-HLA-BC, or anti-HLA-Bw6 mAb, the last of which reacts to a subgroup of HLA-B containing HLA-B62 molecules, and was not inhibited by anti-HLA class II, anti-CD4, or anti-CD14 mAb (Fig. 6D).

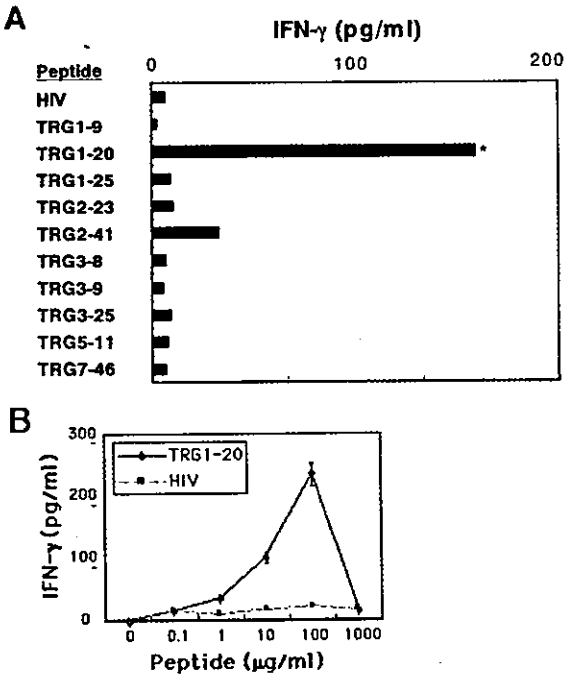

Fig. 4. Identification of TRG-derived CTL epitopes. (A) Determination of the antigenic peptide. Each of the ten different TRG-derived peptides was loaded onto SS-EBB cells at a concentration of 10 μM. GK-B-CTL were cultured with the peptide-loaded SS-EBB for 18 h, and the culture supernatant was harvested to measure IFN-γ production. Values represent the means of triplicate assays; $^*p<0.05$ by Student's *t*-test. (B) Dose dependency of the TRG1–20 peptide. TRG1–20 or irrelevant control (HIV) peptides at the indicated doses were loaded onto SS-EBB cells, and the ability of the peptide to stimulate IFN-γ production by GK-B-CTL was tested. Values represent the means ± SD of triplicate assays. The background IFN-γ production by the GK-B-CTL (41 pg) in response to peptide-unloaded SS-EBB cells was subtracted from the values.

3 Discussion

TRG is located in the intron of the putative tumor suppressor gene Testin in the FRA7G region at 7q31.2. The FRA7G region is one of the chromosomal common fragile sites [28]. Common fragile sites such as FRA3B (3p14.2), FRA7G (7q31.2), FRA7H (7q32), FRA6E (6q26), FRA16D (16q23), and FRAXB (Xp22) appear to be involved in oncogenesis through their deletion, translocation, and methylation [29]. Indeed, deletions of FRA7G have been observed in many epithelial cancers (uterus, ovary, prostate, pancreas, breast, stomach, kidney, colorectum, and head and neck cancers) and leukemias [29]. Therefore, many tumor suppressor genes are predicted to lie within these fragile sites. For example, the fragile histidine triad gene (FHIT), which exists in FRA3B, has been well studied as a tumor suppressor gene [30]. Apoptosis and growth inhibition were reported in tumor cell lines that were transfected with adenoviral FHIT [30]. Deletion and inactivation of FHIT are seen in a number of human premalignant and malignant lesions containing lung cancers [30]. Thus, common fragile sites, including FRA7G, could be appropriate target molecules for a novel cancer therapy.

Although Testin mRNA is expressed in all normal human tissues, its expression is suppressed in many malignant cells by methylation of the 5' end [28]. Testin has also been reported to play a regulatory role in the cell cycle of rat Sertoli cells [31]. However, no direct proof that Testin acts as a tumor suppressor gene has yet been obtained. In the present study, we detected TRG mRNA expression in the majority of cancer cells. TRG expression, in contrast to that of Testin, was relatively limited in cancer cells. TRG was scarcely expressed in the majority of normal tissues and only low-level expression of TRG was detected in the heart, liver, and pancreas.

Recent studies have suggested that introns of some genes are cryptically transcripted to mRNA in malignant cells, and their translated products consequently provide new tumor-specific antigens [32–34]. Examples of such tumor antigens include an antigenic peptide encoded by an intron of N-acetylglucosaminyltransferase V (GnT-V), and two peptides encoded by different introns of tyrosinase-related protein (TRP)-2 [32–34]. Transcripts of these introns were also detected exclusively in tumors; that is, not in normal tissues or normal cells. The other type of abnormal transcription detected in tumor cells was reported in renal cell carcinomas. An antigenic peptide encoded by a reverse strand of the RU2 gene was identified as a tumor antigen recognized by CTL in an HLA-B7-restricted manner [35]. Expression of this antisense transcript (RU2AS) was restricted in malignant cells, and it was absent in most normal tissues (*i.e.* except in the testis and kidney). Therefore, these kinds of tumor antigens are more tumor-specific, and more appropriate targets for immunotherapy, than the authentic transcripts of the mother genes are.

This study showed that TRG encoded a CTL-epitope for HLA-B52-restricted CTL established from the tumor-infiltrating lymphocytes of a lung cancer patient. We also showed that one TRG peptide had the ability to induce HLA-B locus-restricted CTL cytotoxic to TRG$^+$ tumor cells from PBMC of the majority of HLA-B52$^+$ and HLA-B62$^+$ epithelial cancer patients. All these results suggest that TRG and its peptide could be applicable to specific immunotherapy for epithelial cancer patients.

Several antigenic peptides recognized by tumor-reactive CTL in an HLA-B locus-restricted manner have already

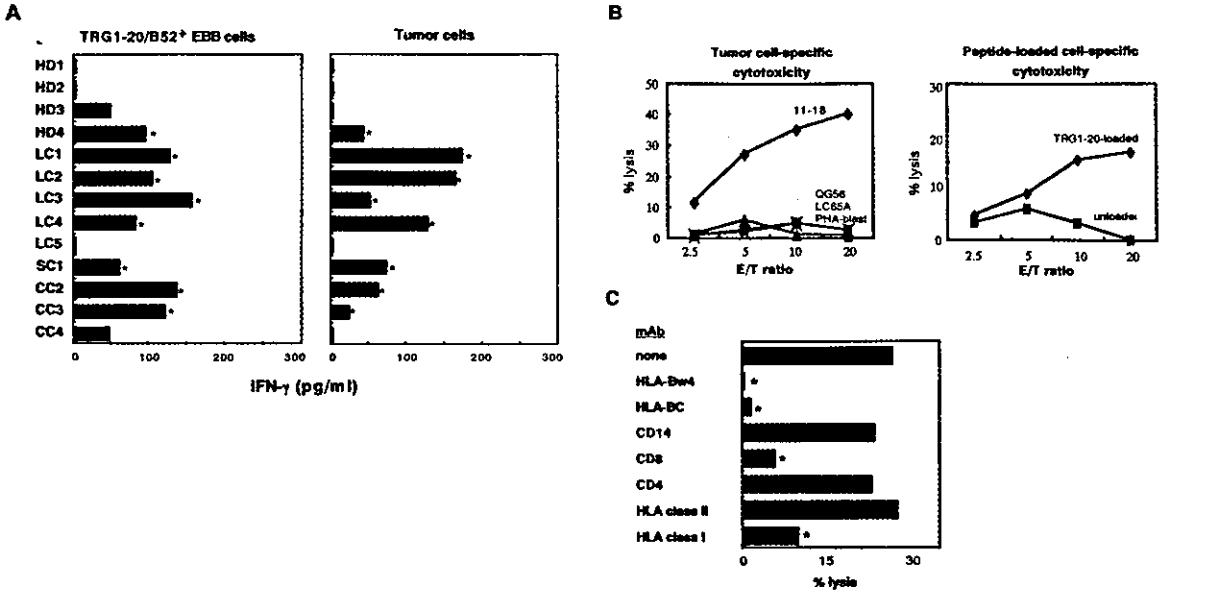

Fig. 5. CTL induction by the TRG peptide. (A) TRG1–20 peptide was tested for its ability to induce CTL in PBMC from nine different HLA-B5201$^+$ cancer patients (five patients with lung cancer, LC; one with stomach cancer, SC; and three with colon cancer, CC) and four HD. The PBMC were stimulated four times every 3 days with the TRG peptide or control peptides (Flu for positive and HIV for negative controls), and the cells were tested for their ability to produce IFN-γ in response to peptide-loaded SS-EBB cells or HLA-B5201$^+$TRG$^+$ tumor cells. Values represent the means of triplicate assays. The background IFN-γ production by the cells in response to HIV peptide-loaded SS-EBB cells (43 pg/ml) or HLA-B5201$^-$ QG56 lung cancer cells (12 pg/ml) was subtracted from the values; $^*p<0.05$ by Student's *t*-test. (B) Cytotoxicity of the peptide-stimulated PBMC against HLA-B5201$^+$TRG$^+$ cancer cells (11–18), HLA-B52$^-$TRG$^+$ cancer cells (QG56), HLA-B52$^+$TRG$^-$ cancer cells (LC65A), and HLA-B52$^+$TRG$^+$ normal cells (PHA-blast), respectively, were measured by a 6-h ^{51}Cr-release assay at different E/T ratios (left panel). The cytotoxicity against SS-EBB cells loaded or unloaded with peptides is also shown (right panel). (C) Effect of mAb on the cytotoxic activity of TRG1–20-stimulated PBMC against HLA-B5201$^+$TRG$^+$ 11–18; $^*p<0.05$ by Student's *t*-test.

been identified [19–25]. Such peptides include those of MAGE-3 and tyrosinase presented by HLA-B44; RAGE-1 and MAGE-A1 peptides by HLA-B7; MART-1 peptide by HLA-B45; and carcinoembryonic antigen (CEA) peptide by HLA-B27 molecules [19–24]. A shared peptide of MAGE-3 and MAGE-12 presented by HLA-B40 has also been reported [25]. However, no clinical trials of peptide vaccine presented by HLA-B molecules for cancer patients have yet been reported in the literature. One reason for this is that the frequency of each HLA-B allele is relatively low. Among the various HLA class I alleles, HLA-B alleles have the greatest diversity. Therefore, the frequency of each HLA-B allele is generally less than that of other loci, such as HLA-A or HLA-C. For example, the antigen frequencies of HLA-B52 and HLA-B62 in Japanese are 21.4% and 16.6%, whereas those of HLA-A24 and HLA-A2 are approximately 60% and 38%, respectively.

The relatively low frequency of the B alleles presents a disadvantage for the development of HLA-B locus-restricted peptide vaccines. However, in the present study, we demonstrated that the TRG-derived peptide was applicable for use as a peptide vaccine for cancer patients with the HLA-B52 or the HLA-B62 allele. Thus, we estimate that this peptide can be used for more than 34% of Japanese, 25% of Korean, 27% of Chinese, and 13% of North American Caucasian patients with cancer. In addition, due to its ubiquitous expression on epithelial cancer cells and its limited expression on normal cells, this TRG peptide might be useful for the treatment of relatively large numbers of cancer patients.

4 Materials and methods

4.1 Cell lines

A lung adenocarcinoma cell line, 11–18, was used for preparation of the cDNA library. COS7 and EBV-transformed B cell lines (SS-EBB, AY-EBB, and OC72-EBB) were used for transfection and peptide pulse experiments, respectively. The other cell lines used in this study were as follows: lung adenocarcinomas (LK77, PC-9), lung squamous cell carci-

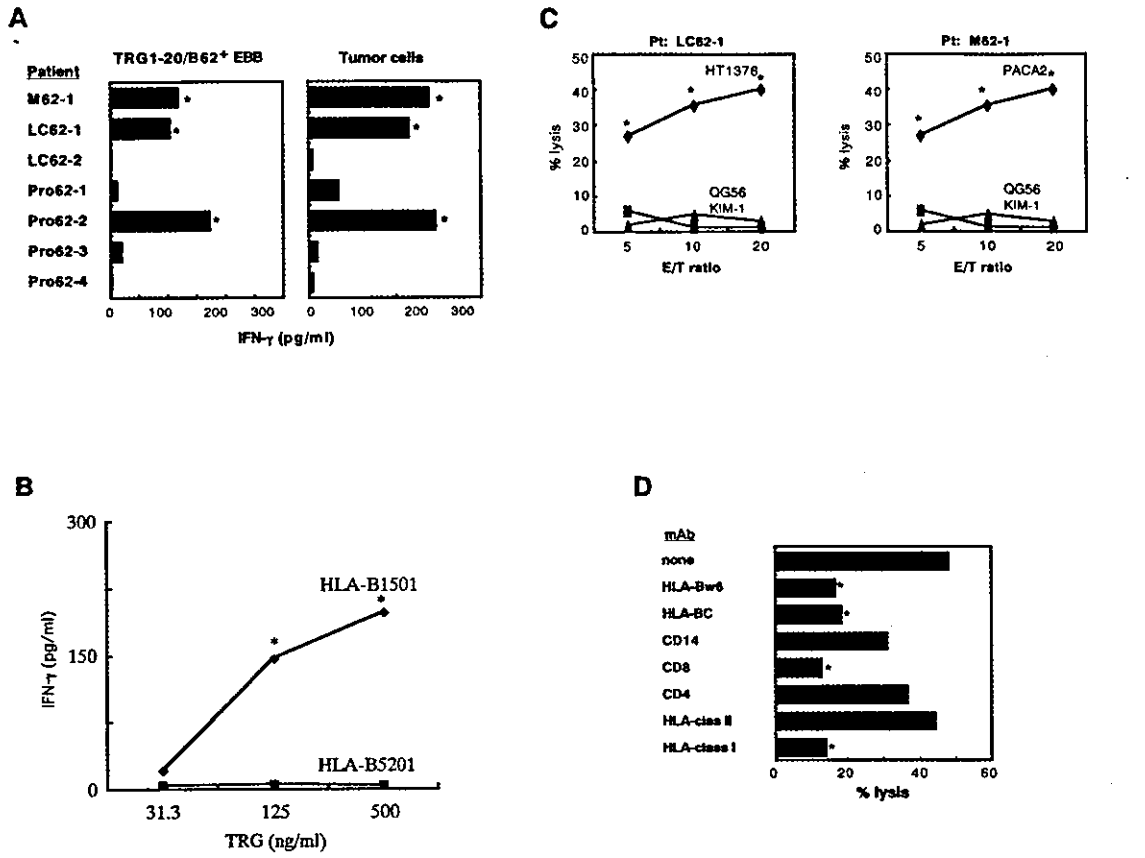

Fig. 6. Induction of HLA-B62⁺-restricted CTL by TRG1–20 peptide. (A) PBMC of seven HLA-B62⁺ patients (two with non-small-cell lung cancer, four with prostate cancer, and one with melanoma) were repeatedly stimulated with TRG1–20, and their reactivities to TRG1–20-loaded HLA-B1501 (B62)⁺ EBV-transformed B cells (AY-EBB) and HLA-B1501⁺TRG⁺ bladder carcinoma HT1376 cells, respectively, were tested. Values represent the means of triplicate assays. The background IFN-γ production (50 pg/ml) by the cells in response to HIV peptide-loaded AY-EBB cells or HLA-B1501⁻ QG56 lung cancer cells was subtracted from the values. (B) Reactivity of TRG1–20-induced CTL in PBMC of an HLA-B62⁺ prostate cancer patient (Pro62–2) to COS7 cells transfected with various doses of TRG along with 100 ng of HLA-B1501 or HLA-B5201 cDNA. (C) Cytotoxicity of TRG1–20-induced CTL against HLA-B1501⁺TRG⁺ (HT1376 or PACA2), HLA-B1501⁺TRG⁻ (KIM1), and HLA-B1501⁻TRG⁺ cancer cells (QG56), respectively, are shown. (D) Effect of mAb on the cytotoxic activity of the TRG1–20-induced CTL (Pro62–2) against HT1376 cells. *$p<0.05$ by Student's *t*-test.

nomas (QG56, Sq-1), lung large cell carcinoma (LC-99A), lung small-cell carcinoma (LC-65A), colon cancers (colo201, SW620), esophagus cancer (KE4), bladder cancer (HT1376), pancreas cancer (PACA2), liver cancers (KYN-2, KYN-3, KIM-1), renal cell carcinomas (KUR11, Caki-1, RC93), and non-tumorous cells (COS7 and VA-13). The origins and HLA genotypes of these cell lines have been described previously [10–16].

4.2 Gene identification

The expression gene-cloning method was used to identify a gene that encodes tumor rejection antigens recognized by an HLA-B52-restricted and tumor-reactive CTL line (GK-B-CTL), which had been established from a lung cancer patient (HLA-A0206/2402, B39/52, Cw7). For preparation of the cDNA library, mRNA of the 11–18 lung adenocarcinoma cell line was converted to cDNA, ligated to the SalI adapter, and inserted into the expression vector pCMV-SPORT2.0 (Life Technology, Gaithersburg, MD). A total of 1×10^5 clones from the cDNA library was divided into 1,000 wells (the expected number of clones/well was 100) and subjected to the first screening. Purified DNA from the divided pools and 100 ng of HLA-B52011 cDNA were cotransfected into COS7 cells (5×10^3 cells/well) and analyzed for their activity to stimulate IFN-γ production by the GK-B-CTL. Finally, one cDNA clone, clone 12A, was obtained. Nucleotide sequencing of the clone was performed by the dyedeoxynucleotide sequencing method using an Auto Read Sequencing Kit (Perkin-Elmer, Foster, CA) and analyzed by an ABI prism 377 (Perkin-Elmer).

4.3 Northern blot analysis

Total RNA (10 μg/lane) extracted from various cells and normal tissues using RNAzol B (TEL-TEST, Friendswood, TX) was separated on formaldehyde agarose gel and transferred to nylon membranes (Hybond-N+; Amersham, GB). The membranes were further hybridized overnight at 65°C in a hybridization buffer (7% SDS, 1 mM EDTA, 0.5 M NaH_2PO_4, p H7.2) containing a ^{32}P-labeled 951-bp fragment of EcoRI/NcoI cut clone 12A as a probe. The membranes were washed three times at room temperature and once at 65°C with a washing buffer (1% SDS, 40 mM NaH_2PO_4, pH 7.2) and then autoradiographed. Human β-actin cDNA (Clontech, Tokyo, Japan) was used as a control probe.

4.4 Quantitative PCR

Gene expression was quantified by real-time PCR using ABI prism 5700 (Applied Biosystems, Foster City, CA) as previously described [36]. Clone 12A and full-length β-actin probe (BD Biosciences Clontech, Palo Alto, CA) were used as DNA standards. Real-time PCR of cDNA specimens (Human MTC panel I, II, and Human tumor MTC panel; BD Biosciences Clontech) and DNA standards were conducted in a total volume of 25 μl with 1× TaqMan Master mix (Applied Biosystems), primers at 400 nM, and probes at 160 nM. Primers and TaqMan probes used in this study are as follows: TRG forward primer 5'-AAGGTGGGCGAATCATGAGG-3'; TRG reverse primer 5'-TCCTGCTTCAGCCTCCCAAG-3'; TRG probe FAM-CCAACATGGTGAAACCCCGTCTCTA-TAMARA; β-actin forward primer 5'-GGCACCCAGCACAATGAAG-3'; β-actin reverse primer 5'-GCCGATCCACACGGAGTACT-3'; β-actin probe FAM-TCAAGATCATTGCTCCTCCTGAGCGC-TAMARA. Thermal cycler parameters included 2 min at 50°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

4.5 Peptides and assays

BIMAS (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) software was used to search for synthetic peptides derived from the deduced amino acid sequence of TRG with binding motifs for the HLA-B5201 molecules as described in the literature [37]. Ten peptides were purchased (purity >95%) from Biologica (Nagoya, Japan) based on information that these motifs included Q at position 2, and I, V, M, and F at positions both 8 and 9. An HIV envelope protein-derived peptide (LPCKIKQII) and an influenza-derived peptide (LPFERATIM) capable of binding to HLA-B5201 were used as negative and positive controls, respectively. The peptide-binding motifs for HLA-B62 (B1501) were Q and L at position 2 and F and Y at position 9 [37]. The estimated scores of the half-time of dissociation of TRG peptides for the HLA-B5201 and HLA-B1501 molecules were calculated using BIMAS software [38].

For detection of antigenic peptides recognized by the GK-B-CTL, the peptides were loaded onto SS-EBB (HLA-B5201/54) or AY-EBB (HLA-B1501/67) cells at 37°C at a concentration of 10 μM, unless stated otherwise. Two hours later, the supernatant was removed, and the GK-B-CTL (1×10^5) were added to the culture, which was then incubated at 37°C for additional 18 h. Culture supernatants were measured for IFN-γ levels by ELISA (limit of sensitivity, 10 pg/ml) in triplicate assays as reported previously [11]. A two-tailed Student's *t*-test was used for statistical analysis.

4.6 CTL induction

PBMC (1×10^5/well) obtained from HLA-B52$^+$ HD (n=4), and HLA-B52$^+$ epithelial cancer patients (n=10; five patients with non-small-cell lung, four with colon, and one with stomach cancer) or HLA-B62$^+$ cancer patients (n=7; two patients with non-small-cell lung cancer, four with prostate cancer, and one with melanoma), were incubated with 10 μM of the peptide in a 96-well plate in the presence of 100 U/ml IL-2, as reported previously [14–16]. At culture days 3, 6, and 9, the culture supernatant of each well was removed and resuspended in fresh medium containing 10 μM of the corresponding peptide for restimulation of the cells. At day 12, the culture supernatant of each well was removed, resuspended in fresh medium, and separated into two wells. One of each pair of wells was stimulated with the corresponding peptide, the other with control HIV peptide.

After 18 h of culture, culture supernatants were measured for IFN-γ levels by ELISA in triplicate assays. In this culture system, antigenic peptides are presented by the initially contained antigen-presenting cells (APC) in the PBMC, and no more addition of exogenous APC is necessary during entire culture periods [39]. Background level of IFN-γ in culture stimulated by HIV peptide-loaded EBB cells, or by HLA-B5201$^-$B1501$^-$TRG$^+$ QG56 cells, was subtracted from that of peptide-loaded EBB cells, or that of HLA-B5201$^+$ or HLA-B1501$^+$TRG$^+$ tumor cells (11–18, Sq-1, HT-1376, or PACA2), respectively. The peptide-stimulated PBMC were further cultured in the presence of irradiated autologous PBMC (2×10^5 cells/well) as APC that had been pulsed by a corresponding peptide, followed by additional culture with IL-2 alone for 7–10 days to obtain a large number of effector cells. The cells were harvested on culture days 28–42, and their cytotoxic activity was measured by a 6-h ^{51}Cr-release assay at different E/T ratios as reported previously [14, 16]. For the inhibition test, 20 μg/ml of anti-HLA class I (W6/32; IgG2a), anti-HLA class II (H-DR-1; IgG2a), anti-CD4 (Nu-Th/i; IgG1), anti-CD8 (Nu-Ts/c; IgG2a), anti-CD14 (H14; IgG1), anti-HLA-BC (B1–23; IgG2a; this mAb cross-reacts to HLA-A23, HLA-A24, HLA-A32), anti-HLA-Bw4 (IgM), and HLA-Bw6 (IgM) mAb were used. Anti-HLA-Bw4 and anti-HLA-Bw6 were purchased from One Lambda, Canoga Park, CA.

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Immunological evaluation of peptide vaccination for patients with gastric cancer based on pre-existing cellular response to peptide

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There is no standard treatment modality for advanced gastric cancer (GC) at the present time. To develop a new treatment modality, we investigated the immunological responses of advanced GC patients (n=13, 9 non-scirrhous and 4 scirrhous types) vaccinated with peptides to a regimen under which pre-vaccination peripheral blood mononuclear cells (PBMCs) were screened for their reactivity *in vitro* to each of 14 peptides on HLA-A24 or 16 peptides on -A2 allele, then only the reactive peptides (maximum: 4) were administered *in vivo*. This regimen was generally well tolerated, although grade I levels of fever and local skin reactions were observed in several patients. Delayed-type hypersensitivity (DTH) to the vaccinated peptides was observed in 4 patients. Increased cellular and humoral immune responses to the vaccinated peptides were observed in post-vaccination PBMCs from 4 of 8 patients and in post-vaccination sera of 8 of 10 patients tested, respectively. Prolonged survival was observed in patients showing cellular and humoral immune responses to the vaccinated peptides in the post-vaccination samples, including all 4 patients with the scirrhous type. These results encourage further development of peptide-based immunotherapy for GC patients. (Cancer Sci 2003; 94: 802–808)

Gastric cancer (GC), a leading cause of cancer-related death worldwide throughout the past century, now ranks second only to lung cancer, with an estimated 750,000 new cases diagnosed annually around the world.[1)] Except in Japan and a few East Asian countries, the prognosis of GC remains poor, and the overall 5-year survival rate ranges from 5 to 15% despite many clinical trials of chemotherapy and other new therapies. In particular, there is no current treatment modality for disseminated GC histologically of the scirrhous type.[2)] Therefore, development of a new treatment modality is needed, and one such modality could be specific immunotherapy, given that recent advances in tumor immunology have resulted in identification of many tumor antigens and epitopes recognized by HLA-class-I-restricted cytotoxic T lymphocytes (CTLs) from various cancers, including GC.[3–6)] However, clinical trials with these peptides have rarely obtained major clinical responses.[7–9)] This failure could have been due to an insufficient induction of anti-tumor responses by these vaccine regimens, since peptide-specific memory T cells were not measured in pre-vaccination peripheral blood mononuclear cells (PBMCs). We speculated that vaccination based on pre-vaccination measurement of peptide-specific CTLs in the circulation might be able to induce potent anti-tumor immune responses in cancer patients.[8–13)] In this report, we describe the safety of such a regimen and the immune responses to peptides and tumor cells in advanced GC patients vaccinated with peptides based on pre-existing cellular response.

Materials and Methods

Patients and eligibility criteria. The Institutional Ethical Review Boards of Hokkaido University and Kurume University approved this clinical protocol (#2031). Complete written informed consent was obtained from all of the patients at the time of enrollment. According to the protocol, patients were required to be positive for HLA-A24 or -A2. All patients were pathologically confirmed to have gastric adenocarcinoma. Eligibility criteria included an age of 85 years or less, serum creatinine of less than 1.4 mg/dl, bilirubin of less than 1.5 mg/dl, platelet count of 100,000/μl or more, hemoglobin of 8.0 g/dl or more, and total WBC of 3000/μl or more. Hepatitis B surface antigen and hepatitis C antibody were required to be negative. The patients were untreated for at least 4 weeks before entry to the study, and had to be an Eastern Cooperative Oncology Group performance status of 0 to 2 at the time of entry. Patients with evidence of serious illness, immunosuppression, or autoimmune disease were excluded. Treatment was carried out at Hokkaido University and Kurume University Hospitals from August 2001 through June 2003.

Screening of peptide-specific CTL-precursors. Thirty millilters of peripheral blood was obtained before and after every 3 vaccinations, and PBMCs were isolated by means of Ficoll-Conray density gradient centrifugation then used for CTL-precursor assay as reported previously.[10)] In brief, PBMCs (1×10^5 cells/well) were incubated with a peptide at 10 μM in wells of U-bottomed 96-well microculture plates (Nunc, Roskilde, Denmark) in 200 μl of culture medium containing 100 U/ml of interleukin-2 (IL-2). Half of the medium was removed and replaced with fresh medium containing a corresponding peptide (20 μM) every 3 days. After incubation for 12 days, these cells were harvested and tested for their ability to produce IFN-γ in response to CIR-A2402 cells for HLA-A24 or to T2 cells for HLA-A2, after pre-loading with either a corresponding peptide or a HIV peptide (RYLRQQLLGI for HLA-A24 and SLYNTVATL for HLA-A2) as a negative control. The level of IFN-γ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/ml). A two-tailed Student's t test was employed for the statistical analyses.

Peptides and vaccination. The peptides used in the present study were prepared under the conditions of Good Manufacturing Practice by Multiple Peptide System (San Diego, CA). The sequences of the peptides are shown in Table 2. All of these peptides have proven ability to induce HLA-A24- or HLA-A2-restricted and tumor-specific CTL activity in the PBMCs of cancer patients.[3–6, 10)] Montanide ISA-51 adjuvant (known as in-

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complete Freund's adjuvant (IFA)) was purchased from Seppic, Inc. (Franklin Lakes, NJ). The peptides were supplied in vials containing 3 mg/ml sterile solution for injection. One milliliter of solution was added in a 1:1 volume to the Monotide ISA 51, then the solution was mixed in a Vortex mixer (Fisher, Inc., Alameda, CA). The resulting emulsion was injected subcutaneously in the lateral abdominal wall using a glass syringe. The interval of vaccination was 2 weeks and a total of 3 injections were performed. For patients with a favorable clinical course, vaccination was repeated thereafter to further evaluate adverse events, immunological responses, and clinical responses.

Immunological assays. Skin tests were performed by intradermal injection of 50 μg of each peptide using a tuberculin syringe and a 27-gauge needle. Saline was used as a negative control. Immediate- and delayed-type hypersensitivity (DTH) reactions were determined at 20 min and 24 h after the skin test, respectively. At least 5 mm of induration or 10 mm of erythema read 24 h after injection was needed to score the skin test as positive. Cytotoxic activity was measured with a standard 6-h ^{51}Cr-release assay, as reported previously.[10] In brief, cryopreserved pre- and post (3rd to 12th)-vaccination PBMCs were thawed at the same time, then cultured in the medium with IL-2 in the absence of any peptides. On the 21st to 25th days of culture, the cells were harvested and used for assay. SSTW-9 (HLA-A24$^+$A2$^-$ stomach adenocarcinoma), KWS (HLA-A24$^-$A2$^+$ stomach adenocarcinoma), SW620 (HLA-A24$^+$A2$^+$ colon adenocarcinoma), Panc-1 (HLA-A2$^-$A2$^+$ pancreatic adenocarcinoma), and phytohemagglutinin (PHA)-blastoid T cells(HLA-A24$^+$ or HLA-A2$^+$) were used as target cells.

The serum levels of peptide-specific IgG were measured by ELISA as previously reported.[10-12] In brief, 100 μl/well of serum samples diluted with 0.05% Tween 20-Block Ace were added to the peptide (20 μg/well)-immobilized plate. After incubation for 2 h at 37°C the plate was washed and further incubated for another 2 h with a 1:1000-diluted rabbit anti-human IgG (γ-chain-specific, DAKO, Glostrup, Denmark). The plate was washed, then 100 μl of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish peroxidase-dextran polymer (En Vision, DAKO) was added to each well, and the plate was incubated for 40 min. After washing, 100 μl/well of tetramethylbenzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by an addition of 1 *M* phosphoric acid. To estimate the peptide-specific IgG levels, the absorbance (*A*) values of each sample were compared with those of serially diluted standard samples, and the values were shown as *A* units/ml.

Evaluation of clinical response. All known sites of disease were evaluated by computed tomography (CT)-scan or X-ray examination before and after every 3 vaccinations. Patients were assigned to a response category according to the response evaluation criteria for solid tumors, a revised version of the WHO criteria published in June 1999 in the WHO Handbook for reporting results of cancer treatment. Kaplan-Meier curves were drawn and survivals were compared using the logrank test.

Results

Patients' characteristics, peptide screening, and adverse events. Thirteen patients with advanced GC were enrolled in this phase I study. The characteristics of these patients are summarized in Table 1. The median age of the patients was 64, ranging from 48 to 78. Six patients had peritoneal dissemination, 6 patients had liver metastasis, 5 patients had lymph node metastases, and 1 patient had bone metastases. Eight cases were recurrence, 4 were stage IV, and the remaining 1 was stage IIIA based on the Japanese Classification of GC 13th edition. Four cases (cases 2, 8, 9, and 13) had histologically scirrhous (diffuse) cancers, and the remaining 9 cases had non-scirrhous cancers. All patients underwent surgical resection of the primary lesion, and 11 of them had previously undergone failed chemotherapy. Pre-vaccination PBMCs were provided for peptide screening. Representative results of three cases (cases 2, 11, and 13) are shown in Fig. 1. One of the four wells of pre-vaccination PBMCs of one patient (case 2) responded to the SART3-315, SART2-161, SART2-93, and CypB91 peptide with criteria of A, A, B, and C, respectively. Two of the four wells of pre-vaccination PBMCs of other patient (case 11) responded to the SART3-315 and lck208 peptide with criteria of AD and ArA, respectively. In contrast, pre-vaccination PBMCs of case 13 responded to lck422 (D) and ppMAPkkk294 (D), respectively. Collectively, CTL precursors reacting to 7, 5, 4, 3, and 2 peptides were detected in pre-vaccination PBMCs of 2, 3, 4, 1, and 3 patients, respectively. Subsequently, 4, 3, and 2 peptides were injected in 7, 1, and 5 patients, respectively. It is of note that the profiles of vaccinated peptides were entirely different among these 13 patients (Table 2). Eleven patients completed the first 3 vaccinations after having given informed consent, and 9 of them received more vaccinations (5 to 37) after providing additional informed consent (Table 1). The peptides used were as follows: SART3-109 and lck208 for 6 of 11 patients; SART3-315, and lck488 for 4 patients; SART2-93, SART2-899, and ART4-75 for 3 patients; SART1-690, SART2-161, and CypB91 for 2 patients; lck486, ART4-13, SART3-309, lck422, ppMAPkkk294, and WHSC2-141 for one patient. Neither CypB84 nor ART1-170 was vaccinated due to immediate-type hypersensitivity in the pre-vaccination skin-test.

All 13 patients were evaluated for adverse events. The vaccinations were generally well tolerated. Fever with mild flu-like symptoms was observed in 3 patients (grade I); it was transient and self-limiting. Grade I local redness and swelling at the injection sites, diarrhea, and pruritus was observed in 2, 1, and 1 patients, respectively. No severe (grade III or IV) toxicity was observed. There was no clinical evidence of an autoimmune reaction as determined by symptoms, physical examination, or laboratory tests.

Cellular immune responses. No DTH reaction against peptides was observed before vaccination in any patient. Peptide-specific DTH reactions were observed in 4 patients (cases 2, 6, 11, and 13) by the 9th vaccination, as indicated in Table 3. For example, in case 2, DTH reactions to SART3-315 and SART2-161 were observed after the 9th vaccination. For evaluation of cellular immune responses to the vaccinated peptides, the post (6th)-vaccination PBMCs from all the cases available in this study (n=8) were provided for the assay of peptide-specific CTL precursors. Three representative cases (cases 2, 11, and 13) are shown in Fig. 1. Three wells of the post-vaccination PBMCs of case 2 responded to SART3-315 and SART2-93 with the criteria of AAA and AAA, respectively, and one well to SART2-161 with the criteria of A. In a patient (case 11), 3, 2, 1, and 1 well of post-vaccination PBMCs each responded to SART3-109, lck208, SART3-315, and ART4-75, respectively. Similarly, 3 wells of the post-vaccination PBMCs of case 13 responded to lck422. Collectively, the post (6th)-vaccination PBMCs of 4 of 8 patients (cases 2, 5, 11, and 13) showed an increased response to at least one of the vaccinated peptides based on either increased numbers of positive wells or higher-grade criteria. In contrast, those of the remaining 4 patients (cases 1, 6, 8, and 9) showed decreased responses or no change. The results are summarized in Table 3.

The pre- and post-vaccination PBMCs from the available 7 patients were expanded *in vitro* in culture with IL-2 alone for 21–25 days followed by measurement of their cytotoxicity against various tumor cells by a 6-h ^{51}Cr release assay.

However, increased levels of HLA-A2-restricted cytotoxicity against tumor cells were not detectable in post-vaccination PB-

Table 1. Patients' characteristics

No.	Age/Sex	HLA type	PS	Stage[1]	Site of metastases	Previous treatments		No. of vaccination
						Surgery[3]	Chemotherapy[4]	
1	54 M	A24	1	T4N1M0H1 IV	liver peritoneum	DG	TS1	9
2	59 M	A24	0	T3N1M0P1 IV	peritoneum	TG	—	37
3	48 F	A24	1	T2N1M0H1 IV	liver para-aortic LNs[2]	DG	TS1	2
4	78 M	A24	2	Recurrence	bone para-aortic LNs	DG (T2N0M0StageIb)	5'DFUR	3
5	59 M	A24	1	Recurrence	liver para-aortic LNs peritoneum	TG+PS (T4N2M0StageIV)	5FU+LV 5FU+CDDP+LV	7
6	53 M	A24	1	Recurrence	peritoneum	PG (T2N3M0StageIV)	5FU+CDDP TS1	10
7	66 M	A24	1	Recurrence	liver para-aortic LNs	DG (T1N2M0StageII)[1] MCT[2]	5FU+CDDP TS1 5FU+MTX+LV	5
8	68 M	A24	1	T3N2M0P1 IV	peritoneum	TG	—	14
9	78 M	A24	1	Recurrence	peritoneum abdominal wall	TG+PS (T4N2M0StageIV)[1] bypass (jejunojejunostomy)[2]	5FU+CDDP	6
10	78 M	A24	2	Recurrence	liver	DG (T2N1M0StageII)	5'DFUR	2
11	72 F	A24	0	T2N2M0 IIIA		TG	5'DFUR	13
12	49 M	A2	0	Recurrence	liver	DG (T1N0M0StageIA)	5FU+CDDP	3
13	70 M	A2	0	Recurrence	para-aortic LNs	TG+PS (T2N2M0StageIIIA)	5FU+CDDP	37

1) Japanese Classification of Gastric Carcinoma (the 13th edition).
2) LNs, lymph nodes.
3) DG, distal gastrectomy; TG, total gastrectomy; PG, proximal gastrectomy; PS, pancreatosplenectomy; MCT, microwave coagulation therapy for liver metastasis.
4) TS1,1 *M* tegafur-0.4 *M* 5-chloro 2,4-dihydroxypyridine-1 *M* potassium oxonate; 5'DFUR, doxifluridine; 5FU, fluorouracil; LV, calcium leucovorin; CDDP, cisplatin; MTX, methotrexate.

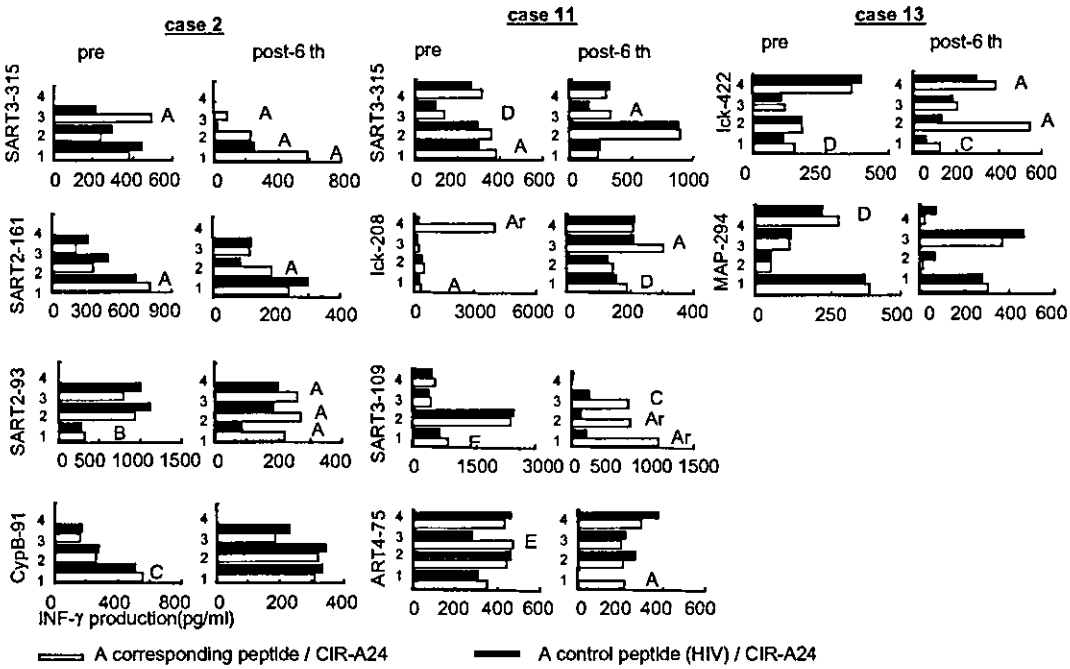

Fig. 1. Peptide-specific CTL precursors. Peptide-specific CTL precursors were measured in pre- and post (6th)-vaccination PBMCs of all 13 patients. Representative results of IFN-γ production in cases 2, 11, and 13 are shown in this figure. A detailed summary is provided in Table 3.

Table 2. Peptide and screening of the peptide-specific CTL precursor cells

Peptide name	Sequence	Patient No.													Positive case	Vaccinated case
		1	2	3	4	5	6	7	8	9	10	11	12	13		
	(HLA-A24)															
SART1-690	EYRGFTQDF			A			E	+/B							4/11	2/11
SART2-93	DYSARWNEI	+[1]/B[2]	+/B	+/AAAAr[3]			+/B								3/11	3/11
SART2-161	AYDFLYNYL		+/A	+/AAAA											2/11	2/11
SART2-899	SYTRLFLIL			+/AAAA		−/AAAAr			+/D		+/D				4/11	3/11
SART3-109	VYDYNCHVDL	+/D				+/A	+/AE		+/A	+/		+/E			5/11	6/11
SART3-315	AYIDFEMKI		+/A				+/D			+/C		+/AD			4/11	4/11
CypB84	KFHRVIKDF				−/AE			−/A							2/11	0/11
CypB91	DFMIQGGDF	+/A	+/C	A											3/11	2/11
lck208	HYTNASDGL	+[1]/AA[3]		+/A	E	+/BC			+/B	+/		+/ArA			6/11	6/11
lck486	TFDYLRSVL			A			E		+/B						3/11	1/11
lck488	DYLRSVLEDF				+/C	+/A	+/C			+/A					4/11	4/11
ART1-170	EYCLKFTKL				−/AA		D			−/C					3/11	0/11
ART4-13	AFLRHAAL	−[4]/A								−/AE	+/Ar				3/11	1/11
ART4-75	DYPSLSATDI				+/E			+/A		B		+/E			4/11	3/11
	(HLA-A2)															
SART3-302	LLQAEAPRL														0/2	0/2
SART3-309	RLAEYQAYI												+/B		1/2	1/2
CypB129	KLKHYGPGWV														0/2	0/2
CypB172	VLEGMEVV														0/2	0/2
lck246	KLVERLGAA														0/2	0/2
lck422	DVWSFGILL													+/D	1/2	1/2
EIF-4EBP51	RIIYDRKFL														0/2	0/2
ppMAPkkk294	GLLFLHTRT													+/D	1/2	1/2
ppMAPkkk432	DLLSHAFFA														0/2	0/2
WHSC2-103	ASLDSDPWV														0/2	0/2
WHSC2-141	ILGELREKV												+/AB		1/2	1/2
UBE2V43	RLQEWCSVI														0/2	0/2
UBE2V85	LIADFLSGL														0/2	0/2
UBE2V208	ILPRKHHRI														0/2	0/2
HNRPL140	ALVEFEDVL														0/2	0/2
HNRPL501	NVLHFFNAPL														0/2	0/2

1) The peptide-specific CTL precursor cells were evaluated by quadruplicate assay using the following criteria. Ar: peptide-specific IFN-γ production>500 pg/ml, and *P* value<0.10; A: 50<IFN-γ production<500 pg/ml, and *P* value<0.05; B: 25<IFN-γ production<50 pg/ml, and *P* value<0.05; C: IFN-γ production>50 pg/ml, and 0.05<*P* value<0.10; D: 25<IFN-γ production<50 pg/ml, and 0.05<*P* value<0.10; E: IFN-γ production>100 pg/ml, and 0.10<*P* value<0.30.
2) Plus (+) mark indicates the vaccinated peptide.
3) "AA" means that 2 wells meet criteria A, and the remaining 2 wells are negative. "AAAAr" means that 1 well meets criteria Ar, and the others meet criteria A.
4) Minus (−) mark indicates no vaccination due to immediate-type hypersensitivity in the skin test.

MCs of any patient except for those from case 13 (data not shown).

Humoral immune responses. Humoral immune responses to the vaccinated peptides were measured in both the pre- and post (3rd to 12th)-vaccination sera. Representative results are shown in Fig. 2, and a summary is given in Table 3. IgG reactive to SART3-109 was induced in the post-vaccination sera of cases 1, 5, 6, and 8, and that reactive to SART3-315 was induced in the post-vaccination sera of cases 2 and 9. Similarly, IgGs reactive to SART1-690, SART2-93, lck208, lck486, lck488, and ppMAPkkk294 were induced in the sera of cases 1, 6, 11, 8, 9, and 13, respectively. In contrast, there were no detectable levels of peptide-reactive IgG in either pre- or post-vaccination sera of the remaining 2 patients (cases 4, 7). The details of the peptide-specificity of each antibody evaluated by means of the absorption test have been published elsewhere.[10–12] Collectively, peptide-specific humoral immune responses were induced in the post-vaccination sera of 8 of the 10 patients tested (cases 1, 2, 5, 6, 8, 9, 11, and 13).

Clinical responses and prognostic factor analysis. It is difficult to draw definitive results from this small-scale phase I study with regard to clinical responses and prognostic factor analysis. Nevertheless, the available results may be relevant from the perspective of development of a suitable peptide vaccine. Objective tumor regression was observed in one patient (case 13). Namely, a necrotic change in the para-aortic lymph node metastasis was observed (Fig. 3). Of the 11 patients eligible for evaluation, 5 (cases 2, 8, 9, 11, and 13) were diagnosed with stable disease (SD) and the remaining 6 patients (cases 1, 4, 5, 6, 7, and 12) were diagnosed with progressive disease (PD) at 3 months after the first vaccination. Four patients (cases 2, 8, 11, and 13) of 11 were diagnosed with SD at the 6-month evaluation, and 2 patients (cases 2, 13) with scirrhous type GC were surviving at over 1 year. The median time to progression (TTP), median survival time (MST), and median 1-year survival rate of 13 patients were 81.8±79.0 (standard error) days, 211.8±213.1 days, and 15.4%, respectively. Prognostic factor analysis indicated that overall survival seemed to correlate, though the correlation was not statistically significant primarily due to the small number of cases, with peptide-specific immune responses in the post-vaccination samples on the basis of positive DTH response, positive IFN-γ release, and IgG production (Fig. 4). Among these three markers, positive IFN-γ release seemed to be superior to the other two markers, since the pa-

Table 3. Peptide-specific immune responses and clinical responses

Pt. No.	Peptide	Peptide-specific CTLp[1]		Antibody to peptide		DTH		Clinical response at[4]		TTP[5] (months)	OS[6] (months)
		Pre	Post	Pre	Post	Pre	Post	3 month	6 month		
1	CypB91	A	–	+	+	–	–	PD	dead	2	4
	Ick208	AA	–	+	+	–	–				
	SART1-690	B	–	–	+(6)[2]	–	–				
	SART3-109	D	–	–	+(6)	–	–				
2	SART3-315	A	AAA	–	+(12)	–	+(9)[3]	SD	SD		22+[6]
	SART2-161	A	A	–	–	–	+(9)				
	CypB91	C	–	+	+	–	–				
	SART2-93	B	AAA	–	–	–	–				
3	SART2-93	AAAAr	NT[7]	NT	NT	–	–	dead	–	1	2
	SART2-161	AAAA	NT	NT	NT	–	–				
	SART2-899	AAAA	NT	NT	NT	–	–				
	Ick208	A	NT	NT	NT	–	–				
4	Ick488	C	NT	–	–	–	–	dead	–	2	3
	ART4-75	E	NT	–	–	–	–				
5	SART3-109	A	AAAr	–	+(6)	–	–	dead	–	1	3
	Ick208	BC	A	–	–	–	–				
	Ick488	A	AE	–	–	–	–				
6	SART3-109	AE	–	–	+(9)	–	–	PD	PD	3	7
	Ick488	C	–	–	–	–	+(3)				
	SART3-315	D	–	–	–	–	–				
	SART2-93	B	–	–	+(3)	–	–				
7	SART1-690	B	NT	–	–	–	–	dead	–	1	2
	ART4-75	A	NT	–	–	–	–				
8	SART3-109	A	–	–	+(6)	–	–	SD	SD	7	8
	SART2-899	D	–	–	–	–	–				
	Ick208	B	D	–	–	–	–				
	Ick486	B	–	–	+(6)	–	–				
9	SART3-315	C	–	–	+(3)	–	–	SD	PD	3	7
	Ick208	–	Ar	+	+	–	–				
	SART3-109	–	–	+	+	–	–				
	Ick488	A	E	–	+(6)	–	–				
10	SART2-899	D	NT	–	NT	–	–	dead	–	1	1
	ART4-13	Ar	NT	–	NT	–	–				
11	SART3-109	E	ArArC	–	–	–	–	SD	SD	7	12
	SART3-315	AD	A	–	–	–	+(3)				
	Ick208	ArA	AD	–	+(6)	–	+(3)				
	ART4-75	E	A	–	–	–	–				
12	SART3-309	B	NT	–	NT	–	–	dead	–	1	1
	WHSC2-141	AB	NT	–	NT	–	–				
13	ppMAPkkk294	D	–	–	+(6)	–	+(4)	SD	SD		22+
	Ick422	D	AAC	–	–	–	–				

1) The peptide-specific CTL precursor cells were evaluated by quadruplicate assay.
2) Number of the vaccination when IgG to the peptide was detected for the first time.
3) Number of the vaccination when DTH to the peptide was detected for the first time.
4) SD, stable disease; PD, progressive disease.
5) TTP, the median time to progression.
6) OS, overall survival; plus (+) mark, patients are arive (2003. 6. 30).
7) NT, not tested.

tients showing both positive IFN-γ release and IgG production (n=4) had a better prognosis than those having IgG production alone (n=4) (Fig. 4, upper right column). Further, from the viewpoint of overall survival, peptide vaccination seemed to provide clinical benefits to patients with the scirrhous type (22M+, 22M+, 8M, and 7M) as compared to patients with non-scirrhous type (12M, 7M, 4M, 3M, 3M, 2M, 1M, and 1M).

Discussion

Increased cellular responses to some of the vaccinated peptides were observed in the post-vaccination PBMCs of 4 of the 8 patients tested, and increased CTL responses to tumor cells were observed in these PBMCs in only 1 of 7 patients tested. This failure to obtain cellular immune responses to either peptides or tumor cells in substantial numbers of the vaccinated patients might in part be due to limited sensitivity (1/3000 to 1/10,000), as well as to the relatively low reproducibility of this CTL assay, as discussed elsewhere.[8-14] Alternatively, CTLs activated by the vaccination might infiltrate into various tissues, including tumors, and induce immune responses to tumor cells, which in turn could be responsible for the appearance of CTL precursors reactive to other peptides. Another explanation might be that CTL activity in the circulation does not always reflect that at tumor sites, and rather few CTLs can infiltrate tumor sites.[15]

In contrast to the findings on CTL assays, increased humoral responses (IgG) to the vaccinated peptides were observed in the post-vaccination sera from the majority of patients tested. Simi-

lar results were obtained in the peptide vaccination trials of patients with hormone refractory prostate cancer and advanced lung cancer.[10–12] Further, induction of the IgG levels by peptide vaccination is well correlated with long survival in patients with advanced stages of lung cancer,[10] whereas the IgG class has been found to be either lacking or unbalanced in atopic dermatitis patients,[16] suggesting its positive role in host defense. However, the role of these peptide antibodies in anti-tumor immunity is as yet unclear, and should be clarified by future basic and clinical studies. The mechanisms of peptide-specific IgG production, including the involvement of CD4 T helper cells and MHC restriction, also need to be clarified.

One of our objectives was to find an appropriate immunological marker for the prediction of clinical benefits in response to peptide vaccination. Only 13 patients were enrolled in this small-scale phase I study with limited observation periods, and therefore it is impossible to obtain a statistically valid correlation between the clinical prognosis and immunological responses. Moreover, post-vaccination samples from 5 patients were not available for the study because of the rapid disease progression. Despite these limitations, the present results showed that measurement of peptide-specific immune responses relative to IFN-γ release might be available as a marker for prediction of prolonged overall survival. The results also suggest that peptide vaccination contributed to the prolonged survival of advanced GC patients with scirrhous type. Tumor cells of the scirrhous type do not cluster, but rather, they are surrounded by fibroblast cells or by other normal cells, which in turn might allow CTLs, Ig, and cytokines to attack tumor

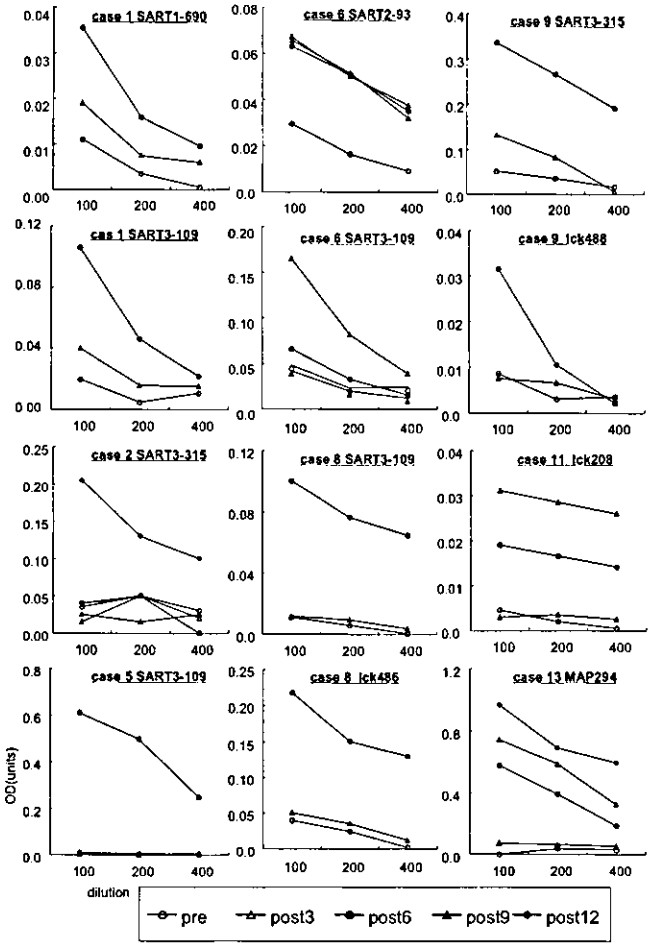

Fig. 2. Peptide-specific IgG antibody. Pre- and post (3rd to 12th)-vaccination sera were provided for the assay, and all positive results for anti-peptide IgG antibodies in the 8 patients are shown in this figure. There were no detectable levels of peptide-reactive IgG in 2 patients (cases 4, 7).

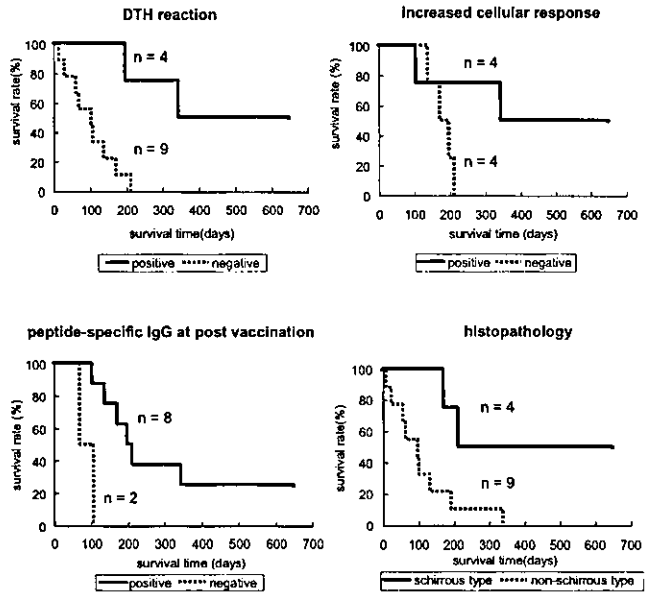

Fig. 4. Correlation between overall survival and laboratory markers.

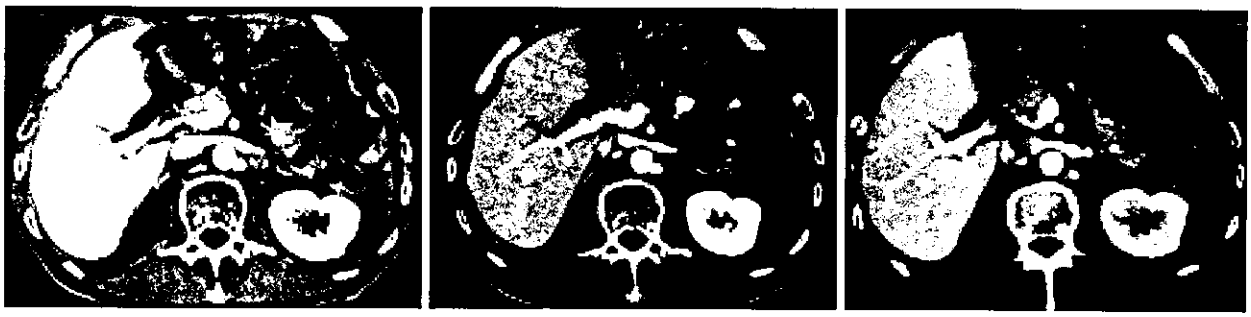

Fig. 3. CT images of case 13.

cells. In contrast, tumor cells of the non-scirrhous type always cluster and create a tumor-mass with a wall, which may in turn hamper the infiltration of immunocompetent cells and any subsequent immune response.[17] If this is the case, the scirrhous type of GC could be one of the most suitable target cancers for peptide vaccination. Currently no standard treatment modality is available for disseminated scirrhous GC.[2] Further studies with a large numbers of cases, however, are required to confirm each of these assumptions.

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In Vivo Evidence That Peptide Vaccination Can Induce HLA-DR-Restricted CD4$^+$ T Cells Reactive to a Class I Tumor Peptide[1]

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Vaccination with class I tumor peptides has been performed to induce tumor-reactive CD8$^+$ T cells in vivo. However, the kinds of immune responses that vaccination might elicit in patients are not fully understood. In this study we tried to elucidate the mechanisms by which vaccination of class I binding tumor peptides into an HLA-A2$^+$ lung cancer patient elicited dramatic amounts of IgG1 and IgG2 specific to a nonamer peptide, ubiquitin-conjugated enzyme variant Kua (UBE2V)$_{43-51}$. The UBE2V$_{43-51}$ peptide contains cysteine at the sixth position. HLA-DR-restricted and UBE2V$_{43-51}$ peptide-recognizing CD4$^+$ T cells were induced from postvaccination, but not from prevaccination, PBMCs of the cancer patient. In addition, a CD4$^+$ T cell line (UB-2) and its clone (UB-2.3), both of which recognize the UBE2V$_{43-51}$ peptide in the context of HLA-DRB1*0403 molecules, were successfully established from postvaccination PBMCs. The peptide vaccination increased the frequency of peptide-specific T cells, especially CD4$^+$ T cells. In contrast, mass spectrometric analysis revealed that the vaccinated UBE2V$_{43-51}$ peptide contained both monomeric and dimeric forms. Both forms, fractionated by reverse phase HPLC, were recognized by UB-2 and UB-2.3 cells. Recognition by these CD4$^+$ T cells was observed despite the addition of a reduction reagent or the fixation of APC. Overall, these results indicate that vaccination with class I tumor peptides can induce HLA-DR-restricted CD4$^+$ T cells in vivo and elicit humoral immune responses, and that a cysteine-containing peptide can be recognized by CD4$^+$ T cells not only as a monomer, but also as a dimer. *The Journal of Immunology,* **2004, 172: 2659–2667.**

Recent advances in molecular biology and tumor immunology have resulted in the identification of many tumor Ags and antigenic peptides recognized by CD8$^+$ T cells (1, 2). Numerous clinical trials have shown that vaccination of class I binding tumor peptides can increase peptide-specific CD8$^+$ T cells in the periphery of cancer patients (3, 4). Because vaccination with class I binding tumor peptides has been performed to induce tumor-reactive CD8$^+$ T cells in vivo, the analysis of peptide-induced immune responses has focused on CD8$^+$ T cells. Therefore, it remains unclear what kinds of immune responses, other than CD8$^+$ T cells, might be elicited in peptide-vaccinated cancer patients.

We have identified a panel of antigenic peptides capable of inducing tumor-reactive CTLs in patients with epithelial cancers (5–8) and have used these peptides in peptide-based immunotherapy (9, 10). In conducting our clinical trials, we observed cases in which peptide-specific IgG was dramatically elicited after the peptide vaccination. This observation led us to test the possibility that vaccination with class I binding tumor peptides would induce peptide-recognizing CD4$^+$ Th cells, as in vivo generation of Ag-specific IgG generally requires a cytokine from Th cells as a result of cognate interaction between T and B cells (11). In the present study we provide evidence that vaccination with class I binding tumor peptides induced the in vivo generation of HLA-DR4-restricted CD4$^+$ T cells in a cancer patient, and that a cysteine-containing peptide can be recognized by CD4$^+$ T cells not only as a monomer, but also as a dimer.

Materials and Methods

Patient and peptides

At the time of the study, the patient (male; EBL-101; HLA-A*0201$^+$ and HLA-DRB1*0403/0403) was 69 years old with non-small cell lung cancer and bone marrow metastasis. The following peptides were used for the clinical trial and were prepared under conditions of good manufacturing practice by Multiple Peptide Systems (San Diego, CA): cyclophilin B-derived peptide (CypB$_{172-179}$;[3] VLEGMEVV), Lck-derived peptide (Lck$_{422-430}$; DVWSFGILL), ubiquitin-conjugated enzyme variant Kua (UBE2V)-derived peptide (UBE2V$_{43-51}$; RLQEWCSVI), and Wolf-Hirschhorn syndrome candidate 2 protein (WHSC2)-derived peptide (WHSC2$_{141-149}$; ILGELREKV). These four peptides have the ability to induce tumor-reactive CTL activity in the PBMCs of HLA-A2$^+$ cancer patients (6–8). UBE2V was identified as an epithelial cancer-related Ag by the screening of a cDNA library from a pancreatic adenocarcinoma cell line, panc-1 (6). The other HLA-A2-binding peptides used as negative controls were an HIV-derived peptide (HIV gag$_{77-85}$; SLYNTYATL), an EBV-derived peptide (BMLFI$_{259-267}$; GLCTLVAML), and a Flu peptide (t-A-MP-MI$_{58-66}$; GILGFVFTL). These peptides, of >90% purity, were purchased from the Biologica (Nagoya, Japan). All peptides were dissolved with DMSO at a dose of 10 mg/ml.

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[3] Abbreviations used in this paper: CypB, cyclophilin B; TFA, trifluoroacetic acid; UBE2V, ubiquitin-conjugated enzyme variant Kua; WHSC2, Wolf-Hirschhorn syndrome candidate 2 protein.

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Peptide vaccination

One milliliter of the peptide solution at 1 mg/ml was mixed with an equal volume of IFA (Montanide ISA-51; Seppic, Paris, France), emulsified in 5-ml sterilized syringes, and injected s.c. into the lateral thigh. Immediate- and delayed-type hypersensitivity reactions were determined at 20 min and 24 h after the skin test, respectively. The patient received the vaccinations at 2-wk intervals. This protocol was approved by the Kurume University review board and the independent ethical committee. The patient gave written informed consent before the trial.

Cell lines and stable transfectants

HM-LCL is an HLA-DRB1*0403+ EBV-transformed lymphoblast cell line (provided by Dr. N. Emi, Nagoya University, Nagoya, Japan). To prepare HLA-DRA1*0101-expressing 293T cells, the *HLA-DRA1*0101* gene was cloned into the pCR3.1 vector (Invitrogen, Carlsbad, CA) by the TA cloning method and was electroporated into 293T cells. To obtain *HLA-DRB1*0403*, *HLA-DRB1*0405*, and *HLA-DRB4*0103* genes, each *HLA-DR* gene was cloned into a pcDNA3.1/V5-His TOPO vector (Invitrogen) by TA cloning. These *DR* genes were cut with *Hin*dIII and *Xba*I enzymes and were inserted into the pcDNA3.1/Hygro (Invitrogen). These genes were electroporated into HLA-DRA1*0101-expressing 293T cells, followed by selection with hygromycin B (Invitrogen) at a dose of 150 μg/ml.

Detection of peptide-specific IgG

Peptide-specific IgG levels in the plasma were measured by ELISA, as previously reported (12). In brief, after a 2-h incubation of the samples in the peptide-immobilized plates, the plates were washed and further incubated for 2 h with 1/1000 diluted rabbit anti-human IgG (γ-chain-specific; DAKO, Glostrup, Denmark). After washing, 100 μl of 1/100 diluted goat anti-rabbit Ig-conjugated HRP (En Vision; DAKO) was added to each well. After washing, 100 μl/well tetramethylbenzidine substrate solution (Kirkegaard & Perry Laboratories, Guildford, U.K.) was added, and the reaction was stopped by the addition of 1 M phosphoric acid. To determine IgG subclasses, rabbit Abs against human IgG1 and IgG2 were used.

Culture of PBMCs and establishment of a $UBE2V_{43-51}$ peptide-recognizing T cell line and its clone

PBMCs (2×10^6) of the patient EBL-101 before and after peptide vaccination were depleted of $CD8^+$ T cells using Dynabeads M-450 (Dynal, Oslo, Norway). The remaining cells were cultured in 48-well plates with the $UBE2V_{43-52}$ peptide (10 μg/ml) in RPMI 1640 medium with 5% human serum. On days 3 and 7, IL-2 was added to a final dose of 20 U/ml. On day 10, the cultured cells were harvested and then cultured with autologous PBMCs (1×10^4 cells/well), which were preloaded with or without the $UBE2V_{43-51}$ peptide (10 μg/ml). After 20-h incubation, the supernatants were collected, and the levels of IFN-γ were determined by ELISA. In some groups, 10 μg/ml of anti-HLA-A2 (BB7.2; mouse IgG2b), anti-HLA-DR (L243; mouse IgG2a), or anti-HLA-DP (B7/21; mouse IgG1) mAb was added to the wells. The $UBE2V_{43-51}$ peptide-stimulated PBMCs were seeded into 96-well, round plates at a cell dose of 10 cells/well in the presence of irradiated allogeneic PBMCs and PHA (10 μg/ml) with a culture medium consisting of 45% RPMI 1640 medium, 45% AIM-V medium (Life Technologies, Gaithersburg, MD), 10% FCS, 100 U/ml human rIL-2, and 0.1 mM MEM nonessential amino acid solution (Life Technologies). After 2 wk in the culture medium, reactivity to the $UBE2V_{43-51}$ peptide was examined by ELISA for IFN-γ, and a cell line designated UB-2 was established. Thereafter, limiting dilution at a cell dose of 0.5 cells/well was conducted, and a clone, designated UB-2.3, was established.

Assay of recognition

The UB-2 and UB-2.3 cells were incubated separately with the indicated stimulator cells, which were pulsed with peptides for 90 min. After a 20-h culture, the level of IFN-γ in the supernatant was determined by ELISA. To block dimerization of the $UBE2V_{43-51}$ peptide, the peptide was cultured with 2 mM DTT (Molecular Probes, Eugene, OR) in a cysteine-free DMEM medium (Life Technologies, Gaithersburg, MD; catalogue no. 21013-0247) without serum for 2 h in CO_2 incubator. HM-LCL cells were then added and cultured for additional 90 min. Thereafter, these peptide-pulsed HM-LCL cells were fixed with 1% paraformaldehyde for 5 min. After thoroughly washing the cells, these HM-LCL cells were cultured with effector cells. In some experiments HM-LCL cells were fixed with 1% paraformaldehyde before pulsation of the peptide to inhibit internalization of the pulsed peptide.

Flow cytometric analysis

To examine the phenotypes of cells, they were stained with anti-CD8, anti-CD4, anti-HLA-A2, or anti-HLA-DR mAb, followed by FITC-conjugated goat anti-mouse IgG. The results were analyzed by the CellQuest program (BD Biosciences, Mountain View, CA).

Assay of peptide-specific T cell precursors

The assay of peptide-specific $CD8^+$ T cell precursors was performed based on a previously reported culture method (13). Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μg/ml of the $UBE2V_{43-15}$ peptide in the wells of U-bottom, 96-well microculture plates (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The culture medium consisted of 45% RPMI 1640 medium, 45% AIM-V medium, 10% FCS, 100 U/ml IL-2, and 0.1 μM MEM nonessential amino acid solution. Half the medium was removed and replaced with new medium containing a corresponding peptide (20 μg/ml) every 3 days. After incubation for 14 days, these cells were harvested and tested for their ability to produce IFN-γ in response to T2 cells that were preloaded with either the $UBE2V_{43-51}$ peptide or the HIV peptide as a negative control. To estimate the frequency of $CD4^+$ T cells, $CD8^+$ T cell-depleted cells (1×10^5 cells/well) were incubated with 10 μg/ml of the $UBE2V_{43-15}$ peptide in the wells of U-bottom, 96-well microculture plates in 200 μl of culture medium consisting of RPMI 1640 medium with 5% human serum. On day 2, IL-2 was added to a final dose of 10 U/ml. On day 7, half the medium was removed and replaced with new medium containing the $UBE2V_{43-15}$ peptide (20 μg/ml), and 10 U/ml IL-2 was added on day 8. After incubation for 14 days, these cells were harvested and tested for their ability to produce IFN-γ in response to HLA-DRB1*0403-expressing 293T cells, which were preloaded with either the $UBE2V_{43-51}$ peptide or HIV peptide, in the presence of 50 U/ml IL-2. Both assays were performed in 12 wells, and cultured cells in one well were divided into four wells. Thereafter, two wells were used for control HIV peptide-pulsed cells, and the other two wells were used for the $UBE2V_{43-51}$ peptide-pulsed cells. Thus, cytokine release was assessed in two different ELISA wells.

Frequency assay of peptide-specific T cells

$CD8^+$ or $CD4^+$ T cells from the pre- or postvaccination PBMCs were positively isolated using the CD8 Positive Isolation Kit (Dynal, Oslo, Norway) or the CD4 Positive Isolation Kit (Dynal). $CD8^+$ or $CD4^+$ T cells (1600, 400, 100, and 25 cells/well) were then cultured with irradiated allogeneic PBMCs (5×10^4 cell/well), 10 μg/ml PHA, and 100 U/ml IL-2 in 24 wells of 96-well, round-bottom plates. On day 10, these cultured cells were harvested and stimulated with T2 cells (for CD8) or 293TDRB1*0403 cells (for CD4), which were pulsed with either $UBE2V_{43-51}$ peptide or the HIV peptide. The cultured cells in one well were divided into four wells, with two being used as control HIV peptide-pulsed cells and the other two being used for the $UBE2V_{43-51}$ peptide-pulsed cells. The level of IFN-γ in the supernatant was determined by ELISA. The wells that produced significantly ($p < 0.05$) IFN-γ in response to the $UBE2V_{43-51}$ peptide were judged to be positive.

Mass spectrometric analysis and peptide fractionation

The vaccinated $UBE2V_{43-51}$ peptide was analyzed by liquid chromatography (Gold Nouveau; Beckman, Palo Alto, CA) with a Vydac C_{18} (218TP54) column. Solution A was 0.1% trifluoroacetic acid (TFA), and solution B was 0.1% TFA/acetonitrile. The flow rate was 1 ml/min. To separate monomeric and dimeric forms, the $UBE2V_{43-51}$ peptide was fractionated by a 625LC liquid chromatography system (Waters, Milford, MA) using reverse phase HPLC with a Finepak SIL300 C_{18} T-7 column (Nippon Bunkou Kogyo, Tokyo, Japan). Solution A was 0.1% TFA, and solution B was a mixture of solution A and acetonitrile at a ratio of 2:3. The peptide was eluted at a flow rate of 1 ml/min.

Statistics

The statistical significance of the data was determined by two-tailed Student's t test, and $p < 0.05$ was considered statistically significant throughout the study.

Results

Marked elicitation of IgG against the vaccinated $UBE2V_{43-51}$ peptide

The patient EBL-101 was vaccinated with four different HLA-A2-binding peptides, $CypB_{172-179}$, $Lck_{422-430}$, $UBE2V_{43-51}$, and

$WHSC2_{141-149}$, with a 2-wk interval at a dose of 3 mg after confirmation that the peptide-specific CTL precursors were present in the PBMCs (data not shown). The patient's disease remained stable for 18 mo after the peptide vaccination. The serum levels of IgG specific to the administered peptides were measured, as IgG reactive to class I-binding tumor peptides can be detected in peptide-vaccinated cancer patients (9). As a result, IgG specific to the $UBE2V_{43-51}$ peptide, but not to the $CypB_{172-179}$ peptide, drastically increased after the third vaccination (Fig. 1*A*). No IgG against the other two peptides was detected, and the patient developed a strong delayed-type hypersensitivity reaction only against the $UBE2V_{43-51}$ peptide (data not shown). IgG specific to the $UBE2V_{43-51}$ peptide was absorbed by culturr in peptide-coated plates in an Ag-specific manner (Fig. 1*B*). In addition, IgG specific to the $UBE2V_{43-51}$ peptide consisted of both IgG1 and IgG2 subclasses (Fig. 1*C*), indirectly suggesting the in vivo elicitation of both Th1-type and Th2-type T cell responses.

Induction of $UBE2V_{43-51}$ peptide-recognizing $CD4^+$ cells after peptide vaccination

To investigate the mechanisms of elicitation of IgG against the $UBE2V_{43-51}$ peptide after the peptide vaccination, we tested the possibility of the participation of peptide-recognizing $CD4^+$ Th cells. Then we determined whether $UBE2V_{43-52}$ peptide-recognizing $CD4^+$ T cells could be detected after the peptide vaccination (Fig. 2). To exclude the influence of $UBE2V_{43-51}$ peptide-recognizing $CD8^+$ T cells, $CD8^+$ T cells were depleted before the in vitro culture. In the prevaccination PBMCs, the percentages of $CD4^+$ T cells were 24.9 and 32.6% before and after $CD8^+$ T cell depletion, respectively. In the PBMCs after the third vaccination, the percentages of $CD4^+$ T cells were 17.5 and 23.8% before and after $CD8^+$ T cell depletion, respectively. In both cases the percentage of $CD8^+$ T cells after $CD8^+$ T cell depletion was $<1\%$ (data not shown). As a result, IFN-γ-producing $CD4^+$ T cells in response to the $UBE2V_{43-51}$ peptide were detected in the PBMCs after the third peptide vaccination. IFN-γ production was inhibited by the addition of anti-HLA-DR mAb, but not by the addition of anti-HLA-DP mAb. Peptide-specific IL-6 production was not observed in this assay (data not shown). These results suggest that the vaccine-induced elicitation of $UBE2V_{43-51}$ peptide-specific IgG was the result of in vivo induction of peptide-specific $CD4^+$ Th cells.

A $UBE2V_{43-51}$ peptide-recognizing Th1-type $CD4^+$ T cell line and its clone

To further investigate the $UBE2V_{43-51}$ peptide-recognizing $CD4^+$ T cells, we tried to establish T cell lines recognizing the $UBE2V_{43-51}$ peptide. Because the patient homozygously expressed HLA-DRB1*0403 molecules, an HLA-DRB1*0403-expressing B lymphoblastoid cell line, HM-LCL, was used for the screening. Although the $UBE2V_{43-51}$ peptide has the ability to bind HLA-A2 molecules (6), the HM-LCL cells were negative for HLA-A2 molecules (Fig. 3). An established T cell line, designated UB-2, was positive for CD4, but not for CD8. In addition, UB-2 cells produced IFN-γ in response to the $UBE2V_{43-51}$ peptide when pulsed on the HM-LCL cells or on autologous PBMCs, whereas they failed to produce IFN-γ when pulsed on T2 cells (Fig. 4*A*). We further confirmed their HLA-DR restriction using 293T cells expressing specific HLA-DRB1 molecules (Fig. 4*B*). UB-2 and its clone, UB-2.3, produced a significant level of IFN-γ in response to the $UBE2V_{43-51}$ peptide only when pulsed on 293TDRB1*0403 cells; neither UB-2 nor UB-2.3 produced IL-4 or IL-6 (data not shown). These results indicate that peptide recognition of these Th1-type UB-2 and UB-2.3 cells was restricted to HLA-DRB1*0403 molecules.

We next determined whether the cloned UB-2.3 cells could recognize 293TDRB1*0403 cells, which were pulsed with various doses of the $UBE2V_{43-51}$ peptide (Fig. 5). As a result, UBE-2.3

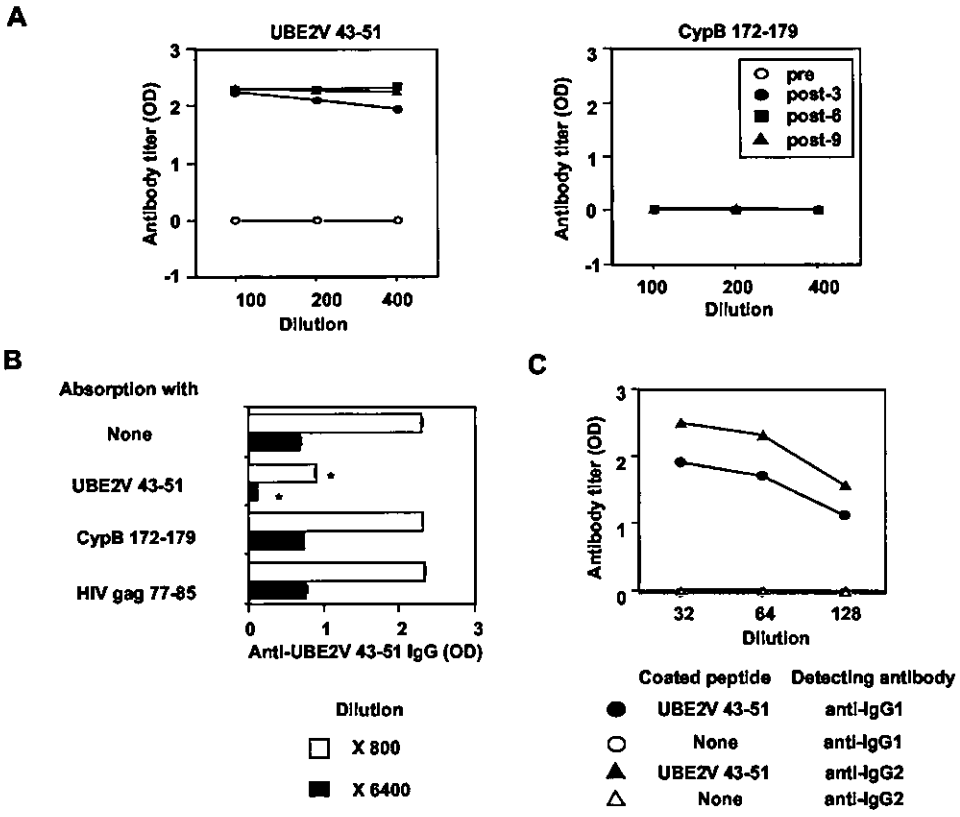

FIGURE 1. Elicitation of $UBE2V_{43-51}$ peptide-specific IgG after the peptide vaccination. *A*, The levels of IgG specific to either the $UBE2V_{43-51}$ peptide or the $CypB_{172-179}$ peptide in plasma of the patient EBL-101 pre- or postvaccination (third, sixth, and ninth) were determined by ELISA. *B*, Peptide-specific IgG in the plasma was absorbed in culturing in peptide-coated wells, and the levels of IgG specific to the $UBE2V_{43-51}$ peptide in the resultants were determined by ELISA. *, $p < 0.05$ was considered statistically significant. *C*, IgG subclasses reactive to the $UBE2V_{43-51}$ peptide in plasma after the ninth vaccination were determined by ELISA.

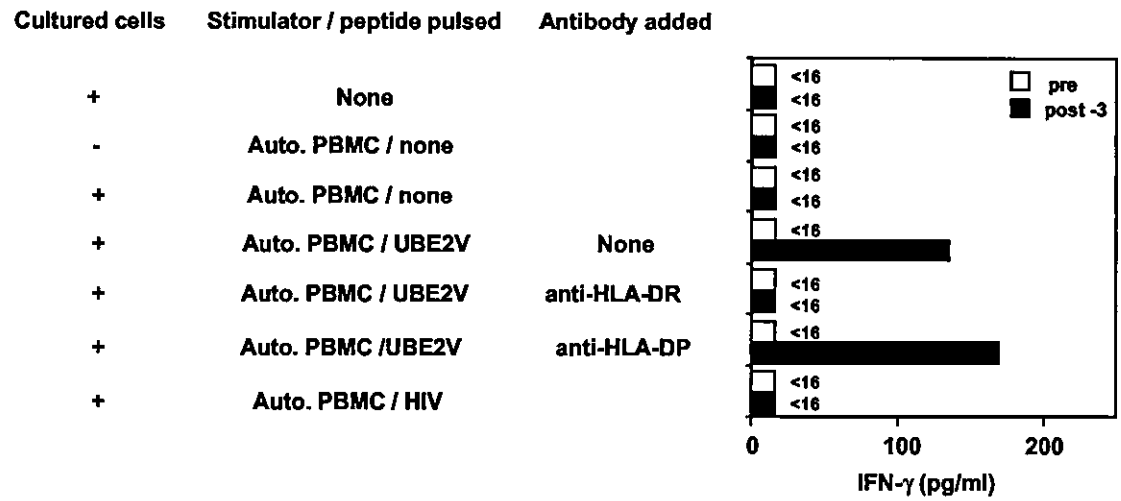

FIGURE 2. In vivo induction of HLA-DR-restricted CD4+ T cells. CD8+ T cell-depleted PBMCs before and after the third peptide vaccination were cultured with the $UBE2V_{43-51}$ peptide (10 μg/ml), as described in *Materials and Methods*. On day 10, the responding cells were stimulated with autologous PBMCs pulsed with either the $UBE2V_{43-51}$ peptide or the HIV peptide for 1 day. In some groups, anti-HLA-DR or anti-HLA-DP mAb was added at a dose of 10 μg/ml. The level of IFN-γ in the supernatant was determined by ELISA.

cells produced IFN-γ when 293TDRB1*0403 cells were pulsed with a relatively high dose (>1 μg/ml) of the $UBE2V_{43-51}$ peptide. In addition, we examined the expression of the UBE2V gene in 293T cells using the RT-PCR method. The result was that the 293T cells were positive for the *UBE2V* gene (data not shown).

Increased frequency of the UBE2V peptide-specific T cell precursors after peptide vaccination

It is important to kinetically estimate the frequency of CD8+ or CD4+ T cells reactive to the $UBE2V_{43-51}$ peptide after the peptide vaccination. We first estimated the frequency of peptide-specific CD8+ T cells by using our previously reported culture protocol (13). Whole PBMCs from the patient were repeatedly stimulated with the $UBE2V_{43-51}$ peptide in the presence of 100 U/ml IL-2. Although CD4+ T cells were not depleted, the reactivity of T cells against peptide-pulsed T2 cells could be judged to depend on HLA-A2-restricted T cells because HLA-DRB1*0403-restricted CD4+ T cells showed no reactivity against $UBE2V_{43-51}$ peptide-pulsed T2 cells (Fig. 4*A*). As shown in Fig. 6*A*, the reactivity of HLA-A2-restricted CD8+ T cells against the $UBE2V_{43-51}$ peptide was detected in one of 12 wells in the prevaccination PBMCs, but it was detected in two and three of 12 wells after the 6th and 12th peptide vaccinations, respectively. With regard to CD4+ T cells, CD8+ T cell-depleted cells were in vitro cultured with the $UBE2V_{43-51}$ peptide and were examined for their IFN-γ production in response to 293TDRB1*0403 cells that were prepulsed with the HIV peptide or the $UBE2V_{43-51}$ peptide. The result was that the HLA-DRB1*0403-restricted and $UBE2V_{43-51}$ peptide-reactive CD4+ T cells were induced only after the peptide vaccination. The reactivity of HLA-DR4-restricted CD4+ T cells against the $UBE2V_{43-51}$ peptide was not detected in the prevaccination PBMCs, but was detected in two, one, and two of 12 wells after the 3rd, 6th, and 12th peptide vaccinations. We performed a limiting dilution assay to accurately determine the frequencies of peptide-specific CD4+ or CD8+ T cells in the pre- and postvaccination PBMCs (Fig. 6*B*). Positively isolated CD8+ or CD4+ T cells from the pre- or postvaccination PBMCs were used for the assay. The positive percentage of CD8+ or CD4+ T cells was >90% (data not shown). As a result, the frequency of peptide-specific CD8+ T cells slightly increased after the 6th and 12th vaccinations, and the increase in the frequency of peptide-specific CD8+ T cells after the 12th vaccination was ~2-fold. In contrast, the frequency of peptide-specific CD4+ T cells increased prominently after the 3rd, 6th, and 12th vaccinations, and the increase in the frequency of peptide-specific CD4+ T cells after the 12th vaccination was >12-fold. These results indicate that the peptide vaccination increased the frequency of peptide-specific T cells, especially CD4+ T cells.

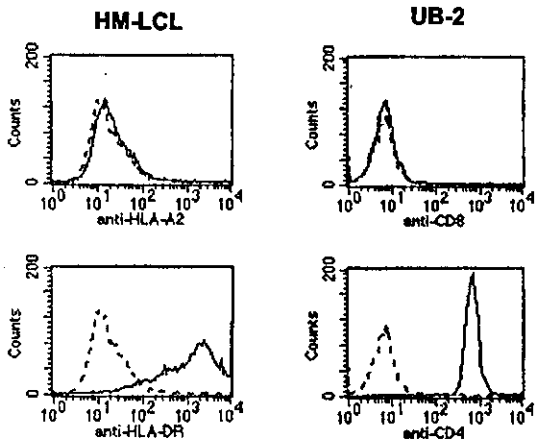

FIGURE 3. Flow cytometric analysis of HM-LCL and UB-2 cells. HM-LCL and UB-2 cells were stained with anti-HLA-A2 mAb, anti-HLA-DR mAb, anti-CD8 mAb, or anti-CD4 mAb, followed by FITC-conjugated goat anti-mouse IgG. The dotted lines represent staining without the first mAb.

Recognition of both monomeric and dimeric forms of the $UBE2V_{43-51}$ peptides

The $UBE2V_{43-51}$ peptide carries cysteine at the 6th position. Chromatography and mass spectrometric analysis were conducted on the vaccinated $UBE2V_{43-51}$ peptide (Fig. 7). Two peaks, with retention times of ~8.52 and ~10.33, were observed in chromatographic analysis. With regard to the mass spectrometric analysis, the peak at retention time ~8.52 had $(M+H)^+ = 1133.6$ and $(M + 2H)^{2+}/2 = 567.3$. The peak at retention time ~10.33 had $(M2 + 2H)^{2+}/2 = 1133.0$ and $(M2 + 3H)^{3+}/3 = 755.6$, and the presence of the latter fragment indicated that the peak at the later retention time (~10.33) was a dimer. We next investigated

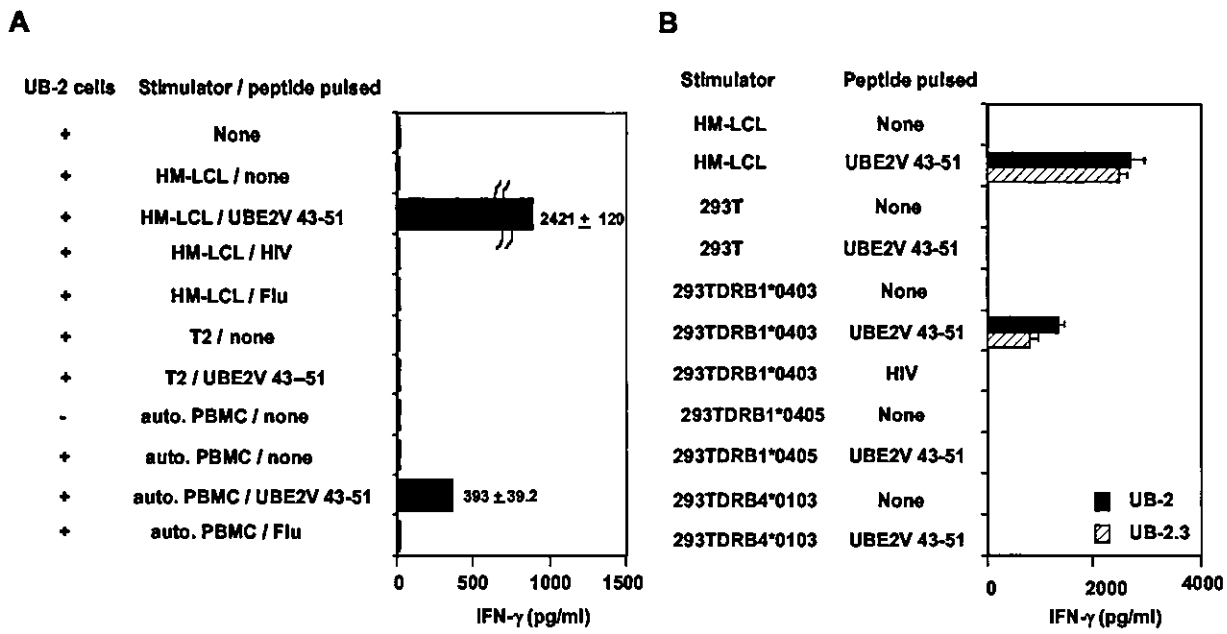

FIGURE 4. HLA-DRB1*0403-restricted recognition of the $UBE2V_{43-51}$ peptide by UB-2 and UB-2.3 cells. *A*, UB-2 cells were cultured with the indicated stimulator cells, which were pulsed with the indicated peptides (10 μg/ml) for 1 day. The level of IFN-γ in the supernatant was determined by ELISA. *B*, UB-2 and UB-2.3 cells were cultured with 293T cells or its stable transfectants, which were pulsed with or without the $UBE2V_{43-51}$ peptide (10 μg/ml) for 1 day. The level of IFN-γ in the supernatant was determined by ELISA.

whether replacement of the 6th cysteine with alanine or serine would have any influence on recognition by UB-2 and UB-2.3 cells and found that the substitution resulted in loss of recognition by these cells (data not shown).

To directly investigate which forms of $UBE2V_{43-51}$ peptide could be recognized by the $UBE2V_{43-51}$ peptide-recognizing $CD4^+$ T cells, monomeric and dimeric peptides were fractionated using reverse phase HPLC (Fig. 8*A*). As shown in Fig. 8*B*, two peaks were observed in IFN-γ production by UB-2 and UB-2.3 cells in response to the HM-LCL pulsed with each fraction. Next, the effect of a reduction reagent, DTT, on recognition of the fractionated monomeric $UBE2V_{43-51}$ peptide (fractions 15–17 in Fig. 8*B*) by UB-2 and UB-2.3 cells was examined to exclude the possibility that the fractionated monomeric $UBE2V_{43-51}$ peptide became dimeric during the in vitro culture. However, both UB-2 and UB-2.3 cells recognized the DTT-treated monomeric $UBE2V_{43-51}$ peptide (Fig. 9*A*). There remains yet another possibility, i.e., that the dimeric $UBE2V_{43-51}$ peptide was internalized into HM-LCL cells, reduced, and subsequently presented in monomeric form in the context of HLA-DRB1*0403 molecules, as intracellular processing can induce reduction (14). However, both UB-2 and UB-2.3 cells produced IFN-γ in response to the fractionated dimeric $UBE2V_{43-51}$ peptide (fractions 25–27 in Fig. 8*B*) even when they were pulsed on the fixed HM-LCL cells (Fig. 9*B*). These lines of evidence indicate that these $CD4^+$ T cells recognized the $UBE2V_{43-51}$ peptide in either the monomeric or dimeric form.

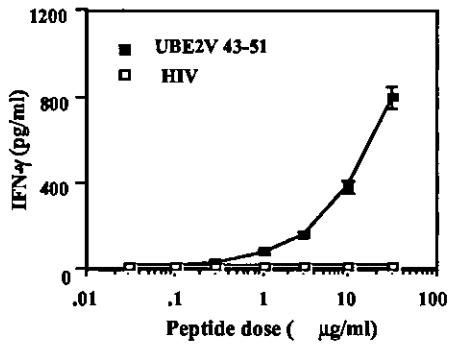

FIGURE 5. Dose-dependent recognition of the $UBE2V_{43-51}$ peptide by the cloned $CD4^+$ T cells. The cloned UB-2.3 cells were incubated for 18 h with 4×10^4 293TDRB1*0403 cells, which were cultured with the indicated peptides at the indicated doses for 90 min. The level of IFN-γ in the supernatant was determined by ELISA. The HIV peptide was used as a control.

Discussion

A notable finding of the present study was that vaccination with tumor peptides binding to HLA-A2 molecules resulted in the in vivo induction of HLA-DR4-restricted $CD4^+$ T cells in an HLA-$A2^+$ lung cancer patient. After observing the dramatic induction of IgG specific to the $UBE2V_{43-51}$ peptide after peptide vaccination, we investigated the possibility that $CD4^+$ Th cells participated in this induction based on the following. First, in vivo generation of Ag-specific IgG generally requires a cytokine from Th cells (11). Second, marked elicitation of IgG specific to the $UBE2V_{43-51}$ peptide was observed, but there was no apparent induction of IgG specific to the other three peptides. This finding indicates that the dramatic induction of IgG specific to the $UBE2V_{43-51}$ peptide was not the result of nonspecific stimulation of B cells. In this study we found that HLA-DRB1*0403-restricted and $UBE2V_{43-51}$ peptide-recognizing $CD4^+$ Th cells were induced in the patient. Although peptides binding to MHC class II molecules have been suggested to be 12–25 aa in length, the core sites anchored to MHC class II molecules are sufficient even at a length of only about nine amino acids (15). The amino acid sequence of the $UBE2V_{43-51}$ peptide could conform to the motif for HLA-DRB1*0403 molecules. Judging from the binding motif of HLA-DRB1*0406 molecules (16), although this motif is different by one amino acid from that of HLA-DRB1*0403 molecules, the leucine at the first position and serine at the sixth position are thought to be the amino acids anchored to the HLA-DRB1*0403 molecules. Several researchers

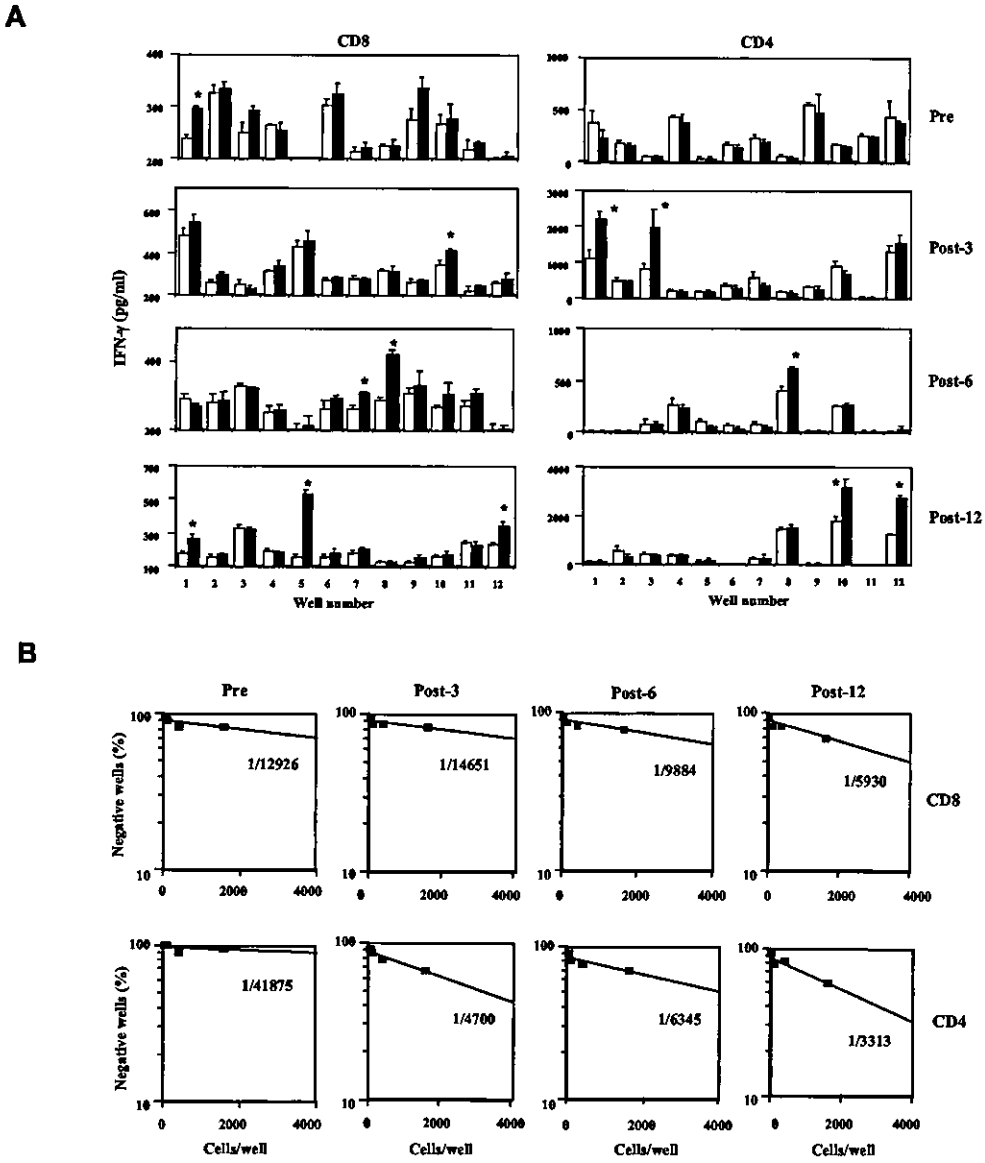

FIGURE 6. Increased frequency of peptide-specific T cell precursors after peptide vaccination. *A*, Whole PBMCs, for an assay of $CD8^+$ T cell precursors, and $CD8^+$ T cell-depleted PBMCs, for an assay of $CD4^+$ T cell precursors, before and after the 3rd, 6th, and 12th peptide vaccinations were cultured with the $UBE2V_{43-51}$ peptide, as described in *Materials and Methods*. On day 14, these cultured cells were harvested and stimulated with T2 cells (for CD8) or 293TDRB1*0403 cells (for CD4), which were pulsed with either the HIV peptide (■) or the $UBE2V_{43-51}$ peptide (■). These cultured cells in one well were divided into four wells, with two being used for control HIV peptide-pulsed cells and the other two being used for the $UBE2V_{43-51}$ peptide-pulsed cells. Thus, cytokine release was assessed in two different ELISA wells. The level of IFN-γ in the supernatant was determined by ELISA. *, $p < 0.05$ was considered statistically significant. *B*, The several doses of $CD8^+$ or $CD4^+$ T cells were cultured in 24 wells of 96-well, round-bottomed plates, as described in *Materials and Methods*. On day 10, these cultured cells were harvested and stimulated with T2 cells (for CD8) or 293TDRB1*0403 cells (for CD4), which were pulsed with either $UBE2V_{43-51}$ peptide or the HIV peptide. The cultured cells in one well were divided into four wells, with two being used as control HIV peptide-pulsed cells and the other two being used for the $UBE2V_{43-51}$ peptide-pulsed cells. The level of IFN-γ in the supernatant was determined by ELISA, and the wells that produced IFN-γ significantly ($p < 0.05$) in response to the $UBE2V_{43-51}$ peptide were judged to be positive. The fraction numbers in the figures are the frequency of peptide-specific T cells.

have reported that $CD4^+$ T cells can recognize nine or 10 aa in the context of MHC class I molecules (17, 18), and long peptides recognized by both $CD8^+$ T cells and $CD4^+$ T cells have been identified (19, 20). Nevertheless, the present study provides the first evidence that vaccination with class I binding peptides can induce HLA-DR4-restricted $CD4^+$ T cells in vivo, and that a nonamer peptide can be recognized by both Ab and T cells with either CD8 or CD4 molecules.

Another notable finding is that a dimeric form of the nonamer peptide was recognized by HLA-DR4-restricted $CD4^+$ T cells. Because the $UBE2V_{43-51}$ peptide has a cysteine at the sixth position and cysteine can be easily oxidized as a result of disulfide bondage, we investigated the influence of dimerization on recognition by $CD4^+$ T cells. MHC class I binding peptides containing cysteine have been reported to decrease immunogenicity for T cells as a result of either cysteinylation or dimerization (17, 21). In the present study we demonstrated that both monomeric and dimeric peptides could be recognized by HLA-DR-restricted $CD4^+$ T cells. Although the precise structure of the complex formed by binding of dimeric peptides to HLA-DR molecules is