

Phase 1 Clinical Study of Cyclophilin B Peptide Vaccine for Patients With Lung Cancer

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Summary: Cyclophilin B (CypB) possesses two antigenic epitopes (CypB_{84–92} and CypB_{91–99}) recognized by HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes (CTLs). To determine the safety of CypB-derived peptides and its ability to generate antitumor immune responses, patients with advanced lung cancer received subcutaneous vaccinations of these peptides or their modified peptides. All 16 patients were vaccinated with CypB_{91–99} or its modified peptide, whereas only two patients were vaccinated with the modified CypB_{84–92}, as immediate-type hypersensitivity to CypB_{84–92} or its modified peptide was observed in the remaining patients. No severe adverse events were associated with the vaccination. No significant increase in cellular responses to either peptides or tumor cells was observed in the postvaccination PBMCs by the conventional CTL assays in any patients tested. These results suggest that the vaccination of CypB_{91–99} peptide was safe, but failed to induce objective immune responses at this regimen. **Key Words:** Cytotoxic T lymphocyte-peptides—Immunotherapy—Lung cancer—Phase 1 study.

Recent advances in molecular biology and cellular immunology in the field of tumor immunology have resulted in identification of a large number of antigens and epitopes recognized by HLA class I restricted cytotoxic T lymphocytes (CTLs) from melanomas and epithelial cancers (1–6), thereby opening the door to new peptide-based specific immunotherapy of cancers. Several subsequent clinical studies of patients with melanoma have shown increased immune responses to vaccinated peptides and tumor cells in PBMCs during the postvaccination period (7–11). To our knowledge, however, there have been no reports of peptide-based immunotherapy for patients with lung cancer. Lung cancer is among the most commonly occurring malignancies in the world and is one of the few that continues to show an increasing

incidence (12). The prognosis for advanced lung cancer is extremely poor, despite recent clinical trials using gene therapy and numerous chemotherapeutic agents (13,14). Therefore, the development of new treatment modalities is needed, one of which should be specific immunotherapy. We have previously reported that the two cyclophilinB (CypB)-derived peptides and their modified peptides, which are recognized by the HLA-A24-restricted CTLs established from T cells infiltrating into lung adenocarcinoma, can induce CTLs from patients with cancer (15). In this report, we investigated the safety and immune responses in patients with advanced lung cancer vaccinated with CypB peptides combined with IFA.

MATERIALS AND METHODS

Peptide Selection

The peptides used in the current study were prepared under Good Manufacturing Practice conditions by the

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Multiple Peptide System (San Diego, CA, U.S.A.): CypB-derived peptide at sequence positions 84–92 (CypB_{84–92}, KFHRVIKDF), its modified peptide in which tyrosine replaced phenylalanine at peptide position 2 (CypB_{84–92}2F-Y, KYHRVIKDF), at positions 91–99 (CypB_{91–99}, DFMIQGGDF), and its modified peptide in which tyrosine replaced phenylalanine at position 2 (CypB_{91–99}2F-Y, DYMIQGGDF). Peptides were > 95.5% pure with endotoxin levels of less than 0.1 endotoxin units per mL. The peptides were supplied as a white powder soluble in dimethylsulfoxide (DMSO), except for CypB_{84–92}2F-Y, which was soluble in saline. Each peptide was dissolved in DMSO or saline at 20 mg/mL, aseptically aliquoted, and stored at –80°C. Stock solutions were diluted with saline for CypB_{84–92} and CypB_{84–92}2F-Y, or with 0.004 N NaOH-saline for CypB_{91–99} and CypB_{91–99}2F-Y just before use. The other peptides used for in vitro assays as controls were an HIV-derived peptide with an HLA-A24-binding motif (RYLRQQLGI), and an Lck-derived peptide at position 488 to 497 (Lck_{488–497}, DYLRSVLEDF), which was shown to induce HLA-restricted and tumor-specific CTLs (16). An EBV-derived peptide with an HLA-A24-binding motif (TYGPVFMCL) was also used as a control peptide.

Eligibility Criteria and Clinical Protocol

All patients had histologically confirmed lung cancers and underwent a complete clinical evaluation including

x-rays and CT scans of all tumor sites. All patients were confirmed to be HLA-A24⁺ by a conventional serologic method and by staining of PBMCs with anti-A24 mAb, as reported previously (4–6). Patient characteristics are listed in Table 1. No patients had received any treatments, steroids, or any other immunosuppressive drugs in the 4 weeks before the study. For the skin test, 10 µg of CypB_{84–92} and CypB_{91–99} peptides for the six patients, or 10 µg of their modified peptides for the other six patients in 50 µL of saline was independently injected intradermally using a tuberculin syringe with a 26-gauge needle. Ten µg was chosen since this was 1/100 of 1 mg, and 1/100 dose was generally used for the skin test of the other drugs. Immediate- and delayed-type hypersensitivity reactions were determined at 20 minutes and 24 hours after the skin test, respectively. If immediate-type hypersensitivity was negative, the peptide was injected as follows: group 1 patients (n = 3) received 1 mg each of the native peptides; group 2 patients (n = 5) received 3 mg each of the native peptides; group 3 patients (n = 3) received 1 mg each of the modified peptides; and group 4 patients (n = 5) received 3 mg each of the modified peptides. For groups 1 and 3, 1.5 mL of the peptide solution at 1 mg/mL was mixed with an equal volume of the IFA (Montanide ISA-51, Seppic, France) and emulsified in 5-mL sterilize-glass syringes, and 2 mL was injected into the subcutaneous tissue of the anterior thigh. For groups 2 and 4, 2 mL of the peptide solution at 2 mg/mL was mixed with an equal volume of IFA and

TABLE 1. Demographics, adverse events, and clinical evaluation of CypB vaccination

Group (patient no.)	Therapy before vaccination*	Peptide (dose)	Number of vaccination	Age	Sex	PS	Stage†	Adverse events	Clinical response at		Time to progression (weeks)	Overall survival (weeks)
									5 weeks	11 months		
1 (001)	OP/Chemo	CypB _{91–99} (1 mg)	18	63	Female	0	IIb	Local reaction	SD	PD	53	92+
1 (002)	Chemo	CypB _{91–99} (1 mg)	14	63	Female	1	IV	Local reaction	SD	PD	27	89+
1 (003)	Chemo/Rad	CypB _{91–99} (1 mg)	5	41	Male	1	IV	Local reaction	SD	PD	8	33
2 (004)	OP/Chemo	CypB _{91–99} (3 mg)	2	47	Female	2	IV	None	NE§	NE	NE	3
2 (005)	OP	CypB _{91–99} (3 mg)	4	74	Female	0	IV	Local reaction	SD	SD	—	77+
2 (006)	OP/Chemo	CypB _{91–99} (3 mg)	1	65	Female	1	IV	None	NE	NE	NE	43
2 (007)	Chemo/Rad	CypB _{91–99} (3 mg)	8	55	Male	2	IV	None	SD	PD	11	38
2 (008)	OP	CypB _{91–99} (3 mg)	10	78	Female	0	IV	Local reaction	SD	SD	—	75+
3 (011)	OP	CypB _{91–99} 2F-Y (1 mg)	5	66	Male	0	IV	Local reaction	SD	PD	8	53+
3 (012)	OP/Chemo/Rad	CypB _{84–92} 2F-Y and CypB _{91–99} 2F-Y (1 mg)	4	50	Male	1	IV	Local reaction	SD	PD	5	40+
			6									
3 (013)	Chemo	CypB _{91–99} 2F-Y (1 mg)	6	50	Male	2	IV	Local reaction	SD	PD	8	21
4 (014)	Chemo	CypB _{84–92} 2F-Y (3 mg)	2	64	Male	2	IIIa	Local reaction	NE	NE	NE	7
4 (015)	Chemo/Rad	CypB _{84–92} 2F-Y (3 mg)	5	70	Male	1	IV	Local reaction	SD	PD	20	38+
4 (016)	OP	CypB _{84–92} 2F-Y and CypB _{91–99} 2F-Y (3 mg)	3	63	Male	1	IV	Local reaction	SD	PD	5	16
			6									
4 (017)	Chemo	CypB _{91–99} 2F-Y (3 mg)	4	77	Female	1	IV	Local reaction	SD	PD	3	17
4 (018)	Chemo	CypB _{91–99} 2F-Y (3 mg)	6	45	Male	1	IV	Local reaction	SD	PD	4	15

* Therapy before vaccination was noted. OP, Surgery, Chemo, Chemotherapy; Rad, radiotherapy.

† UICC-TNM classification of malignant tumors was used for determination of clinical stage.

‡ Toxicity-grading criteria of the Japanese Clinical Oncology Group were used for grading of adverse events.

§ Not eligible for evaluation.

emulsified in the 5-mL glass syringes, and 3 mL was injected into the subcutaneous tissue. It was intended that all patients would receive at least three vaccinations at 2-week intervals. This protocol was reviewed and approved by the Kurume University Review Board and the Independent Ethical Committee (Protocol numbers 9905 and 9910). All patients were required to comprehend and sign an informed consent form before the clinical trial.

Response Evaluation

All known sites of disease were evaluated 7 days after every third vaccination, and these findings were compared with those obtained before the vaccinations. Cellular immune responses in the postvaccination PBMCs were evaluated 7 days after every third vaccination. Patients who received less than three vaccinations were excluded from clinical and immunologic evaluations. Patients were assigned a response category according to the following response criteria: (i) a complete response was considered to be a complete disappearance of all clinical evidence of the indicated lesion for a minimum of 4 weeks; (ii) a partial response was defined as a decrease of $\geq 50\%$ in the product of the perpendicular diameters of the indicator lesion for a minimum of 4 weeks; (iii) progressive disease was defined as an increase of $\geq 25\%$ in the product of the perpendicular diameters of the indicator lesion or as an appearance of tumors in a new region; and (iiii) stable disease (SD) was defined as any variation of the indicator lesion not meeting the above three criteria.

Cellular Immune Responses

PBMCs were cultured in vitro for 7 days with or without the corresponding peptide (10 $\mu\text{g/mL}$) used for in vivo vaccination in wells of a 24-well culture plate (2 mL/well). These cells were harvested, then tested for their ability to produce IFN- γ in response to HLA-A24⁺11–18 lung adenocarcinoma cells or HLA-A24⁺QG56 lung squamous cell carcinoma cells, or to produce IFN- γ in the absence of tumor cells as a negative control in triplicate assays at three different E:T ratios by ELISA (limit of sensitivity: 10 pg/mL). The effector cells were also tested for their ability to produce IFN- γ in response to C1R-A2402 cells alone as a negative control, or to those pulsed with either a corresponding peptide or an irrelevant HIV peptide in triplicate assays at three different E:T ratios. The effector cells were further expanded in the complete medium for 21–25 days, and were tested for their surface phenotypes and their cytotoxic activity by a 6-hour ⁵¹Cr-release assay at different

E:T ratios in triplicate assays. The target cells were 11–18, QG56 cancer cells, VA13 fibroblast cells, and HLA-A24⁺ PHA-activated normal T cells.

RESULTS

Demographics, Skin Tests, Adverse Events, and Clinical Evaluation

Sixteen patients with advanced lung cancer (inoperable stages IIIa, IIIb, and IV) were treated in this regimen, and their demographics are given in Table 1. The histologic diagnosis was adenocarcinoma (nos. 001–008, 011, 012, and 016–018), squamous cell carcinoma (no. 013), large-cell carcinoma (015), or small-cell carcinoma (014). Immediate-type hypersensitivity to CypB_{84–92} and its modified peptide was observed in skin tests before vaccination in all but two (nos. 012 and 016) patients. A representative skin reaction is shown in Figure 1. Subsequently, all 16 patients received vaccinations with CypB_{91–99} (n = 8) or its modified peptide (n = 8), while two patients (nos. 012 and 014) received the CypB_{84–92}F-Y peptide. The numbers of injections varied among patients from 1 to 18, with this number being primarily dependent on patients' own requests and also on clinical conditions as evaluated by the corresponding physicians. No delayed-type hypersensitivity was observed in any of the patients before or after vaccination.

All 16 patients were evaluated for all common toxicities; the overall toxicities are given in Table 1. The CypB peptide vaccine was well tolerated, but 13 of 16 patients had grade I local reactions at the injection sites. No medication was needed for these local immune reactions. No other adverse events were observed in this clinical trial. The 13 patients receiving more than three vaccinations were eligible for evaluation of clinical responses at two time points, 5 weeks after the first vaccination and at 11 months, which was the median observation time for all 16 patients (Table 1). All 13 patients were evaluated as having SD at 5 weeks. At 11 months after the first vaccination, two patients (nos. 005 and 008) vaccinated with 3-mg injections of CypB_{91–99} peptide were clinically SD, whereas the other 11 patients showed progressive disease (PD). The median time to progression of the four patients vaccinated with the CypB_{91–99} peptide or of the seven patients vaccinated with the modified peptides was 25 or 8 weeks, respectively. The overall survival of the six patients vaccinated with the CypB_{91–99} peptide or the seven patients vaccinated with the modified peptides was 67+ and 29+ weeks, respectively.

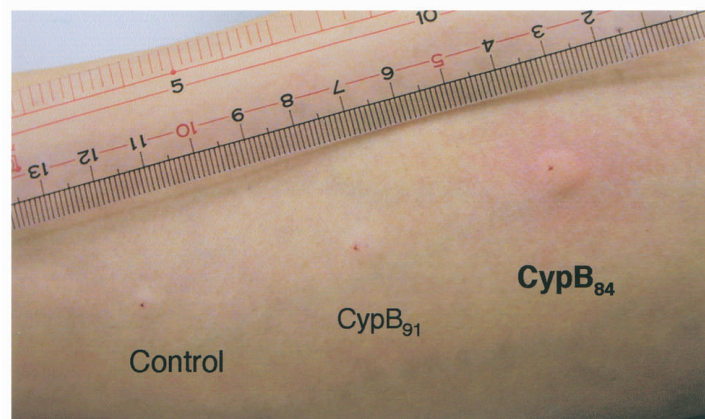
**A. Pre-injection****B. 20 min after**

FIG. 1. Immediate type hypersensitivity to the CypB₈₄₋₉₂ peptide. A skin test on one patient (no. 001) is shown. (A) Before injection, and (B) 20 minutes after injection. CypB₈₄, CypB₈₄₋₉₂; CypB₉₁, CypB₉₁₋₉₉.

Cellular Immune Responses

Levels of IFN- γ production in response to HLA-A24⁺ tumor cells by the postvaccination PBMCs from three patients (nos. 005, 008, and 017) were significantly higher ($p < \text{at least } 0.05$) than either those in response to QG-56 tumor cells or levels of IFN- γ production by prevaccination PBMCs in response to both 11-18 and QG-56 tumor cells (Fig. 2A). In the other nine patients, no significant levels of IFN- γ production in response to HLA-A24⁺ tumor cells were observed. Significant levels of IFN- γ production in response to the vaccinated peptide were not observed in any of the samples under the experimental conditions used. Similarly, an increase in HLA-A24-restricted and tumor-specific CTL activity by a 6-hour ⁵¹Cr-release assay was not observed in the postvaccination PBMCs of any patients, except for one (no. 008) who received 3-mg injection of CypB₉₁₋₉₉ (Fig.

2B). Pre and postvaccination PBMCs from patient no. 001 showed equal levels of HLA-A24-restricted and tumor-specific cytotoxicity (data not shown). Low levels of HLA-nonrestricted cytotoxicity against tumor cells, but not against PHA-blast cells, were observed in the pre and postvaccination PBMCs of two patients (nos. 002 and 007) (data not shown). No cytotoxicity was observed in either the pre or postvaccination PBMCs of the remaining three patients (nos. 003, 005, and 011).

Pre and postvaccination PBMCs of the six patients of groups 1 and 2 were tested for their ability to produce IFN- γ and IL-6 to study whether the vaccination induced or increased a Th1-like response to the vaccinated peptide. There was no significant difference between the pre and postvaccination samples with regard to the relative ratios of the amount of IFN- γ and that of IL-6 in the majority of patients tested, and the result of one representative case is shown in Figure 2C.

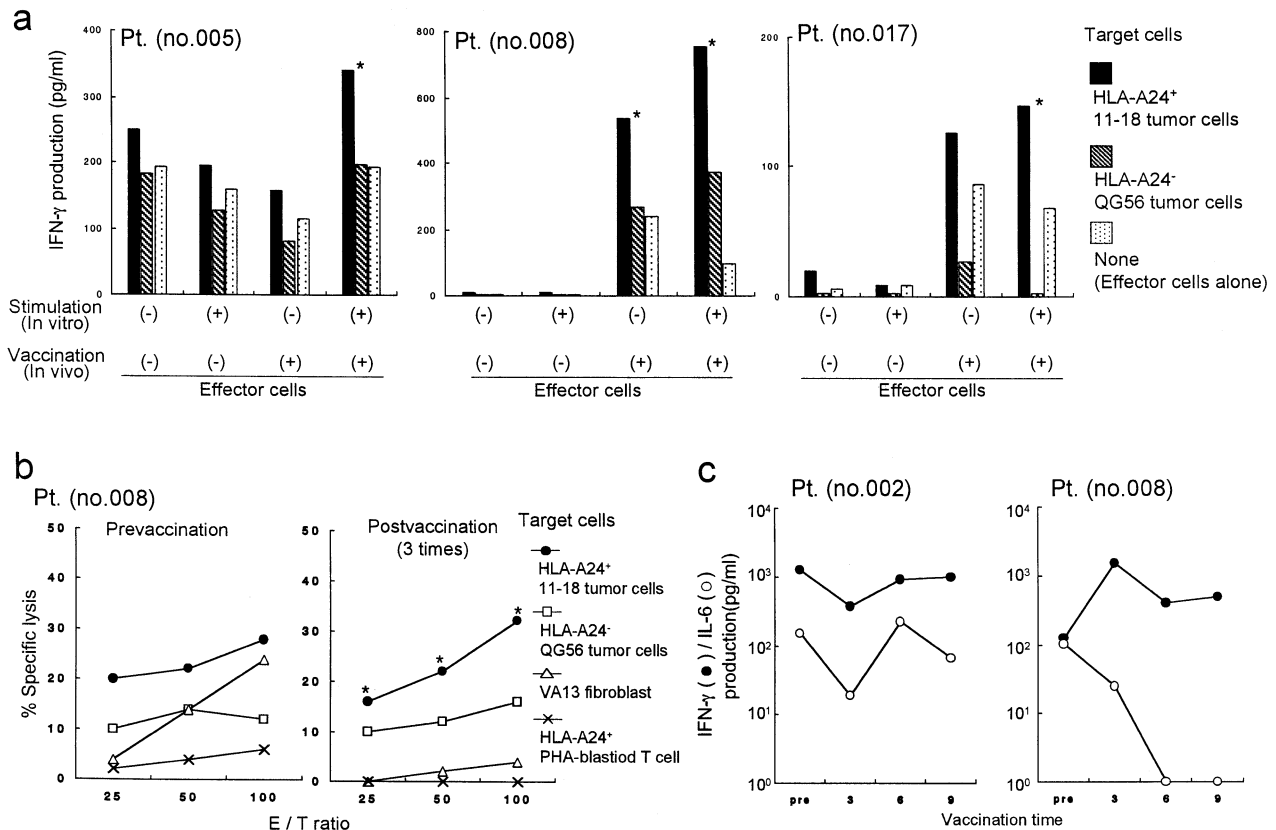


FIG. 2. CTL responses to tumor cells. (A) Pre and postvaccination (3 times) PBMCs from three patients (nos. 005, 008, and 017) were stimulated for 7 days in vitro with or without the vaccinated peptide and were tested for their ability to produce IFN- γ by recognition of HLA-A24⁺ or HLA-A24⁻ lung cancer cells at E:T ratios of 4:1 in triplicate assays. The mean value is shown in the figure. *Mean value of IFN- γ by recognition of HLA-A24⁺ tumor cells was significantly ($p < 0.05$) higher than that by recognition of HLA-A24⁻ tumor cells or that produced by effector cells alone. A two-tailed Student t test was used for the statistical analysis. (B) Cytotoxicity by a 6-hour Cr-release assay in the pre and postvaccination PBMCs of one patient (no. 008) is shown. Values represent the means of % specific lysis of the triplicate determinants. *Cytotoxicity against HLA-A24⁺ tumor cells was significantly ($p < 0.05$) higher than that against the other target cells. (C) Pre and postvaccination PBMCs of the six patients of groups 1 and 2 were incubated in vitro with 10 μ mol/L of the CypB₉₁₋₉₉ peptide for 5 days, and IFN- γ and IL-6 levels in the cell-free supernatants were measured by ELISA, as reported previously (15,16). The results of two patients (nos. 002 and 008) are shown.

DISCUSSION

Some adverse reactions to the CypB vaccination were expected, as CypB is a self-antigen playing an important role in protein folding and having peptidyl-prolyl isomerase activity, which is crucial to the cellular proliferation of T lymphocytes and other cell types (17,18). The region of CypB at positions 85–100, which includes the two peptides used in the vaccinations, is important because of its biologic activity of signal transmission, and, thus, is highly conserved in other members of the family of human cyclophilins and also in those of many other species, including fungi (19,20). However, no adverse reaction, except for grade I local reactions at the injection sites, was observed with this regimen. Cyclophilin has been reported to be one of the self-antigens involved in autoallergy of atopic dermatitis (21,22). Furthermore, the

sequences of CypB₈₄₋₉₂ and CypB₉₁₋₉₉ show high homology to the corresponding sequence of cyclophilins of other species including fungi and other bacteria that are well known as allergens. The CypB₈₄₋₉₂ peptide also induces immediate hypersensitivity not only patients with lung cancer, but in the majority of healthy donors (23). Furthermore, these species-derived peptides induce immediate hypersensitivity in healthy donors. These results suggest that the CypB₈₄₋₉₂ peptide contains epitopic regions recognized by human B cells and, thus, is responsible for immediate-type hypersensitivity in humans. Environmental exposure to these species-derived sequences corresponding to the CypB₈₄₋₉₂ might facilitate the constitutional production of anti-CypB₈₄₋₉₂ IgE.

Increased cellular responses to tumor cells were rarely detectable in postvaccination (three times) PBMCs by the conventional bulk-culture method with IFN- γ release

assay. Further, the increased cellular responses were not observed in any patients other than patient no.008 by the standard ^{51}Cr -release assay. These results indicate that the CypB vaccination failed to induce objective immune responses to either peptide or tumor cells under the conventional CTL assays at the used regimen. An assay using the labeled HLA class I-A tetramer was recently carried out to monitor immune responses (24,25). Therefore, we prepared a labeled HLA-A24 tetramer loaded with CypB₉₁₋₉₉ peptide and used it to monitor the post-vaccination PBMCs of two patients (nos. 001 and 005) in this study, but the percentage of positive cells was < 0.5% of the gated CD8⁺ cells. This tetramer assay was not conducted for the other ten cases because only a limited number of PBMCs was available for in vitro analyses.

The present results indicated that the vaccination of CypB₉₁₋₉₉ peptide was safe, but failed to induce immune responses at the used regimen. Further clinical studies with different regimen shall be taken to determine whether CypB peptide is suitable in use as cancer vaccine for the treatment of patients with lung cancer.

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