Nonmutated Self-Antigen-Derived Cancer Vaccine Peptides Elicit an IgE-Independent but Mast Cell-Dependent Immediate-Type Skin Reaction without Systemic Anaphylaxis[1]

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We previously reported an unexpected phenomenon, i.e., several cancer vaccine peptides, including a cyclophilin B-derived peptide (CypB-84), elicited an immediate-type skin reaction in prevaccination skin tests. These peptides were prohibited in the subsequent vaccinations because of a possible induction of systemic anaphylaxis. In this study, we investigated mechanisms involved in the peptide-elicited inflammatory reactions in BALB/c mice whose MHC class I molecule (K^d) shared similar binding motifs with the human HLA-A24 molecule. Among 11 peptides tested, all of which had been scheduled for use in clinical trials with HLA-$A24^+$ cancer patients, three peptides (CypB-84, ART1-170, and ART4-13) elicited immediate footpad reactions in BALB/c mice similar to the skin reactions in humans. The footpad reaction was also observed in C57BL/6, athymic *nu/nu*, and CB17-SCID mice, but not in mast cell-deficient $WBB6F_1{}^{w/wv}$ mice, indicating the reaction was not mediated by specific immunity, but was mast cell-dependent. Furthermore, the reactions were not correlated to in vivo antitumor effects of the peptides. An anaphylaxis was not elicited when the peptides were systemically injected due to a very rapid clearance of the peptides from the plasma by in vivo degradation. These results suggest that certain peptides of cancer vaccine candidates exhibit an IgE-independent but mast cell-dependent inflammatory response with no elicitation of systemic anaphylaxis, and may provide new insights for further development of peptide-based vaccinations for cancer patients. *The Journal of Immunology,* **2006, 176: 857–863.**

Recent progress in immunology has enabled the identification of cancer Ags recognized by tumor-specific CTL. Our group, along with others, previously investigated the tumor Ags recognized by class I-HLA-restricted CTLs established from T cells infiltrating into cancer tissues, and identified numerous cancer Ags (1, 2). Our clinical protocols for cancer vaccine trials include prevaccination skin tests to prevent an anaphylactic response in patients (3, 4). Several vaccine peptides expressed positive reactions in these prevaccination tests, and thus a substantial number of the vaccine peptides could not be used for the actual vaccination (3, 4). For example, the CypB-84 peptide for HLA-A24-positive patients elicited an immediate skin reaction in 100% (16 of 16) of the lung cancer patients as well as in 95.1% (39 of 41) of healthy volunteers, and thus CypB-84 was excluded from the vaccine candidate peptides in further clinical trials (4). However, it is not clear whether the skin test-positive peptides will actually elicit anaphylaxis. To find out, we tried to establish murine models of human cancer vaccines for HLA-A24-positive patients using H-2^d haplotype BALB/c mice, because both the human HLA-A2402 and mouse H-$2K^d$ molecules share similar Ag peptide-binding motifs (5). Both the antitumor effects of the vaccination and local immediate hypersensitivity to the vaccine peptides were confirmed in the models, and the systemic anaphylactic reaction was further analyzed.

Materials and Methods

Peptides

The peptides used in this study were prepared for clinical use, under the conditions of good manufacturing practice by Multiple Peptide Systems. The following peptides were used: the human SART1-derived peptide at positions 690–698 (SART1-690, EYRGFTQDF), SART2-derived peptides at positions 899–907 (SART2-899, SYTRLFLIL), SART3-derived peptides at positions 109–118 (SART3-109, VYDYNDHVDL) and 315–323 (SART3-315, AYIDFEMKI), lck-derived peptides at positions 208–216 (lck-208, HYTNASDGL) and 486–494 (lck-486, TFDYLRSVL), cyclophilin B-derived peptides at positions 84–92 (CypB-84, KFHRVIKDF) and 91–99 (CypB-91, DFMIQGGDF), ART1-derived peptide at position 170–178 (ART1-170, EYCLKFTKL), and ART4-derived peptides at positions 13–20 (ART4-13, AFLRHAAL) and 75–84 (ART4-75, DYPSL SATDI). All of these peptides possessed the ability to induce HLA-A24-restricted and tumor-reactive CTLs (6–12), and thus are currently being used in clinical trials as peptide vaccines for cancer patients (3, 4, 13–17). These peptide sequences were conserved between humans and mice. An HIV-derived-peptide (RYLRDQQLL) was used as a negative control. Each peptide was dissolved in DMSO (WAKO) at a concentration of 20 mg/ml and stored at −80°C until use.

Mice, vaccination protocol, and tumor transplantation

Male or female CD-1 (ICR), BALB/c, C57BL/6, BALB/c *nu/nu*, and CB17.SCID mice were purchased from Charles River Japan. $WBB6F_1{}^{w/wv}$ mice were obtained from SLC. The mice were maintained under specific pathogen-free conditions, and 8- to 12-wk-old mice were used for the experiments. The vaccination of the mice was performed as follows: a stock solution of the peptides was diluted with saline at a concentration of 2 mg/ml, and emulsified with an equal volume of IFA (ISA-51; SEPPIC) or

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CFA supplemented with 0.5 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Rv. The BALB/c mice were s.c. injected into an inguen with the peptide emulsion (0.1 ml/mouse) with CFA. Two weeks after the initial injection, the mice were injected three times a week with the same peptide emulsified with IFA (0.1 ml/mouse). Cells (2×10^5 cells) of colon 26, a colon tumor cell line established from H-2^d mice, were s.c. injected into the back of the mice 1 wk after the last vaccination. The study protocols were approved by the Ethical Committee for Animal Experiments at the Kurume University School of Medicine (Kurume, Japan).

Immediate type footpad reaction

Fifty microliters of peptide solution (10 μg/50 μl) and control 2% DMSO-saline were injected into the left and right hind footpads, respectively, by 26-gauge needles. Footpad swellings were measured 15 min after the injection, unless stated otherwise, using a dial thickness gauge (Teraoka). The degree of swelling in the footpads was calculated using the following formula: swelling ($\times$0.1 mm) = (thickness of peptide-injected footpad) $-$ (thickness of DMSO-saline-injected footpad).

Histological analyses and assessment of mast-cell degranulation

The footpads or ears of the mice were obtained after the mice were sacrificed. One-micrometer sections of Formalin-fixed and paraffin-embedded footpads or Epon-embedded ears were stained with toluidine blue and examined by an observer who was not aware of the identity of the individual specimens. The mast cells were classified as extensively degranulated (>50% of the cytoplasmic granules exhibiting fusion, staining alterations, and extrusion from the cells), moderately degranulated (10–50% of the granules exhibiting fusion or discharge), or normal. The lungs, hearts, and livers were also obtained, and 4-μm paraffin-embedded sections were subjected to H&E staining for histological observation. For the anaphylactic controls, 100 μg of anti-DNP IgE (clone SPE-7; Valeant Pharmaceuticals) were i.v. injected into BALB/c mice for passive sensitization. One day after the sensitization, 500 μg of $DNP_{6.4}$-BSA (dinitrophenylated BSA; molar ratio of DNP to BSA was 6.4) was i.v. injected into the anti-DNP IgE-sensitized mice. DNP-BSA was prepared as follows: 10 mg/ml BSA in 0.15 M potassium carbonate was incubated with 5 mg/ml sodium dinitrobenzene sulfonate overnight at 4°C and further purified by Sephadex G-25 PD-1 column (Amersham Biosciences). The molar ratio of DNP to BSA of the conjugate was calculated by the following formula: molar ratio = (OD at 360 nm) $\times$ (0.067×10^{-3} M DNP)/(BSA concentration mg/ml) $\times$ 67,000.

Quantitation of histamine in the culture supernatant of mast cells

Rat peritoneal mast cells were prepared as follows: 20 ml of Tyrode's solution (0.2% glucose, 0.1% BSA, 134 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4)) was injected into peritoneal cavity of specific pathogen-free Sprague Dawley rats (Kyudo) and the peritoneal cells were recovered as cell suspension. After centrifugation at 300 $\times$ *g* for 2 min at room temperature, cell pellet was suspended with Tyrode's solution and the mast cells were subsequently enriched by the density sedimentation method using 22.3 w/v% Histodenz (Sigma-Aldrich)-20 vol% Tyrode's solution. The cell suspension was overlaid on the Histodenz solution and centrifuged at 600 $\times$ *g* for 15 min at room temperature. Cell pellet was recovered, resuspended with Tyrode's solution, and used as mast-cell-enriched fraction. Purity of mast cells in the fraction was >95%. Cells (1 million cells per milliliter) of the freshly prepared mast-cell-enriched fraction were further incubated with 10^{-4} or 10^{-5} M of the peptide at 37°C for 30 min. Substance P (10^{-5} M; Sigma-Aldrich) was used as positive control. Histamine content of the culture supernatants was measured by Histamine ELISA kit (IBL). To determine the complete release of histamine, the cells were incubated with 1% Triton X-100. The percentage of histamine release was calculated as follows: percent histamine release = (experimental $-$ blank)/(complete $-$ blank) $\times$ 100.

Quantitation of peptides in the plasma

CypB-84, ART1-170, or ART4-13 was injected into CD-1 mice through the ocular venous plexus or tail vein, and the blood was collected in tubes containing 10 ml of 0.5 M EDTA (final concentration; ~5 mM) at the indicated periods after the injection. For each group, plasma samples were taken from two mice, pooled, and subjected to further quantitation. One volume (0.1 ml) of the pooled plasma samples was mixed with 9 vol of ice cold 5% trichloroacetic acid and incubated for 5 min on ice to precipitate proteins. The samples were subsequently centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatants were collected. The peptides were further purified using Empore C18 disk cartridges (3M Bioanalytical) as follows: the entire volume (~1 ml) of the supernatants was adjusted to pH 3–4 by addition of 1 N NaOH and applied to methanol/water preconditioned Empore cartridges. The cartridges were sequentially washed with 0.3 ml each of 0.1% trifluoroacetic acid (TFA),[3] water, and 10% acetonitrile-0.1% TFA. Binding peptides, including the injected peptides, were subsequently eluted by 0.1 ml of 40% acetonitrile-0.1% TFA. The eluates were concentrated by a Speed Vac (Savant) for 10 min at 60°C to evaporate acetonitrile, and the final volume of the specimens was adjusted to 0.1 ml by adding 0.1% TFA. Quantitation of the peptides was performed by a high-pressure liquid chromatography-mass spectrometry (LC-MS) system (JMS-700; JEOL) with reverse phase C18 column (Inertsil Peptide C18, 2.1 $\times$ 150 mm; GL Science). The detection limit of the peptide was 10 ng/ml under the present conditions. To examine the stability of the peptides in the murine serum, 300 μg of CypB-84, ART1-170, or ART4-13 was added to 1 ml of the freshly isolated pooled murine serum and incubated for 3, 10, or 30 min at 37°C. The peptides were recovered and subsequently quantify the peptide contents by the HPLC with UV (215 nm) detector.

Radiolabeling and organ distribution of peptides

Peptides were labeled with ^{125}I using IODO-BEADS iodination reagent (Pierce) according to the manufacturer's protocol. In brief, 37 MBq of $Na^{125}I$ (IMS-30; Amersham Biosciences) was preincubated with IODO-BEADS at room temperature for 5 min in 0.1 ml of PBS. Peptide solution (1 mg/ml in PBS) was subsequently added, and the mixture was further incubated at room temperature for 10 min. In the case of the peptide CypB-84 and ART4-13, both of which have histidine but not tyrosine residues, the pH of the PBS was adjusted to 8.2 for the iodination of histidine residues. After incubation, iodinated peptides diluted with 0.8 ml of 0.1% TFA were further purified using Empore C18 disk cartridges. Approximately 1,000,000 cpm of the radiolabeled peptides was injected into CD-1 mice through the ocular venous plexus. Mice were sacrificed by bleeding at 30 s after the injection and the radioactivities of the blood and major organs were measured by a gamma counter. The blood samples were further fractionated by HPLC with a reverse phase C18 column (Inertsil Peptide C18, 4.6 $\times$ 150 mm; GL Science).

Results

Induction of immediate skin reaction by cancer vaccine peptides

Our previous clinical studies indicated that CypB-84, ART4-13, and ART1-170 frequently induced immediate skin reaction in humans (13–18). Therefore, we examined whether these peptides induced an immediate footpad reaction in the nonimmune H-$2K^d$ BALB/c mice (Fig. 1*A*). As expected, CypB-84, ART4-13, and ART1-170 induced immediate footpad reactions in nonimmune mice. Our previous study showed the presence of the IgG class of natural Abs to CypB-84 and SART3-109, but not to the other nine peptides shown in Fig. 1*A*, including ART4-13 and ART1-170, in nonimmune mice (19). Therefore, the immediate hypersensitivity to the peptides seems to be unrelated to the presence of the IgG Ab to the corresponding peptides as a natural Ab. As shown in Fig. 1*B*, CypB-84, ART4-13, and ART1-170 induced immediate footpad reactions in a dose-dependent manner. A representative kinetics of the CypB-84-elicited footpad reaction is shown in Fig. 1*C*. The response reached its maximum 15 min after the injection, and the footpad swelling subsequently subsided and reached normal levels 24 h after the injection. In contrast, no such reaction was observed in the control SART3-109-injected group. Kinetics similar to that shown in the figure was obtained for the responses of ART4-13 and ART1-170 (data not shown).

Vaccine peptide-induced immediate skin reaction in various strains of mice

The immediate footpad reaction to CypB-84, ART4-13, and ART1-170 was further analyzed in the H-2^b haplotype C57BL/6, athymic *nu/nu*, CB17-SCID, and mast cell-deficient $WBB6F_1{}^{w/wv}$ mice (Fig. 1*D*). Footpad reactions to the three peptides found in the BALB/c mice were confirmed in C57BL/6 mice with different H-2

[3] Abbreviations used in this paper: TFA, trifluoroacetic acid; LC-MS, liquid chromatography-mass spectrometry.

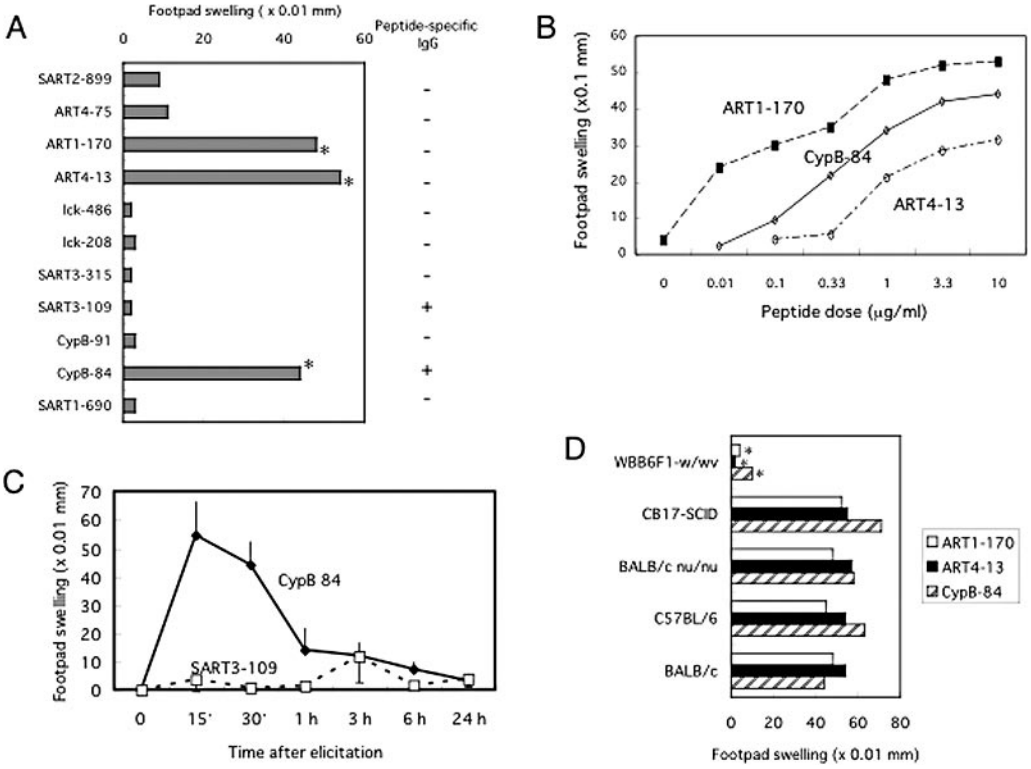

FIGURE 1. Vaccine peptide eliciting an immediate footpad reaction. Fifty microliters of the indicated peptides and control 2% DMSO-saline were injected into the left and right hind footpads, respectively. *A*, Footpad swelling in response to the cancer vaccine peptides was measured at 15 min after the injection. Presence of the peptide-specific IgG as a natural Ab in sera of nonimmune mice is also shown. *, $p < 0.05$. *B*, Dose response of CypB-84-, ART1-170-, or ART4-13-elicited footpad reaction measured 15 min after the injection. *C*, Time course of the CypB-84-elicited footpad reaction. Ten micrograms of CypB-84 or control SART3-109 peptide was injected into the footpads, and the swelling was measured at the indicated periods. *D*, CypB-84-, ART1-170-, or ART4-13-elicited footpad reactions in BALB/c (H-2^d), C57BL/6 (H-2^b), BALB/c *nu/nu* (athymic, no T cells), CB17-SCID (no T cells, no B cells), and WBB6$F_1^{w/wv}$ (no mast cells). *, $p < 0.05$.

haplotypes, suggesting the reactions are not restricted by H-2. Furthermore, the reactions were also observed in the *nu/nu* and SCID mice. In contrast, no such reactions were detected in the WBB6$F_1^{w/wv}$ mice. These results suggest that the immediate footpad reaction induced by each of the three peptides is an H-2 nonrestricted response that is T cell- and B cell-independent, but mast cell-dependent. The positive reactions in the SCID mice also suggest that there was no contribution from Igs, including IgG and IgE.

Histological analysis of vaccine peptide-induced immediate skin reaction

Formalin-fixed tissue sections of the footpad reaction sites were stained with toluidine blue and analyzed under microscopy. Representative photographs of the injection sites of CypB-84 and the control saline are shown in Fig. 2. Degranulation of the mast cells was observed in the CypB-84-injected group, whereas no such degranulation was seen in the saline controls. Similar degranulation of the mast cells was observed in the ART4-13- and ART1-170-injected groups (data not shown).

Direct activation of mast cells

Direct effects of CypB-84, ART1-170, and ART4-13 peptides on in vitro mast cell activation were analyzed. Peritoneal mast cells of rats were incubated with the peptides (10^{-4} or 10^{-5} M) for 1 h at 37°C, and histamine content of the supernatant of the cells were measured. Substance P and Lck-486 peptides were used as positive and negative controls, respectively. Significant levels of histamine release were observed in the culture of cells stimulated with 10^{-4} M of CypB-84, ART1-170, or ART4-13 peptide, as well as that stimulated with 10^{-5} M of substance P (Fig. 3). In contrast, no significant histamine release was observed in both 10^{-4} and 10^{-5} M of lck-486 stimulated groups. The histamine release induced by the three peptides was only observed when the cells were stimulated with a relatively high concentration (10^{-4} M) of peptides,

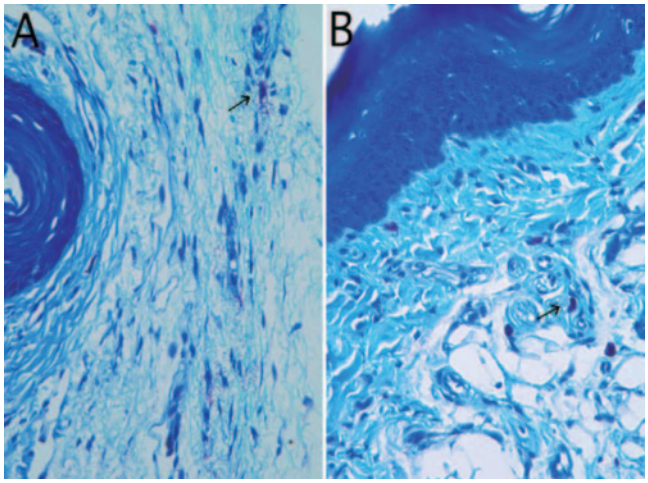

FIGURE 2. Toluidine blue-stained footpad sections from CypB84 (*A*)- and control 2% DMSO-saline (*B*)-injected BALB/c mice. Arrows indicate the mast cells.

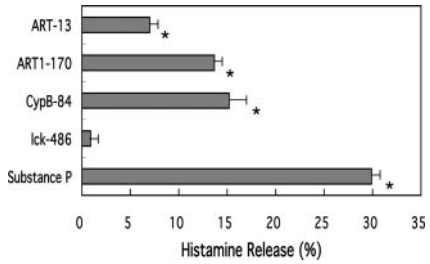

FIGURE 3. Direct effects of CypB-84, ART1-170, and ART4-13 peptides on in vitro mast cell activation. Peritoneal mast cells of rats were incubated with 10^{-4} or 10^{-5} M of CypB-84, ART1-170, ART4-13, or lck-486 peptide for 30 min at 37°C, and histamine content of the supernatant of the cells were measured. Substance P (10^{-6} M) was used as positive control. Representative results of 10^{-4} M peptides are shown. Histamine content of the supernatant of the cells without any peptide stimulation was also measured as spontaneous release of histamine and the value (10.5% in the present experiment) was already subtracted from the data. *, $p < 0.05$.

equivalent to that used for the skin reaction, and no such effects were observed at 10^{-5} M. These results suggest that the three peptides directly activated the mast cells.

Systemic effect of vaccine peptides

An immediate skin reaction is a phenotype of local hypersensitivity. The type of skin tests we used in this study is often used to forecast systemic anaphylaxis. Therefore, we examined whether each of the three peptides also induced anaphylaxis when they were injected systemically. One hundred or 500 μg of the peptides were i.v. injected to BALB/c mice, and their body temperature was recorded. Five hundred micrograms of DNP-BSA was i.v. injected to anti-DNP IgE-sensitized mice as a positive control. The changes in body temperature after the peptide injection are shown in Fig. 4*A*. A marked decrease of body temperature was observed after the DNP-BSA injection into the anti-DNP IgE-sensitized group. In contrast, no significant alteration of body temperature was observed in the peptide-injected groups. After the measurement of body temperature at 30 min after injection, the mice were sacrificed, and their livers, lungs, and hearts were subjected to histological analysis (Fig. 4*B*, *A–C*). No remarkable changes were found in the livers of the peptide-injected groups (Fig. 4*BB*) and saline controls (Fig. 4*BA*). In contrast, extensive degenerative changes of the hepatocytes, such as unclear trabecular structure and granular appearance in the cytoplasm with indistinct cellular margins, were observed in the liver of the DNP-BSA-injected groups (Fig. 4*BC*). The histological appearance of the lungs and

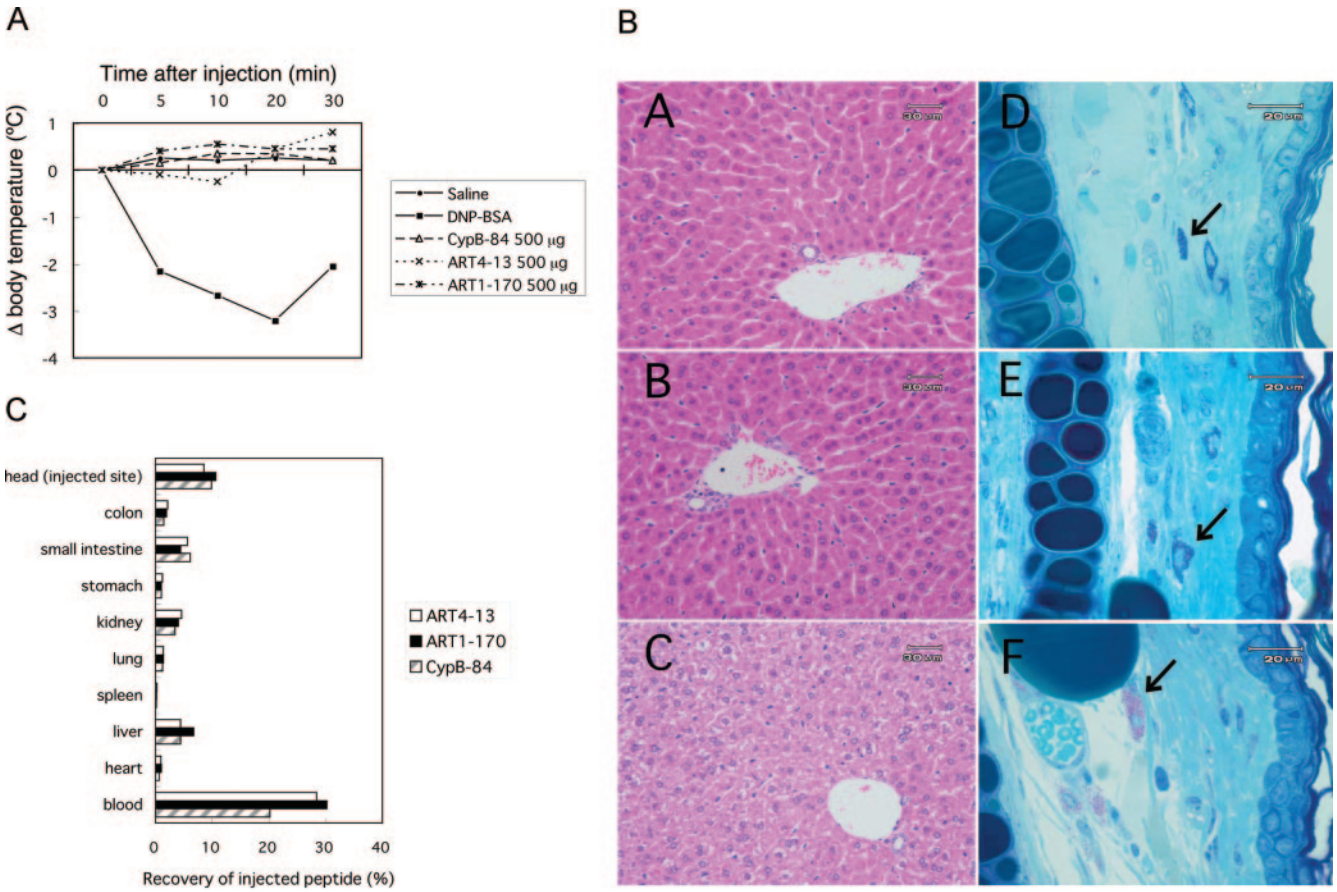

FIGURE 4. Systemic effects of the peptide after i.v. injection. *A*, Alteration of body temperature after systemic injection of the peptides. Either 100 or 500 μg of the indicated peptides were i.v. injected into BALB/c mice, and their body temperature was recorded. For anaphylactic controls, 500 μg of DNP-BSA was i.v. injected into the anti-DNP IgE-sensitized mice. Results of 500 μg of the peptide-injected groups are shown. *B*, Histological analyses of mice after systemic injection of the peptides. The liver (*A–C*) and ear skin (*D–F*) sections from mice i.v. injected with 2% DMSO-saline (*A* and *D*) and 500 mg of CypB-84 (*B* and *E*), or anti-DNP IgE-sensitized and DNP-BSA-injected mice (*C* and *F*). Arrows indicate the mast cells. *C*, ^{125}I-labeled peptides were injected into CD-1 mice through the ocular venous plexus, and the mice were sacrificed by bleeding at 30 s after the injection. Radioactivities of the blood and organ samples were measured by gamma counter.

Table I. *Mast cell degranulation of the ear skins after i.v. injection of peptides*

	Percentage of Degranulated or Normal Mast Cells[a]		
	Extensive	Moderate	Normal
Control (saline)	1.1 ± 1.2	3.1 ± 1.9	95.8 ± 1.4
CypB-84 (500 μg)	1.3 ± 1.3	2.4 ± 1.4	96.3 ± 2.4
CypB-84 (100 μg)	0.7 ± 1.3	2.1 ± 2.2	97.1 ± 3.4
Anti-DNP IgE/DNP-BSA	89.4 ± 1.2	2.0 ± 1.3	8.6 ± 2.2
ART1–170 (500 μg)	1.5 ± 1.7	5.2 ± 3.1	93.3 ± 3.8
ART1–170 (100 μg)	0 ± 0	3.9 ± 0.2	96.1 ± 0.2
ART4–13 (500 μg)	1.0 ± 0.2	3.1 ± 1.6	95.9 ± 1.4
ART4–13 (100 μg)	1.2 ± 1.1	3.1 ± 3.6	95.7 ± 4.0

[a] The mast cells were classified as extensively degranulated (>50% of the cytoplasmic granules exhibiting fusion, staining alterations, and extrusion from the cell), moderately degranulated (10–50% of the granules exhibiting fusion or discharge), or normal.

hearts did not differ among the groups (data not shown). The ears of the mice were also subjected to histological analysis to quantitate the degranulation of mast cells in the skin. The results on the extent of mast cell degranulation in the ear skin are shown in Table I. The mast cells in the ear skin of the peptide-injected or saline control groups showed a normal appearance (Fig. 4*B*, *D* and *E*), whereas most of the mast cells in the DNP-BSA-injected anaphylactic controls were extensively degranulated, with >50% of the granules exhibiting staining changes and/or extrusion from the cells (Fig. 4*BF*). All of these results suggest that injection of the CypB-84, ART4-13, and ART1-170 peptides can elicit a local skin reaction only, but not systemic anaphylaxis.

Pharmacokinetics of i.v. injected peptides

The plasma concentration of CypB-84, ART1-170, and ART4-13 after an i.v. injection was measured by LC-MS. One or 0.5 mg of the peptide was injected through the ocular venous plexus or tail vein and the blood was collected at 15 s to 5 min after the injection. Each of the three peptides was detectable in the plasma at 30 s and 1 min after the injection (Table II); unexpectedly, however, the levels of the peptides were very low (ranging between 1440 and 32 ng/ml). The expected initial concentration of the peptide after 1 mg of peptide injection is ~0.5 mg/ml for a plasma volume of 2 ml. Therefore, only 1:200 to 1:2000 of the initially injected peptide was found in the plasma after 30 s under this estimation. At 3 and 5 min after the injection, the plasma levels of CypB-84 reached the levels of background noise (<10 ng/ml). The organ distribution of the peptides immediately (30 s) after the injection was further analyzed by using radiolabeled peptides (Fig. 4*C*). Approximately 20–30% of the injected radioactivity was recovered from the blood. The other major distributed organs were the livers, kidneys, and small intestines, and their radioactivity levels were 4–7% of the injected amount, respectively. Total recovery of the radioactivity in these experiments was ~60–70% of the injected samples. These results suggest that the injected peptides are very rapidly degraded to small molecules in the plasma and distributed to the carcass. Plasma samples separated from the blood of ^{125}I-labeled peptide-injected mice were also analyzed by HPLC, and the radioactivity of each fraction was measured. However, no significant radioactivity was recovered from the plasma samples at the same time points at which the original labeled peptides were retained in the earlier trial (data not shown). These results suggested that the injected peptides are very rapidly degraded in vivo. To confirm the degradation of the peptides in the plasma, the peptides were added to freshly prepared pooled-serum of mice and incubated for 3, 10, or 30 min at 37°C. Peptide content of the each sample was further analyzed by HPLC. As expected, CypB-84, ART1-170, and ART4-13 peptides were rapidly degraded in the serum and their half-life were 7.4, 31.9, and 1.7 min, respectively (Fig. 5).

Antitumor effect of cancer vaccine peptides in a murine model

To understand a correlation between antitumor effect and the immediate-type skin reaction, antitumor effect of the cancer vaccine peptides was analyzed in BALB/c mice. Seven of the 11 peptides that were used in the phase I/IIa clinical trials of cancer vaccines performed in Kurume University Hospital, all of which possessed sequence similarities to the corresponding murine homologs, were used in this study. The seven peptides used were derived from five different mother proteins. The sequence similarity of each peptide to the corresponding murine homolog was 100%, except in the case of SART3-109, whose similarity was 80%. Two amino acids at positions 1 and 9 of the SART3-109 peptide (10-mer) were mismatched to the corresponding sequence of the murine homolog; however, these positions were different from both the agretope (anchor motif) and putative CTL-epitope. The BALB/c mice were primed with one of the seven peptides or saline-emulsified with CFA. Beginning at 2 wk after the initial priming, the mice were boosted once a week for 3 wk by the corresponding peptide emulsified with IFA. Colon 26 cells were s.c. inoculated into the mice 1 wk after the last immunization. A summary of the effects of the peptide immunization on tumor growth and overall survival are shown in Table III. The tumor masses became a measurable size (>25 mm^2) 13 days or later after the tumor inoculation under the present conditions. The median time it took for the tumors to grow to that size (median onset time) in the saline controls was 14 days, and 14–19 days in all of the other groups except for the SART3-315-immunized group, in which the onset time was 24 days (Table III). Growth inhibition of the tumors and subsequent prolongation of overall survival time was also observed in the SART3-315-immunized group, but not in the other groups. It is of note that two

Table II. *Plasma concentration of peptides after i.v. injection*[a]

Peptide Injection			Plasma Peptide Concentration (ng/ml)					
Peptide	Amount (mg)	Injection Site	Preinjection	15 s	30 s	1 min	3 min	5 min
CypB-84	1	Ocular venous plexus	<10	—[b]	229	138	<10	<10
CypB-84	0.5	Tail vein	<10	—	138	76	<10	<10
ART1–170	0.5	Ocular venous plexus	<10	674	117	32	<10	<10
ART4–13	0.5	Ocular venous plexus	<10	—	1440	166	<10	<10

[a] Indicated peptides were injected to CD-1 mice through ocular venous plexus or tail vein, and the blood was collected to tubes containing EDTA (final concentration was ≈5 mM) at the indicated periods after the injection. Plasma samples were deproteinized by a TCA precipitation, and the peptides were further purified by Empore C18 disk cartridge. Quantitation of the peptide was performed by LC-MS and the detection limit of this method was 10 ng/ml. Plasma samples of each group consisting of two mice were pooled and subjected for quantitation.

[b] Not tested.

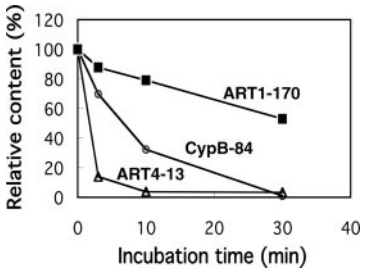

FIGURE 5. Stability of the peptides in the murine serum. Three hundred micrograms of CypB-84, ART1-170, or ART4-13 was added to 1 ml of the freshly isolated murine serum and incubated for 3, 10, or 30 min at 37°C. The peptide contents in the samples were measured by HPLC.

of five mice immunized with SART3-315 were tumor free during the entire period. Similar results were obtained when the experiment was repeated (data not shown). Furthermore, the antitumor effect of the SART3-315-immunization was confirmed by Winn assay. Namely, lymph node cells from the SART3-315-immunized mice inhibited both the initial tumor mass formation and subsequent growth, resulting in a prolongation of the overall survival of the mice (data not shown). Therefore, the immediate hypersensitivity to the CypB-84, ART4-13, or ART1-170 peptide seems to be unrelated to in vivo antitumor effect in mice.

Discussion

We showed that the 3 peptides (CypB-84, ART4-13, and ART1-170) among the 11 peptides elicited an immediate skin reaction in murine models. All of the 11 peptides are derived from nonmutated self Ags involved in cellular proliferation and thus are preferentially expressed in tumor cells and some proliferating normal cells (6–11). In addition, all the 11 peptides used in this study share the similar amino acid sequences at the position 2 (Y or F) and position 9 (F, L, or I) with the ability to induce HLA-A24-restricted and tumor-reactive CTLs (6–11). Therefore, there was no difference in the levels of mother proteins or the ability to induce CTL activity between the former three (CypB-84, ART4-13, and ART1-170), which elicited immediate skin reactions in both humans and mice, and the remaining eight peptides, which failed to do so. The immediate reaction to the peptides seems to be unrelated to either antitumor effect in mice or the presence of the IgG Ab to the corresponding peptides as natural Ab.

Histological analyses have suggested that the reaction found in mice was a typical mast cell-dependent immediate hypersensitivity reaction, similar to a type I allergy. However, in the present study, analyses in *nu/nu* and SCID mice suggested that the reaction was different from a typical type I allergy mediated by IgE, or IgG1 in the case of mice, as the levels of both classes of Ig were very low or negative in the *nu/nu* or SCID mice, respectively. We further examined whether the CypB-84, ART4-13, and ART1-170 peptides elicited systemic anaphylaxis when i.v. injected into mice. Anti-DNP IgE presensitized and DNP-BSA-injected controls expressed typical anaphylactic reactions, such as a decrease in body temperature, mast cell degranulation in skin, and degenerative changes of the hepatocytes in the liver. However, none of the peptides induced these alterations in mice, even when 0.5 mg of peptides, which is a dose that is comparable to the 500-fold excess of clinical trial dose per body weight in humans, was i.v. injected into the mice. We further demonstrated that the i.v. injected peptides were very rapidly degraded in vivo and the plasma concentrations of the peptides at 30 s after the injection were ~1:2000 of the injected amounts. The rapid degradation of the peptides in the plasma was also confirmed in vitro. Plasma contains many different kinds of peptidases, and these peptidases might contribute to the rapid clearance of the peptides from the plasma. These results suggest that three peptides—CypB-84, ART4-13, and ART1-170—might be safely used for vaccinations without anaphylaxis, at least in mice and possibly in humans. We further demonstrated the direct activation of mast cells by the three peptides in vitro using peritoneal mast cells. Although heterogeneity of the mast cells in the different tissues is well known, we showed the activation of two different types of mast cells by the peptides, i.e., an activation of peritoneal mast cells in vitro and degranulation of skin mast cell in vivo. These results suggest that one of the main mechanisms by which the short self-peptides elicited immediate skin reactions is a direct activation of skin mast cells by the peptides. Substance-P is known as a neuropeptide that is released from sensory nerve endings in the skin, and induces mast cell degranulation (20). The amino acid sequence of substance-P is conserved in various species, and, therefore, the action can be detected in both humans and mice, like the action of the CypB-84, ART4-13, and ART1-170 peptides. Recently, we found that the skin-reaction-inducing capacity of the peptides is correlated to their electric charge, i.e., basic charge of the peptides is essential for the induction of skin reactions as well as mast cell activation (T. Ono, Y. Komohara, K. Itoh, and A. Yamada, manuscript in preparation).

The role of mast cells in host defense against tumors has not been well characterized. Mast cells release various kinds of bioactive components, such as histamine, eicosanoids, preformed TNF-α, newly synthesized cytokines, tryptases, and chemokines (21). Some of them cause vasodilatation and extravasation of fluid and initiate subsequent inflammation. Recruitment of lymphocytes, including CTLs, to the inflammation sites, also occurs. It is known that tumor cells produce various kinds of cytokines and

Table III. *Antitumor effect of cancer vaccine peptides in BALB/c mice*

Peptides	Median Onset Time (day)[a]	Size at Day 34 (mm^2)			Median Survival Time (day)	Tumor Free (%)
		Median	Mean	Percent inhibition		
Saline control	14	283	298		74	0
SART1–690	14	275	282	5	69	0
SART3–109	14	359	341	–14	66	0
SART3–315	24	94	151	49	108	40
ART4–75	16	305	299	0	81	0
lck-208	14	285	289	3	83	0
CypB-84	19	221	219	27	87	0
CypB-91	16	255	302	–1	66	0

[a] Time when inoculated tumor become a measurable mass size (>25 mm^2).

chemokines that are mitogenic and/or chemoattractants for granulocytes, mast cells, monocytes/macrophages, fibroblasts, and endothelial cells (22). These facts suggest that tumor masses contain substantial numbers of mast cells, and it is likely that mast cells play a positive role in antitumor host defense. Therefore, it is conceivable that the injection of CypB-84 or other mast cell-activating peptides into the tumor mass enhances CTL infiltration to the tumor tissues.

To understand a correlation between antitumor effect and the immediate-type skin reaction, antitumor effect of the cancer vaccine peptides was analyzed in BALB/c mice and antitumor effect of SART3-315 was shown. However, none of the other peptides expressed apparent antitumor effects. Therefore, any correlation between the antitumor effects and the immediate-type skin reaction was observed.

In conclusion, we showed that cancer vaccine peptides derived from cyclophilin B, ART1, and ART4, elicited IgE-independent, but mast cell-dependent, immediate-type skin reaction without inducing systemic anaphylaxis. These results may provide new insights for further development of peptide-based vaccinations for cancer patients.

Disclosures

The authors have no financial conflict of interest.

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