

Antitumor Effect of New HER2 Peptide Vaccination Based on B Cell Epitope

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Abstract. *Background:* While the benefit of passive immunotherapy is commonly accepted, active immunization may have advantages for the patient's quality of life. We identified a new epitope of Mab CH401 against Her-2/neu extracellular domain (N: 167-175), and evaluated the effect of active immunization of the 20mer peptide containing the epitope (CH401 peptide). *Materials and Methods:* Epitope-mapping was performed using ELISA with Her-2/neu-related multiple antigen peptides (MAP). BALB/c mice were transplanted with Her-2/neu-expressing lymphoma cell line and immunized with the peptides. For monitoring the condition, ELISA and flow cytometry was performed. *Results:* CH401 peptide induced Her-2/neu-specific IgG antibody. Tumor growth in immunized mice was suppressed and tumor-infiltrating lymphocytes comprised more CD8⁺ T-cells, which secreted larger amounts of interleukin-2 after the peptide re-stimulation. *Conclusion:* The new Her-2/neu peptide contained epitopes for CD4⁺ and CD8⁺ T-cells, which contributes to the suppressive effect on Her-2/neu-expressing tumor cell growth.

Molecular targeting reagents have been extensively developed for cancer therapy. Among them, antibodies against tumor-associated antigen (TAA) are considered to be powerful tools through their multiple functions, such as antibody-dependent cell cytotoxicity and blocking of signals

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for cell proliferation (1-3). The antitumor effect of anti-TAA antibody has been investigated in two ways: *via* passive immunization with monoclonal antibody (Mab) and active immunization with TAA vaccination (4, 5). However, the processes including the clinical application of Mab therapy require huge amounts of antibodies, and consequently may impose considerable physiological, psychological and financial burdens upon the patients.

As for the strategy for overcoming the disadvantage of passive immunization with Mab, active immunization with tumor-specific antigen may avoid adverse effects induced by transfusing large amounts of antibodies repeatedly. However, most of the trials with immunization of whole TAA molecules have not been successful (4), partially because the antibodies sometimes function positively for cancer growth (6-9). Thus, peptides composed of limited amino acid sequences, which do not produce tumor-promotory antibodies, may be useful for effective antitumor antibody production. In past studies, however, the B-cell epitope was tentatively estimated simply from the biochemical/structural nature of the antigen, which is still problematic because epitopes may include the region agonistic to tumor growth (10, 11). Otherwise, B-cell epitopes or mimotopes were artificially found by screening with phage display (12-14). Thus, identifying an epitope of TAA-specific monoclonal antibody, which is already characterized to induce an antitumor effect, and designing a peptide that includes the epitope sequence might be an effective protocol for the peptide design of anti-TAA vaccination.

In contrast, T-cell epitope, particularly of CD8⁺ cytotoxic T-cells (CTL), has been well investigated since the characterization/identification of TAA (15). The techniques for purifying peptide presented on major histocompatibility complex (MHC) class I and II molecules have identified numerous anchoring peptides and categorized (15, 16).

Table I. Synthetic MAP peptides from human *c-erbB-2* protein sequence selected for fine CH401 epitope mapping. Each amino acid in the multiple antigen peptides is indicated by a single letter code. B; *t*-butoxycarbonyl residue (Boc). Each peptide in the bracket was conjugated with Boc* and lysine residues. The numbers in the peptide section indicate the position of the sequence in the Her-2/neu molecule.

Peptide	Sequence	Peptide	Sequence
N:143-162	(RSLTEILKGGVLIQRNPQLC-BBB)8-K4K2KB	N:253-272	(LACLHFNHSGICELHCPALV-BBB)8-K4K2KB
N:153-172	(VLIQRNPQLCYQDTILWKDI-BBB)8-K4K2KB	N:263-282	(ICELHCPALVYNTDTFESM-BBB)8-K4K2KB
N:163-182	(YQDTILWKDIFHKNNQLALT-BBB)8-K4K2KB	N:273-292	(TYNTDTFESMPNPEGRYTFG-BBB)8-K4K2KB
N:173-192	(FHKNNQLALTLIDTNRSRAC-BBB)8-K4K2KB	N:283-302	(PNPEGRYTFGASCVTACPYN-BBB)8-K4K2KB
N:183-202	(LIDTNRSRACHPCSMPCCKGS-BBB)8-K4K2KB	N:292-310	(GASCVTACPYNYLSTDVGS-BBB)8-K4K2KB
N:193-212	(HPCSMPCCKGSRGWGESSEDC-BBB)8-K4K2KB	N:300-321	(PYNYLSTDVGSCTLVCPHNLQE-BBB)8-K4K2KB
N:203-222	(RCWGESSEDCQSLTRTVAC-BBB)8-K4K2KB	N:312-330	(TLVCPHNLQEVTAEDGTQR-BBB)8-K4K2KB
N:213-232	(QSLTRTVACAGGCARCKGPLP-BBB)8-K4K2KB	N:322-341	(VTAEDGTQRCEKCSKPCARV-BBB)8-K4K2KB
N:223-242	(GCARCKGPLPTDCCHEQCAA-BBB)8-K4K2KB	N:332-351	(EKCSKPCARVCYGLGMEHLR-BBB)8-K4K2KB
N:233-252	(TDCCHEQCAAGCTGPKHSDC-BBB)8-K4K2KB	N:343-361	(YGLGMEHLREVRVAVTSANI-BBB)8-K4K2KB
N:243-263	(GCTGPKHSACLHLFNHSG-BBB)8-K4K2KB	N:352-370	(EVRVAVTSANIQEFAGCKKI-BBB)8-K4K2KB

*Boc: *t*-butoxycarbonyl residue.

Vaccination with the peptide containing T-cell epitope has been vigorously performed in CTL induction, which is considered to be vital for antitumor activity. However, clinical responses were very poor when only CTL was targeted (17). Concerning this evidence, the participation of CD4⁺ T-cells has recently been highlighted in TAA-specific cellular immunity, as CD4⁺ helper T-cells are critical for the supply of activated dendritic cells (DC) and for generation and persistence of CTL (18-21). As helper T-cells are activated by peptides presented on MHC class II molecules, peptides designed to be restricted to both MHC class I and class II molecules might be powerful tools for T-cell-based antitumor therapy. At the same time, as helper T-cell activity is essential for IgG antibody production with high affinity, vaccination with helper peptide is useful for inducing effective antibody-mediated antitumor therapy (22).

In this study, we identified the antibody epitope of CH401, a Mab raised by immunizing Her-2/neu-expressing tumor cells, which was reported to possess cytotoxicity against Her-2/neu-expressing tumor cells (23). The peptide spanning the antibody epitope contained putative anchoring motifs for helper and cytotoxic T-cells. Our aim was to evaluate the immunogenicity to produce specific IgG antibody and antitumor effect to reduce tumor growth of Her-2/neu-positive cells of the epitope peptide. We found that the new Her-2/neu epitope peptide containing epitopes for B-cells and both helper and cytotoxic T-cells can induce not only TAA-specific antibodies but also antitumor effect related to cytotoxic T-cells.

Materials and Methods

Mice. Female BALB/c mice, 8 weeks old, were purchased from Japan-SLC Co. Ltd. (Hamamatsu, Japan). RAG-2 knockout (KO) mice were provided by the Central Institute for Experimental Animals (Kawasaki, Japan). All of the animal works in this study were in accordance with international regulations and were approved by Tokai University School of Medicine.

Peptide synthesis. All amino acid sequences used for epitope mapping are shown in Table I. Peptides were synthesized from Rink amide resin (0.4-0.7 mmol/g) using a peptide synthesizer ACT357 (Advanced ChemTech, Louisville, KY, USA), resulting in the formation of amide at the carboxyl termini. Multiple antigen peptides (MAPs) (7) were formed for all the peptide antigens and used for immunization and enzyme-linked immunosorbent assay (ELISA).

Cell lines. Mouse lymphoma cell line A20 was transfected with pME18S/*c-erbB-2*-derived human ERBB2/neu cDNA-transduced MIGR1, an MSCV-IRES-EGFP retroviral construct, using a potent retrovirus packaging cell line named Platinum-E. Her-2/neu-expressing A20 cells (Her-2-A20) were purified by cell sorting of green fluorescent protein (GFP)-positive cells. These cells were cultured and maintained in Dulbecco's-Modified Eagle Medium with 10% fetal calf serum (FCS), 1% L-glutamine and 1% antibiotics at 37°C, 95% humidity and 5% CO₂.

Antibodies. Anti-mouse CD3-PerCP-Cy5.5, anti-mouse CD4-PE, anti-mouse CD8-FITC, anti-human IgG-PE and anti-mouse IgG-PE monoclonal antibodies were purchased from BD-Bioscience (San Jose, CA, USA). Herceptin was purchased from Chugai Pharmaceutical co. LTD (Tokyo, Japan). CH401 was prepared as reported previously (24).

Immunization. BALB/c mice were immunized with synthetic MAP peptide (100 µg/head, n=2 for each kind of peptide) with Freund's complete adjuvant (FCA) purchased from Becton Dickinson (Franklin Lakes, NJ, USA) and boosted 2 times biweekly with the same amount of peptide with Freund's incomplete adjuvant (FIA) (Becton Dickinson). One week after the 3rd immunization, peripheral blood was collected and titers were checked by ELISA.

Flow cytometric analysis. A20 transfectants or tumor-infiltrating lymphocytes (TIL) were prepared and incubated with labeled primary Mab for 15 min at 4°C and washed with 1% (w/v) bovine serum albumin (BSA)-containing phosphate-buffered saline (PBS). In some cases, cells were re-incubated with labeled secondary

antibody. Flow cytometry was performed using FACSCalibur (Becton Dickinson). The MAbs used were purchased from BD Biosciences (Franklin Lakes, NJ, USA). TIL index was calculated as $CD4^+$ cell % / $CD3^+$ cell % or $CD8^+$ cell % / $CD3^+$ cell%. All the data used for the calculation of TIL index were obtained from the tumor-infiltrating cells in the lymphocyte gate.

Transplantation. BALB/c mice (8 weeks old) were transplanted with A20 cells (5×10^5) or Her-2-A20 cells (5×10^5) subcutaneously on both sides. Tumors were measured by calipers every 7 days, and the products of perpendicular diameters were recorded. Blood was collected from the orbit biweekly. Thirty-five or 42 days later, mice were sacrificed and the weight and mass of tumors were analyzed. T-cells or B-cells purified by magnetic cell sorting (MACS) system with anti-CD3 or anti-CD19 antibodies were transplanted to RAG-2 KO mice with BALB/c background. Two weeks after the Her-2-A20 transplantation, peptide antigen immunization was performed.

Cell sorting. Her-2-A20 was harvested and re-suspended in PBS with 1% BSA and 0.1% sodium azide. GFP-positive cells were sorted using FACS Vantage (BD Biosciences). Gates were set on the basis of forward and side scatter profiles. Cut-off values for the quadrants were set using positive and negative control samples and the appropriate mouse IgG isotype controls. Subsequently, cells were analyzed in a FACSCalibur (BD Biosciences) using Cell Quest software.

In vitro culture. Spleen cells from BALB/c mice were stained with antibodies against mouse CD4, CD8 or CD3 followed by reacting with magnetic bead-conjugated anti-rat IgG or anti-hamster IgG. After purification by the magnetic cell sorting system, $CD4/CD8$ T-cells and $CD3^+$ cell-depleted spleen cells were co-cultured ($CD4/CD8$:antigen-presenting cells=4:1). After 24 h, culture supernatants were collected and IL-2 quantified as follows.

ELISA. As for the antigenicity of MAPs, antisera were collected from the mice immunized with each MAP, and the titers of the specific antibodies were assayed by ELISA. Microwells were coated with each peptide (1 mg/ml) diluted in carbonate buffer (pH 9.5). Wells were washed with PBS-Tween 20 (0.05% v/v) and blocked with 3% BSA-PBS at room temperature for 2 h. Following three washes with PBS-Tween 20, serial ten-fold dilutions of mice sera were added and the plates were incubated for 2 h at room temperature. Plates were washed three times and horseradish peroxidase (HRP)-conjugated goat anti-mouse Igs-HRP (BD Bioscience) (1:3000 v/v) was added. After 1.5-h incubation at room temperature, plates were washed and TMB peroxidase EIA substrate kit solution (Bio-Rad Laboratories, Hercules, CA, USA) was added. Reaction was stopped by 10% HCl and absorbance was measured at 450 nm. For epitope mapping, CH401 Mab was used as primary antibody instead of antisera. For the assay of CH401 MAP-specific subclass antibody, HRP-conjugated antibodies against IgM, IgG1, IgG2a, IgG2b, and IgG3 were used as secondary antibodies (BD Biosciences). For IL-2 assay, an Opt-EIATM kit (BD-Bioscience) was used according to the manufacturer's instructions.

Statistics. Values were compared using unpaired, two-tailed Student's *t*-test. Data are expressed as the mean \pm SD.

Results

Epitope mapping of CH401 monoclonal antibody (Mab) against Her-2/neu-expressing tumor cells. Firstly, we tried to select an epitope peptide of anti-Her-2/neu antibody in order to induce Her-2/neu-epitope specific antibodies *in vivo*. The Mab, CH401, was previously obtained from hybridoma constructed with spleen cells of BALB/c mice immunized with *her-2/neu* gene-transfected cells (23). Binding analysis showed that CH401 Mab reacts to *her-2/neu* gene-transfected cells on their surface but not to the transfectant of truncated *her-2/neu* gene (region between N:143-N:370 on the extracellular domain of *her-2/neu* was deleted). In this study, we further examined the epitope recognized by CH401 Mab in detail. First, based on the region covering from N:143 to N:370, we generated 22 kinds of synthetic MAPs containing 20 amino acid residues as shown in Table I. Then using the MAPs as antigens, the cross-reactivity of CH401 Mab to each 20mer MAP was screened by ELISA. As shown in Figure 1A, only N:163-N:182 peptide showed a prominent reactivity with CH401 Mab. Moreover, a further series of 20mer MAPs were prepared by a 2 amino acid shift of the peptide sequence from N:143-N:192 (Figure 1B) in order to define the exact epitope sequence. Screening of the newly prepared peptide set revealed that the cross-reactivity was limited to the peptide including N:167-N:175 9mer so as to be an epitope of CH401 Mab (Figure 1C).

Immunogenicity of Mab epitope. We then examined whether the 20mer MAP (N:163-182) including the CH401 epitope, termed CH401 MAP, was capable of inducing specific antibody production *in vivo*. BALB/c mice were immunized with each of the 22 MAPs. One week after the fourth immunization, sera from each mouse were submitted to estimation of the titer of the antibody specific for the immunized peptide. The serum level of the specific antibody was markedly high only in the mouse administered CH401 MAP including the N:163-182 sequence, but it was not increased with the other MAPs (Figure 1D). The increased specific antibody was mainly IgG1 (data not shown, and Figure 2), suggesting that CH401 MAP was able to induce at least helper T-cell function *in vivo*, which is required for IgG antibody production. To confirm this, we examined whether the CH401 MAP contains anchoring motifs of mouse class II MHC molecule using the algorithm of Southwood *et al.* (25). As shown in Table II, CH401 MAP was predicted to be presented on H2-A^d and H2-E^d molecules of BALB/c mouse cells. Since such anchoring motifs of mouse and/or human class II MHC are considered to be essential for antigen recognition by helper T-cells, it is suggested that CH401 MAP also contains $CD4^+$ T-cell epitope, resulting in efficient antibody production in immunized mice.

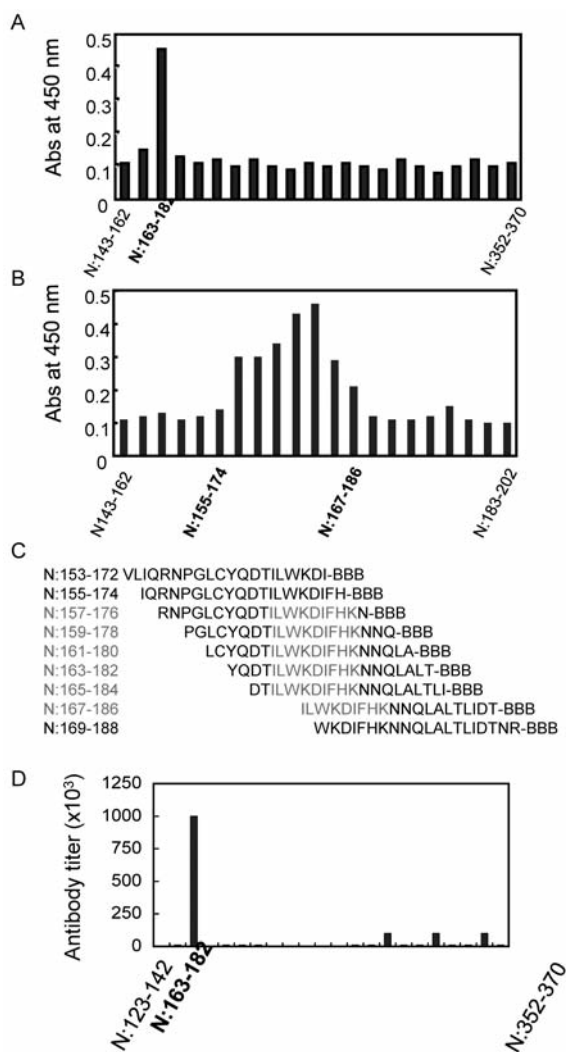


Figure 1. Epitope-mapping of CH401 by Her-2 synthetic peptides. A: Epitope mapping of CH401 by 22 kinds of Her-2/neu-related MAPs. Her-2 synthetic MAP peptides were used for epitope screening of CH401 by ELISA. Absorbance at 450 nm is shown in the panels. B: The results of more detailed peptide sequence screening. The 20mer MAPs covering N:143-N:202 peptide were used for epitope mapping by ELISA. Main peptides are shown beneath the vertical bars. C: The epitope of CH401 predicted by epitope mapping is shown in gray letters. D: Induction of specific antibodies in BALB/c mouse by Her-2 epitope peptide. Antigens were injected into BALB/c mice (n=2 each) with Freund's complete adjuvant followed by booster with Freund's incomplete adjuvant biweekly. Antibody titer was assayed by ELISA with serially diluted antisera. Data are mean values of titers of two mice each. Data are representative of three independent experiments.

TAA-specific antitumor effect by immunization of epitope peptide. We then examined whether immunization of CH401MAP peptide induces antitumor activity in BALB/c mice. We prepared BALB/c-derived B lymphoma cells expressing Her-2/neu, which were generated by introducing

her-2/neu cDNA as described in the Materials and Methods. The transfectant of the lymphoma cell line, termed Her-2-A20, was specifically stained by anti-CH401 MAP polyclonal antibody and Herceptin (Figure 2A).

BALB/c mice were subcutaneously transplanted with 5×10^5 Her-2-A20 cells and simultaneously immunized with CH401 MAP plus adjuvant or adjuvant alone according to the schedule shown in Figure 3B. Both tumor size and serum level of the produced antibody were measured weekly in mice with and without CH401 MAP immunization. Growth speed of the transplanted tumor mass was delayed in immunized mice compared to non-immunized mice, and on day 35, tumor size was significantly smaller in the MAP-immunized mice (Figure 2C). In contrast to the tumor suppression, the serum level of specific IgG1 antibody in tumor-bearing mice gradually increased in immunized mice but was almost undetectable in mice receiving adjuvant alone (Figure 2D). Tumor size on day 35 was inversely correlated with the serum level of IgG1-specific antibody but not of IgM-specific antibody (Figure 3E, and data not shown). These results imply that immunization of epitope-containing peptide in tumor-bearing mice induces the peptide-specific immune response in B-cells and presumably T-cells, targeting tumors expressing TAA including the immunized peptide. In fact, the mice transplanted with A20 parent cells showed no significant tumor suppression even if they were immunized with CH401 MAP (data not shown).

Tumor growth is suppressed in B-cell-depleted mice. To examine whether CH401 MAP vaccine causes the activation of T-cells to suppress tumor growth, we generated B-cell- and T-cell-depleted mice; BALB/c spleen cells after depletion of T-cells or B-cells were transplanted into RAG-2 KO mice with the same background. These reconstituted RAG-2 KO mice were transplanted with Her-2 A20 and immunized with CH401 MAP.

The results showed that the suppressive effect on growth of transplanted Her-2-A20 was not significantly different between the reconstituted mice with T-cell-depleted spleen cells and those with whole spleen cells (Figure 3A). On the contrary, tumor growth was delayed in RAG-2 KO mice reconstituted with B cell-depleted spleen cells. This likely resulted from the fact that T-cells and/or DCs stimulated with administered peptide made a great contribution to tumor suppression independently of tumor-specific antibody. For suppression of tumor growth, CTL developed with T-cell help is generally a major player if $CD8^+$ T-cells recognize antigen peptides anchoring to MHC class I molecule. In our CH401 peptide, algorithmic analysis (Table II) predicted that the peptide theoretically contains class I MHC-specific anchoring motif. In fact, the ratio of tumor-infiltrating $CD8^+$ T-cells was increased in the peptide-immunized mouse tumors (Figure 3B). We also examined the IL-2 production

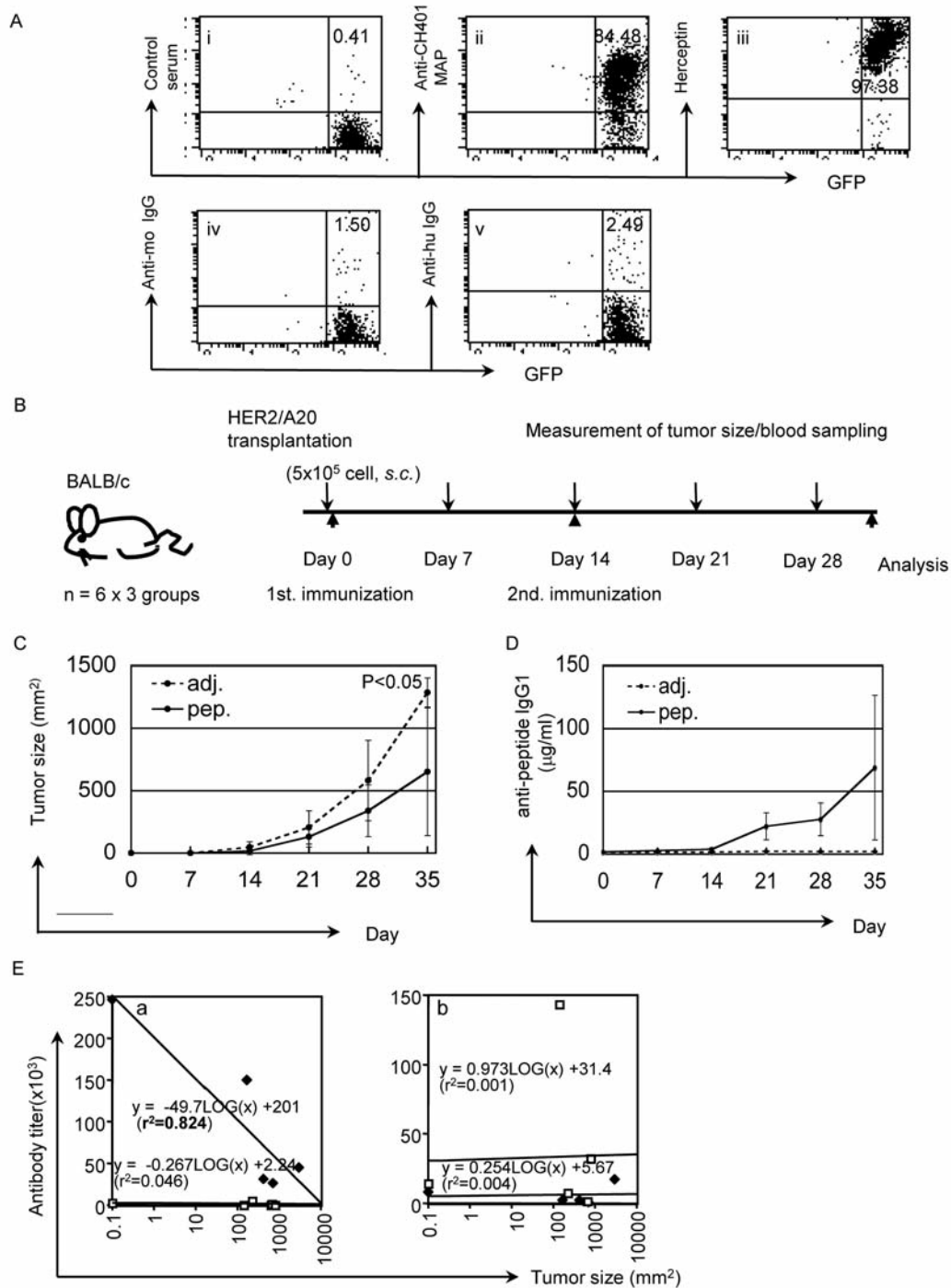


Figure 2. Antitumor effect induced by epitope peptide immunization in BALB/c mouse. **A**: Her-2/neu-transfectant recognized by anti-Her-2/neu antibodies. Her-2/neu-expressing A20 stained with Her-2-recognizing antibodies and secondary antibodies and analyzed by FACSvantage. i. control mouse serum, ii. anti-CH401 peptide antisera, iii. Herceptin, iv. anti-mouse IgG-PE, v. anti-human IgG-PE. Percentages of GFP-positive/anti-Her-2/neu-positive cells are shown in each panel. **B**: Transplantation of Her-2-A20 and immunization with epitope peptide protocol of BALB/c mice. Epitope peptide was immunized simultaneously with Her-2-A20 transplantation, and tumor size was measured weekly. Booster was given after 14 days. Tumor size (mm²) (**C**) and anti-epitope peptide IgG1 concentration in mouse sera (**D**) are shown. Adj: Adjuvant only administered; pep: epitope peptide administered. **E**: Correlation of epitope-specific antibody titer and tumor size. Correlation of subclasses and titer of anti-peptide antibodies are shown (n=6 each). As control peptide, #15 MAP in Figure 2 (N:283-302) was used. Regression curves with regression values under each panel are shown. Open squares represent sera of mice immunized with control peptide and closed diamonds represent those immunized with epitope peptide. Panels show: IgG1 concentrations of (i) anti-CH401 epitope MAP and (ii) anti-#15 MAP.

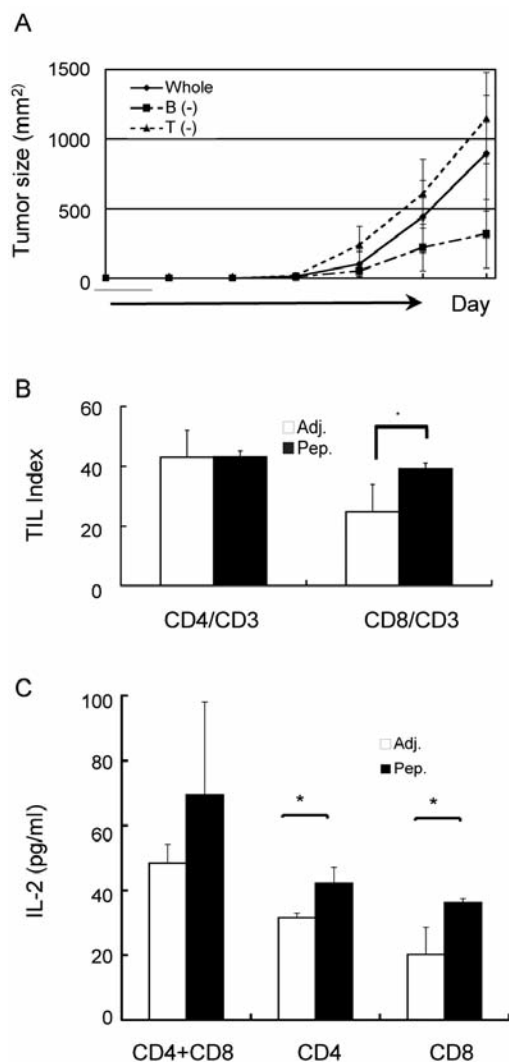


Figure 3. Effect of CH401 MAP immunization on Her-2-A20-transplanted BALB/c-RAG2KO mice. A: Transplantation of Her-2-A20 and immunization with epitope peptide BALB/c-RAG2KO mice transplanted with T-/B-/whole spleen cells. BALB/c spleen T-/B-cells were sorted with a magnetic bead system and transplanted to RAG2 KO mice with BALB/c background. Epitope peptide was immunized simultaneously with Her-2-A20 transplantation to these mice (each group, n=5), and tumor size was measured weekly. Booster was given after 14 days. Tumor size (mm²) of the mice is shown. The mean value and values of each group of mice are shown with standard errors. B: Tumor-infiltrating lymphocytes were prepared from 35-day tumors and the ratios of CD4⁺ and CD8⁺ T-cells were determined by flow cytometry. Percentages of CD4⁺ cells and CD8⁺ cells in the CD3⁺ cell fraction of TIL (TIL index) were calculated and compared between the mice immunized with adjuvant only (adj) and with epitope peptide (pep). The mean value and values of each group of mice are shown with standard errors (adjuvant, n=6; epitope peptide, n=9). C: CD4⁺ and CD8⁺ T-cells were prepared from immunized mice spleens. CD4⁺ cells, CD8⁺ cells, or both were co-cultured with T-cell-depleted antigen-presenting cells of non-stimulated BALB/c mouse spleen in the absence or presence of CH401 epitope peptide. IL-2 concentration (pg/ml) in the culture supernatant was determined by ELISA. Data shown are the mean±SD (n=3). *p<0.05.

potential of CD8⁺ T-cells obtained from mice primed with CH401 peptide, and a significant increase in IL-2 production in the CH401-stimulated CD4⁺ and CD8⁺ T-cells was noted (*p*<0.05), although the level was not as high as in the whole T-cell fraction (Figure 3C). These results suggested that tumor suppression in B-cell-depleted RAG-2 KO mice may occur in collaboration with cytotoxic and helper T-cells stimulated with CH401 peptide.

Taken together, it is suggested that CD4⁺ and CD8⁺ T-cells recognize an epitope on CD401 MAP which might induce antitumor activity in MAP-immunized mice and contribute to tumor suppression in a B-cell antibody-independent manner.

Discussion

The design of suitable peptides is required to achieve an effective peptide-based vaccination for antitumor therapy. In this study, we identified a new B-cell epitope by using a Mab, CH401, that reacts to a certain portion of the Her-2 extracellular domain (23). The epitope was revealed to be near the N-terminus of Her-2/neu (N:167-175, CH401 epitope) and was different from the previously reported epitopes that were mainly located at the juxta-membrane portion. In addition, CH401 epitope was precisely determined from the IgG Mab by epitope mapping and immunogenicity search. The B-cell epitopes in previous reports were almost tentatively predicted by the biochemical and structural features of the antigen character. In general, B-cell epitopes are sometimes non-immunogenic and may include TAA agonistic epitopes, a situation considered problematic. Our CH401 epitope was predicted by algorithmic analysis to contain anchoring motif of MHC class II molecule, meaning CD4⁺ T-cell epitope (Table II). This is consistent with our results that the 20mer peptide (N: 163-182) including CH401 epitope (CH401 epitope peptide) was immunogenic, causing a high titer of peptide-specific IgG antibody in immunized BALB/c mice (Figure 1D), for which activated helper T-cells are essential. In other words, the CH401 epitope peptide may contribute to the efficient production of target antibody because it contained epitopes for both B-cells and helper T-cells.

In mice immunized with CH401 epitope-peptide, tumor growth was significantly suppressed when Her-2/neu-positive tumor cells were transplanted. Such tumor suppression in the individual has been scarce in past reports when they received the peptide containing putative B-cell epitope, except a few reports stating that metastasis of injected cells and engraftment of injected tumors decreased in mice and rats in which vaccination protocols were somehow designed; they used chimera peptide containing B-cell and helper T-cell epitopes or protein with predicted helper T-cell epitope, which were immunized with cytokines (11, 26). In the

Table II. Anchoring motif of CH401 epitope peptide for mouse and human class-I and class-II MHC. SYFPEITHI database (<http://www.syfpeithi.de/scripts/MHCServer.dll/Epitope Prediction>) was used for the epitope prediction of mouse and human MHC. Only high-score MHC is shown.

Mouse Class-II	SYFPEITHI score	Human Class-II	SYFPEITHI score
H-2-A ^d	9	HLA-DRB1*0101	18
H-2-A ^k	10	HLA-DRB1*0301	21
H-2-E ^d	22	HLA-DRB1*0401	28
H-2-E ^k	15	HLA-DRB1*0701	16
		HLA-DRB1*1101	24
		HLA-DRB1*1501	20
Mouse Class-I	SYFPEITHI score	Human Class-I	SYFPEITHI score
H-2-K ^d	21	HLA-B*4101	21
H-2-D ^b	14	HLA-A*01	20
H-2-L ^d	14	HLA-B*4501	20
		HLA-B*5001	20
		HLA-B*2705	17
		HLA-A*1101	15
		HLA-B*2709	15

majority of the past studies, antitumor effects of specific antibodies induced by B-cell epitope peptides were estimated by *in vitro* systems, such as blocking of cell proliferation and cell lysis induction, while the vaccine peptides were designed to be chimeric with multi-epitopes of B-cells and helper T-cells (10, 27).

Tumor growth was not increased after immunization with CH401 epitope peptide in B-cell-depleted mice (Figure 3), although IgG1 antibody against immunized CH401 peptide was negatively correlated with tumor growth (Figure 2). From this we can infer that antibodies induced by the TAA immunization may possess multiple functions against tumor cells *in vivo*, such as cell lysis through antibody-dependent cell cytotoxicity and blocking signals for proliferation, with occasional enhancement for cell proliferation (27, 28). The vaccination of CH401 epitope peptide might cause *in vivo* antitumor effect through peptide-specific T-cells in an antibody-independent fashion. The CH401 epitope-peptide contained the putative MHC class I binding site, in addition to epitopes for B-cells and helper T-cells (Table II). Accordingly, it may be suggested that the tumor suppression induced by vaccination of CH401 epitope peptide mainly occurs in collaboration with helper T-cells and CTL. In regard to this hypothesis, the ratio of CD8⁺ T-cells in TIL of CH401 peptide-immunized mice was larger than in control mice (Figure 2E), although the apoptotic activity of the peptide-induced CTL against Her-2-positive tumor cells was not investigated in this study. Moreover, CD8⁺ T-cells increased secretion of IL-2 after the epitope peptide stimulation *in vitro* compared to non-stimulated T-cells, suggesting that the peptide did not induce anergy for the cytotoxic T-cells.

For the peptide vaccination, we prepared a certain peptide based on the exact B-cell epitope identified from anti-Her-2 Mab, and identified putative epitopes for helper T-cells and for CTL in the peptide spanning the B-cell epitope. As we prepared the peptide containing these epitopes, high production of endogenous-specific IgG was observed. Moreover, tumor suppressive activity may be induced in individuals receiving the vaccination even if the induced antibody does not possess the potential for antitumor activity itself. Therefore, it is suggested that CH401 MAP induces an efficient antitumor effect in human immunity. If a B-cell epitope peptide with high specificity includes both class I and class II MHC anchoring motifs, it may be adopted for cancer therapy by inducing effective cytotoxic T-cells and antibodies.

References

- 1 Stern M and Hermann R: Overview of monoclonal antibodies in cancer therapy: present and promise. *Crit Rev Oncol Hematol* 54: 11-29, 2005.
- 2 Borghaei H, Smith M and Campbell K: Immunotherapy of cancer. *Eur J Pharmacol* 625: 41-54, 2009.
- 3 Campoli M, Ferris R, Ferrone S and Wang X: Immunotherapy of malignant disease with tumor antigen-specific monoclonal antibodies. *Clin Cancer Res* 16: 11-20, 2010.
- 4 Jager D and Knuth A: Antibodies and vaccines-Hope or illusion? *Breast* 14: 631-635, 2005.
- 5 Reuschenbach M, von Knebel Doeberitz M and Wentzensen N: A systematic review of humoral immune responses against tumor antigens. *Cancer Immunol Immunother* 58: 1535-1544, 2009.
- 6 Shawver L, Mann E, Elliger S, Dugger T and Arteaga C: Ligand-like effects induced by anti-c-erbB-2 antibodies do not correlate

- with and are not required for growth inhibition of human carcinoma cells. *Cancer Res* 54: 1367-1373, 1994.
- 7 Tam JP: Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system. *Biochemistry* 85: 5409-5413, 1988.
 - 8 Tan T-T and Coussens L: Humoral immunity, inflammation and cancer. *Curr Opin Immunol* 19: 209-216, 2007.
 - 9 Andreu P, Johansson M, Affara N, Pucci F, Tan T, Junankar S, Korets L, Lam J, Tawfik D, DeNardo D, Naldini L, de Visser K, De Palma M and Coussens L: FcR γ activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* 17: 121-134, 2010.
 - 10 Dakappagari N, Douglas D, Triozzi P, Stevens V and Kaumaya P: Prevention of mammary tumors with a chimeric Her-2 B-cell epitope peptide vaccine. *Cancer Res* 60: 3782-3789, 2000.
 - 11 Dakappagari N, Pyles J, Parihar R, Carson W, Young C and Maumaya P: A chimeric multi-human epidermal growth factor receptor-2 B cell epitope peptide vaccine mediates superior antitumor responses. *J Immunol* 170: 4242-4253, 2003.
 - 12 Riemer A, Klinger M, Wagner S, Bernhaus A, Mazzucchelli L, Pehamberger H, Scheiner O, Zielinski C and Jensen-Jarolim E: Generation of peptide mimics of the epitope recognized by Trastuzumab on the oncogenic protein Her-2/neu. *J Immunol* 173: 394-401, 2004.
 - 13 Jiang B, Liu W, Qu H, Meng L, Song S, Ouyang T and Shou C: A novel peptide isolated from a phage display peptide library with Trastuzumab can mimic antigen epitope of HER-2. *J Biol Chem* 280: 4656-4662, 2005.
 - 14 Itoh K, Yamada A, Mine T and Noguchi M: Recent advances in cancer vaccines: An Overview. *Jpn J Clin Oncol* 39: 73-80, 2009.
 - 15 Boon T, Coulie P, van den Eynde B and van der Bruggen P: Human T cell responses against melanoma. *Annu Rev Immunol* 24: 175-208, 2006.
 - 16 Oka Y, Tsuboi A, Oji Y, Kawase I and Sugiyama H: WT1 peptide vaccine for the treatment of cancer. *Curr Opin Immunol* 20: 211-220, 2008.
 - 17 Rosenberg S: Progress in human tumor immunology and immunotherapy. *Nature* 411: 380-384, 2001.
 - 18 Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D and Levitsky H: The central role of CD4(+) T cells in the antitumor immune response. *J Exp Med* 188: 2357-2368, 1998.
 - 19 Rocha B and Tanchot C: Towards a cellular definition of CD8+ T-cell memory. *Curr Opin Immunol* 16: 259-263, 2004.
 - 20 Kennedy R and Celis E: Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunol Rev* 222: 129-144, 2008.
 - 21 Kobayashi H, Celis E: Peptide epitope identification for tumor-reactive CD4 T cells. *Curr Opin Immunol* 20: 221-227, 2008.
 - 22 Humphreys R, Hillman G, von Hofe E and Xu M: Forcing tumor cells to present their own tumor antigens to the immune system: a necessary design for an efficient tumor immunotherapy. *Cell Mol Immunol* 1: 180-185, 2004.
 - 23 Ishida T, Tsujisaki M, Hinoda Y, Imai K and Yachi A: Establishment and characterization of mouse-human chimeric monoclonal antibody to erbB-2 product. *Jpn J Cancer Res* 85: 172-178, 1994.
 - 24 Sasaki S, Tsujisaki M, Jinnohara T, Ishida T, Sekiya M, Adachi M, Takahashi S, Hinoda Y and Imai K: Human tumor growth suppression by apoptosis induced with anti-ErbB-2 chimeric monoclonal antibody. *Jpn J Cancer Res* 89: 562-570, 1998.
 - 25 Southwood S, Sindy J, Kondo A, del Guercio M-F, Appella E, Hoffman S, Kubo R, Chesnut R, Grey H and Sette A: Several common HLA-DR types share largely overlapping peptide binding repertoires. *J Immunol* 160: 3363-3373, 1998.
 - 26 Renard V, Sonderbye L, Ebbehoj K, Rasmussen P, Gregorius K, Gottschalk T, Mouritsen S, Gautam A and Leach D: Her-2 DNA and protein vaccines containing potent Th cell epitopes induce distinct protective and therapeutic antitumor responses in Her-2 transgenic mice. *J. Immunol* 171: 1588-1595, 2003.
 - 27 Montgomery R, Makary E, Schiffman K, Goodell V and Disis M: Endogenous anti-Her2 antibodies block Her2 phosphorylation and signaling through extracellular signal-regulated kinase. *Cancer Res* 65: 650-656, 2005.
 - 28 Peipp M, Dechant M and Valerius T: Effector mechanisms of therapeutic antibodies against ErbB receptors. *Curr Opin Immunol* 20: 436-443, 2008.

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