**Abstract**

**Purpose:** We developed a complex of tumor antigen protein with a novel nanoparticle antigen delivery system of cholesteryl pullulan (CHP). To target HER2 antigen, we prepared truncated HER2 protein 1-146 (146HER2) complexed with CHP, the CHP-HER2 vaccine. We designed a clinical study to assess the safety of the vaccine and HER2-specific T-cell immune responses measured by the newly developed enzyme-linked immunospot assay with mRNA-transduced phytohemagglutinin-stimulated CD4+ T cells in HLA-A2402-positive patients with therapy-refractory HER2-expressing cancers.

**Experimental Design:** Nine patients with various types of solid tumors were enrolled. Each patient was s.c. vaccinated biweekly with 300 μg of CHP-HER2 vaccine for three times followed by booster doses. HER2-specific T-cell responses were evaluated by enzyme-linked immunospot assay by targeting autologous phytohemagglutinin-stimulated CD4+ T-cells transduced with 146HER2-encoding mRNA to cover both identified peptides and unknown epitopes for MHC class I and class II that might exist in the sequence of the vaccine protein.

**Results:** CHP-HER2 vaccine was well tolerated; the only adverse effect was grade 1 transient skin reaction at the sites of vaccination. HER2-specific CD8+ and/or CD4+ T-cell immune responses were detected in five patients who received four to eight vaccinations, among whom both T-cell responses were detected in these patients. In four patients with CD8+ T-cell responses, two patients reacted to previously identified HER263-71 peptide and the other two reacted only to 146HER2 mRNA-transduced cells.

**Conclusions:** CHP-HER2 vaccine was safe and induced HER2-specific CD8+ and/or CD4+ T-cell immune responses.

**Protein-based cancer vaccine may potentially activate both CD8+ T cells and CD4+ T cells by presenting multiple epitopes. However, although exogenous soluble protein antigens incorporated by antigen-presenting cells (APC) are in general efficient in sensitizing CD4+ T cells, they are inefficient in sensitizing CD8+ T cells because these proteins are hardly processed by MHC class I pathway. To overcome this, we have developed a novel protein antigen delivery system consisting of cholesteryl pullulan (CHP) nanogels complexed with soluble protein molecules in an attempt to present peptides that can bind both MHC class I and class II molecules in APCs (1, 2).**

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**Note:** Sh. Kitano and S. Kageyama contributed equally to this work.

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peptide derived from 146HER2, an epitope we had previously defined, restricted to murine MHC class I, K\(^d\) (1, 6). Immunized mice rejected HER2-expressing tumors in a CD8\(^{+}\) T-cell-dependent manner (1). Similar immunization with CHP-HER2 complex also led to generation of multiple CD4\(^{+}\) T-cell responses. We prepared a CHP-HER2 vaccine of clinical grade and did a phase I clinical trial in HER2-expressing cancer patients to evaluate safety of the vaccine and investigate specific T-cell immune responses with the newly developed enzyme-linked immunospot (ELISPOT) assay (8, 9). This is the first report of the clinical application of CHP-based protein vaccine.

Materials and Methods

The CHP-HER2 vaccine. Truncated HER2 cDNA encoding an NH\(_2\)-terminal portion (amino acids 1-146) was PCR amplified and inserted into pET15b (Novagen, Madison, WI). Escherichia coli strain JM109 was transformed with the resulting plasmid and induced by isopropyl-L-thio-B-D-galactopyranoside to produce 6His-tagged truncated HER2 protein. The recombinant protein of truncated HER2 was produced stably by E. coli (Asahi Glass Co., Tokyo, Japan). CHP was synthesized by the reaction of pullulan with cholesterol isocyanate in pyridine and DMSO (Nippon Oil and Fat Co., Tokyo, Japan). After purification by extraction and precipitation, the resultant CHP was emulsified in water and subsequently freeze dried. In solution in water, CHP was spontaneously assembled to form nanoparticles (nanogels). These nanogels (20-50 nm in diameter; Supplemental Figure 1) contain hydrophobic domains of cholesterol groups that associate with the hydrophobic regions of the 146HER2 protein, forming a stable complex in solution. The combined complex is used as CHP-HER2 vaccine (3–5).

All processes were done under current Good Manufacturing Practice conditions. In preclinical animal studies, CHP-HER2 complex did not show any significant toxicity, except local mild skin swelling at the site of injection. The vaccine material was regularly examined for its biochemical and biological stability.

Clinical trial. A clinical trial of CHP-HER2 vaccine (LUD01-016) was designed as a phase I clinical trial to evaluate its toxicity and the specific immune reactions to HER2 antigen. The eligibility criteria included the following: (a) HER2-positive cancers refractory to standard chemotherapy or patients with metastatic disease who rejected further therapy, (b) positivity for HLA-A2402, and (c) HER2 expression in tumor cells scored as 1\(^{+}\) or more, confirmed by immunohistochemistry (HercepTest, DAKO Corp., Carpinteria, CA).

CHP-HER2 vaccine was injected s.c. every 2 weeks for three times at a dose of 300 \(\mu\)g 146HER2 protein. Two weeks after the third cycle of vaccination, the patient was evaluated for safety. Based on the patient’s decision, injections of CHP-HER2 vaccine were continued with an adjuvant of either recombinant human granulocyte macrophage colony-stimulating factor (Leukine, Berlex, Richmond, CA) or OK-432, an immunomodulator (Chugai Pharmaceutical, Tokyo, Japan; refs. 10, 11). Granulocyte macrophage colony-stimulating factor was given at each dose of 75 \(\mu\)g for 5 consecutive days, or 0.02 mg OK-432 was given on the day of vaccination. The toxicity was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0, 2003). The tumor responses were determined by the Response Evaluation Criteria in Solid Tumors criteria. This trial was approved by the Ethics Committees of Mie and Nagasaki Universities, and it was conducted in full conformity with the current revision of the Declaration of Helsinki. Written informed consent was obtained from each patient enrolled in the trial.

Cell line. T2-A24 is a transfected cell line with HLA-A2402 cDNA construct from the original T2 cell line, which is deficient in TAP transporter proteins.

Peptides. HER2\(_{63-71}\) peptide (6, 12), TYLPTNASL, and MAGE-A4124-132 peptide (8), KYRAKELVT, were synthesized at a purity exceeding 90% and supplied by Sawady Technology (Tokyo, Japan). Peptides were dissolved in DMSO at concentration of 10 mg/mL and stored in aliquots at –80°C before use.

Preparation of phytohemagglutinin-stimulated CD4\(^{+}\) T cells. Phytohemagglutinin (PHA)-stimulated CD4\(^{+}\) T cells (T-APC) were prepared as described previously (8, 9, 13). Briefly, CD4\(^{+}\) T cells were separated...
Transcription was done with T7 polymerase (mMESSAGE mMACHINE). Plasmids were linearized with appropriate restriction enzymes. In vitro CD8+ T cells or CD4+ T cells and also used as target cells in ELISPOT (Qiagen, Hilden, Germany). Plasmids were purified by using Qiagen EndoFree Plasmid Mega kit (Ludwig Institute for Cancer Research, Brussels, Belgium). These plasmids were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) was a gift from Dr. T. Boon (Ambion, Austin, TX). Full-length MAGE-A4 cDNA cloned into pTRIamp18 (Ambion, Austin, TX) were cloned into pcDNA3.1 (BD Bioscience Clontech, Palo Alto, CA) were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) was a gift from Dr. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). These plasmids were purified by using Qiagen EndoFree Plasmid Mega kit (Qiagen, Hilden, Germany).

Preparation of mRNA. Before in vitro mRNA synthesis, the plasmids were linearized with appropriate restriction enzymes. In vitro transcription was done with T7 polymerase (mMESSAGE mMACHINE T7 kit, Ambion), and in vitro–transcribed RNA was polyadenylated using poly(A) polymerase [Poly(A) Tailing kit, Ambion] according to the instructions supplied by the manufacturer. The resulting capped and tailed RNA was resuspended in water and stored at −80°C before use.

mRNA electroporation. T-APCs were harvested and washed twice and resuspended in X-VIVO 20 (Takara Bio, Inc., Shiga, Japan). mRNA (10 μg) was mixed with 200 μL suspensions of 2.5 × 10^6 to 5 × 10^6 cells and transferred to a 2-mm gap cuvette (Genetronics, Inc., San Diego, CA). Using the ECM 830 square wave electroretropator (BTX, San Diego, CA), mRNA was electroporated into the cells. The cells were immediately suspended in 5.0 mL X-VIVO 20 and cultured 18 to 24 h in six-well plates at 37°C in a CO2 incubator until use.

In vitro sensitization for monitoring T-cell immune responses. After separating CD8+ T cells or CD4+ T cells from peripheral blood mononuclear cells using MACS CD8 or CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), the T cells (0.5 × 10^6 to 1 × 10^6 per well) were cultured with autologous 146HER2 mRNA-transduced and irradiated (30 Gy) T-APC (1 × 10^6 cells per well) in 24-well plates in RPMI 1640 supplemented with 10% AB serum and then stimulated with autologous 146HER2 mRNA-transduced and irradiated T-APC on weekly basis for two cycles. On day 8, half of the medium was replaced with complete medium containing interleukin-2 (20 units/mL; kindly provided by Takeda Pharmaceutical Corp., Osaka, Japan) and resuspended in X-VIVO 20 and cultured 18 to 24 h in six-well plates at 37°C in a CO2 incubator until use.

Table 1. Clinical characteristics and events

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Disease</th>
<th>HER2 expression</th>
<th>Status at enrollment</th>
<th>Adverse events (grade)</th>
<th>Clinical responses*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>M</td>
<td>Lung</td>
<td>2+</td>
<td>PD after multi-CT</td>
<td>Skin reaction (1)</td>
<td>SD</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>F</td>
<td>Breast</td>
<td>3+</td>
<td>SD after multi-CT</td>
<td>Skin reaction (2)</td>
<td>SD</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>F</td>
<td>Breast</td>
<td>3+</td>
<td>SD after multi-CT</td>
<td>Skin reaction (1)</td>
<td>SD</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>F</td>
<td>Breast</td>
<td>2+</td>
<td>PR after CT</td>
<td>Skin reaction (1)</td>
<td>NE</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
<td>M</td>
<td>Pancreas</td>
<td>2+</td>
<td>Nonresectable tumor</td>
<td>Skin reaction (1)</td>
<td>SD</td>
</tr>
<tr>
<td>6</td>
<td>58</td>
<td>M</td>
<td>Urinary pelvis</td>
<td>2+</td>
<td>Recurrence after surgery</td>
<td>Skin reaction (1)</td>
<td>SD</td>
</tr>
<tr>
<td>7</td>
<td>62</td>
<td>M</td>
<td>Nasal cavity</td>
<td>1+</td>
<td>PD after multi-CT</td>
<td>Skin reaction (1)</td>
<td>PD</td>
</tr>
<tr>
<td>8</td>
<td>66</td>
<td>F</td>
<td>Lung</td>
<td>1+</td>
<td>PD after multi-CT</td>
<td>Skin reaction (1)</td>
<td>SD</td>
</tr>
<tr>
<td>9</td>
<td>42</td>
<td>F</td>
<td>Breast</td>
<td>1+</td>
<td>Recurrence after surgery</td>
<td>Skin reaction (1), dry skin (1)</td>
<td>SD</td>
</tr>
</tbody>
</table>

Abbreviations: PD, progressive disease; SD, stable disease; PR, partial response; CT, chemotherapy; NE, not evaluable.

*Clinical responses were evaluated at the completion of three cycles of CHP-HER2 vaccinations.

from peripheral blood mononuclear cells using MACS CD4 microbeads (Miltenyi Biotec, Auburn, CA) and seeded into 24-well plates (Nunc, Roskilde, Denmark) at a density of 1 × 10^6 to 2 × 10^6 per well in 1 mL RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 25 mmol/L HEPES, 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. On day 0, 10 μg/mL PHA (Lectin, Sigma Chemical Co., St. Louis, MO) was added to the culture. On day 3, half of the medium was replaced with complete medium containing interleukin-2 (20 units/mL; kindly provided by Takeda Pharmaceutical Corp., Osaka, Japan) and interleukin-7 (40 ng/mL; R&D Systems, Minneapolis, MN), and it was repeated twice weekly. The activated CD4+ T cells (T-APC) were electroporated with antigen mRNA and used for in vitro stimulation of CD8+ T cells or CD4+ T cells and also used as target cells in ELISPOT assay around days 14 to 21 of the culture (Fig. 1).

Plasmids. Truncated HER2 cDNA encoding an NH2-terminal portion (amino acids 1-146) and enhanced green fluorescent protein cDNA (BD Bioscience Clontech, Palo Alto, CA) were cloned into pTRIamp18 (Ambion, Austin, TX). Full-length MAGE-A4 cDNA cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) was a gift from Dr. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). These plasmids were purified by using Qiagen EndoFree Plasmid Mega kit (Qiagen, Hilden, Germany).

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Table 2. Immune responses and clinical outcomes

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>No. vaccinations*</th>
<th>HER2-specific immune responses†</th>
<th>TTP (mo)</th>
<th>Follow-up (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>Detected after vaccination 8</td>
<td>5</td>
<td>23 (died)</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>Detected after vaccination 8</td>
<td>6</td>
<td>22+</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>ND</td>
<td>5</td>
<td>13 (died)</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>ND</td>
<td>&gt;25+</td>
<td>25+</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>ND</td>
<td>3</td>
<td>6 (died)</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>Detected after vaccination 9</td>
<td>&gt;19+</td>
<td>19+</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>Detected after vaccination 5</td>
<td>2</td>
<td>4 (died)</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>ND</td>
<td>3</td>
<td>10 (died)</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>ND</td>
<td>16</td>
<td>16+</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not detected; TTP, time to progression.

*Granulocyte macrophage colony-stimulating factor or OK-432 was coadministered after the fourth vaccination.
†The vaccination cycle when T-cell responses were first detected. A positive immune response was defined as spots more than three times those of the negative controls in ELISPOT assay.
**ELISPOT assay.** Human IFN-γ ELISPOT assay was done as described previously with some modifications (8, 9). Briefly, 96-well nitrocellulose ELISPOT plates (MAHA S4510, Millipore, Bedford, MA) were coated overnight at 4°C with 2 μg/ml anti-human IFN-γ monoclonal antibody (1-D1K, Mabtech, Nacka, Sweden). The wells were washed with PBS containing 0.05% Tween 20 and blocked with 10% human AB serum RPMI 1640 for 2 h at 37°C. Sensitized 5 × 10^8 (or 2 × 10^7) CD8⁺ T cells or CD4⁺ T cells and 1 × 10^5 peptide-pulsed T2-A24 cells or antigen mRNA-transduced T-APCs were placed in each well of the ELISPOT plate at a final volume of 200 μL. After incubation for 22 h at 37°C in a CO₂ incubator, the plate was washed thoroughly with PBS containing 0.05% Tween 20 and then supplemented with 0.2 μg/mL biotinylated anti-human IFN-γ monoclonal antibody (7-B6-1, Mabtech) and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween 20, they were reacted with 1 μg/mL streptavidin-alkaline phosphatase conjugate (Mabtech) in 100 μL PBS for 90 min at room temperature. The wells were washed twice with PBS containing 0.05% Tween 20 and then stained with an alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, CA). The reaction was stopped by rinsing the plates with distilled water. After drying the plates, the spots were counted using a dissecting microscope (Carl Zeiss Vision, Hallbergmoos, Germany). A positive immune response was defined as spots more than three times those of the negative controls.

**Results**

**Safety of CHP-HER2 vaccine and clinical outcome.** Nine patients were enrolled in this clinical trial, including eight patients who were refractory to standard therapies and one in partial remission after chemotherapy (patient no. 4; Table 1). All patients developed mild transient skin reactions at the sites of vaccine injection. These included erythema-like lesions with an approximate diameter of 2 to 3 cm after 2 h of vaccine injection, which lasted 20 to 30 h and then resolved within 48 h without any treatment. Other adverse effects included grade 1 general fatigue, diarrhea, and dry skin in one patient (Table 1), but they did not require any treatment or hamper further vaccinations.

All patients were evaluated for the safety of CHP-HER2 after three cycles of vaccinations. Because the safety of vaccination was determined in each patient, we were able to provide booster injections. There were no significant additional adverse events in repeated vaccinations from 6 to 38 injections, except grade 2 skin reaction in patient no. 2 (Tables 1 and 2).

No tumor regression was observed in any of the patients throughout CHP-HER2 vaccinations. During the first 8 weeks, seven patients remained in stable disease, although patient no. 7 showed disease progression (Table 1). In the long-term follow-up, disease progression was noted in seven patients within a median of 5 months (range, 2-16 months), and five patients died. The remaining two patients (nos. 4 and 6) have shown no disease progression over a period of 19 and 25 months, respectively. Four patients were followed up for a period of 16 to 25 months (Table 2).

**Detection of CD8⁺ T cells specific for HER2 antigen following vaccination.** To monitor the immune responses induced by antigen protein vaccine, we applied the newly developed ELISPOT assay using T-APC electroporated with antigen mRNA as APC (8, 9). As shown in Fig. 1, CD4⁺ T cells were isolated and stimulated by PHA and then cultured for 10 to 14 days. As T-APC expresses not only MHC class I and class II molecules but also CD80 and CD86, they are also considered to be suitable target cells in ELISPOT assay (9). These T-APCs were used in this ELISPOT assay to detect overall T-cell immune responses covering not only readily identified epitopes but also yet undefined epitopes that might exist in 146HER2. CD8⁺
T-cell responses were assayed by ELISPOT over the CHP-HER2 vaccinations in nine patients, whose peripheral blood CD8⁺ T cells were stimulated in vitro with 146HER2 mRNA, HER253-71 peptide-pulsed T-APCs, and the T-APCs were also used as target cells. There was no detectable CD8⁺ T-cell response against 146HER2 antigen before vaccinations as shown in Fig. 2. Specific CD8⁺ T cells became detectable in four patients, nos. 1, 2, 6, and 7, after five to nine vaccinations (Table 2; Fig. 2). Figure 3A-D shows the results of ELISPOT assays in individual patients when specific responses were detected for the first time as described in Table 2. Enhanced green fluorescent protein mRNA-transduced T-APC, MAGE-A4 mRNA-transduced T-APC, and untreated T-APC were used as target cells in ELISPOT assays.
T-APC were used as controls. Each column represents the number of IFN-γ-releasing cells in 5 × 10⁵ (or 2 × 10⁵) CD8⁺ T cells, which were derived from independently cultured wells.

Because previously defined HLA-A2402-restricted HER2₆₃₋₇₁ peptide is a part of 146HER2, we analyzed in parallel HER2₆₃₋₇₁ peptide-specific responses in these four 146HER2 responders. Patient nos. 1 and 6 responded to HER2₆₃₋₇₁ peptide-pulsed T2-A24 cells as shown in Fig. 3E and F, whereas the other two patients, nos. 2 and 7, did not.

Detection of CD4⁺ T cells specific for HER2 antigen following vaccination. Similar to CD8⁺ T-cell responses, there was no detectable specific CD4⁺ T-cell response before vaccinations (Fig. 2). It became detectable in four patients, nos. 1, 6, 7, and 9, after four to eight vaccinations as shown in Table 2 and Fig. 2. Results of ELISPOT assays when specific CD4⁺ T cells became detectable for the first time are shown in each patient (Fig. 3G-J).

In three patients (nos. 1, 6, and 7), HER2-specific immune responses were detected in both CD8⁺ T cells and CD4⁺ T cells (Table 2; Fig. 2).

Changes in HER2-specific T-cell immunity by boosting vaccinations. We analyzed the chronological responses of HER2-specific CD8⁺ T cells and CD4⁺ T cells in patient no. 1 who received long-term booster vaccinations. As shown in Fig. 4, the CD8⁺ T cells became detectable after the eighth vaccination, where only a single culture well out of six independently cultured wells (initial 1 × 10⁶ CD8⁺ T cells) was positive for 146HER2 mRNA-transduced T-APC. At the 14th vaccination, 146HER2-specific CD8⁺ T cells were positive in three of four wells, and the CD4⁺ T cells were positive in two of four wells. After 19 vaccinations, 9 of 10 wells (initial 5 × 10⁵ CD8⁺ T cells) were positive for 146HER2 mRNA-transduced T-APC.

Discussion

We recently developed a system for analysis of T-cell responses to any antigen molecule in individuals regardless of their HLA types by the use of T-APC as APC in ELISPOT assay (8, 9). These T-APCs express CD80 and CD86, in addition to...
MHC class I and class II molecules (9). To deliver cognate whole antigen molecules into APC, we also attempted to use mRNA encoding antigens. The PHA-stimulated mRNA-transduced CD4+ T cells were found to be suitable target cells for ELISPOT assay. In addition, these PHA-stimulated antigen mRNA-transduced CD4+ T cells could also sensitize T cells in vitro to generate antigen-specific T cells (9).

HER2 protein, once complexed with CHP, could elicit HER2-specific CD8+ T cells as well as CD4+ T cells in HER2-expressing cancer patients. We found five responding cases out of nine vaccinated patients, in whom four were positive for CD8+ T cells and also four were positive for CD4+ T cells specific against 146HER2 by ELISPOT assay. Among them, in three patients, both CD8+ T cells and CD4+ T cells were detected. In four cases with CD8+ T cells detectable, we analyzed the immune responses to a previously identified epitope, HER263-71 peptide restricted to HLA-A2402 as well (12). We found two cases with HER263-71 peptide-specific responses, whereas in the other two patients CD8+ T cells were reactive only to 146HER2 mRNA-transduced target cells. These two patients were considered to have responded to either undefined HLA-A2402-restricted epitopes other than HER263-71 peptide or peptides for other HLA alleles. Hence, to monitor overall specific T-cell responses covering whole epitopes of an antigen, the method of antigen mRNA-transduced CD4+ T cells will be a method to be applied.

HER2-specific immune responses by CD8+ T cells as well as CD4+ T cells became detectable as a consequence of multiple vaccinations of CHP-HER2. This clearly indicates that CHP functions in vivo as a protein antigen delivery system to bring cognate peptides to both MHC class I and class II pathways as expected from our previous analysis of in vitro human systems and in animal systems (1, 2). We used granulocyte macrophage colony-stimulating factor or OK-432 as immunoadjuvants with

![Graphs showing ELISPOT results](image-url)

**Fig. 4.** ELISPOT at the repeated vaccinations for 146HER2-specific CD8+ T-cell and/or CD4+ T-cell responses in patient no. 1. Columns: IFN-γ-releasing cells in independent culture wells that responded to 146HER2-mRNA, enhanced green fluorescent protein-mRNA, and MAGE-A4-mRNA-transduced T-APC.
boosting doses from the fourth vaccinations. OK-432 is a penicillin-killed and lyophilized preparation of a low-virulence strain of *Streptococcus pyogenes* (group A; refs. 10, 11). It is not yet clear whether and how granulocyte macrophage colony-stimulating factor or OK-432 contributed to induction of HER2-specific immune reactions in this trial. It will be required to design a trial to show their advantage in CHP-protein cancer vaccine. Our result might suggest that frequent vaccinations would be required to obtain maximum magnitude of T-cell immunity.

There have been several reports of HER2-targeting cancer vaccine using peptides (14–18). HLA-A0201–binding peptide for CD8+ T cell, E75 peptide of 369 to 377 amino acids, has been often investigated. It was reported that the peptide could elicit cellular responses (14–18), and in a phase II trial for resected breast cancer patients at high risk of disease recurrence, the induced HER2 immunity seems to reduce the recurrence rate (17). Cancer vaccine targeting HER2 antigen would be considered to be clinically beneficial. Disis et al. (19) applied protein-based vaccine, HER2 intracellular domain of 676 to 1,255 amino acids. They allocated the vaccine doses to three groups, low dose (25 µg), intermediate dose (150 µg), and high dose (900 µg), and reported that the dose did not predict the magnitude of the T-cell response but that the time to development of detectable HER2-specific immunity was significantly earlier for the high-dose vaccine group. We observed in our trial that five to nine vaccinations with the time of 2.5 to 4.5 months were needed to detect HER2-specific CD8+ T cells in peripheral blood. It is not clear yet whether 300 µg 146HER2 protein in our setting is an optimal dose to induce HER2-specific CD8+ T cells or higher doses could be more immunogenic and induce earlier immune reactions. To cancer patients who are in a refractory phase to standard chemotherapy, rapid induction of immune responses is naturally desirable. We found that booster vaccinations with >14 times increased the proportion of HER2-specific CD8+ T cells in patient no.1. This result suggests that frequent vaccinations would be required to obtain maximum magnitude of T-cell immunity. It should be examined whether it could be shortened with higher vaccine doses or with use of immunoadjuvant, which could be given simultaneously with the vaccine.

Protein-based cancer vaccines using antigen delivery systems, such as liposome and ISCOMATRIX, have been reported (20–24). Liposome is a delivery consisting of bilayer phospholipid, which is broadly used as various drug delivery systems. Liposome vaccine that carries idiotype protein and adjuvant cytokines for follicular lymphoma was developed (20, 21). In a clinical trial using the vaccine combined with interleukin-2, both CD8+ and CD4+ T-cell immunities were induced along with specific antibody production (21). Another antigen delivery device is ISCOMATRIX with a diameter of 50 nm (22–24). It induced significant CD8+ and CD4+ T-cell responses not only to viral antigens, including influenza virus, hepatitis C virus, and human papillomavirus, but also to tumor antigen, NY-ESO-1 antigen (22–24). The similarity of these protein-based vaccines to our CHP vaccine in terms of antigen delivery capacity is still unknown, although they are all able to present MHC class I and class II peptides, inducing antigen-specific CD8+ and CD4+ T cells.

CHP vaccine is a simple complex of tumor antigen protein and carbohydrates with cholesteryl moieties, presuming to be harmless. As we showed in this trial, CHP-HER2 was well tolerated and feasible even in long-term vaccinations. The only common adverse event was mild skin reaction at the site of injection. As the lesions do not deteriorate by additional vaccinations, they do not seem to interfere with repetitive CHP-HER2 vaccinations. CHP-protein complex vaccine is technically simple to formulate and chemically stable in solution for over a period of years (data not shown). These characteristics may facilitate wide distribution of CHP vaccine once its clinical usefulness is established.

In conclusion, CHP-HER2 vaccine is safe and induces specific CD8+ and CD4+ T-cell responses against HER2 as expected in preclinical studies. CHP-HER2 vaccine trial is considered to be a role model for the development of efficient cancer vaccine that can be applied for various categories of tumor antigens.

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**References**


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