cursors, as previously reported (Prieyl & LeBien, 1996; Napolitano *et al*, 2003; Parrish *et al*, 2009). IL-15 mainly stimulated the $CD7^-CD56^+$ NK cell generation. Notch ligands DLL1 and DLL4, which are important for T cell development in mice (Koch *et al*, 2008; Koch & Radtke, 2011), were expressed in hTERT stromal cells (*DLL1/GAPDH*, $8{\cdot}62 \pm 0{\cdot}72 \times 10^{-4}$: *DLL4/GAPDH*, $6{\cdot}65 \pm 6{\cdot}60 \times 10^{-5}$). However, the soluble form of Notch ligand has been shown to be much less effective in activating Notch signalling than membranous or immobilized forms of Notch ligand (Varnum-Finney *et al*, 2000). Consistently, the expression level of one Notch target gene, *HES1*, in haematopoietic progenitors was not remarkably changed by coculture with the stromal cells (data not shown). Further studies are required to identify the critical factors required for early B and T/NK lymphopoiesis.

The coculture assays allowed us to demonstrate that flt3L simultaneously promotes the generation of not only early B and T/NK lymphoid precursors but also myeloid and plasmacytoid dendritic cells from haematopoietic progenitors on stromal cells. Flt3L has a positive effect on the generation of $CD19^+$ B cell precursors (Rawlings *et al*, 1997; Parrish *et al*, 2009), $CD34^+CD7^-CD122$ (IL-2/15 receptor β)$^+$ NK cell precursors (Yu *et al*, 1998), and myeloid or plasmacytoid dendritic cells (Chen *et al*, 2004; Rossi & Young, 2005; Ueno *et al*, 2011). The present study confirms these observations, and further demonstrates that flt3L also enhances the development of $CD7^+$ lymphoid precursors with T cell potential without significantly affecting their differentiation. The single-cell assays suggest that flt3L may directly stimulate the growth of lymphohaematopoietic progenitors. The plasma concentrations of flt3L are as low as approximately 20 pg/ml under steady-state conditions, but increase by more than 100 times during the haematopoietic recovery phase after chemotherapy or transplantation (Wodnar-Filipowicz *et al*, 1996). From these findings, we speculate that flt3L is a critical stimulator of B and T/NK lineage cell generation, at least when large numbers of B, T and NK cells need to be rapidly regenerated after myelosuppression. Notably, in the serum-free cultures, even $CD19^-cyCD79a^+$ early B cell precursors were rarely observed in the absence of cytokines, whereas $CD19^+cyCD79a^+$ pro-B cells, in addition to a significant number of $CD19^-cyCD79a^+$ cells, were generated in the presence of flt3L. These observations imply that flt3L is particularly important for human B cell development from haematopoietic progenitors.

SCF and TPO are involved in the survival and proliferation of primitive haematopoietic progenitors (Heike & Nakahata, 2002). Unlike flt3L, SCF or TPO alone had little or no effect on the generation of B and T/NK lymphoid precursors. However, SCF or TPO, in combination with flt3L, significantly augmented the generation of B and T/NK lymphoid precursors without considerably affecting their differentiation. These findings indicate that flt3L is a principal cytokine involved in early B and T/NK cell development, and that SCF and TPO act synergistically with flt3L to enhance lymphoid lineage development, presumably by stimulating the growth of primitive haematopoietic progenitors with lymphoid differentiation potential.

Not only telomerized but also primary bone marrow stromal cells supported the generation of early B and T/NK cell precursors from human haematopoietic progenitors, and similar stimulatory effects by flt3L were observed in cocultures with both types of stromal cells. These data are consistent with the notion that the telomerized human bone marrow stromal cells maintain the phenotype of primary human bone marrow stromal cells (Kawano *et al*, 2003; Kobune *et al*, 2005). Altogether, our findings indicate that the telomerized stromal cells provide an excellent culture system for assessing the lymphoid differentiation potential of primary or leukaemic haematopoietic progenitors. For example, in single cell assays with the telomerized stromal cells, we observed that B and/or T/NK lymphoid precursors were generated in accompaniment with $CD14^+$ cells. These findings are consistent with the observation that human multipotent lymphoid progenitors retain the potential to generate monocytic cells (Doulatov *et al*, 2010). These investigations will yield novel insights into the mechanisms involved in human normal and malignant haematopoiesis.

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Author's contributions

Y.N. and B.L. performed the research and analysed data; K. O. designed the research, analysed the data, and wrote the manuscript; K.S., K.I., T.M., M.M. analysed and interpreted the data; H.N. and H.S. analysed the data; H.H. contributed essential reagents or tools; N.K designed the research, analysed the data, and wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression of surface or cytoplasmic lymphoid antigens in freshly isolated $CD34^+$ cells.

Fig. S2. The effect of flt3L on the generation of dendritic lineage cells from haematopoietic progenitor cells cultured on telomerized stromal cells.

Fig. S3. The effect of flt3L, SCF, and TPO on the differentiation of lymphoid cells in serum-containing cultures.

Fig. S4. IL-7 has little to no effect on lymphopoiesis.

Fig. S5. The effect of IL-15 on lymphopoiesis.

Fig. S6. The effect of flt3L, SCF, and TPO on the generation of lymphoid cells in serum-free cultures.

Table SI. The generation of $CD19^+$, $CD7^+$, and/or $CD14^+$ cells in single-cell assays with or without flt3L.

Table SII. (A) Cell growth in single cell cultures with SCF + flt3L + TPO. (B) The generation $cyCD79a^+$, $CD7^+$ and/or $CD14^+$ cells in the single-cell assay.

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A phase I study of vaccination with NY-ESO-1f peptide mixed with Picibanil OK-432 and Montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen

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We conducted a phase I clinical trial of a cancer vaccine using a 20-mer NY-ESO-1f peptide (NY-ESO-1 91–110) that includes multiple epitopes recognized by antibodies, and CD4 and CD8 T cells. Ten patients were immunized with 600 µg of NY-ESO-1f peptide mixed with 0.2 KE Picibanil OK-432 and 1.25 ml Montanide ISA-51. Primary end points of the study were safety and immune response. Subcutaneous injection of the NY-ESO-1f peptide vaccine was well tolerated. Vaccine-related adverse events observed were fever (Grade 1), injection-site reaction (Grade 1 or 2) and induration (Grade 2). Vaccination with the NY-ESO-1f peptide resulted in an increase or induction of NY-ESO-1 antibody responses in nine of ten patients. The sera reacted with recombinant NY-ESO-1 whole protein as well as the NY-ESO-1f peptide. An increase in CD4 and CD8 T cell responses was observed in nine of ten patients. Vaccine-induced CD4 and CD8 T cells responded to NY-ESO-1 91–108 in all patients with various HLA types with a less frequent response to neighboring peptides. The findings indicate that the 20-mer NY-ESO-1f peptide includes multiple epitopes recognized by CD4 and CD8 T cells with distinct specificity. Of ten patients, two with lung cancer and one with esophageal cancer showed stable disease. Our study shows that the NY-ESO-1f peptide vaccine was well tolerated and elicited humoral, CD4 and CD8 T cell responses in immunized patients.

Key words: NY-ESO-1, cancer vaccine, long peptide, immune response

Additional Supporting Information may be found in the online version of this article

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The NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum.[1,2] NY-ESO-1 expression is observed in a wide range of human malignancies,[3,4] but the expression is restricted to germ cells in the testes in normal adult tissues.[1,3] Therefore, NY-ESO-1 has emerged as a prototype of a class of cancer/testis (CT) antigens.[5]

More than 100 patients with NY-ESO-1-expressing tumors have received the NY-ESO-1 vaccine either as full-length recombinant protein given as protein alone, with ISCOMATRIX® or cholesterol-bearing hydrophobized pullulan (CHP), delivered in a recombinant vaccinia or fowlpox vector, or as the NY-ESO-1b peptide given with various adjuvants.[6–11] These studies established safety with various preparations of the NY-ESO-1 vaccine, showing toxicity to be limited to Grade 1 or 2 injection-site reactions or flu-like

symptoms, *e.g.*, fever and malaise. Vaccination with these preparations has been shown to enhance or generate NY-ESO-1 immune responses in the majority of patients by immune monitoring using sera and peripheral blood lymphocytes.

CHP is a newly developed antigen delivery vehicle that can be used to formulate nanoparticles, including protein antigens.[12,13] Both CD4 and CD8 T cells are efficiently activated by DCs pulsed with a complex of CHP and NY-ESO-1 protein (CHP-NY-ESO-1) *in vitro*.[14] In a phase I clinical trial, we immunized nine cancer patients with CHP-NY-ESO-1 and showed that the vaccine had potent capacity to induce the NY-ESO-1 antibody in all of nine vaccinated patients.[15] The regions in the NY-ESO-1 molecule recognized by antibodies from vaccinated patients were similar to those recognized by antibodies in nonvaccinated cancer patients with spontaneous immunity. Especially, we showed that NY-ESO-1 91–108 was recognized in six of nine vaccinated patients and in eight of nine nonvaccinated, seropositive patients.[15] This region was defined as the most dominant serological antigenic epitope. A CHP-NY-ESO-1 vaccine also elicited CD4 and CD8 T cell responses in immunized patients.[16] An increase in the CD4 and CD8 T cell responses was observed in all of two initially seropositive and five of seven initially seronegative patients after vaccination. Analysis of T cell responses against overlapping peptides spanning the NY-ESO-1 molecule revealed that two dominant NY-ESO-1 regions, regions II (73–114) and III (121–144), were recognized by CD4 and CD8 T cells in most patients irrespective of their HLA type. Importantly, the most dominant peptide region (91–108) eliciting an antibody response was also included in region II. Essentially similar findings were obtained by studies using other preparations of NY-ESO-1 protein vaccine.[9,11]

Protein vaccines containing multiple epitopes appear to be promising in eliciting strong immune responses, but there are several constraints against their general use. To produce sufficient amounts of recombinant protein for a vaccine, a huge fermentation facility is necessary. Operating such facilities at GMP grade is extremely costly. Furthermore, there are several technical difficulties to be overcome to obtain highly purified protein at a sufficient yield such as removing bacterial or other contaminants from the preparation.

CD8 and CD4 T cells induced by immunization with NY-ESO-1 class I and II short epitope peptides, respectively, have been shown to be of low affinity and do not recognize naturally processed NY-ESO-1.[17] However, it has recently been shown that a long peptide is capable of inducing antibody, CD4 and CD8 T cell responses *in vivo* as the protein antigen.[18,19]

On the basis of these findings, in our study, we investigated the immunogenicity of a long peptide spanning a peptide region NY-ESO-1 91–110 for use as a vaccine. We examined the safety of repeated vaccinations with NY-ESO-1f peptide at a dose of 600 μg mixed with immune adjuvants Picibanil® OK-432 and Montanide® ISA-51. Furthermore, we monitored the humoral, CD4 and CD8 T cell responses in patients receiving NY-ESO-1f peptide vaccine and recorded tumor responses.

Material and Methods

NY-ESO-1f peptide vaccine

NY-ESO-1f peptide (NY-ESO-1 91–110: YLAMPFATP-MEAELARRSLA) was manufactured by CLINALFA, Merck Biosciences (Läufelfingen, Switzerland) and provided by the Ludwig Institute for Cancer Research, New York. The vaccine, consisting of 600 μg of NY-ESO-1f peptide, 0.2KE OK-432 (Picibanil™; Chugai Pharmaceutical, Tokyo, Japan) and 1.25 ml ISA-51(Montanide™; Seppic, Paris, France), was emulsified under sterile conditions. All synthesis, production, formulation and packaging of the investigational agent were in accordance with applicable current Good Manufacturing Practices and met the applicable criteria for use in humans.

Study design

A phase I clinical trial of the NY-ESO-1f peptide vaccine was designed to evaluate the safety, immune response and tumor response. Patients with advanced cancers that were refractory to standard therapy and expressed NY-ESO-1 as assessed by immunohistochemistry (IHC) were eligible. Cancer patients including six patients with esophageal cancer, three patients with non-small-cell lung cancer and one patient with gastric cancer were enrolled in a washout period of at least 4 weeks after surgery, chemotherapy or radiation therapy. The vaccines were administered subcutaneously once every 3 weeks in six doses. Four weeks after the last administration, the safety, immune response and tumor response were evaluated. Thereafter, the vaccine was administered additionally. The ten patients received 5–21 immunizations.

The protocol was approved by the Ethics Committee of Osaka, Tokyo and Okayama Universities in light of the Declaration of Helsinki. Written informed consent was obtained from each patient before enrolling in the study. The study was conducted in compliance with Good Clinical Practice. The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000001260) on July 24, 2008 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

Blood samples

Peripheral blood was drawn from the patients before vaccination, at each time point of immunization and 4 weeks after the last immunization. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated by density gradient centrifugation using lymphoprep (Axis Shield PoC AS, Oslo, Norway). A CD8 T cell-enriched population was obtained from PBMCs using CD8 microbeads with a large-scale column and a magnetic device (Miltenyi Biotec, Auburn, CA). A CD4 T cell-enriched population was then obtained from the residual cells using CD4 microbeads. The final residual cells were used as a

CD4- and CD8-depleted population. These populations were stored in liquid N_2 until use. HLA typing of PBMCs was done by sequence-specific oligonucleotide probing and sequence-specific priming of genomic DNA using standard procedures.

Overlapping peptides

The following series of 28 overlapping NY-ESO-1 18-mer peptides spanning the protein were used: 1–18, 7–24, 13–30, 19–36, 25–42, 31–48, 37–54, 43–60, 49–66, 55–72, 61–78, 67–84, 73–90, 79–96, 85–102, 91–108, 97–114, 103–120, 109–126, 115–132, 121–138, 127–144, 133–150, 139–156, 145–162, 149–166, 153–170 and 156–173. A 30-mer peptide, 151–180, was also used. These peptides were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl chemistry on a Multiple Peptide Synthesizer (AMS422; ABIMED, Langenfeld, Germany) at Okayama University.

ELISA

Recombinant NY-ESO-1 protein was prepared as described previously.[1] Recombinant NY-ESO-1 protein (1 μg/ml) or NY-ESO-1f peptide (10 μg/ml) in a coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6) was adsorbed onto 96-well PolySorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Plates were washed with PBS and blocked with 200 microliters per well of 5% FCS/PBS for 1 hr at room temperature. Then, 100 μl of serially diluted serum was added to each well, and it was incubated for 2 hr at room temperature. After extensive washing, horseradish peroxidase-conjugated goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells, and the plates were incubated for 1 hr at room temperature. After washing and development, absorbance at 490 nm was read. Recombinant murine Akt protein[20] and ovalbumin (OVA, albumin from chicken egg white; Sigma, St. Louis, MO) were used as control proteins.

In vitro stimulation of CD4 and CD8 T cells

Frozen cells were thawed and resuspended in AIM-V (Invitrogen, Carlsbad, CA) medium supplemented with 5% heat-inactivated pooled human serum (CM) and kept at room temperature for 2 hr. CD4- and CD8-enriched populations (2×10^6) were cultured with irradiated (30 Gy), autologous CD4- and CD8-depleted PBMCs (2×10^6) in the presence of the 28 18-mer overlapping peptides and a 30-mer C-terminal peptide spanning the entire NY-ESO-1 protein (1 μg/ml for each peptide) in 2 ml of CM supplemented with 10 U/ml rIL-2 (Takeda Chemical Industries, Osaka, Japan) and 10 ng/ml rIL-7 (Peprotech, London, UK) in a 24-well culture plate at 37°C in a 5% CO_2 atmosphere for 12 days. For the second stimulation, 1×10^6 instead of 2×10^6 responder cells were used in the culture described above.

IFNγ capture assay

The IFNγ capture assay[21,22] was carried out according to the manufacturer's protocol (Miltenyi Biotec). Briefly, 2×10^5 responder CD4 and CD8 T cells were stimulated for 4 hr at 37°C in a 5% CO_2 atmosphere with paraformaldehyde (PFA, 0.2%)-treated autologous CD4- and CD8-depleted PBMCs (2×10^5) prepulsed with the peptides. The cells were then washed and suspended in 100 μl of cold RPMI medium and treated with bispecific CD45 and IFNγ mouse antibodies (IFNγ catch reagent) (2 μl) for 5 min on ice. The cells were then diluted in AIM-V medium (1 ml) and placed on a slow rotating device (Miltenyi Biotec) to allow IFNγ secretion at 37°C in a 5% CO_2 atmosphere. After incubation for 45 min, the cells were washed with cold buffer and treated with 7AAD (7-amino-actinomycin D, Becton Dickinson, Mountain View, CA), PE-conjugated anti-IFNγ (detection reagent) and FITC-conjugated anti-CD4 or CD8 mAbs for staining. After incubation for 10 min at 4°C, the cells were washed and analyzed with a FACS Calibur (Becton Dickinson). Dead cells were sorted by 7AAD staining. The data were analyzed with FlowJo software (Tree Star, Ashland, OR). A net population of IFNγ-captured CD4 and CD8 T cells of more than 0.1% was considered significant.

Immunohistochemistry

IHC was performed as described previously.[3] E978[23] and EMR8-5 (Funakoshi, Tokyo, Japan)[24] mAbs were used to analyze NY-ESO-1 and HLA class I expression, respectively. The reaction was evaluated as +++ (>50% stained cells), ++ (25–50% stained cells), + (5–25% stained cells) and − (<5% stained cells).

Results

Patient characteristics

Table 1 shows a list of the ten patients enrolled in the study. They included six patients with esophageal cancer, three with non-small-cell lung cancer and one with gastric cancer who were refractory to the standard therapy. Expression of NY-ESO-1 and MHC class I in the tumor was confirmed in biopsy or surgical specimens by IHC in all patients upon entry into the study. Nine patients completed the study with six injections of the NY-ESO-1f peptide with Picibanil and Montanide, but patient OS-f01 was withdrawn from the study after five doses of the vaccine because of disease progression. All patients were considered evaluable for toxicity, immunological and clinical responses. Six patients with a prolonged disease course were allowed to continue vaccination after a cycle of six doses of the vaccine.

Toxicity

Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events v.3.0.[25] As shown in Table 1, six patients showed Grade 1 fever (38–39°C) that subsided within a few days without any medication. All patients except OY-f04 developed an injection-site reaction (Grade 1 or 2). TK-f01, TK-f04 and TK-f05 developed a Grade 2 injection-site reaction early after the first vaccination. The reaction appeared 48–72 hr after

Table 1. Patient characteristics

ID	Age/Sex	Cancer/Histology	Vaccination	Vaccine-related toxicity
OY-f04	59/M	Esophageal cancer Squamous cell carcinoma	6	Fever (Grade 1)
OS-f01	66/M	Esophageal cancer Squamous cell carcinoma	5	Fever (Grade 1), injection-site reaction (Grade 1)
OS-f03	61/M	Gastric cancer Adenocarcinoma	26	Fever (Grade 1), injection-site reaction (Grade 1), induration (Grade 1)
OS-f06	51/M	Esophageal cancer Squamous cell carcinoma	6	Injection-site reaction (Grade 1), induration (Grade 1)
OS-f08	69/M	Esophageal cancer Squamous cell carcinoma	13	Injection-site reaction (Grade 1), induration (Grade 1)
TK-f01	59/M	Lung cancer Adenocarcinoma	12	Fever (Grade 1), injection-site reaction (Grade 2), induration (Grade 2)
TK-f02	67/M	Lung cancer Adenocarcinoma	12	Fever (Grade 1), injection-site reaction (Grade 2), induration (Grade 2)
TK-f03	72/M	Esophageal cancer Squamous cell carcinoma	7	Injection-site reaction (Grade 1)
TK-f04	37/F	Lung cancer Adenocarcinoma	6	Fever (Grade 1), injection-site reaction (Grade 2), induration (Grade 2)
TK-f05	71/M	Esophageal cancer Squamous cell carcinoma	11	Injection-site reaction (Grade 2), induration (Grade 2)

injection, and erythema was accompanied by swelling. Grade 2 induration occurred thereafter without retraction. In patient TK-f02, erythema was first observed after the third injection and accompanied induration after the fifth injection (Supporting Information Fig. 1). The induration gradually subsided during the course of the treatment. No augmentation of the reaction intensity was observed at previous injection sites. No severe adverse events related to the drug were observed.

Antibody response to the NY-ESO-1 whole protein and NY-ESO-1f peptide

The NY-ESO-1 antibody response in the patients vaccinated with NY-ESO-1f peptide with Picibanil and Montanide was evaluated by ELISA using recombinant NY-ESO-1 protein and the NY-ESO-1f peptide. Figure 1 shows the results of ELISA with sera from each patient obtained at the baseline and after each vaccination. The patients include two baseline seropositive patients (OS-f03 and TK-f03) and eight baseline seronegative patients. The sera from two seropositive patients also reacted to the NY-ESO-1f peptide, consistent with our previous observation that the NY-ESO-1f peptide represents an immunodominant B cell epitope.[15]

In the seropositive patients, an increase in the NY-ESO-1 antibody response was observed after vaccination. In seven of eight baseline seronegative patients, the NY-ESO-1 antibody response was induced after three to six vaccinations and increased gradually thereafter. The response against NY-ESO-1 protein could be detected in higher dilutions of sera than that against the NY-ESO-1f peptide. The kinetics of the responses against NY-ESO-1 protein and NY-ESO-1f peptide were basically the same.

CD4 and CD8 T cell responses in patients after NY-ESO-1f peptide vaccination

CD4 and CD8 T cell responses were evaluated in the ten patients by the IFNγ capture assay. Patient HLA genotypes are listed in Table 2. CD4 and CD8 T cell-enriched populations were cultured for 12 days with irradiated autologous CD4- and CD8-depleted PBMC in the presence of a mixture of 28 overlapping 18-mer peptides and a 30-mer C-terminal peptide spanning the entire NY-ESO-1 protein (1°IVS). The cells from the stimulation culture were then assayed for IFNγ secretion by stimulating them for 4 hr with PFA-treated CD4- and CD8-depleted PBMC prepulsed with the peptide. To confirm the response, the cells were also analyzed after secondary *in vitro* stimulation (2°IVS). Figure 2 shows the representative FACS plot results from the two patients for three different time points before and after vaccination in 1° and 2°IVS. The net percentage of IFNγ-secreting cells of the total number of CD4 and CD8 T cells in cultures was determined. Values >0.1% were considered significant. As shown in Figure 3 and Table 3, a CD4 T cell response was detected in nine of ten patients in 1°IVS. In seropositive patient

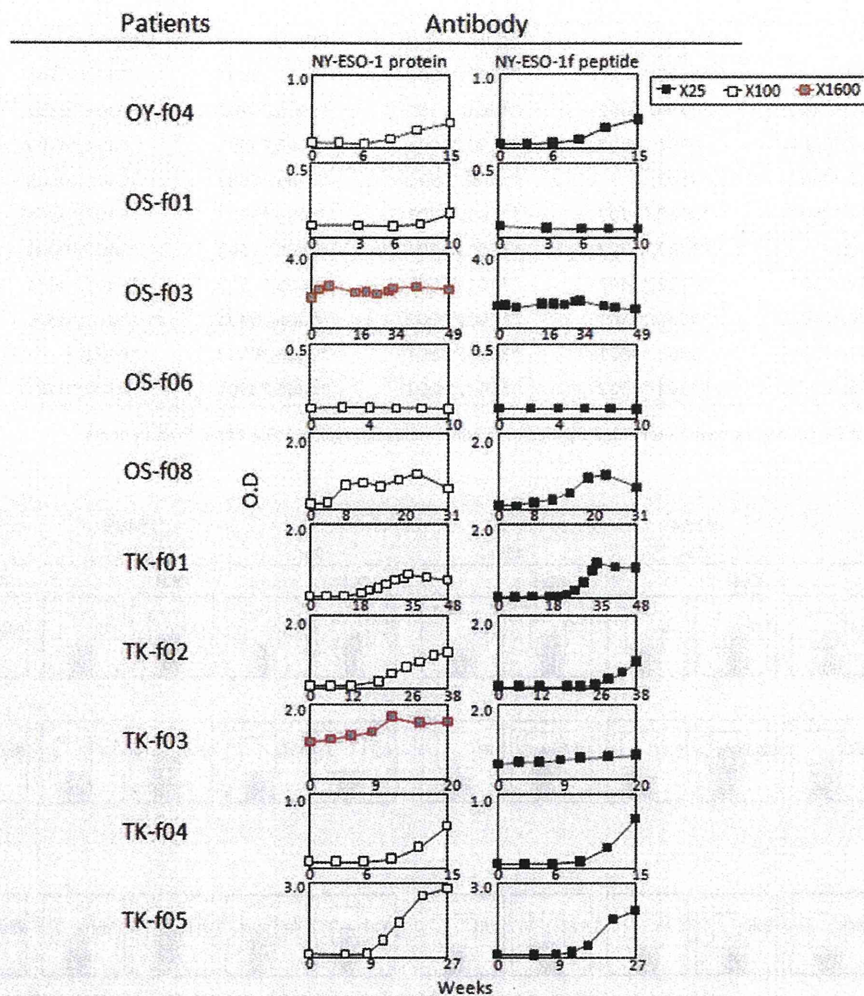

Figure 1. Antibody response to the NY-ESO-1 protein or NY-ESO-1f peptide. Sera obtained at the baseline and after each vaccination were used for ELISA. The O.D. values (490 nm) for the NY-ESO-1f peptide at a serum dilution of 1:25 (closed) and for NY-ESO-1 protein at a serum dilution of 1:100 (open) for seronegative patients or 1:1,600 (gray) for seropositive patients are shown. The O.D. values of the control protein (Akt) were less than 0.05. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TK-f03, a strong CD4 T cell response was observed before vaccination and increased after vaccination. In another seropositive patient, OS-f03, and a seronegative patient, OS-f01, a strong CD4 T cell response was observed after vaccination. In the remaining six seronegative patients, a moderate CD4 T cell response was induced after vaccination. The frequency of IFNγ-producing CD4 T cells increased and reached a plateau after repeated vaccinations in all patients except OS-f01 and OS-f08. In OS-f01, the response could be examined only with the cells taken after the first and third vaccinations. In OS-f08, the response was transient. In patient OS-f06, the CD4 T cell response was barely detectable.

As shown in Figure 3 and Table 3, a CD8 T cell response was also detected in nine of ten patients in 1°IVS. In seropositive patient TK-f03, IFNγ-producing CD8 T cells were detected before vaccination and their frequency increased after vaccination. In another seropositive patient, OS-f03, and seronegative patients TK-f01, TK-f02 and TK-f04, a robust and sustained CD8 T cell response was induced after vaccination. Even a single vaccination elicited a response in these patients. In patient OS-f08, an increase in CD8 T cell response was observed after the seventh vaccination. In patients OY-f04 and TK-f05, the CD8 T cell response was transient. No CD8 T cell response was detected in patient OS-f01.

Determination of NY-ESO-1 peptides recognized by CD4 and CD8 T cells in patients vaccinated with NY-ESO-1f peptide with Picibanil and Montanide

CD4 and CD8 T cell responses for individual overlapping peptides were analyzed by an IFNγ capture assay. As shown

Table 2. Patient HLA

ID	A	C	B	DR	DQ	DP
OY-f04	*2402, –	*0702, –	*0702,*4001	*0101,*0901	*0303,*0501	*0201,*0402
OS-f01	*0201,*1101	*0304,*0401	*1301,*1501	*0406,*1202	*0301,*0302	*0201,*0501
OS-f03	*1101,*2402	*0401,*0801	*1501,*4006	*0406,*0901	*0302,*0303	*0201,*0501
OS-f06	*0201,*2402	*0102, –	*5401,*5901	*0405,*0803	*0401,*0601	*0201, –
OS-f08	*1101,*2402	*0303,*1202	*3501,*5201	*0403,*1502	*0302,*0601	*0201,*0901
TK-f01	*2402, –	*0303,*1202	*3501,*5201	*0405,*1502	*0401,*0601	*0201,*0901
TK-f02	*2402,*3101	*1202,*1402	*5101,*5201	*0405,*1502	*0401,*0601	*0301,*0901
TK-f03	*0201,*2402	*0303,*0401	*1501,*4002	*0406,*0901	*0302,*0303	*0201,*0501
TK-f04	*0201,*1101	*0303,*0801	*3501,*4801	*0406,*0802	*0302, –	*0201,*0501
TK-f05	*0206, –	*0304,*0702	*3902,*4002	*0405,*0901	*0303,*0401	*0301,*1301

HLA-A, C, B, DR, DQ and DP genotypes were determined by high-resolution molecular typing using PBMC from patients.

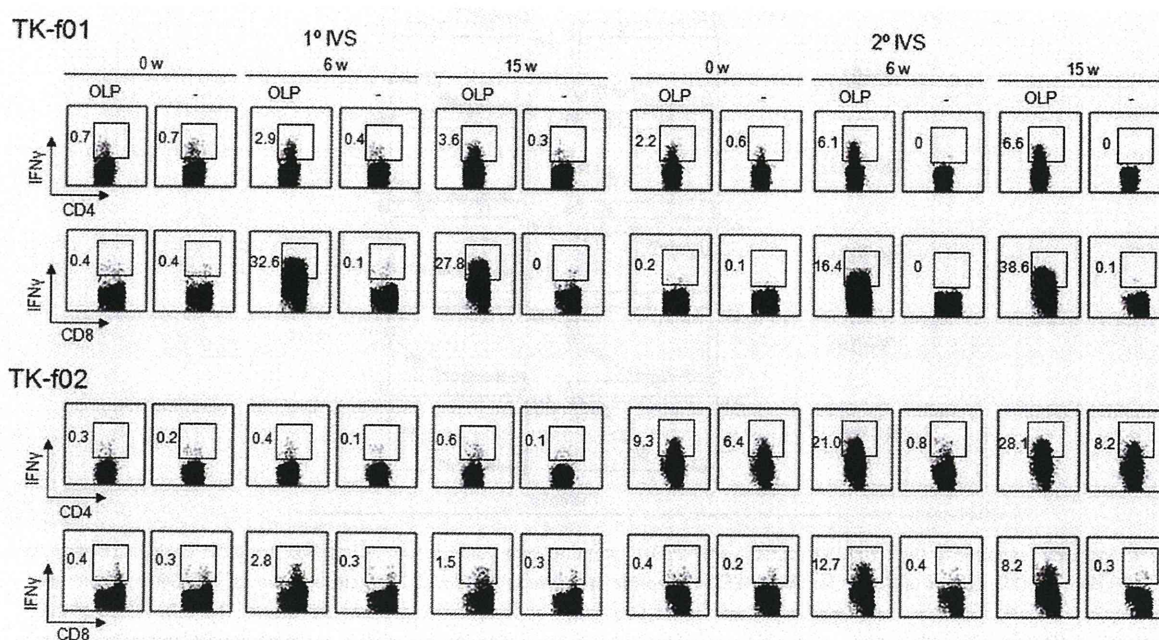

Figure 2. IFNγ capture assay of CD4 and CD8 T cells. MACS beads-purified CD4 and CD8 T cells (2×10^6) obtained from PBMC of vaccinated patients at three time points were stimulated once (1°IVS) for 12 days or twice (2°IVS) for 24 days with irradiated autologous CD4- and CD8-depleted PBMC (2×10^6) in the presence of a mixture of 28 18-mer overlapping peptides and a 30-mer C-terminal peptide spanning the entire NY-ESO-1 protein (1 μg/ml for each peptide). The cells (2×10^5) from the stimulation culture were assayed for IFNγ secretion by stimulating them for 4 hr with PFA-treated CD4- and CD8-depleted PBMC (2×10^5) prepulsed or not prepulsed with a mixture of the peptides (OLP) using FACS. The net percentage of IFNγ-secreting cells of the total number of CD4 and CD8 T cells in cultures was determined. Values >0.1% were considered significant.

in Supporting Information Figure 2, the NY-ESO-1f peptide vaccine-induced CD4 T cells showed a response to peptide 16 (NY-ESO-1 91-108) in all six patients analyzed and to peptide 15 (NY-ESO-1 85-102) or 17 (NY-ESO-1 97-114) in two patients, respectively. Similarly, vaccine-induced CD8 T cells showed a response to peptide 16 in all six patients analyzed and to peptide 15 (NY-ESO-1 85-102) in two patients. These patients showed different HLA types (Table 2). The results indicated that the 20-mer NY-ESO-1f peptide includes multiple HLA class II and class I binding epitopes recognized by CD4 and CD8 T cells, respectively, with distinct specificity (manuscript in preparation). No recognition of other peptides was observed except for CD4 T cells from OS-f08, which showed a moderate response to peptides 2 and 20 and rather lower responses to other multiple peptides, probably because of the high background.

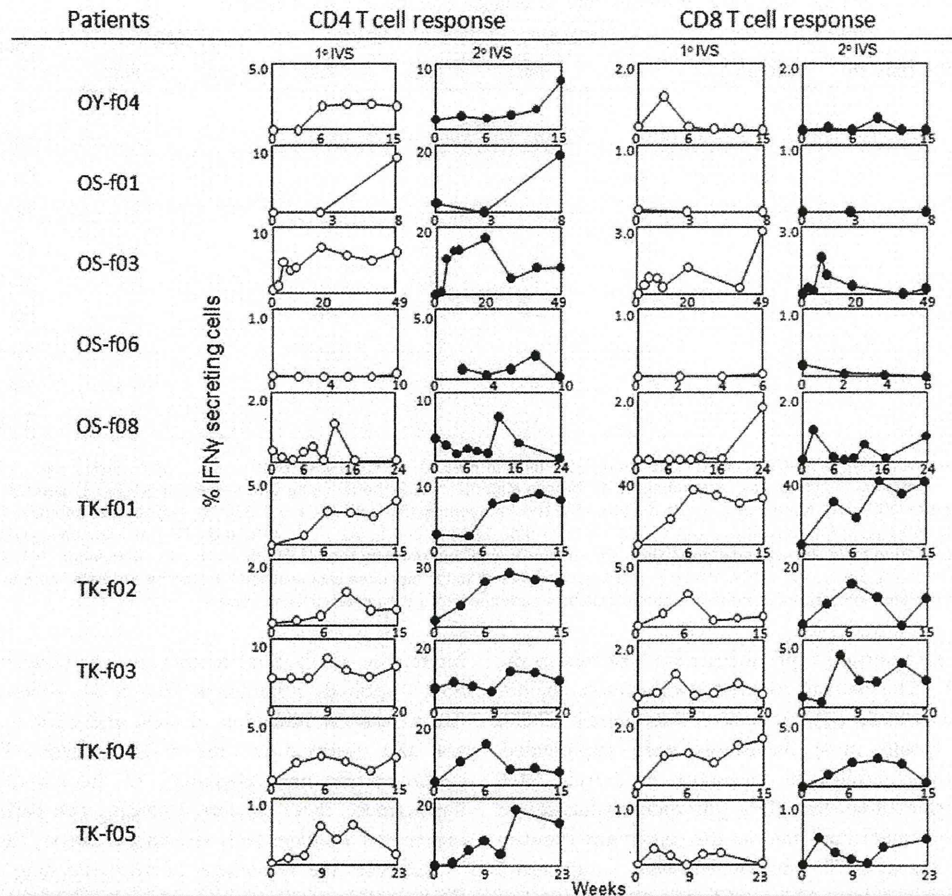

Figure 3. CD4 and CD8 T cell responses determined by the IFNγ capture assay. The net percentage of IFNγ-secreting cells of the total number of CD4 and CD8 T cells in cultures was plotted at the baseline and after each vaccination.

Clinical responses

Table 3 summarizes the immune and clinical responses in all patients. Stable disease (SD) was observed in three patients, including two patients with lung cancer and one patient with esophageal cancer. Lung cancer patient TK-f01 received a right middle lobectomy in October 2004, followed by postoperative adjuvant chemotherapy with Tegafur-Uracil (UFT) for 6 months. Since recurrence was detected in the left lung and a right hilar lymph node by CT scan in April 2007, he received three courses of combination chemotherapy with carboplatin and paclitaxel. As the tumor continued to grow despite the chemotherapy, he was enrolled in the study in June 2008. After initiating the vaccine, the tumor remained stable for 6 months and was classed as SD at the end of the sixth vaccination (Supporting Information Fig. 3*a*). The patient subsequently received another cycle of six vaccinations. However, the tumor started to grow after the eighth vaccination, consistent with an accelerated elevation in the serum CEA level. A greater than 20% increase in the sum of target lesion diameters was detected after the 11th vaccination, and this was evaluated as progressive disease (PD).

Lung cancer patient TK-f02 received a right upper lobectomy in January 2001, followed by postoperative adjuvant chemotherapy with UFT. Recurrence was noticed in March 2002. After that he received cryoablation surgery and chemotherapy including S-1 (oral fluoropyrimidine), combination chemotherapy with carboplatin and paclitaxel, Gefitinib and Erlotinib, one after the other. The nodule in the right middle lobe continued to grow, and the serum CEA level increased despite these therapies. Therefore, he was enrolled in the study in August 2008. After initiating the vaccine, growth of the tumor evaluated by CT scan, and the increase in the serum CEA level slowed down during the initial course of six vaccinations (Supporting Information Fig. 3*b*). Although a small nodule was detected as a new lesion in the left lower lobe after the fifth vaccination, another cycle of six vaccinations was given. During the second cycle of vaccinations, the sum of target diameters was almost unchanged from 24.8 to 27 mm (less than a 10% increase).

Esophageal cancer patient TK-f05 received surgery in January 2006, followed by two courses of postoperative adjuvant chemotherapy comprising CDDP and 5-fluorouracil. In July

Table 3. Study summary: Immune and tumor responses after vaccination with the NY-ESO-1f peptide

ID	IHC[1]		Antibody[2]		CD4[3]		CD8[3]		Clinical response[4]
	MHC class I	NY-ESO-1	Pre	Post	Pre	Post	Pre	Post	
OY-f04	+++	+++	−	+	−	++	−	++	PD
OS-f01	+++	+++	−	+	−	+++	−	−	PD
OS-f03	++	++	+++	+++	−	+++	−	++	PD
OS-f06	+++	+++	−	−	−	−	−	+	PD
OS-f08	++	+	−	++	+	++	−	++	PD
TK-f01	+++	++	−	++	−	++	−	+++	SD (→PD)
TK-f02	+++	++	−	++	−	++	−	++	PD (→SD)
TK-f03	+++	+	++	++	++	+++	+	++	PD
TK-f04	++	+++	−	++	−	++	−	++	PD
TK-f05	++	+	−	+++	−	+	−	+	SD (→PD)

[1]IHC was performed using EMR8–5 mAb for MHC class I and E957 mAb for NY-ESO-1. IHC-positive cells: $+++ > 50\%$; $50\% \geq ++ > 25\%$; $25\% \geq + > 5\%$; $5\% \geq -$. [2]Antibody response was determined by ELISA (see Material and Methods) using O.D. values for NY-ESO-1f peptide at a serum dilution of 1:25 and for NY-ESO-1 protein at a serum dilution of 1:100 for seronegative patients or 1:1,600 for seropositive patients. Antibody response against protein is shown. Antibody: $+++ > 2$; $2 \geq ++ > 0.5$; $0.5 \geq + > 0.1$; $0.1 \geq -$. [3]CD4 and CD8 T cell responses were determined by IFNγ capture assay in once *in vitro* stimulation (1°IVS). The response was confirmed by that of two times *in vitro* stimulation (2°IVS). IFNγ-positive cells: $+++ > 5\%$; $5\% \geq ++ > 1\%$; $1\% \geq + > 0.1\%$; $0.1 \geq -$. [4]Clinical response was assessed according to RECIST criteria at Weeks 20–22 and after additional injections (described in parentheses) in patients with a prolonged disease course.

2008, enlarged para-aortic lymph nodes were observed in the upper abdomen. The patient received combination chemotherapy with docetaxel, cisplatin and 5-fluorouracil (DCF); however, the lymph node metastases were exacerbated. Therefore, he was enrolled in the study in March 2009. Uptake of fluorine-18-labeled FDG (fluorodeoxyglucose) in para-aortic lymph node measured as the maximum standardized uptake value by PET (positron emission tomography) was initially increased from 12.7 in February 2009 to 14.7 in May 2009, but gradually deceased to 10.3 in September 2009 (Supporting Information Fig. 3*c*). The CT scan showed that some low-density areas corresponding to necrotic change appeared in the lymph nodes (Supporting Information Fig. 3*c*). The clinical response was evaluated as SD after the sixth vaccination, and he received another cycle of vaccinations. However, bone metastasis in the sternum was suspected by PET-CT (Supporting Information Fig. 3*c*, red arrow). Finally, new lesions appeared in the lung in November and he was withdrawn from the study.

Discussion

In our study, we immunized patients with NY-ESO-1-expressing tumors by injecting the NY-ESO-1f peptide (600 μg) mixed with Picibanil OK-432 (0.2 KE) and Montanide ISA-51 (1.25 ml) subcutaneously once every 3 weeks for six doses and evaluated the safety and immunological responses. The study population consisted of ten patients, including six patients with esophageal cancer, three patients with non-small-cell lung cancer and one patient with gastric cancer. As vaccine-related adverse events Grade 1 fever, Grade 1 and 2 injection-site reactions and Grade 2 induration were observed. The treatment was considered to be well tolerated. Vaccination with the NY-ESO-1f peptide with Picibanil and Montanide resulted in an increase or induction of an NY-ESO-1 antibody response in nine of ten patients immunized. An increase or induction of CD4 and CD8 T cell responses was also observed in nine of ten patients. These findings confirmed the immunogenicity of the NY-ESO-1f peptide. Furthermore, three patients, including two patients with lung cancer and a patient with esophageal cancer, showed SD.

Recently, the advantage of synthetic long peptides over short peptides for use as vaccines has been acknowledged.[19] Long peptides do not bind to MHC class I molecules directly, and the antigen is presented after processing by dendritic cells. Therefore, use of long peptides prevents the antigen peptides from direct binding to MHC class I molecules on nonprofessional antigen-presenting cells such as B cells and T cells, which may cause transient activation of CTLs followed by their subsequent anergy in the absence of appropriate costimulatory signals.[26]

Furthermore, because Th cells licensed DCs for their efficient antigen presentation and stimulation capacity, introduction of a Th epitope into the vaccine or physical linking of Th and CTL epitope peptides facilitated increased immunogenicity of CTL vaccines.[27,28] Interestingly, Th and CTL epitopes are sometimes located in close proximity or are even overlapped, in the molecules, for example, in the case of the human papillomavirus[29] and Her-2/neu.[30] Zeng *et al.*[31] reported that NY-ESO-1 157–170 (SLLMWITQCFLPVF) was recognized by both NY-ESO-1-reactive CD4 and CD8 T cells. The synthetic long peptide containing overlapping CD4 and CD8 T cell epitope sequences in the antigens is expected to generate both CD4 and CD8 T cell responses as a vaccine.

Recently, we identified regions II (73–114) and III (121–144) in the NY-ESO-1 molecule that were frequently recognized by either CD4 or CD8 T cells irrespective of the patients'

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HLA type.[16] Moreover, the most dominant peptide region (91–108) eliciting an antibody response was also included in region II.[15] Our study showed that a long peptide, NY-ESO-1f, spanning a peptide region 91–110 was immunogenic and induced antibody, CD4 and CD8 T cell responses in patients.

In our study, Picibanil® OK-432 was chosen as an adjuvant. Picibanil is dried penicillin-treated *Streptococcus pyogenes*, which has been shown to activate the immune cells of both the innate and adaptive immune system.[32,33] Montanide® ISA-51[34], which causes inflammation at the injection site and is believed to be helpful in attracting immune cells, was used as the vehicle to deliver the vaccine containing NY-ESO-1f peptide and Picibanil® OK-432. Montanide® ISA-51 also forms a local depot that allows persistence of antigens resulting in prolonged immune activation. This formula induced Grade 1 fever (38–39°C) in six of ten patients that subsided within several days without any medication. In addition, the vaccine induced a robust skin reaction when it was injected close to the dermis of the skin. The reaction caused erythema and induration at the site of the vaccine injection within 48–72 hr. The intensity of the skin reaction was augmented by repeated vaccinations as shown in TK-f02 (Supporting Information Fig. 1), suggesting the reaction was a delayed-type hypersensitivity reaction against Picibanil or the NY-ESO-1f peptide in this patient. The induration was sustained during the course of the treatment, but it subsided gradually. Surgical specimens for histological examination were not available in our study.

The NY-ESO-1f peptide vaccine elicited humoral, CD4 and CD8 T cell responses in the immunized patients (Table 3). The increase or induction of an NY-ESO-1 antibody response was observed in nine of ten immunized patients. The sera from NY-ESO-1f peptide-immunized patients reacted with NY-ESO-1 protein as well as the NY-ESO-1f peptide, suggesting elicitation of an antibody response by a long peptide vaccine including a dominant B cell epitope. The increase and induction of CD4 and CD8 T cell responses were also detected after NY-ESO-1f peptide vaccination in nine of ten patients. Although the number of patients was small, the responses were comparable or even stronger in terms of the frequency and characteristics of the immune response, when compared with various preparations of NY-ESO-1 protein vaccine such as NY-ESO-1/ISCOMATRIX,[35] NY-ESO-1 vaccinia/fowlpox,[36] NY-ESO-1/CpG/Montanide[11,37] and CHP-NY-ESO-1 vaccines.[16]

It has been reported that vaccination with the NY-ESO-1 protein with CpG and Montanide elicited detectable CD8 T responses in half of the immunized patients (9/18), and vaccine-induced CD8 T cells mostly recognized NY-ESO-1 81–110 restricted by either HLA-B35 or HLA-Cw3.[11,37] Consistently, we also observed that vaccination with the NY-ESO-1f peptide elicited CD8 T responses in patients OS-f08, TK-f01, TK-f03, TK-f04 and TK-f05, who were shown to be positive for HLA-B35 and/or HLA-Cw3 (Table 2). In addition, NY-ESO-1f peptide vaccination induced CD8 T responses in patients OY-f04, OS-f03, OS-f06 and TK-f02, who were shown to be negative for HLA-B35 and HLA-Cw3. Thus, it is not necessary for the NY-ESO-1f peptide vaccine to exclude patients who are negative for HLA-B35 and Cw3.

B35-binding peptide epitopes 94–102 and 94–104 and Cw3-binding peptide epitopes 92–100 and 96–104 have been described.[38,39] Analysis of the CD8 T cell response using OLPs revealed that the NY-ESO-1f peptide (NY-ESO-1 91–110) vaccine elicited a response to peptide 16 (NY-ESO-1 91–108) in all six patients analyzed with or without B35 and/or Cw3. The vaccine elicited a CD8 T cell response to peptide 15 (NY-ESO-1 85–102) in two of six patients to a lesser extent. Although a full-length protein vaccine can potentially induce multiple immune responses restricted to different HLA molecules in a patient, the presence of an immunodominant epitope may shift the response to a dominant one. If the NY-ESO-1f peptide is a subdominant epitope in a given patient, the peptide can be efficiently recognized by T cells in the absence of a dominant epitope. The fact that NY-ESO-1f peptide vaccine elicited CD8 T cell responses in patients with various HLA types suggests the advantage of a long peptide over the whole protein for vaccination. Extending our study using a single NY-ESO-1f peptide as a vaccine, we are now conducting a clinical trial of a cancer vaccine using multiple overlapping long peptides spanning NY-ESO-1 79–173 that includes highly immunogenic regions II (73–114) and III (121–144).

In our study, we observed SD in three of ten patients enrolled, including two patients with lung cancer and a patient with esophageal cancer (Supporting Information Fig. 3). Integrated antibody, CD4 and CD8 T cell responses were detected in all of these patients. Although patient TK-f01 expressed both HLA-B35 and Cw3, patients TK-f02 and TK-f05 expressed none of these antigens.

It is now accepted that an immune-related tumor response should be evaluated by different criteria from that for a tumor response induced by cytotoxic agents.[40] Clinical response resulting from immunotherapy can be appreciated generally after an initial increase in tumor volume sometimes associated with the appearance of new lesions evaluated as PD by the Response Evaluation Criteria in Solid Tumors (RECIST) or WHO criteria. Thus, immune-related response criteria (irRC) were proposed recently.[41] For TK-f02 in our study, the increase in tumor diameter measured by CT images was less than 20% of the initial tumor burden, and a reduced increase in serum CEA level was observed during the initial course of six vaccinations. However, a small new lesion was noticed in the left lower lobe after the fifth vaccination (Supporting Information Fig. 3*b*). Patient TK-f02 was PD according to the RECIST criteria, but irSD according to irRC. As both CD4 and CD8 T cell responses were detected even after the first NY-ESO-1f peptide vaccination, we decided to give another cycle of six vaccinations to this patient, resulting in sustained SD with good quality of life.

In summary, the NY-ESO-1f peptide is a dominant region in the NY-ESO-1 molecule that includes multiple epitopes frequently recognized by antibody, CD4 and CD8 T cells.

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Therefore, the use of the NY-ESO-1f peptide as a cancer vaccine will practically allow inclusion of most, if not all, patients into a study irrespective of their HLA type. The finding that the NY-ESO-1f peptide vaccine caused little toxicity and strong humoral and cellular immune responses suggests the usefulness of long peptide vaccines in the clinical management of cancer patients.

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Heteroclitic serological response in esophageal and prostate cancer patients after NY-ESO-1 protein vaccination

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NY-ESO-1 is a prototypic cancer/testis antigen. In a recent phase I clinical trial, we vaccinated 13 patients bearing NY-ESO-1-expressing tumors with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1) and showed efficient induction of NY-ESO-1 antibody, and CD4 and CD8 T cell responses using peripheral blood from the patients. In our study, we analyzed heteroclitic serological responses in those patients after vaccination. Serological response against 11 tumor antigens including MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2, XAGE1B and p53 was examined by enzyme-linked immunosorbent assay (ELISA) using sera from ten vaccinated patients. Expression of tumor antigens was determined by reverse transcription-polymerase chain reaction or immunohistochemistry. Eight of nine patients who showed antibody responses against NY-ESO-1 also showed an antibody response against at least 1 of these 11 tumor antigens after vaccination. In one patient, seven tumor antigens were recognized. Specificity analysis of the antibody response by ELISA using control recombinant proteins and synthetic peptides and by Western blot showed that the response was not against His6-tag and/or bacterial products included in a preparation of CHP-NY-ESO-1 used for vaccination. Thus, heteroclitic serological responses appear to be indicative of the overall immune response against the tumor, and their analysis could be useful for immune monitoring in cancer vaccine.

NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum[1,2] and has been shown to be strongly immunogenic. Patients bearing NY-ESO-1-expressing tumors often show antibody and CD4 and CD8 T cell responses spontaneously.[3–5] Clinical trials using NY-ESO-1 peptide,[6–9] protein[10,11] and viral constructs[12] as cancer vaccine have been conducted. Those studies have demonstrated efficient induction of antibody, and CD4 and CD8 T cell responses, and have also shown in some sporadic cases efficacy of immunotherapy.[2,6–12]

In a recent phase I clinical trial, we vaccinated 13 patients including eight Stage IV esophageal cancer patients, four

Key words: NY-ESO-1, cancer vaccine, cancer testis antigen, heteroclitic antibody response

Abbreviations: CHP: cholesterol-bearing hydrophobized pullulan; CMV: Cytomegalovirus; CT antigen: cancer/testis antigen; CTL: cytotoxic T lymphocytes; DHFR: dihydrofolate reductase; EBV: Epstein–Barr virus; OD: optical density; OLPs: overlapping peptides; RT-PCR: reverse transcription-polymerase chain reaction; SEREX: serological expression cloning

Additional Supporting Information may be found in the online version of this article

The authors declare that there is no conflict of interest

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Stage D3 prostate cancer patients and a Stage IV malignant melanoma patient with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1).[13–16] We showed that the vaccine was well tolerated and had a potent capacity to induce NY-ESO-1 antibody and CD4 and CD8 T cell responses using peripheral blood from patients. Beneficial effects such as tumor regression or no tumor growth for a prolonged period were observed in six of seven disease-evaluable patients. However, all patients died eventually. Immunohistochemical analysis of the tumors that grew after vaccination suggested involvement of different mechanisms resulting in immune impairment.[15] NY-ESO-1-antigen loss was observed in a patient, disappearance of tumor-infiltrating CD4 and CD8 T cells was observed in two patients and an increase in the number of $CD68^+$ macrophages was observed in another patient. Further study will be necessary to elucidate cellular mechanisms including regulatory T cells causing immune suppression at the local tumor site.

It has been shown that cytotoxic T cell responses to tumor antigens other than the antigen used for immunization occur after vaccination.[17–26] This heteroclitic immune response following the initial response to a specific antigen was originally described in autoimmune disease and has been suggested to play a crucial role in clinical responses mediated by cancer vaccines.[27,28] Occurrence of CD8 T cell responses to unrelated tumor antigens was shown in studies of vaccination with MAGE-A1 and/or MAGE-A3,[19,20,25] HER-2/neu,[17,18,21,22] MART-1/Melan-A[23–25] and gp100.[26]

In our study, we analyzed the heteroclitic serological response against a panel of tumor antigens. Specificity analysis of antibody response was performed by enzyme-linked immunosorbent assay (ELISA) using control recombinant proteins and synthetic peptides, and by Western blot. The findings indicate frequent occurrence of heteroclitic serological responses in patients after CHP-NY-ESO-1 vaccination.

Material and Methods

Patients and sera

Eight advanced esophageal cancer patients (E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8) and two prostate cancer patients (P-2 and P-3) were enrolled in the clinical trial (protocol LUD 2002-005 of the Ludwig Institute for Cancer Research, New York, NY) (http://clinicaltrials.gov/ct2/show/NCT00106158?term=CHP+NY-ESO-1&rank=2).[13–16] Peripheral blood was drawn from patients with written informed consent with the permission of the ethics committees of Osaka and Okayama Universities. Sera from MAGE-A4 seropositive esophageal cancer patients (EC-1, EC-2 and EC-3) and five healthy donors were drawn with written informed consent with the permission of the ethics committees of Osaka University. Sera were stored in −80°C freezer until use.

Preparation of a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein (CHP-NY-ESO-1)

Preparation of recombinant NY-ESO-1 protein for vaccine was described elsewhere.[29] A complex of CHP and NY-ESO-1 protein (CHP-NY-ESO-1) and the schedule of vaccine were described previously.[14] Briefly, patients with advanced cancers expressing NY-ESO-1 were injected 2–31 times subcutaneously at biweekly intervals with 100 μg of NY-ESO-1 recombinant protein formulated with 2 mg of CHP.

Recombinant protein and overlapping peptides

N-His6-tagged recombinant proteins, NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B, dihydrofolate reductase (DHFR) and Akt,[30–32] were produced by using pQE30 vector (QIAGEN, Hilden, Germany) and expressed in M15 *E. coli* cells. N-His6-tagged p53 produced in a *Baculovirus* system was purchased (Enzo Life Sciences, New York, NY). N-His6-tagged CCDC-62 protein was produced in a *Baculovirus* system at Okayama University.[33]

A series of 22 25-mer MAGE-A4 overlapping peptides (OLPs) were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl chemistry on an ABIMED Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University. MAGE-A4 OLPs: 1 (1–25), 2 (15–39), 3 (29–53), 4 (43–67), 5 (57–81), 6 (71–95), 7 (85–109), 8 (99–123), 9 (113–137), 10 (127–151), 11 (141–165), 12 (155–179), 13 (169–193), 14 (183–207), 15 (197–221), 16 (221–235), 17 (225–249), 18 (239–263), 19 (253–277), 20 (267–291), 21 (281–305), 22 (295–317).

MAGE-A4 transfectants

CMS5a, a murine fibrosarcoma cell line from a strain of BALB/c origin, was stably transfected with pcDNA3.1 MAGE-A4 plasmid as described previously.[34] The plasmid was provided by Dr. A. Kuroda and Dr. M. Miyamoto (Hokkaido University, Sapporo, Japan) and purified using plasmid Mini kit.

ELISA

Serially diluted sera were added to 96-well plates coated overnight at 4°C with 1 μg/ml recombinant protein or 5 μg/ml peptide solution and blocked for 2 hr at room temperature. After overnight incubation, plates were extensively washed with PBS containing 0.2% Tween 20. For determining reciprocal titer by ELISA, shown in Figure 1, serum IgG bound to antigens was detected by alkaline phosphatase-conjugated specific monoclonal antibody (Southern Biotech, Birmingham, AL). After addition of ATTOPHOS substrate (Fisher Scientific, Waltham, MA), absorbance was measured using a Cytofluor Series 4000 fluorescence reader (PerSeptive Biosystems, Framingham, MA). A reciprocal titer was calculated for each sample as the maximal dilution still significantly reacting to a specific antigen. Specificity was determined by

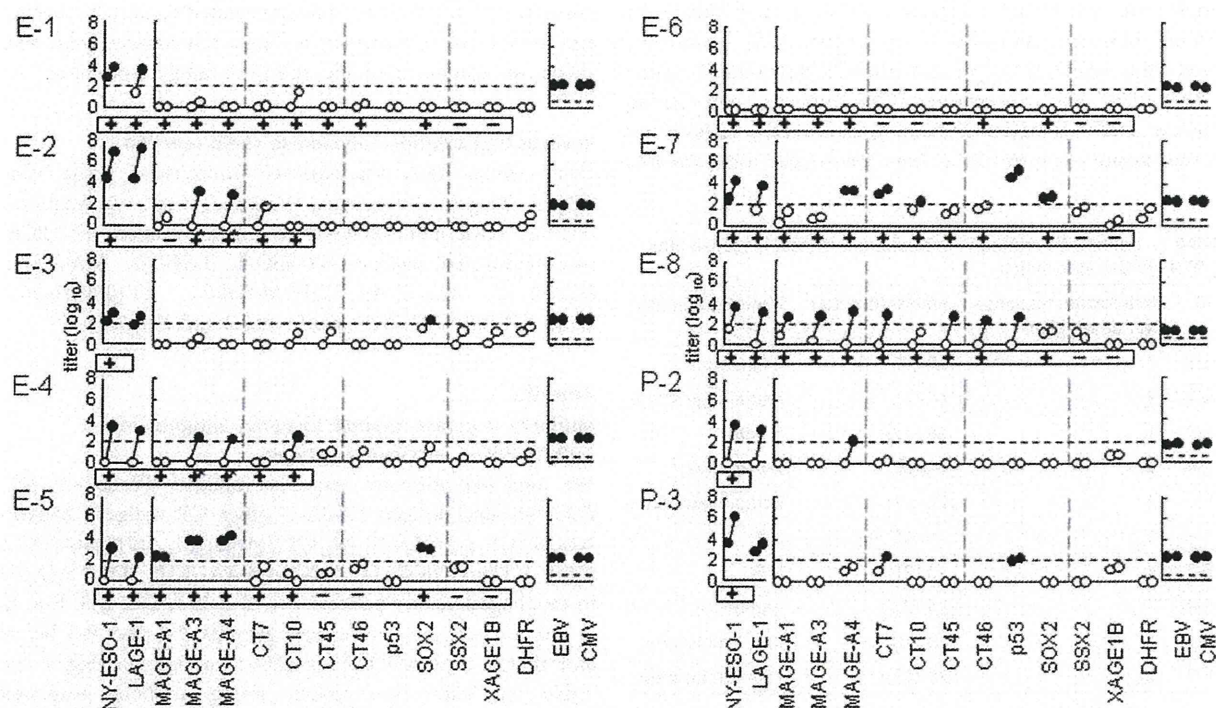

Figure 1. Reciprocal antibody titer against 13 tumor antigens in sera from patients vaccinated with CHP-NY-ESO-1 by ELISA. Serially diluted sera obtained before and after vaccination were assayed against N-His6-tagged recombinant proteins NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B and DHFR. The reciprocal titer was the maximal dilution showing significant reaction (open and closed circles). Closed circles indicate reciprocal titers exceeding 100 (positive reaction). In each assay, antibody positive and negative sera were included as controls. Positive (+) and negative (–) expression of tumor antigens indicated in boxes under each panel was analyzed by RT-PCR and/or IHC when sample was available (see Supporting Information Table). Titer of EBV and CMV antibody in sera were measured by EBV and CMV kits, respectively. Values exceeding 4.0 were positive by manufacturer's indication. +*; 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.

comparing seroreactivity among the various antigens tested.[31] In each assay, positive and negative control sera were included. A positive result was defined as reciprocal titers >100. For conventional ELISA, peroxidase-conjugated goat anti-human IgG or IgM (Jackson Immuno Research Laboratory, West Grove, PA) was added to the wells for second antibody. After washing, signals were developed with *o*-phenylene diamine dihydrochloride, and absorbance at 490 nm was read using an ELISA reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA). Positivity was defined as sample optical density (OD) greater than three times that of the value for irrelevant control protein. Titers of Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) antibody in sera were measured by EBV VCA kit (Denka Seiken, Tokyo, Japan) and CMV kit (Denka Seiken), respectively.

Western blot

Recombinant protein (20 ng) or cell lysate (20 μg) in sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS and 1 mM dithiothreitol) was boiled for 5 min and subjected to SDS-PAGE with 10–20% polyacrylamide BioRad Ready-Gels (Bio-Rad). After electrophoresis, the membrane (Hybond-P membrane, Amersham Pharmacia Biotech, Buckinghamshire, UK) was blocked with 5% FCS/PBS and then incubated with patients' sera diluted 1:1,000 for recombinant protein or 1:200 for cell lysate for 1 hr at room temperature. After washing, alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratory) was added to the membrane. Signals were developed with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chromogenic substrate kit (Bio-Rad). Polyclonal rabbit anti-MAGE-A1 serum (Abcam, Cambridge, UK) and monoclonal anti-MAGE-A4 (clone 3D12; Abnova, Taipei, Taiwan), anti-p53 (clone PAb421; Enzo) and anti-His6-tag (clone OGHis; MBL, Nagoya, Japan) antibodies were used for positive controls at 1:1,000 dilution.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using formalin-fixed paraffin-embedded specimens. Monoclonal antibodies

used were anti-MAGE-A1 (clone MA454), anti-MAGE-A3 (clone M3H67), anti-MAGE-A4 (clone 57B), anti-CT7/MAGEC1 (clone CT7-33) and anti-CT10/MAGEC2 (clone LX-CT10.5). For cancer-testis (CT) antigens, only strong nuclear and/or cytoplasmic staining as observed in testicular tissue (positive control) in at least 5% of cells was scored as positive. 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.[35,36]

Table 1. Heteroclitic antibody response and clinical response after CHP-NY-ESO-1 vaccination

ID	Heteroclitic response No. of antigens	Weeks (the No.)	Clinical response
E-1	0	89 (31)	Regression
E-2	2	14 (7)	Partial regression
E-3	1	28 (12)	Stable
E-4	3	12 (6)	Progressive
E-5	2	22 (11)	Partial regression
E-6	0	4 (3)	N.E.
E-7	4	2 (2)	N.E.
E-8	7	54 (27)	Stable
P-2	1	28 (10)	PSA stabilization
P-3	2	29 (13)	PSA stabilization

Abbreviations: Weeks (the No.): weeks after the start of vaccination and the number of vaccinations given; N.E.: not evaluable.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted from frozen tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Conventional reverse transcription-polymerase chain reaction (RT-PCR) was performed against NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2 and XAGE1B.[30,31]

Results

Antibody response against 13 tumor antigens in CHP-NY-ESO-1-vaccinated patients

We analyzed antibody responses against NY-ESO-1, NY-ESO-1-related antigen LAGE-1, other CT antigens MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SSX2 and XAGE1B, SOX2 and p53 in esophageal cancer patients E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8 and prostate cancer patients P-2 and P-3 before and after a cycle of CHP-NY-ESO-1 vaccination (Fig. 1 and Table 1). Before vaccination, strong antibody responses against NY-ESO-1 and/or LAGE-1 were observed in E-2 and P-3 and defined as baseline seropositive. Additionally,

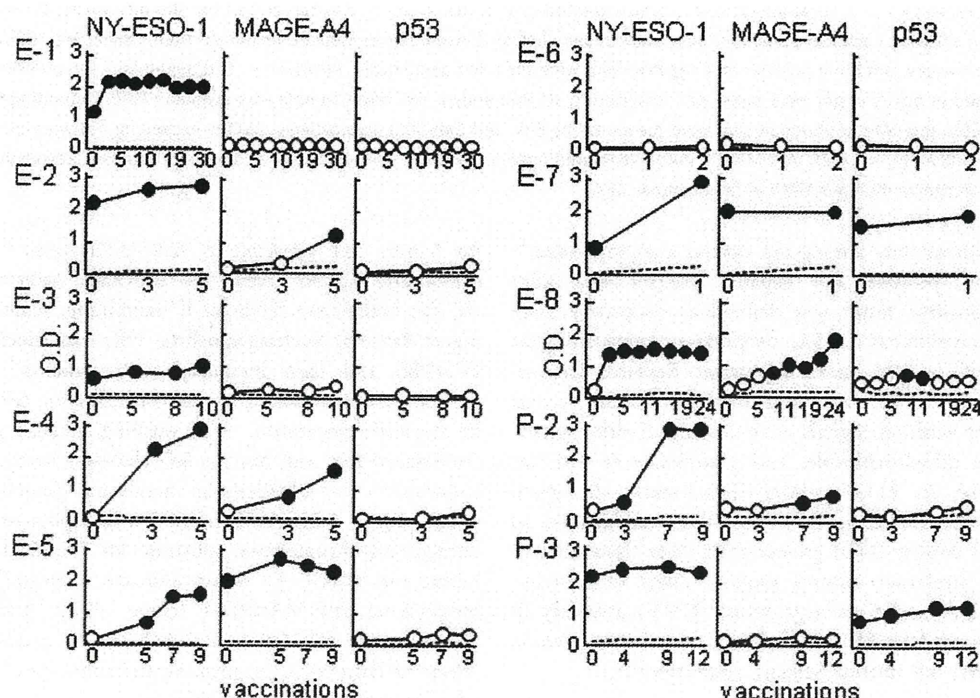

Figure 2. IgG antibody response against NY-ESO-1, MAGE-A4 and p53 in sera from patients before and after CHP-NY-ESO-1 vaccination by ELISA. Sera diluted at 1:100 were assayed against N-His6-tagged recombinant proteins NY-ESO-1, MAGE-A4 and Akt produced in *E. coli* and recombinant proteins p53 and CCDC-62 produced in *Baculovirus*. Akt and CCDC-62 were included as negative control (dotted line). Positive reaction (closed circles) represented the OD values exceeding three times the control OD value.

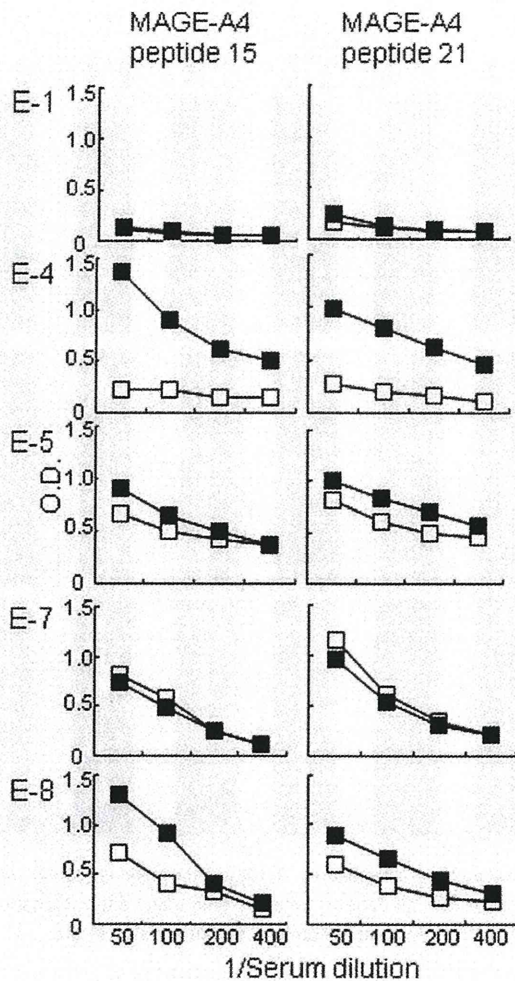

Figure 3. Serially diluted sera from patients before (open squares) and after (closed squares) CHP-NY-ESO-1 vaccination were assayed against MAGE-A4 peptide 15 and peptide 21 by IgG ELISA.

marginal antibody responses were observed in E-1, E-3 and E-7. In E-5, antibody against MAGE-A1, MAGE-A3, MAGE-A4 and SOX2 was observed. In E-7, antibody against MAGE-A4, CT7/MAGEC1, p53 and SOX2 was observed. In P-3, antibody against p53 was observed. After vaccination, in all patients except E-6, antibody response against NY-ESO-1 and LAGE-1 was increased or induced. In E-2, antibody responses against MAGE-A3 and MAGE-A4 were induced. In E-3, antibody response against SOX2 was induced. In E-4, antibody responses against MAGE-A3, MAGE-A4 and CT10/MAGEC2 were induced. In E-5, antibody responses against MAGE-A3 and MAGE-A4 were increased. In E-7, antibody responses against CT7/MAGEC1, p53 and SOX2 were increased and that against CT10/MAGEC2 was induced. In E-8, antibody responses against MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT45, CT46/HORMAD1 and p53 were induced. In P-2, antibody response against MAGE-A4 was induced. In P-3, antibody response against CT7/MAGEC1 was induced and that against p53 was increased. No antibody against DHFR included as a control was detected in any patient. Furthermore, no increase of antibody response was observed against EBV and CMV after CHP-NY-ESO-1 vaccination.

Expression of 13 tumor antigens in tumor specimens

Expression of NY-ESO-1 was detected by RT-PCR and IHC in tumors from all patients before vaccination. Expression of other tumor antigens except p53 was analyzed by RT-PCR in E-1, E-5, E-6, E-7 and E-8, and expression of MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1 and CT10/MAGEC2 was also analyzed by IHC in E-1, E-2, E-4, E-5, E-6, E-7 and E-8 (Fig. 1 and Supporting Information Table). Mutation of p53 was not determined in our study. Expression of corresponding antigen was confirmed with tumor specimens in patients who showed antibody against tumor antigens.

Antibody response against tumor antigens in CHP-NY-ESO-1-vaccinated patients: No involvement of antibody against His6-tag and the product of E. coli present in the vaccine

Antibody responses against selected tumor antigens were further confirmed in sera obtained at each time during multiple vaccinations. As shown in Figure 2, IgG antibody against MAGE-A4 was detected in sera from E-5 and E-7 before vaccination, and the response was increased or induced in E-2, E-4, E-5, E-8 and P-2 after vaccination. IgG antibody against p53 was detected in sera from E-7 and P-3 before vaccination, and the response was increased or induced in E-7, E-8 and P-3.

Induction of IgM antibody against MAGE-A4 was detected in sera from E-8 after vaccination (Supporting Information Fig. 1). IgM antibody against p53 was detected in sera from E-7 before vaccination. Increase or induction of IgM antibody against p53 was detected in E-7 and E-8 after vaccination. Interestingly, in E-8, transient IgM response against MAGE-A4 and p53 was followed by IgG response.

Recombinant NY-ESO-1 protein used for vaccination has His6-tag in the N-terminus and was produced in *E. coli* as the host cells. All antigens shown in Figure 1 also have His6-tag and were produced in *E. coli*. To exclude the possibility of detecting antibody against His6-tag and/or the product of *E. coli* in the assay that might be raised by vaccination, DHFR was tested as control. No antibody against DHFR was detected (see above). To further exclude the possibility, the antibody response against control antigens was examined by IgG ELISA using serum samples obtained in each time during multiple vaccinations. As shown in Figure 2, antibody against Akt protein with His6-tag and produced in *E. coli* was within a background level (<0.2 OD value). p53 used in the experiments shown in Figure 2 and Supporting Information Figure 1, but not in Figure 1, and CCDC-62 protein

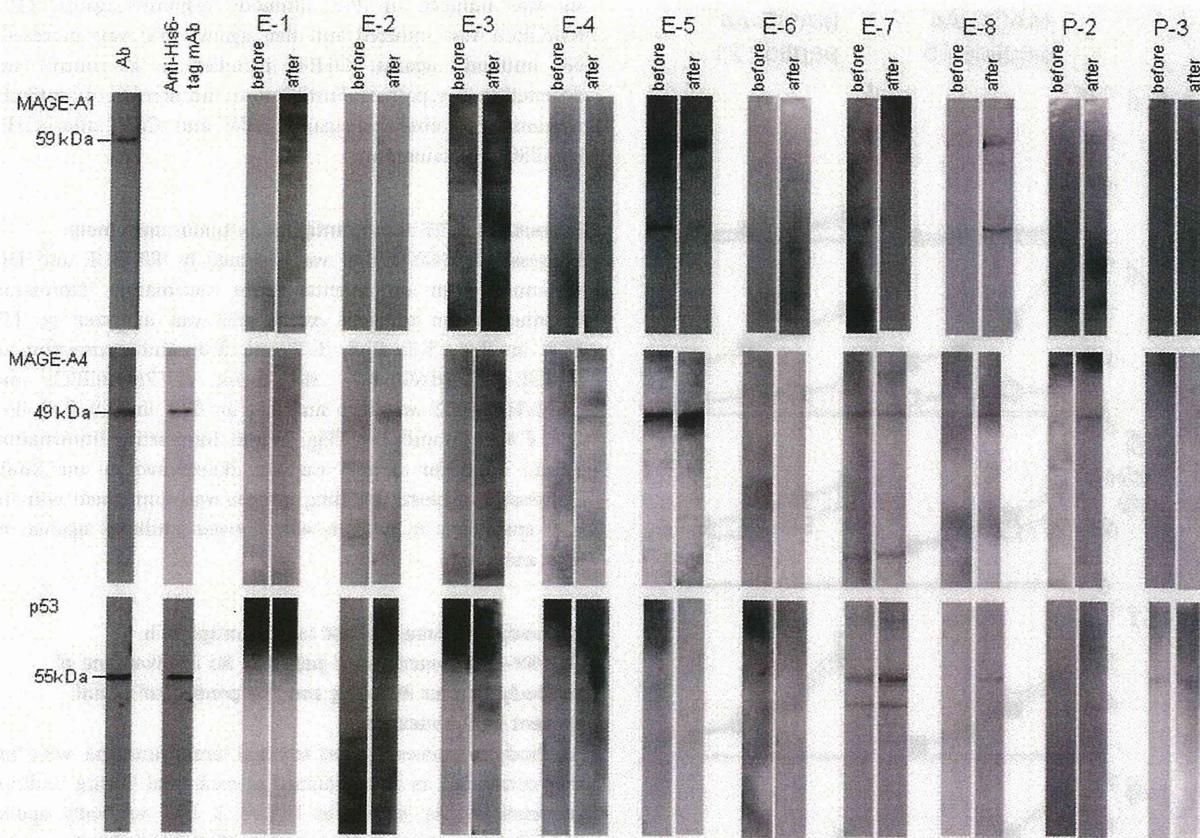

Figure 4. Western blot analysis. Reaction of sera against MAGE-A1, MAGE-A4 and p53 was investigated. Recombinant proteins (20 ng) were run by SDS-PAGE and transferred to a membrane by electrophoresis. Sera (1:1,000) from all patients obtained before and after vaccination were examined. Marker and control bands of each protein detected by monoclonal or polyclonal antibody (1:1,000) are also shown.

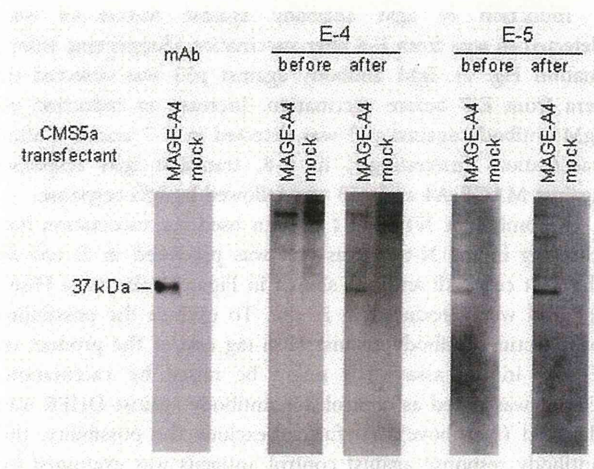

Figure 5. Western blot analysis of sera against MAGE-A4 in lysate of MAGE-A4-transfected CMS5a cells. Cell lysate (20 μg) was run by SDS-PAGE, transferred to a membrane by electrophoresis and sera (1:200) from E-4 and E-5 patients obtained before and after CHP-NY-ESO-1 vaccination were examined. Control band of the protein detected by monoclonal antibody (1:1,000) is shown.

share His6-tag and were produced by *Baculovirus*. Antibody against CCDC-62 was undetectable in sera from any patients.

Next, we synthesized MAGE-A4 OLPs and investigated antibody response by ELISA. Antibody response against MAGE-A4 peptides 15 and 21 was frequently observed in patients showing antibody response against MAGE-A4 protein (Supporting Information Fig. 2). Serially diluted sera from patients E-4, E-5, E-7 and E-8 obtained before and after CHP-NY-ESO-1 vaccination were examined against MAGE-A4 peptides 15 and 21 by IgG ELISA (Fig. 3). Increase or induction of antibody response was observed in E-4, E-5 and E-8, but not E-7 after vaccination. No antibody response was detected in E-1 included as negative control. These results were consistent with those by ELISA using recombinant MAGE-A4 protein in Figure 2.

Western blot analysis

The specificity of antibody against MAGE-A1, MAGE-A4 and p53 in sera from all patients vaccinated was further analyzed by Western blot (Fig. 4). Each antibody as positive control showed the representative band for MAGE-A1 protein at 59 kDa, for MAGE-A4 protein at 49 kDa and for p53 protein