Toxicity, Immunogenicity, and Induction of E75-specific Tumor-lytic CTLs by HER-2 Peptide E75 (369–377) Combined with Granulocyte Macrophage Colony-stimulating Factor in HLA-A2+ Patients with Metastatic Breast and Ovarian Cancer[1]

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ABSTRACT

To determine the toxicity and immunogenicity of the HER-2/*neu*, HLA-A2-restricted peptide E75 in patients with metastatic breast and ovarian cancer, 14 patients were vaccinated with escalating amounts of E75 (100, 500, and 1000 μg) mixed with 250 μg granulocyte macrophage colony-stimulating factor as adjuvant. Each vaccine dose was administered in a total volume of 1.5 ml divided into four intradermal injections and administered weekly for 4 weeks, followed by monthly boosts for a total of 10 injections. Vaccinations were well tolerated without significant toxicity. Blood was drawn before, at 8 weeks, and up to 13–16 months after vaccination for measurement of cellular immunity. Seven of 8 patients tested had significant delayed type hypersensitivity to E75 defined as >5 mm induration. Peripheral blood mononuclear cells from 5 of 9 patients tested proliferated to E75 with a stimulation index of ≥2.0. Of 8 vaccinated patients tested for induction of a CTL response, 4 responded to stimulation by autologous dendritic cells plus cytokines by eliciting E75-specific lytic activity consistent with the presence of activated/memory cells, 2 others after *in vitro* stimulation with E75 + interleukin-12 ± anti-CD152^{33KD}, whereas 2 others did not respond. Four patients with E75-specific CTLs present specifically recognized E75 on indicator tumors as demonstrated by cold-target inhibition of tumor lysis. These 4 patients showed E75-specific IFN-γ production. peripheral blood mononuclear cell from 3 of these patients proliferated to E75, but stimulation indices were higher in the prevaccine samples. All 4 of the patients showed DTH responses to E75. These results demonstrate that vaccination with E75+ granulocyte macrophage colony-stimulating factor can induce both peptide-specific IFN-γ and epitope specific CTLs, which lyse HER-2/*neu*$^{+}$ tumors in stage IV patients.

INTRODUCTION

The HER-2[3] proto-oncogene is amplified on tumors in 20–30% of patients with breast and ovarian cancer. Because HER-2 is an overexpressed nonmutated "self" protein it was believed that it would not be immunogenic in humans. However, HER-2 overexpression was postulated to lead to a higher level of T-cell epitope precursors and epitopes, which can activate T cells *in vivo* and *in vitro* (1). In confirmation of these hypotheses CTLs specific for HER-2 epitopes in the 968–984 area have been identified in ovarian cancer tumor-associated lymphocytes (2). Antibodies reactive with HER-2 have been detected in the serum of breast cancer patients (3). These and additional studies demonstrated preexisting T-cell immunity in patients with HER-2-positive cancers (4, 5) raising the possibility of using HER-2 as a target for cellular immune responses to tumors.

Cancer vaccines that target self tumor antigens are often weaker immunogens for CD8^{+} CTL induction than foreign antigens (6). In general, peptides are considered weak immunogens for CTLs recognizing endogenous epitopes (7, 8). This

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[3] The abbreviations used are: HER-2, HER-2/*neu* protein; DTH, delayed type hypersensitivity; PBMC, peripheral blood mononuclear cell; SI, stimulation index; IL, interleukin; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; mAb, monoclonal antibody; TT, tetanus toxoid; LU, lytic units; NP, not pulsed/no peptide; ST, stimulation; IVS, *in vitro* stimulation; NP-ST, stimulated with no peptide; E75-ST, stimulated with E75; T2-E75, T2 cells pulsed with E75; T2-NP, T2 cells not pulsed with peptide; DC-E75, dendritic cells pulsed with E75; DC-NP, dendritic cells not pulsed with peptide; TTP, time to progression; TCR, T-cell receptor.

Table 1 Patient characteristics and DTH responses to E75

Patient no.	Tumor type	Age	Metastatic site(s)	TTP (wk)	No. of injections	PS[a]	DTH skin test to E75 (mm induration)
1	BR	43	Liver	4	4	0	nd
2	BR	55	Liver, skin	8	5	1	nd
3	BR	41	Bone	1	1	1	nd
4	BR	32	Bone	8	5	0	−(<5)
5	BR	45	Lymph nodes, liver	4	4	0	+(5)
6	BR	39	Bone	16	7	0	+(7)
7	BR	51	Bone	16	7	0	+(10)
8	OV	77	Peritoneum	8	5	1	+(6)
9	BR	45	Bone, supraclavicular	12	6	0	nd
10	BR	61	Bone marrow[b]	52	10	0	+(5)
11	BR	69	Skin	8	5	1	+(15)
12	BR	56	Lymph nodes	4	4	0	nd
13	BR	48	Lymph nodes	3	3	1	nd
14	BR	39	Bone	12	6	0	+(5)

[a] PS, performance status; BR, breast; nd, not determined; OV, ovarian.
[b] Patient in remission after bone marrow transplantation before vaccination.

holds true for peptide vaccines in melanoma, ovarian, and breast carcinoma (9, 10). In a few instances examples of peptide-induced tumor cytolytic T cells were reported in vaccinated ovarian and breast cancer patients (11–13). Peptide vaccines are less likely to induce low dose tolerance and likely can overcome antigen ignorance because of the presence of the antigenic epitopes at 50–100 fold higher concentrations than whole proteins (14). A major concern with tumor-Ag-specific CTL induction by peptide vaccines is that the resulting CTLs in many instances do not recognize tumor cells because of their low affinity for endogenous Ag (9), although the TCR repertoire is present, both in patients and healthy donors (15–17). An alternative possibility is that local immunization with μM amounts of CTL epitopes may induce apoptosis by overstimulation of existent high-affinity, Ag-specific CTLs (12). In patients with advanced metastatic disease, the function of these high-affinity CTLs it is still unclear (16). This raises the questions as to whether peptide vaccines can induce immune responses in patients with advanced disease (Stage IV), and whether the induced responses are limited only to activation of cytokine elaboration or can also activate Ag-specific cytolytic activity against HER-2expressing tumor cells. Induction of IFN-γ and perforin synthesis appears to be mediated by distinct signaling pathways originating from the TCR. In addition, the questions of specificity of *ex vivo* isolated CTLs, of duration of tumor-lytic E75-specific CTLs, and of the frequency of these responses in vaccinated patients have not yet been investigated.

We demonstrated that CTLs expanded from tumor-associated lymphocytes from ovarian and breast cancer specifically recognized HER-2^+ tumors (2, 18). Recognition was associated with several CTL epitopes on HER-2 mapped by peptides C85 (971–979) and E75 (369–377:KIFGSLAFL). Parallel studies demonstrated an additional epitope GP2 (19). Peptides corresponding to C85 and E75 induced CTLs that lysed HER-2^+ HLA-A2+ tumors suggesting that C85 and E75 are immunogenic in healthy individuals (20, 21).

E75 was the antigen of choice for this vaccine because it is a dominant CTL epitope. The optimal adjuvants to use with peptide vaccines have not yet been determined. Studies in rats showed enhancement of DTH response to HER-2 peptides vaccines in which GM-CSF was added (22). GM-CSF is one of the most effective cytokines for activating DCs (22, 23). For these reasons it was selected for use together with E75 in a Phase I trial of breast and ovarian cancer patients. The primary objectives of this study were to determine the vaccine toxicity and its ability to induce E75-specific, tumor-lytic CTLs in breast and ovarian patients with limited stage IV disease, and the duration of this CTL response. An additional objective was to define approaches that allow detection of *in vitro* effector responses to vaccines by *ex vivo* activated CTLs. Toward this goal, IL-12 and α-CTLA-4 were used to costimulate IFN-γ production and cytolytic responses to E75.

PATIENTS AND METHODS

Subjects

Patients with stage IV breast and ovarian cancer were eligible for study (Table 1). All of the patients gave written consent to participate in the study as mandated by the Surveillance Committee at M. D. Anderson Cancer Center. Patients had an Eastern Cooperative Oncology Group performance status of 0–1, and were refractory to standard chemotherapy and/or local radiotherapy. They had been off all chemotherapy for a minimum of 3 weeks before study. Before vaccination all of the patients were tested for immunocompetence using a battery of four skin tests to recall antigens: mumps, TT, histoplasmin, and Candida Albicans. To be eligible for vaccination at least two of the four tests had to be positive as defined by erythema and induration of ≥5 mm in diameter. Patients had to have tumor, which overexpressed HER-2, and peripheral blood lymphocytes, which were positive for HLA-A2 (see below). The use of immunosuppressive drugs such as corticosteroids was prohibited.

Fourteen patients, 13 with breast cancer and 1 with ovarian cancer, were eligible to participate in the trial. Mean age was 50 years. All of the patients had received two or more chemotherapy regimens, and either had stable disease, complete, or partial remissions before receiving vaccinations. Six

had received previous hormonal therapy. Patients had one or two sites of evaluable/measurable disease. Patient 10 was vaccinated after complete recovery of lymphocyte count after high-dose chemotherapy plus autologous peripheral blood stem cell transplant.

Vaccination Schedule

GMP quality E75 peptide was synthesized and purified by Corixa Corporation. Vials of E75 were supplied at concentrations of 100, 500, or 1000 μg/m1 in 2.2 ml of 10 mM sodium acetate buffer (pH 4.0). GM-CSF (Sargramostim) was purchased from Immunex Corporation as sterile, preservative-free lyophilized powder in vials containing 500 μg. Immediately before injection, 1 ml of E75 was mixed with 250 g (0.5 ml) of GM-CSF for a total volume of 1.5 ml (12, 23). Patients received intradermal injections divided among all four extremities (~0.4 ml/injection) weekly for 4 weeks and then monthly for a total of 10 injections. Five patients received 100 μg peptide, 5 received 500 μg, and 4 received 1000 μg. Patients had to receive a minimum of five injections over 2 months to be eligible for determination of immune reactivity to E75 and tumor response. Patients were observed up to 1 h and 24 h after injection for significant side effects. Acute and chronic toxicity were graded according to National Cancer Institute common toxicity criteria. Although not an aim of this study, patients had repeat X-rays, computed tomography scans, and physical exams at every 2-month intervals to evaluate any antitumor activity or disease progression using traditional criteria (12).

Screening Studies Required for Patient Eligibility

HER-2 Expression. HER-2 expression was examined on paraffin block tumor specimens using IMPATH kits with methods as described by the manufacturer. Eligible patients had ≥2± staining based on a graded intensity of 1–4 and at least 20% of tumor cells staining positively for HER-2.

HLA-A2 Positivity. HLA-A2 expression was determined with patient PBMCs isolated from heparinized blood using Ficoll gradients. Immunofluorescence experiments to examine for the presence of HLA-A2 on PBMCs were performed as described using mAb BB7.2 (anti HLA-A2) from supernatants of hybridomas obtained from the American Type Culture Collection (Rockville, MD). The number of fluorescent cells and the fluorescence intensity were examined using an EPICS-V Profile Analyzer (Coulter Corporation, Hialeah, FL; Ref. 18).

Immunological Monitoring

Lymphocyte Proliferation to E75. PBMCs collected from peripheral blood drawn before vaccine treatment and at 8 weeks, just before the fifth vaccination, were cultured in quadruplicate in 96-well flat-bottomed microtiter plates (Costar Corp, Cambridge, MA) at a concentration of 2.5×10^5 cells/well in 100 μl of complete RPMI 1640 with L-glutamine (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO). E75 was added at a final concentration of 25 μg/ml. A weak HLA-A2 binding HER-2 peptide, E72 (HER-2: 828–836, QIAKGMSYL), was used at the same concentration as specificity control. One μg/ml of phytohemagglutinin, 5 μg/ml TT (positive controls), or no peptide were positive and negative controls, respectively. Cells were cultured for 6 days at 37°C in 5% CO (2). [^{3}H]thymidine at l μCi/well was added 16–18 h before the end of culture as described (12). Results were expressed as cpm [^{3}H]thymidine incorporation and as a SI calculated as: (average cpm of lymphocytes cultured with peptide or positive controls) ÷ (cpm of lymphocytes cultured in without peptides).

DTH Responses to E75. In 7 patients, 50 μg of E75 peptide was injected s.c. along with a normal saline control at 8 weeks immediately before the fifth vaccination to determine DTH response to peptide. Responses were not tested before vaccination because of the concern that the Ag might serve to "prime" lymphocytes to E75. Responses were recorded as the maximum diameter of skin induration in mm. Values >5 mm induration (*i.e.*, that used to grade recall antigens) were considered positive.

IFN-γ Induction by Vaccine. Supernatants collected at 24 and 48 h from lymphocyte stimulation assays were used for measurement of IFN-γ levels in duplicate using an ELISA kit with a sensitivity of 10 pg/ml (Biosource, Camarillo, CA). Differences were considered significant if higher than 50 pg/ml.

Detection of CTL Induction by Vaccine and Generation of PBMC-derived DCs. Patient PBMCs were added to 24-well flat-bottomed plastic plates (Costar). After 2-h incubation at 37°C in RPMI 1640 (serum-free), nonadherent cells were removed by repeated washings and frozen in liquid nitrogen at a concentration of 10×10^6 cells/ml in FCS with 5% DMSO. Monocyte-derived DCs were obtained by culturing the adherent cell population in complete RPMI 1640 supplemented with 1000 IU/ml GM-CSF and 500 IU/ml IL-4 (Bioscience International; DC medium) for 5 days (19). DCs were detached with 0.5 mM PBS-EDTA and tested for purity by staining using phycoerythrin-conjugated anti CD13 mAb (Caltag Laboratories, San Francisco, CA) followed by fluorescence-activated cell sorter analysis. More than 90% of cells were CD13 positive. DCs were then replated at 1.2×10^5 cells/well in 24-well plates and pulsed with 25 μg/ml E75 in serum-free medium for 4 h. Tumor necrosis factor α (Chiron Corporation, Emoryville, CA) was added at a concentration of 50 units/ml to DCs for the last hour to stimulate DC maturation and antigen presentation.

Responder PBMCs were resuspended in RPMI 1640 containing 10% human serum and added to autologous DCs at 1.5×10^6 cells/ml (E:T ratio of 12.5:1; Ref. 21). In some experiments IL-12, a gift from Dr. Stanley Wolf, (Genetics Institute, Cambridge, MA), was added 60 min later at a concentration of 3 IU/ml (300 pg/ml) aiming to enhance primary antigen stimulation. All of the stimulations were performed in the presence or absence of IL-12 and/or E75 in the same experiment with PBMCs collected after vaccine and, when available, before vaccine. Similarly, all of the direct CTL assays with effectors from each particular patient were performed in the same experiment using the same targets. After expansion in IL-2, lymphocytes were washed twice and incubated without IL-2 for 20 h in complete medium. They were then tested for their ability to lyse ^{51}Cr-labeled T2 cells and pulsed with various concentrations of E75 (5–25 μl/m1) using as specificity control a high HLA-A2 affinity HER-2 peptide: E71(HER-2, 799–807: QLMPYGCLL; Ref. 18). HLA-A2 expressing

Table 2 Toxicity from vaccine E75+GM-CSF

Side effect	Toxicity grade 1	2	3
Pain at injection site	61[a]	0	0
Fever	12	0	0
Chills	4	1	0
Nausea	9	0	0
Fatigue	3	0	0
Myalgia	3	0	0
Headache		7	0
Itching at injection site	2	0	0
Ulceration at injection site		2	0
Back pain	2	0	0
Abdominal pain	4	0	0

[a] Total number of episodes. No grade 3 toxicity was experienced.

SKOV3 ovarian tumor cells, developed in this laboratory, SKBR3A2 breast tumor cells (a gift from Dr. Mary L. Disis, University of Washington, Seattle, WA), and freshly isolated ovarian tumor cells (HLA-$A2^+$, HER-2^+) were used in cold-target inhibition experiments. Results are expressed as percentage of specific lysis as described (2, 18). In some experiments lymphocytes were stimulated three times with DC-E75 in the presence of IL-12 for up to 3 weeks before testing for CTL activity. Responses were considered positive when the mean ± SD percentage of specific lysis of T2-E75 was significantly greater than the mean ± SD percentage of specific lysis of T2-NP or T2-E71 as described (12).

Data Analysis

Statistical analysis was performed using unpaired Students' *t* test as well as ANOVA for three or more groups.

RESULTS

Toxicity

There was no grade 3 toxicity to E75 and GM-CSF vaccine. (Table 2). The majority of patients had mild pain and erythema at the injection site. Low-grade fever was the second most frequent side effect, followed by grade 1 nausea, fatigue, myalgias, itching at the injection site, and back and abdominal pain. There was one episode of grade 2 chills, seven episodes of headache, and two episodes of moderate ulceration at the injection sites, all occurring in patient 6. No cumulative toxicity was observed. Side effects to vaccine were not dose-dependent.

Patient Status

There were no tumor responses observed. The number of vaccinations received and the TTP are shown in Table 1. All except patient 10 progressed before receiving all 10 of the injections, with a mean TTP of 11 months. Patient 10 remained disease-free for 52 weeks until relapsing in bone marrow. At the insistence of the patient, chemotherapy and traztuzamab were reinstituted after completing all of the vaccinations; hence, the duration of remission because of vaccine alone could not be assessed.

DTH Responses to E75 Peptide

Seven of 8 patients tested (5, 6, 7, 8, 10, 11, and 14) had positive DTH responses to E75. Patient 4 had no response (Table 1). There were no responses to the saline control. The pattern of responses to TT was similar to the pattern of responses to E75.

Lymphocyte Proliferation to E75

Table 3 shows the cpm [^{3}H]thymidine incorporation and SI for lymphocytes from 11 of 14 patients who had PBMCs tested *in vitro* to E75. Data shown are derived from patient PBMCs stimulated before vaccination and at 8 weeks after vaccination. Patients 1 and 3 progressed before 8 weeks and did not have postvaccine blood samples drawn. Patients 4, 13, and 14 did not have a high enough lymphocyte yield from blood drawn for pretesting because of difficulties in venous access, and did not return for posttesting because of marked disease progression and poor performance status before 8 weeks. The PBMCs of 5 of 9 patients proliferated to E75; cpm were significantly increased over proliferation in medium alone. Postvaccination, PBMCs of patient 6 proliferated on *in vitro* exposure to low affinity peptide E72. There were no significant differences in proliferation to E75 from PBMCs studied pre- *versus* postvaccination in 3 patients, in 2 patients there was an increase in SI after vaccination, whereas in 4 patients there was a decrease in SI in postvaccination PBMCs, although significant response to E75 was seen before vaccination. SIs ranged from 0.8 to 10.9 (mean + SD = 2.9 ± 2.3). SIs to phytohemagglutinin ranged from 2 to 106. Because of wide variations between patients, SIs pretreatment values did not differ significantly from posttreatment (pre = 45 ± 43; post = 26 ± 26; $P > 0.05$). Similar results were observed for TT (pre = 6 ± 7; post = 4 ± 3; $P > 0.05$).

In essence, 6 of 7 patients who had positive proliferation responses either pre- or postvaccination had positive DTH. Of the 7 patients with positive DTH responses to E75, 4 (7, 8, 10, and 11; Tables 3 and 5) had positive proliferation responses to peptide before vaccination and 4 had proliferation to E75 postvaccination (6, 8, 10, and 11). Three of 5 patients (7, 8, 10) had no significant change in proliferation postvaccination compared with prevaccination; 2 of the 5 (11, 12) had significant ($P = 0.04$) decrease in proliferation post-treatment compared with pretreatment.

IFN-γ Responses to E75

To determine whether the Th1 cytokine IFN-γ was produced from peptide pulsed lymphocytes blood was drawn before disease progression on patients 6, 7, 9, 10, 11, and 14. Purified PBMCs were stored frozen in liquid nitrogen. Patients 10 and 11 also had additional PBMCs collected and frozen before vaccination. PBMCs were thawed and separated into DCs and nonadherent lymphocytes as described, and incubated with E75 or medium (see "Materials and Methods"). After one or two rounds of IVS, supernatants were collected at 24 and 48 h, and frozen for later analysis of IFN-γ secretion. In the absence of IL-12, only 2 of 6 patients responded by IFN-γ secretion. In the presence of IL-12, 5 of 6 patients responded to E75 *in vitro* by IFN-γ secretion. In 1 patient (patient 10), vaccination induced a significant increase in response to E75 after repetitive IVS. Of

Table 3 Proliferative responses to E75 of PBMCs from vaccinated patients

	Prevaccine				Postvaccine			
Patient no.	NP	E72	E75	SI (E75)	NP	E72	E75	SI (E75)
1	389 ± 122	280 ± 19	615 ± 168	1.6	nd[a]	nd	nd	—
2	295 ± 14	322 ± 46	310 ± 33	1.1	302 ± 70	332 ± 35	890 ± 661	2.9
3	532 ± 202	504 ± 90	793 ± 362	1.5	nd	nd	nd	—
4	nd	nd	nd	—	nd	nd	nd	—
5	309 ± 87	348 ± 121	574 ± 542	1.0	2116 ± 478	1946 ± 731	1685 ± 949	0.8
6	nd	nd	nd	—	663 ± 127	2166 ± 758[b]	1281 ± 405[b]	1.9
7	2249 ± 1249	2205 ± 897	6089 ± 3351[c]	2.5	3262 ± 2678	3951 ± 3000	3751 ± 2191	1.1
8	1758 ± 330	1917 ± 725	4974 ± 1685[c]	2.8	585 ± 346	2135 ± 1241	2431 ± 1485[c]	4.2
9	864 ± 509	571 ± 230	nd	—	709 ± 437	542 ± 266	536 ± 207	0.8
10	363 ± 189	319 ± 273	1533 ± 667[b]	4.2	642 ± 349	402 ± 149	2218 ± 1228[c]	3.5
11	457 ± 222	2482 ± 1136[b]	5002 ± 508[b]	10.9	670 ± 310	1198 ± 713	3149 ± 1032[b]	4.7[a]
12	468 ± 284	737 ± 795	1550 ± 396[c]	3.3	363 ± 190	545 ± 273	726 ± 270	2.0[a]
13	nd	nd	nd	—	nd	nd	nd	—
14	nd	nd	nd	—	nd	nd	nd	—

[a] Significant decrease from prestimulation ($P = 0.04$); nd, not done.
[b] Significant at $P \leq 0.001$.
[c] Significant at $P \leq 0.05$.

Table 4 E75 induced IFN-γ secretion in pre- and postvaccination patient PBMCs

		Stimulation group/IFN-γ (pg/ml)			
		NP[a]	E75	NP	E75
Patient no.	Vaccination status	−IL-12		+IL-12	
6	Post 1	0.0	0.0	0.0	0.0
7	Post 1	0.0	0.0	0.0	65.0
9	Post 1	0.0	0.0	0.0	106.3
10	Pre	0.0	0.0	0.0	405.5
	Post 1	0.0	108.0	479.7	782.2
	Post 2[b]	176	211	194	276
11	Pre	0.0	0.0	45.4	747.0
	Post 1	0.0	0.0	56.5	840.1
	Post 2	0.0	0.0	12.7	733.9
14	Post 2	146	212	316	455

[a] No peptide control.
[b] Supernatant collected after two rounds of stimulation; all determinations represent the highest values of IFN-γ in 24-h supernatants.

3 patients who had no measurable IFN-γ after one IVS with E75-pulsed DCs IL-12 induced measurable IFN-γ levels in 2 of the 3 patients (65 and 106.3 pg/ml, respectively; Table 4). Patients 10, 11, and 14 also had significant increases in IFN-γ secretion after two IVS with E75 in the presence of IL-12: mean ± SE: 605.4 ± 83.6 pg/ml compared with lymphocytes incubated without IL-12 (75.9 ± 38.1 pg/m1; $P < 0.003$). This value was also significantly higher than for lymphocytes incubated without peptide (157.7 ± 68.7pg/m1; $P < 0.006$). Collectively these results suggest that E75-responsive T cells were present in these patients. It is possible, although it needs to be investigated that in stage IV patients E75 was a weak immunogen, which could not activate antigen-presenting cells and required costimulation by IL-12 to induce detectable levels of IFN-γ.

E75 Specific Cytolytic Activity

The objectives of these studies were 4-fold: (*a*) to determine whether patients with metastatic breast cancer developed specific cytolytic responses against E75 and against tumor cells expressing this epitope: (*b*) to determine whether E75-specific tumor-lytic memory CTLs were present in some of these patients and could be detected *in vitro*; (*c*) to determine whether vaccination with E75 enhanced the specific-lytic activity of these CTL; and (*d*) to determine whether the use of IL-12 at priming enhanced the lytic activity against tumor of CTLs from E75+GM-CSF vaccinated patients. To determine CTL activity against T2 targets pulsed with peptides E75 (T2-E75), negative control HER-2 peptide E71 (T2-E71), or experimental negative control, T2 not pulsed with peptide (T2-NP), as well as HLA-A2-transfected tumor cell lines SKOV3 (ovarian) and SKBR3 (breast) lymphocytes were harvested after one round of IVS (patients 6, 7, and 9) or three rounds of IVS (patients 10, 11, 12, 13, and 14) and subsequently tested for lytic activity in 4- and 20-h ^{51}Cr release assays.

CTL Responses by Patient 7. Patient 7 was tested for CTL responses to E75 4 weeks after the seventh vaccination. To increase the sensitivity of detection of E75-specific CTLs, autologous PBMCs were primed *in vitro* with DC-E75 plus IL-12. The results in Fig. 1*A* demonstrated that *in vitro* priming with

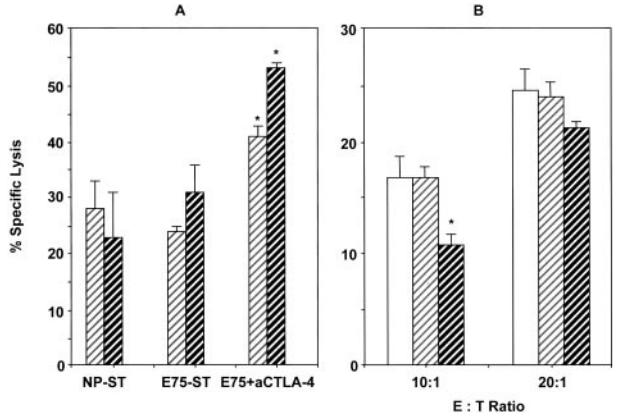

Fig. 1 *A*, Addition of IL-12 and α-CTLA-4 during IVS with E75 enhanced lytic activity of PBMCs from vaccinated patient 7. Specific lysis of E75-ST +IL-12 + α-CTLA4 *versus* NP-ST and E75-ST against NP pulsed T2 (▨); percentage of specific lysis of E75 + IL-12 + αCTLA4 *versus* NP-ST and E75-ST against E75 pulsed T2 (■); ∗, $P < 0.01$. *B*, Cold-target inhibition of lysis by E75-ST+ IL-12+ αCTLA4 of an HLA-A2$^+$ HER2$^+$ tumor by T2-E75 (■), T2-NP (□), and T2-E71 (▨); ∗, $P < 0.05$; *bars*, ±SE.

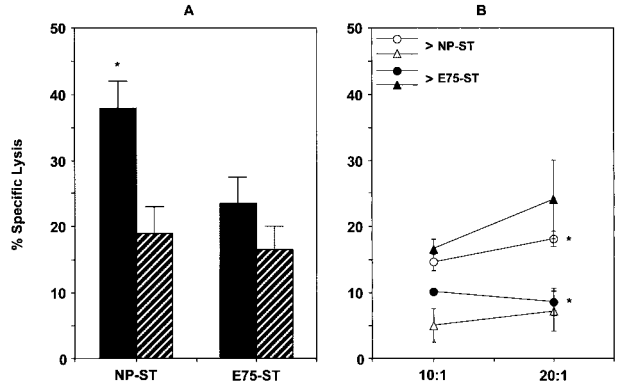

Fig. 2 Decreased recognition of E75 by PBMCs from vaccinated patient 6 (*A*) and patient 9 (*B* and *C*) after stimulation with DC-E75 compared with stimulation with DC-NP. Results of a 5-h CTL assay. *A*, E:T ratio was 30:1; targets, T2- E75 (▨) and T2- E71 (■). ∗, difference in lysis between targets pulsed with E75 and targets pulsed with E71 were significant ($P < 0.05$). *B* and *C*, targets were T2-E75 (*B*) and T2-E71 (*C*); ∗, differences in lysis of T2-E75 *versus* T2-E71 by NP-ST cells were significant ($P < 0.05$). Effectors in *B* and *C* were NP-ST postvaccine (○, △) and E75-ST postvaccine (●, ▲); *bars*, ±SE.

E75+IL-12 did not induce significantly higher E75-specific lytic activity than NP+IL-12. This activity increased when α-CTLA-4 was present ($P < 0.01$), suggesting that a part of E75-reactive CTLs was tolerized. IFN-γ secretion at 20 h also increased in the presence of α-CTLA-4 from 65 to 175 pg/ml. There was no specific recognition of E75 by DC-NP-stimulated PBMCs suggesting that *ex vivo* activated CD8+ cells endowed with lytic function were either absent or below the levels of detection or required E75-stimulation for activation of lytic function. Cold-target inhibition experiments showed that T2-E75 inhibited lysis by E75+IL-12+αCTLA-4 ST cells of an HLA-A2$^+$ HER-2$^+$ breast tumor significantly better than T2 E71 (38% inhibition and 12% inhibition at E:T ratios of 10:1 and 20:1, respectively; Fig. 1*B*), indicating that a subpopulation of *in vitro* E75-primed CTLs recognized endogenously presented E75. To obtain a general estimate of the proportion of the tumor lytic E75-specific cells we calculated LU. LU for tumor lysis by CTLs in the presence of T2-NP or T2-E71 as inhibitors were 24.4/10^6 cells, whereas in the presence of T2-E75 as inhibitors they were 17.6/10^6 cells, indicating that 28% of the tumor lytic effectors recognized endogenous E75.

CTL Responses by Patients 6 and 9. Lymphocytes were collected 2 weeks after the last vaccination. IVS was performed with DC-NP and DCs pulsed with E75 (DC-E75) in the presence of IL-12. The results show that cells stimulated by NP-ST from both patients recognized E75 better than the control HER-2 peptide E71. One IVS with DC-E75 lead to significantly decreased recognition of E75 ($P < 0.05$). E75 recognition was similar (patient 9) or lower (patient 6) than of E71 (Fig. 2, *A* and *B*). This suggested that a subpopulation of *ex vivo* activated E75-reactive CTLs were present in both patients. Additional IVS with E75 lead to its decreased recognition raising the possibility that E75 induced apoptosis of effector CTLs.

CTL Responses by Patients 10 and 11. Preliminary experiments with PBMCs from patients 10 and 11 collected before treatment and after the last vaccination, respectively, showed that *in vitro* priming with DC-NP or DC-E75 was insufficient to induce detectable levels of cytolytic activity (data not shown). These results suggested that the frequency of E75-specific CTLs in these patients may be lower than in patients 6, 7, and 9. For these reasons, the ability of these vaccinated patients to maintain lytic responses to E75 and HLA-A2$^+$ HER-2$^+$ tumors over time was determined after three consecutive IVS with E75 pulsed on autologous DCs.

CTL Responses by Patient 10. In experiments performed using PBMCs collected 5 months after the last vaccination we confirmed that IL-12 was required to induce detectable E75-specific lytic activity at IVS with DC-E75. αCTLA-4 antibody did not increase the CTL activity over the levels of lytic activity induced in the presence of IL-12. However, E75-specific CTL activity by DC-NP ± IL-12-stimulated PBMCs was not detected (Fig. 3*A*). This raised the question of whether activated/memory peripheral effectors were present in insufficient numbers to be detectable in this assay. IVS of prevaccine lymphocytes from this patient did not result in CTLs. Thus, the CTLs after *in vitro* recall with E75 were similar to those of patient 7 but not to those of patients 6 and 9. To address the duration of the presence of tumor lytic CTLs, PBMCs collected 9 months after the last vaccination were stimulated with E75 presented by autologous DCs. Lytic activity against tumors was tested after three IVS with DC-E75. To address whether these cells recognized endogenously presented E75, we performed cold-target inhibition of lysis, using as a target the ovarian tumor SKOV3.A2. The results in Fig. 3*B* show that in a 5-h assay at a high E:T ratio of 30:1, T2-E75 inhibited lysis of SKOV3.A2 cells by 16.6% compared with T2-NP. Again, calculation of LU indicated that 25% of the tumor lytic effectors recognized endogenous E75 [LU/10^6: inhibitor (T2-NP) = 83.4 *versus* LU/10^6: inhibitor (T2-E75) = 62.5].

To address whether memory effector CTLs persisted in this

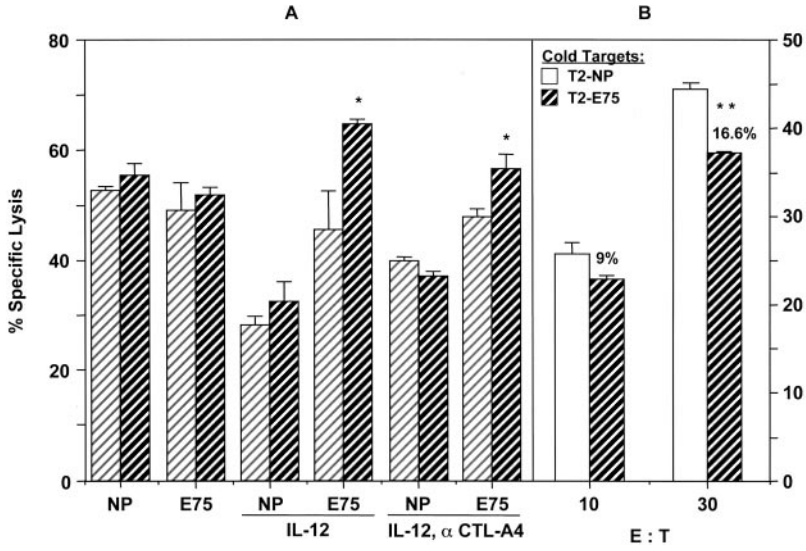

Fig. 3 A, One IVS with DC-E75+IL-12 of PBMCs from patient 10 induced specific recognition of T2-E75 (■) compared with that of DC-E75. ∗, $P < 0.01$. There was no enhanced recognition of T2-NP (▨). αCTLA4 was a weaker costimulator of E75-specific CTL activity compared with IL-12 ($P < 0.05$). *B,* Cold-target inhibition of lysis of SKOV3.A2 tumor cells by patient 10. PBMCs after three IVS with DC-E75 + IL-12 indicate the presence of tumor-reactive CTLs. ∗∗, $P < 0.001$. Inhibitors were T2-NP (□) and T2-E75 (■); *bars,* ±SE.

patient, the experiments were repeated with PBMCs collected 13 months after the last vaccination. To enhance the type 1 response inducing the ability of patient DCs in addition to IL-12, αIL-4, αIL-10, and mAbs against transforming growth factor β were added in all of the stimulation cultures. After three consecutive IVS with DC-NP and DC-E75 the resulting cells were tested in CTL assays. To assure that the number of effectors did not change subsequent to changes in CD8:CD4 ratios, the percentage of expression of $CD8^+$ cells was determined in both effector populations, and the effector numbers were adjusted at equal $CD8^+$ cells numbers in both assays.

The results in Fig. 4*A* show that both three IVS (3×NP-ST and 3×E75-ST) cells recognized T2 cells pulsed with exogenous E75. Recognition of E75 at the lower concentration of 5 μM was higher when 3×E75-ST were used as effectors than when 3×NP-ST cells were used as effectors. Thus, E75-specific CTLs were present in this patient 13 months after the last vaccination. Their reactivation required the presence of autologous MHC and was dependent on cytokines used but did not require, although was enhanced by, restimulation with E75.

This finding suggested that E75-specific "memory-like" CTLs were present in patient 10. To address whether NP-ST memory-like CTLs and E75-ST CTLs differed in their lytic potential, the CTL assay was continued for up to 20 h. The results in Fig. 4*B* show that the lytic activity of 3×E75-ST cells increased over a 20-h period, but the lytic activity of 3×NP-ST cells declined. This raised the possibility that E75-specific memory-like CTLs in this patient were endowed with weak lytic activity, and restimulation with E75 was required to activate their lytic function. Cold target inhibition of tumor lysis experiments showed similar results with the experiments performed 4 months earlier (Fig. 4*C*). In a 5-h assay, T2-E75 inhibited lysis by both NP-ST and E75-ST effectors compared with T2-NP. This supported the hypothesis that E75-specific cytolytic T cells recognizing tumors were present, and their reactivation did not require E75. IVS with E75 increased tumor lysis. 4×E75-ST effectors also lysed the breast tumor line SKBR.3.A2 (Fig. 4*D*). T2-E75 induced 81% and 47.2% inhibition of lysis, respectively, compared with T2-NP, confirming that these cells endogenously recognized epitopes presented by the tumor.

To confirm that vaccination induced E75-specific CTLs, we repeated the experiment using as responders plastic nonadherent PBMCs of patient 11. Responders were collected before and 16 months after the last vaccination. At this time, the patient had progressive disease. In previous experiments we found that E75-specific CTLs were undetectable in the PBMCs collected before vaccination even when E75+IL-12 were used. Addition of αCTLA-4 at priming with DC-NP and DC-E75 did not increase E75 recognition: percentage of specific lysis by NP-ST cells = 35.4 ± 3.6(NP) *versus* 40.5 ± 1.2(E75), whereas by E75-ST cells = 37.7 ± 1(NP) *versus* 47.9 ± 2.5(E75). Targets are indicated in the parentheses. αCTLA-4-induced specific lysis was 10.2 ± 2.5. Memory-like CTL effectors activated by DC-NP showed a similar pattern of reactivity with CTL effectors of patient 10 (*i.e.,* weak specific killing in the 5-h but not in the 20-h CTL assay). These results suggested that memory-like effectors were induced in this patient by vaccination because they were absent in the PBMCs collected before vaccination tested in parallel.

The lytic activity of cells from patient 11 stimulated three times with DC-E75 or DC-NP is shown in Fig. 5, *A–D*. Fig. 5*A* demonstrated that NP-ST PBMCs collected before vaccination had no lytic activity against E75 in the 5-h CTL assay and that lytic activity did not significantly increase after *in vitro* E75 stimulation. This confirmed the results of the previous experiment. PBMCs collected after vaccination (Fig. 5*B*) showed little increase in E75 lytic activity in the 5-h CTL assay. There was a significant increase in lytic activity for NP-ST at 5 h (from 13.2 ± 3.9 to 21.4 ± 0.3; $P < 0.05$) implying the presence of memory cells in this patient. This value was also markedly increased above pre- values (3.4 ± 3.6; $P < 0.001$).

In contrast to what was observed after 5-h incubation, PBMCs collected before vaccination and incubated for 20 h with targets specifically lysed T2-E75 [increase of 75%; 0 μg

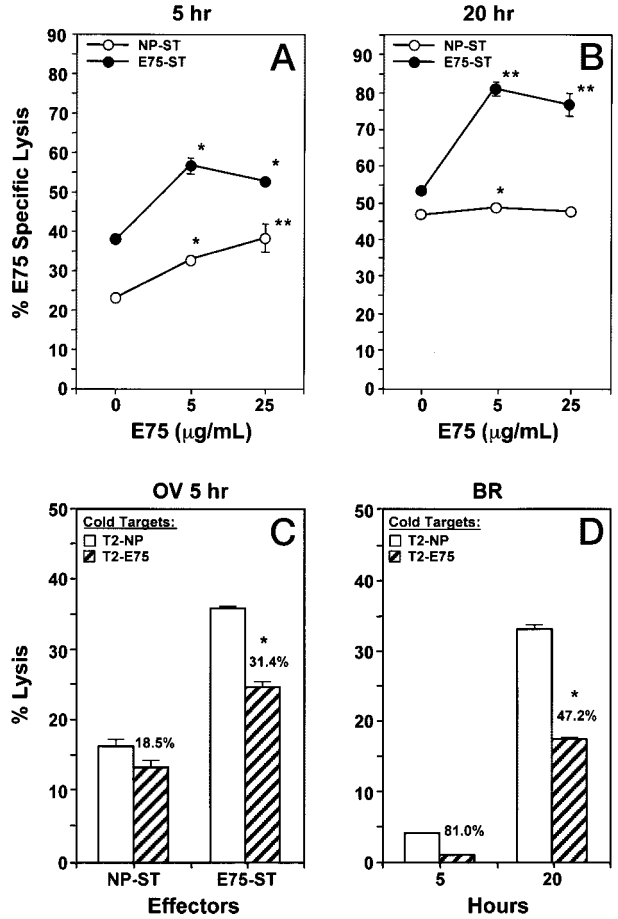

Fig. 4 Tumor-reactive memory-like CTLs were present in patient 10 13 months after last vaccination and were activated by stimulation with autologous DCs in the absence of E75. PBMCs from patient 10 were subjected to three IVS in the presence (E75-ST) and absence (NP-ST) of E75 before being tested in CTL assays. E:T ratio was 18:1. *A* and *B*, concentration-dependent recognition of E75 by NP-ST (○) and E75-ST (●). Differences in lysis of tumor targets stimulated by DC-NP and DC-E75 in the presence of T2-NP *versus* the presence of T2-E75 are all significant; ∗, $P < 0.05$. *A*, 5 h CTL assay; *B*, 20 h CTL assay; *C* and *D*, tumor lysis inhibition assays; *C*, tumor target = SKOV3.A2; *D*, tumor target = SKBR3.A2. Inhibitors: T2-NP (□), T2-E75 (■); *bars*, ±SE.

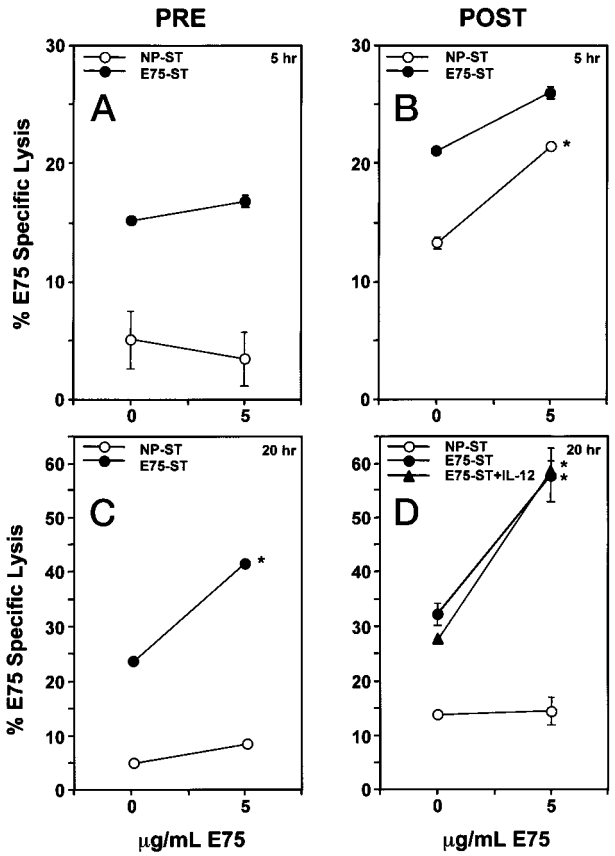

Fig. 5 E75-specific memory-like CTLs are present at low levels in patient 11 6 months after last vaccine. Effectors*:* NP-ST (○) and E75-ST (●) as described in Fig. 4. E:T ratio was 20:1. *A* and *B*, PBMCs collected before vaccination; *C* and *D*, PBMCs collected after vaccination; *A* and *C*, 5 h CTL assays; *B* and *D*, 20 h CTL assays; ∗, $P < 0.01$; *bars*, ±SE.

E75 (NP) compared with 5 μg/ml E75; $P < 0.01$]. Similarly, lysis of T2-E75 by postvaccine E75-ST PBMCs was significantly higher than for T2-NP (57.7 ± 5.0% *versus* 32.2 ± 7.2%; $P < 0.01$). Lysis was also increased for E75-ST + IL-12 (58.7 ± 2.3%; $P < 0.001$), but the lysis was not increased over that of E75-ST cells minus IL-12 (Fig. 5, *C* and *D*).

To confirm that E75-induced CTLs recognized tumors, we repeated the cold-target inhibition experiments with patient 14. PBMCs were collected 3 months after the last vaccination and stimulated in parallel with DC-NP, DC-E75, and DC-E75+IL-12. A caveat of this experiment was that autologous DCs were not sufficient for the entire stimulation protocol, thus allogeneic HLA-A2-matched DCs from a healthy donor were used as antigen-presenting cells for all of the stimulation groups. CTLs from all three of the stimulation groups expressed E75-specific lysis (data not shown).

Cold target inhibition experiments (Fig. 6, *A* and *B*) showed that T2-E75 inhibited lysis of SKOV3.A2 cells by 3×E75-ST but not lysis by 3×NP-ST cells suggesting that E75-specific memory CTLs were absent from this patient. When IL-12 was present at the time of *in vitro* priming with T2-E75, resulting 3×E75+IL-12ST-CTL showed an even higher increase in inhibition of lysis compared with E75-ST-CTL. These results were similar when the assay was extended for 20 h (Fig. 6*B*). This experiment confirmed that a subpopulation of E75-ST cells lysed tumors overexpressing HER-2. CTLs that were stimulated in the presence of IL-12 showed a higher avidity for the E75, as evidenced by the higher inhibition of lysis in the E75+IL-12 group compared with the E75 alone group.

CTL Responses by Patients 12 and 13. Patients 12 and 13 showed no E75-specific CTL responses after three IVS with DC-E75 and with DC-NP, suggesting that E75-specific CTLs were either absent or they could not be expanded by DC-E75 to numbers where their cytolytic activity could be detected. The second possibility is more likely because E75-specific CTLs could be recalled in patient 13 by one IVS with E75 agonist-

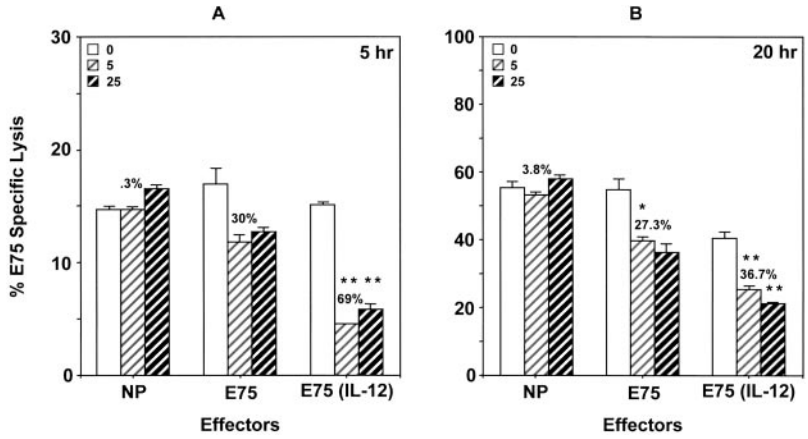

Fig. 6 The presence of IL-12 during IVS enhanced cytolytic activity against tumor by PBMCs from patient 14. E:T ratio was 30:1. *A* and *B*, cold-target inhibition of lysis of SKOV3.A2 by PBMC IVS with DC-NP (*NP*), DC-E75 (*E75*), and DC-E75 + IL-12 (*E75, IL-12*). *Bars* represent μg of E75 pulsed on T2 cells used for cold-target inhibition ± SE. *A*, 5 h CTL assay; *B*, 20 h CTL assay. *Numbers above bars* represent percent inhibition of lysis. *, $P < 0.01$; **, $P < 0.001$.

Table 5 Summary of immunological responses to E75

		Proliferation*		CTL		IFN-γ	
Patient no.	DTH	Pre	Post	Pre	Post	Pre	Post
1	nd[b]	(−)	nd	nd	nd	nd	nd
2	nd	(−)	(+) ↑	nd	nd	nd	nd
3	nd	(−)	(−)	nd	nd	nd	nd
4	(−)	nd	nd	nd	nd	nd	nd
5	(+)	(−)	(−)	nd	nd	nd	nd
6	(+)	nd	(+)	nd	(+) NP	nd	(−)
7	(+)	(+)	(−)	nd	(+) E75, IL-12, αCTLA4, **T**	nd	(+) IL-12
8	(+)	(+)	(+)→	nd	nd	nd	nd
9	nd	(−)	(−)	nd	(+) NP	nd	(−) IL-12
10	(+)	(+)	(+)→	(−) E75, IL-12, αCTLA4	(+) NP, E75, ↑, **T**	(−) IL-12	(+) IL-12 ↑
11	(+)	(+)	(+) ↓	(−) E75, IL-12, αCTLA4	(+) NP, E75, ↑, **T**	(−) IL-12	(+) IL-12→
12	nd	(+)	(+) ↓	nd	(−) NP, E75, IL-12	nd	nd
13	nd	nd	nd	nd	(−) NP, E75, IL-12	nd	nd
14	(+)	nd	nd	nd	(+) E75, IL-12, ↑, **T**	nd	(+) IL-12 ↑

[a] By cpm to E75 in the pre- and postvaccination sample.
[b] nd, not done; +/−, positive/negative response; NP, E75, IL-12, αCTLA4, the IVS agent(s) that induced CTL activity; **T**, specific tumor lysis; ↑, ↓, →, increase, decrease, or the same.

variants presented by HLA-A2-matched DCs or T2 cells in the presence of IL-12 (24). The ability of these variants to activate E75-specific CTLs was confirmed with patients 10 and 11 (24).

In summary, of the 8 vaccinated patients tested for induction of a CTL response to E75 by recall *in vitro* with E75, 4 (patients 7, 10, 11, and 14) showed a specific lytic response to E75. E75-specific CTL responses correlated with significant increases ($P < 0.01$) in IFN-γ secretion in 2 patients with and 2 without the addition of IL-12 (Table 4). In addition, 3 of 3 vaccinated patients tested contained a population of CTLs of which the lytic activity against tumors was inhibited by T2-E75 indicating that they recognized endogenously presented epitopes by the tumor. In 4 of these 8 patients (patients 6, 9, 10, and 11) E75-specific CTLs were detected after stimulation with DC-NP *in vitro* either after priming or restimulation, suggesting the presence of E75-specific memory-like cells in patients with metastatic breast cancer.

A summary of all of the immunological tests performed for each patient is shown in Table 5. Eight patients had results from at least two of four assays. Excluding patient 10 from analysis because of additional treatment after vaccination, the mean + SD of TTP for 8 patients with either positive DTH responses to E75, proliferative responses to E75, or both was slightly greater (10.7 ± 4.8 weeks) than for 5 patients who had negative responses to DTH, proliferation, or both (5.7 ± 3.7 weeks; $P = 0.06$). Five of the 8 also had either specific CTL precursors for E75 and/or IFN-γ production with the addition of exogenous IL-12.

DISCUSSION

The most important findings of the present vaccine study in stage IV patients were: (*a*) repeated vaccinations with E75 plus GM-CSF were well tolerated; (*b*) in 4 of 8 patients E75+GM-CSF induced epitope-specific tumor-lytic CTLs; (*c*) such CTLs were present in several patients for 1–12 months after completion of vaccination; and (*d*) these CTLs could be activated by self-MHC in the presence of inflammation-inducing conditions to produce IFN-γ, and to recognize and lyse tumor cells expressing E75. This functional behavior may be consistent with peripheral memory-effectors.

Although the patients with stage IV disease are not considered the most promising group for treatment by cancer vaccines, compared with patients with disease remission or less advanced disease, this group conceivably could benefit the most from immunotherapy, because the applicability of other approaches is limited. The fact that Ag-specific tumor lytic CTLs were detected after vaccination may address concerns that vaccination with 100-1000 μg/ml of peptide can induce deletion of tumor-lytic effectors. Our study demonstrated that E75-specific cytolytic effectors were induced by E75, were present in the peripheral circulation, and could be detected after one recall with DC+E75 or DCs alone in 4 of 6 (67%) patients tested. Overall they were detected in a high proportion (6 of 8; 75%) of patients tested. The E75-specific repertoire was maintained over time, because such cells could be detected at recall with DC-NP or DC-E75 months after the last vaccination. A fraction of these effectors from 3 of 3 patients tested specifically recognized the endogenously presented E75 by indicator tumor cells. A preliminary estimation of the comparative size of the E75-specific tumor-lytic effectors performed in 2 patients indicated that these effectors represented 25% of the tumor-lytic effectors. This proportion is within the range described for CTLs induced by foreign peptides, which recognize endogenous antigen (7, 8).

These results suggest that E75+GM-CSF delivered at multiple sites is immunogenic either for *de novo* activation of E75-specific tumor-lytic CTLs or stimulate activated/memory cells present in the patients below the levels of detection of currently used methodology. This possibility is supported by reports that peptides E75 and GP2 could activate Ag-specific tumor-lytic CTLs from tumor infiltrating/associated lymphocyte (13, 25–27). Alternatively, the optimization of conditions for *in vitro* immunological monitoring using DCs, IL-12, αCTLA4, and cold-target inhibition of lysis may have increased the sensitivity of detection of these effectors.

A third possibility may be related to the use of GM-CSF at higher doses than in a recent study (12) and the multiple vaccinations at multiple sites. Knutson *et al.* (12) reported recently that HER-2 peptide:369–384 mixed with two other HER-2 peptides plus GM-CSF at one-half the concentration used here induced E75-specific and C85-specific CTLs. PBMCs from 1 patient and a CTL clone from another patient after two to three IVS with Ag lysed targets transfected with HER-2 and HLA-A2 better than nontransfected targets. Similar results (2 of 2 patients tested) were reported when DC-E75 were used as vaccine after three IVS (11). In an earlier study, 2 of 3 patients vaccinated with the same amount of E75 as in this study, plus incomplete Freund's adjuvant, after several IVS with E75 also increased their IFN-γ secretion but not their lytic activity to HLA-A2$^+$ HER-2$^+$ targets compared with their HLA-A2$^-$ HER-2$^-$ counterparts (10). This suggested that E75 in the vaccine could induce partial activation of specific CTLs in the vaccine.

The method for DTH measurements in this study differed significantly from that of Disis *et al.* (28) in the following respects: (*a*) 50 μg of E75 was used compared with 100 μg of a mixture of peptides; (*b*) patients were skin tested 1 month after four weekly injections instead of 6 months after six monthly injections (3), and all of the patients in our study had been heavily treated and had stage IV disease. These differences may explain the lower frequency of DTH responses >10 mm in this study 2 of 8 (25%) *versus* 7 of 14 (50%; Ref. 28). E75 (369–377) appeared to be the strongest inducer of DTH responses because DTH responses >10 mm to longer peptides (688–703 and 971–984) from other HER-2 sites were less frequently observed (21.4% and 14.2%, respectively) than to E75 in this study (28). The majority of patients (4 of 5) with positive DTH of ≥5 mm proliferated to E75 in the prevaccine, postvaccine, or both. Proliferative responses to E75 postvaccination were not significantly higher than pretreatment values. The fact that SI decreased postvaccination in 3 of 4 DTH$^+$ patients suggests that patients were presensitized to E75 before study and that E75 alone provided too weak a stimulus in heavily treated, stage IV patients. There are several possible explanations for differences between DTH responses in this study and other reports: (*a*) this study involved one 9-mer peptide as an immunogen rather than mixtures of three 13–16 mer peptides (12); hence, vaccination with E75, which is an epitope recognized by CD8+ cells on HLA-A2 alone, might be less effective to induce DTH than the cumulative effects of three 13–16 were peptides; (*b*) the contribution of CD4$^+$ cells to DTH could not be excluded in the study by Disis. There was a trend ($P = 0.06$) for patients with positive DTH, proliferation, or both to have longer TTP. This finding must be confirmed in a larger study, preferably a randomized trial.

Collectively these studies indicated that a single peptide, E75, corresponding to a single CTL epitope together with GM-CSF could induce long-lived epitope-specific CTLs that recognized the corresponding epitope on tumor cells. Only 2 of 6 patients tested postvaccination showed a weak IFN-γ response in the absence of IL-12. Testing for IFN-γ induction by ELISPOT performed in another laboratory by a different stimulation method showed no IFN-γ induction in the same 3 patients, which were also found to be negative by ELISA (patients 6, 7, and 9; Table 4). Thus, at this time, because of limitations in the techniques used, we cannot establish whether there was an increase in E75-TCR$^+$, IFN-γ secreting cells and the levels of their differentiation. Of interest, Knutson *et al.* (29) using quantitative ELISPOT reported that vaccination with a helper peptide vaccine consisting of the peptide HER-2:369–384 significantly increased the IFN-γ+ precursor frequency to E75 (369–377) compared with PBMCs collected prevaccine. Specific conclusions and implications of this approach for vaccines applicable to cancer are summarized below.

The Role of Antigen. Vaccination with E75 induced Ag-specific, tumor-lytic CTLs in 3 of 3 patents tested. This was confirmed in three independent systems of testing: (*a*) recall responses by DCs plus cytokines; (*b*) recall response by one stimulation with Ag *in vitro*; and (*c*) recall responses by three stimulations with Ag *in vitro*. Whereas responses in the third system may be considered a result of IVS, responses in the first two systems support the hypothesis that E75-specific, tumor-lytic CTLs were induced *in vivo* by the vaccine.

Vaccination with E75 induced E75 peptide-specific CTLs in 6 of 8 patients tested. An important observation from these studies was that only 4 of these 8 patients responded by higher E75-specific CTL activity to IVS with E75. In 2 patients stimulation with E75 decreased the responses, whereas in 2 others E75 failed to induce CTL responses. This suggests that wild-type tumor Ag vaccines may be immunogenic only in a fraction

of individuals (50% in this case or lower). For other individuals, modified tumor Ag should be used directed by immunological monitoring. For example, it is possible that patients such as patients 6 and 9 will benefit from boosting with "attenuated" or "survival inducing immunogens" (30), whereas patients 12 and 13 may benefit from boosting with enhancer agonists, as observed with patient 13 (24).

The Role of IL-12. Vaccination with E75 required IL-12 as a costimulator for detection of IFN-γ-producing cells within 24 h. IL-12 also enhanced our ability to detect CTL responses *in vitro*. Because E75 appeared to be a weak inducer of IL-12 and/or the frequency of E75-specific cells may be low, the use of IL-12 or proinflammatory agents (CpG) during use of the *in vivo* priming with vaccine may deserve additional consideration. Again, the *ex vivo* IFN-γ and CTL responses need to be monitored to adjust the IL-12 dosage to levels where IL-12 by itself does not induce IFN-γ.

The Role of CTLA-4. αCTLA-4 antibody was tested in 3 patients for activation of CTL and IFN-γ responses. In 1 patient αCTLA-4 enhanced E75-specific CTL activity above the levels observed with E75+IL-12; in the other it decreased the levels of E75-specific lysis compared with E75+IL-12. In a third patient, its CTL potentiating activity was borderline. Although this study was limited, the results suggest that the use of αCTLA-4 in immunological monitoring may be needed to identify the presence of tolerized effectors, and characterize their functional status and ability to respond to E75 by mitosis or apoptosis. On the basis of these determinations, αCTLA-4 may be a significant component of tumor vaccines aiming to induce tumor-specific CTL alone or together with IL-12, but its utility should be determined on a per case (or patient status) basis, particularly in patients with advanced disease who received cytotoxic treatment.

In conclusion, vaccinations with peptide E75 + GM-CSF were well tolerated by patients with advanced disease. Although E75-specific CTLs could be generated in select patients, their frequencies were low requiring multiple IVS with DCs with or without IL-12 and/or αCTLA-4 antibody. These findings argue for the use of IL-12 and/or αCTLA-4 (31), or consensus or specific class II-restricted helper peptides (25) combined with E75 to generate sufficient Th1-inducing cellular immunity, and should be considered in designing other peptide vaccine trials. Newer assays such as ELISPOT and intracellular cytokine measurements may increase the sensitivity of detection of CTL precursors, although they cannot discriminate which precursors have lytic activity. Similarly, the use of carefully selected viral peptides in relation to previous exposure of the patient to such viruses (*e.g.*, influenza, cytomegalovirus, and EBV) may allow useful comparisons to establish the significance of the stimulation protocols *in vitro*, and compare the ability and limitations of tumor Ag to induce differentiation of cytotoxic function compared with the limitations of viruses to mediate similar effects in patients with resolved and unresolved infections. E75 or agonist peptides (24, 26) with higher affinity for the TCR should also be tested in cancer patients in the adjuvant or tumor-free setting where there is less likelihood for tumor immune suppression to occur.

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