Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients

Elke Jäger*†, Julia Karbach*, Sacha Gnjatic‡, Antje Neumann*, Armin Bender*, Danila Valmori§, Maha Ayyoub§, Erika Ritter‡, Gerd Ritter‡, Dirk Jäger¶, Dennis Panicali‖, Eric Hoffman‡, Linda Pan‡, Herbert Oettgen‡, Lloyd J. Old†‡, and Alexander Knuth**

*Ludwig Institute Clinical Trial Center, Medizinische Klinik II, Hämatologie–Onkologie, Krankenhaus Nordwest, Steinbacher Hohl 2–26, 60488 Frankfurt, Germany; ‡Ludwig Institute for Cancer Research, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; §Ludwig Institute Clinical Trial Center, Columbia University College of Physicians and Surgeons, 622 West 168th Street, New York, NY 10032; ¶Medizinische Onkologie, Nationales Centrum für Tumorerkrankungen, Universitätsklinik Heidelberg, Im Neuenheimer Feld 350, 69120 Heidelberg, Germany; ‖Therion Biologics Corporation, 76 Rogers Street, Cambridge, MA 02142-1119; and **Klinik und Poliklinik für Onkologie, Universitätsspital, Rämistrasse 100, 8091 Zürich, Switzerland

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NY-ESO-1 is a cancer/testis antigen expressed in a range of human malignancies, and a number of vaccine strategies targeting NY-ESO-1 are being developed. In the present study, the safety and immunogenicity of recombinant vaccinia-NY-ESO-1 and recombinant fowlpox-NY-ESO-1 were analyzed in a series of 36 patients with a range of different tumor types. Each construct was first tested individually at two different dose levels and then in a prime-boost setting with recombinant vaccinia-NY-ESO-1 followed by recombinant fowlpox-NY-ESO-1. The vaccines were well tolerated either individually or together. NY-ESO-1-specific antibody responses and/or specific CD8 and CD4 T cell responses directed against a broad range of NY-ESO-1 epitopes were induced by a course of at least four vaccinations at monthly intervals in a high proportion of patients. CD8 T cell clones derived from five vaccinated patients were shown to lyse NY-ESO-1-expressing melanoma target cells. In several patients with melanoma, there was a strong impression that the natural course of the disease was favorably influenced by vaccination.

antibody response | NY-ESO-1 recombinant vaccine | T cell response | tumor reactivity

NY-ESO-1 is a cancer/testis antigen that is expressed in a variety of human malignancies but not in normal tissues except the testis (1–3). Spontaneous immune responses involving antibody as well as CD4 and CD8 T cells directed against a broad range of MHC class I- and class II-restricted NY-ESO-1 peptides have been observed in patients with advanced NY-ESO-1-expressing tumors (4–9).

Attempts to induce NY-ESO-1-specific immune responses in cancer patients have included vaccination with synthetic HLA-A2-restricted NY-ESO-1 peptides or recombinant NY-ESO-1 protein administered either alone or in combination with adjuvants (10, 11). HLA-A2-restricted NY-ESO-1 peptides injected intradermally were shown to be safe and immunogenic. Although these trials were designed only to determine safety and immunogenicity, some patients showed tumor regression or stabilization of disease (11). A broad NY-ESO-1-specific immune response including antibody and CD4 and CD8 T cell responses was seen after immunization with recombinant NY-ESO-1 protein combined with ISCOMATRIX adjuvant (CSL Ltd., Parkville, Victoria, Australia) in patients with resected NY-ESO-1-expressing melanoma. This immune response to the vaccine appeared to be associated with long disease-free survival (10).

We conducted a clinical trial using recombinant vaccinia-NY-ESO-1 (rV-NY-ESO-1) and recombinant fowlpox-NY-ESO-1 (rF-NY-ESO-1) constructs in patients with advanced NY-ESO-1-expressing cancers. Vaccinia and related pox viruses have been used to construct vaccines against HIV (12, 13) and cancer-related antigens (14, 15), and immunization with these viral vectors has been found to induce specific humoral and cellular immune responses in clinical trials. The objectives of the present study were to assess the safety of the recombinant vaccines individually at two different dose levels and together in a prime-boost strategy and to examine humoral and cellular NY-ESO-1-specific immune responses before and after vaccination.

Results

Patients. Thirty-five patients were enrolled in the trial, and one additional patient was treated under a single-patient exemption. All patients were evaluable for toxicity. Twenty-three patients completed four vaccinations and were therefore considered evaluable for immunological response and tumor response. Ten of these evaluable patients (all HLA-A2-positive) were treated in cohorts 1 (two patients), 2 (two patients), 3 (three patients), and 4 (three patients). In cohort 5, 13 evaluable patients were enrolled (3 HLA-A2-positive and 10 HLA-A2-negative). Additional patient characteristics are presented in Table 1.

Toxicity. Grade-3 or -4 toxicity was not observed. Erythema and pruritus at the sites of vaccination were seen in all patients and lasted 5–7 days, with a maximum on day 3. Systemic hypersensitivity reactions were not observed.

Immune Response to NY-ESO-1. The three vaccine strategies (rV-NY-ESO-1, rF-NY-ESO-1, or rV-NY-ESO-1 followed by rF-NY-ESO-1) induced an NY-ESO-1-specific immune response in the majority of patients and appeared to be equally effective in the different cohorts. The pattern of immune responses after vaccination can be described in terms of four categories: category I, sero-negative patients who did not develop any detectable immune response; category II, sero-negative patients who remained sero-negative but developed CD4 and/or CD8 T cell responses; category III, sero-negative patients who sero-converted and developed CD4 and/or CD8 T cell responses; and category IV, sero-positive

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Abbreviations: rF-NY-ESO-1, recombinant fowlpox-NY-ESO-1; rV-NY-ESO-1, recombinant vaccinia-NY-ESO-1; ELISPOT, enzyme-linked immunospot; APC, antigen-presenting cell.

†To whom correspondence may be addressed. E-mail: elke.jaeger@licr.org or l.old@licr.org.

Table 1. Characteristics and immune responses of 23 evaluable patients immunized with rV- and rF-NY-ESO-1 vaccine

Patient	Disease	Stage	Pre-study dev.	Metastases	Cohort	No. of vacc	Ab pre	Ab post	CD4 pre	CD4 post	CD8 pre	CD8 post	TTP
1	Melanoma	III	NA	NED	1	4	−	+	−	−	−	+	0
2	Melanoma	IV	PD	LU	2	4	−	−	+	+	−	+	12
6	Sarcoma	IV	PD	P, LN	2	4	−	−	−	−	−	+	0
7	Sarcoma	IV	PD	LU, SC	1	4	+	+	+	+	−	+	0
9	Melanoma	IV	PD	LN, LU	3	4	−	+	−	−	−	+	0
10	Melanoma	III	NA	NED	4	4	−	−	−	−	−	+	9
11	Ovarian cancer	IV	NA	NED	3	4	−	−	−	+	−	+	8
13	Breast cancer	IV	NA	NED	3	4	−	+	−	+	−	+	5
14	Melanoma	IV	PD	SC, P	4	26+	+	+	−	−	+	+	32+
15	Teratoma	IV	PD	LN, LU	4	8	−	−	−	−	−	−	7
17	Melanoma	IV	SD	LN	5	26+	+	+	+	+	+	+	31+
18	Endometrial cancer	IV	PD	LU	5	4	−	+	−	+	−	+	0
19	Melanoma	IV	PD	LN	5	26+	−	+	−	+	−	+	25
21	Melanoma	III	NA	NED	5	6	−	+	−	+	−	−	7
22	Melanoma	IV	PD	LN, bone	5	6	−	−	+	+	−	+	6
23	HNC	IV	PD	LN, skin	5	4	−	−	+	+	+	+	0
24	Sarcoma	IV	PD	LU, SC	5	6	−	−	−	−	−	−	0
26	Prostate cancer	IV	SD	loc recurr	5	4	−	−	−	−	−	+	0
28	HNC	IV	SD	LN, LU	5	6	−	−	−	−	−	−	0
31	Melanoma	IV	PD	LN, LI, P	5	8	+	+	+	+	+	+	0
32	Melanoma	III	NA	NED	5	7	−	−	−	−	−	+	5
35	Sarcoma	IV	NA	NED	5	4	−	−	−	+	−	+	0
36	Melanoma	IV	PD	LN	OP	4	+	+	ND	ND	+	+	28

Ab, antibody; HNC, head and neck cancer; LI, liver; LN, lymph node; loc recurr, local recurrence; LU, lung; NA, not applicable; ND, not done; NED, no evidence of disease; OP, off protocol; P, peritoneal; PD, progressive disease; post, postvaccination; Pre-study dev., prestudy tumor development; pre, prevaccination; SC, subcutaneous; SD, stable disease; TTP, time to progression (in months); vacc, vaccinations.

patients who showed CD4 and/or CD8 T cell reactivity before vaccination that remained stable or was broadened during the course of vaccination (Figs. 1 and 2, Table 2).

Category I. Three sero-negative patients (patients 15, 24, and 28) did not develop any detectable NY-ESO-1-specific immune response after four vaccinations.

Category II. Nine sero-negative patients developed an NY-ESO-1-specific CD4 and/or CD8 T cell response but no antibody response. A CD8 T cell response alone was induced in patients 6, 10, 26, and 32. Both CD4 and CD8 T cell responses were detected after vaccination in patients 2, 11, 22, 23, and 35, with CD4 T cells already present at baseline in patients 2, 22, and 23 and CD8 T cells present

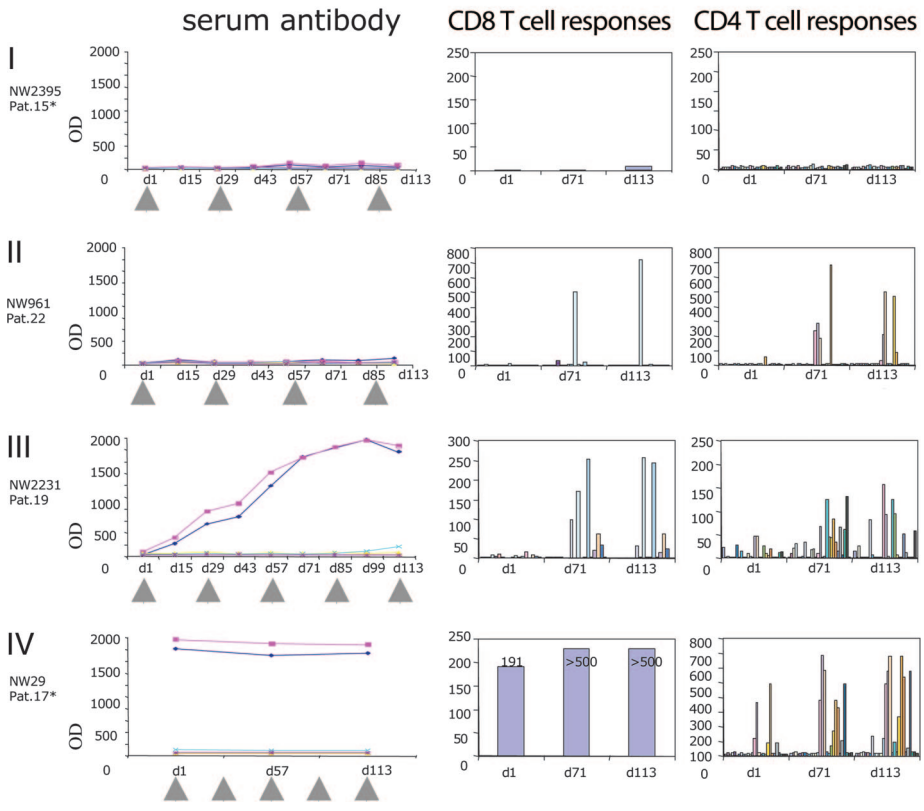

Fig. 1. Development of NY-ESO-1 serum antibody and specific CD8 and CD4 T cell responses in individual patients immunized with rV/rF-NY-ESO-1 vaccine. NY-ESO-1 (pink), LAGE-1 (blue), MAGE-3 (yellow), MAGE-4 (green), and p53 (light blue) serum antibody was assessed by ELISAs before and after vaccination. OD values at a serum dilution of 1:400 are shown. Arrows indicate day of vaccine. CD8 and CD4 T cell responses against NY-ESO-1 epitopes were assessed in ELISPOT assays. Bars show the number of specific spots per 25,000 effector T cells. CD8 T cell responses in HLA-A2-positive patients (indicated by asterisks) are shown tested with the representative NY-ESO-1 p157–165 epitope, and CD8 T cell responses in non-HLA-A2 patients are shown tested with overlapping NY-ESO-1 18- to 20-mer peptides (colored bars). CD4 T cell responses were tested with 18- to 20-mer peptides (colored bars). T cell responses were considered positive when they were at least threefold higher than the background. Pat., patient.

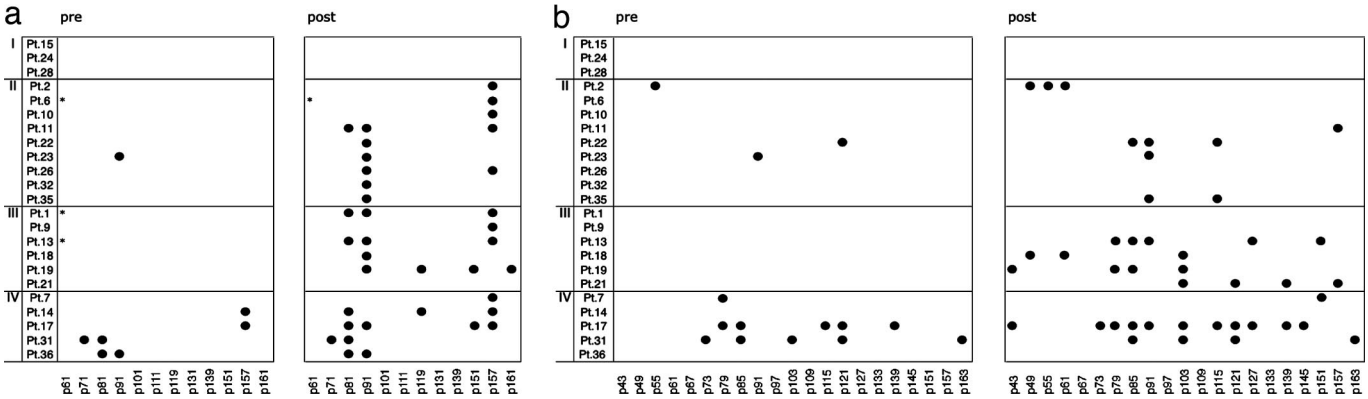

Fig. 2. NY-ESO-1 epitopes (the *x* axis indicates the position of the first amino acid of each 20-mer or 18-mer peptide) recognized by CD8 T cells of 23 evaluable patients grouped into categories I–IV before and after vaccination. (*a*) CD8 epitopes are clustered around NY-ESO-1 regions p81–110 and p151–170. In patients marked with an asterisk, T cell responses were assessed by presensitization with NY-ESO-1 p157–165 only. All other patients were monitored by presensitization of effector cells with Ad2/ESO and, in addition, with NY-ESO-1 p157–165 in HLA-A2-positive patients. (*b*) CD4 epitopes show a broader distribution spanning NY-ESO-1 regions p43–138; additional epitopes recognized less frequently are located between p139–180. Pt., patient.

in patient 23. The transient CD4 response observed in patient 26 on day 71 only was not considered an interpretable response.

Category III. Six patients (patients 1, 9, 13, 18, 19, and 21) showed NY-ESO-1 sero-conversion and developed a CD4 and/or CD8 T cell response. All patients also developed serum antibody against LAGE-1 (an antigen closely related to NY-ESO-1) but not against MAGE-3, MAGE-4, or p53. An NY-ESO-1-specific CD8 T cell response was induced in two patients (patients 1 and 9), a CD4 T cell response was induced in one patient (patient 21), and a CD8 and CD4 T cell response was induced in three patients (patients 13, 18, and 19).

Category IV. The serum of the five patients in this category (patients 7, 14, 17, 31, and 36) showed reactivity with NY-ESO-1 before vaccination and also with LAGE-1 in four cases, but not with MAGE-3, MAGE-4, or p53. An NY-ESO-1-specific CD8 T cell response was detected in four of these patients before vaccination and in all five patients after vaccination. Only four patients in this group were tested for NY-ESO-1-specific CD4 T cell reactivity. Three showed a CD4 T cell response before and after vaccination, and one patient showed no response (patient 14).

Analysis of NY-ESO-1 Epitope Clusters Recognized by Prevaccine and Postvaccine T Cells by Using Overlapping Peptides. CD8 T cell responses were observed in 19 of 23 evaluable patients and were directed against the NY-ESO-1 p81–110 and p157–170. Vaccine-induced and spontaneous CD8 T cell responses were focused on these two primary epitope clustering regions of NY-ESO-1. No CD8 T cell reactivity with NY-ESO-1 region p1–70 was seen (Fig. 2*a*).

CD4 T cell responses were observed in 12 of 22 evaluable patients (no results were available for patient 36) and were directed against a broader region of the NY-ESO-1 protein (p73–138 in most cases). Generally, vaccination induced the same broad range of NY-ESO-1 epitope recognition by CD4 T cells seen in patients with spontaneous NY-ESO-1 reactivity. No reactivity was found against NY-ESO-1 p1–43 (Fig. 2*b*).

Table 2. Four categories of NY-ESO-1 immune responses (see Fig. 1) observed in 23 evaluable patients immunized with NY-ESO-1 recombinant vaccinia/fowlpox vaccine

	NY-ESO-1 antibody		CD4 T cells		CD8 T cells		
Category	Pre	Post	Pre	Post	Pre	Post	Patients
I	–	–	–	–	–	–	**15, 24, 28**
II	–	–	–	–	–	+	**6, 10, 26, 32**
	–	–	–	+	–	+	**11, 35**
	–	–	+	+	–	+	**2, 22**
	–	–	+	+	+	+	**23**
III	–	+	–	–	–	+	**1, 9**
	–	+	–	+	–	–	**21**
	–	+	–	+	–	+	**13, 18, 19**
IV	+	+	+	+	–	+	**7**
	+	+	–	–	+	+	**14**
	+	+	+	+	+	+	**17, 31, 36**

Recognition of Naturally Processed Cell-Surface NY-ESO-1 by Postvaccine NY-ESO-1 Peptide-Specific CD8 T Cell Clones. Recognition of NY-ESO-1, expressed naturally and presented on the surface of tumor cells, by postvaccine CD8 T cell clones was tested in five patients. The NY-ESO-1-specific CD8 T cell clones from HLA-A2-positive patients 1 (category III) and 14 (category IV), generated by presensitization with the NY-ESO-1 p157–165 peptide, were tested in cytotoxicity assays. CD8 T cell clones of both patients efficiently lysed T2 cells pulsed with the respective sensitizing peptide and also the NY-ESO-1-positive melanoma cell line SK-MEL-37 but not untreated T2 cells, K562 cells, or the NY-ESO-1-negative melanoma cell line NW-Mel-145 (Fig. 4, which is published as supporting information on the PNAS web site). To determine recognition of NY-ESO-1-positive tumor cells by T cell clones specific for non-HLA-A2-restricted NY-ESO-1 epitopes, we selected three patients from the three different immune-response categories: patient 22 (category II), who was sero-negative and showed no CD8 T cell response before vaccination but developed a strong CD8 T cell response after vaccination; patient 19 (category III) who sero-converted and developed a strong CD8 T cell response after vaccination; and patient 31 (category IV), who showed antibody and T cell reactivity before vaccination. Postvaccine CD8 effector T cell clones were obtained by *in vitro* stimulation with NY-ESO-1 peptides or Ad2/ESO. For specificity analysis, we selected clone NW961-CD8-74 from patient 22 (which recognized NY-ESO-1 p91–110), clone NW2231-CD8-45 from patient 19 (which recognized NY-ESO-1 p71–90), and clone NW2541-CD8-4 from patient 31 (which recognized both NY-ESO-1 p71–90 and p81–100). As shown in Fig. 3, these T cell clones recognized autologous EBV cells pulsed with the relevant peptide and recognized allogeneic or autologous NY-ESO-1-positive tumor cell lines in cytotoxicity assays but showed no reactivity with NY-ESO-1-nonexpressing cells.

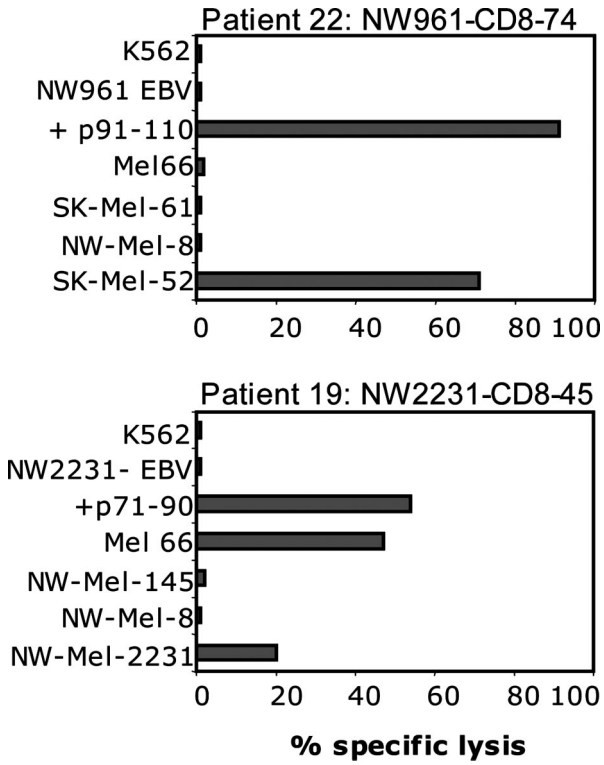

Fig. 3. Specific cytotoxicity of CD8 T cell clones obtained from patient 22 (category II) and patient 19 (category III). Clones were generated by presensitization of postvaccine T cells with Ad2/ESO followed by limiting dilution and restimulation with the relevant NY-ESO-1 peptide epitope recognized after the initial stimulation. The distinct specificity of the T cell clones reflects recognition of different NY-ESO-1 epitopes (p91–110 in patient 22 and p71–90 in patient 19). Cross-reactivity against naturally processed NY-ESO-1 in tumor cells is shown by the specific reactivity against different NY-ESO-1-positive tumor cell lines (SK-Mel-52, Mel66, NW-Mel-2231) and the lack of reactivity against NY-ESO-1-negative tumor cell lines (NW-Mel-8, NW-Mel-145, SK-Mel-61) and K562. The effector-to-target cell ratio is 3:1 for patients 19 and 22.

Tumor Response. Of 23 evaluable patients, 16 had measurable disease and 7 had completely resected disease. In the former group, there were eight patients with melanoma, three patients with sarcoma, two patients with head and neck cancer, and one patient each with teratoma, prostate cancer, and endometrial cancer. The latter group included four patients with melanoma and one patient each with ovarian cancer, sarcoma, and breast cancer.

Patients with measurable disease. Melanoma. In the group of eight patients with measurable melanoma, one patient had a complete response (patient 14), one patient had a minor response (patient 2), and one patient had a mixed response (patient 31). Four patients showed stable disease (patients 17, 19, 22, and 36), and one patient showed disease progression (patient 9).

A complete response was seen in patient 14, a patient with subcutaneous and peritoneal melanoma metastases that had progressed under previous chemotherapy. With continued vaccination, all lesions regressed completely. The duration of the response is 32 months at this point, and the response is ongoing. This patient had a category-IV immune response to NY-ESO-1.

A mixed response was seen in patient 31. This patient showed disease stabilization in liver metastases that had been shown to express NY-ESO-1. After eight vaccinations, the patient developed a peritoneal metastases that was resected and shown to be NY-ESO-1-negative. With continued vaccination for an additional 9 months, the liver metastases have not shown any progression. This patient had a category-IV immune response to NY-ESO-1.

A patient showing impressive disease stabilization is patient 19. The patient had debulking surgery (incomplete resection) for progressing axillary and cervical lymph node metastases. With continued vaccination, the patient has not shown disease progression for 25 months. The patient had a category-III immune response to NY-ESO-1. Three other patients (patients 17, 22, and 36) showed disease stabilization for 31+, 6, and 28 months, respectively. Their respective immune response categories were IV, II, and IV.

Other types of cancer. Disease stabilization was seen in patient 15 with malignant teratoma (24+ months) and in patient 28 with head and neck cancer (11+ months). These patients showed no immune response to NY-ESO-1 (category I). Three patients with sarcoma (patients 6, 7, and 24), one patient with head and neck cancer (patient 23), one patient with prostate cancer (patient 26), and one patient with endometrial cancer (patient 18) showed disease progression. The immune response categories of these individual patients were II, I, IV, II, II, and III, respectively.

Patients with completely resected disease. Melanoma. Three patients (patients 10, 21, and 32) with completely resected stage-III disease remained free of detectable disease after vaccination for 9 months, 7 months, and 5 months. One patient (patient 1), who had repeated resections of rapidly recurring in-transit skin metastases before entering the trial, developed an inguinal node metastasis after the third vaccination. It was resected, and vaccination has continued, first with rV-NY-ESO-1 and then with NY-ESO-1 peptide p157–165. The patient has remained free of disease for >5 years. The immune-response categories of these individual patients were II, III, II, and III, respectively.

Other types of cancer. In this group are one patient with stage-IV ovarian cancer (patient 11), one patient with stage-IV sarcoma (patient 35), and one patient with stage-IV breast cancer (patient 13). These patients remained free of detectable disease for 8 months, 5 months, and 5 months after vaccination. Their immune response categories were II, II, and III, respectively.

Discussion

We have chosen NY-ESO-1 as a prototypic human cancer antigen for the development of antigen-specific human cancer vaccines. The highly restricted expression pattern of NY-ESO-1 in normal tissues (testis), its frequent expression in a wide variety of cancers, and the spontaneous humoral and cellular immune responses elicited by NY-ESO-1 in a subset of patients with NY-ESO-1-expressing tumors are highly favorable characteristics for a vaccine target (1, 5, 6, 16). Immunological assays to detect antibody and CD4 and CD8 T cell responses to NY-ESO-1 are well advanced and provide a secure basis for monitoring spontaneous and vaccine-induced immune responses and for comparing the immunogenicity of different NY-ESO-1 vaccine constructs (4, 16, 17) .

A broad array of approaches to generating NY-ESO-1 vaccines are currently available, and the first challenge is to construct vaccines that induce long-lasting, high-affinity CD8 T cell responses that recognize naturally processed NY-ESO-1. Because of their ready availability, NY-ESO-1 peptides (p157–165) initially identified by reactivity with CD8 T cells from patients with spontaneous NY-ESO-1 immunity were chosen to inaugurate the NY-ESO-1 vaccine program. NY-ESO-1 peptide immunization (in conjunction with granulocyte/macrophage colony-stimulating factor) induced CD8 T cell responses in patients without preexisting NY-ESO-1 immunity (11). Intensive peptide vaccination (repeated injections of NY-ESO-1 peptides over short intervals of time) were particularly effective in generating CD8 T cell responses.[††] However, these peptide-induced CD8 T cell responses were generally of low affinity and did not recognize naturally processed NY-ESO-1 *in vitro* after presensitization of effector cells with peptide (17). To induce a broader range of NY-ESO-1 class I-restricted CD8 as well as class

[††]Biskamp, M., Jäger, E., Karbach, J., Neumann, A., Jäger, D., Gnjatic, S., Pugliese, E., Hoffman, E., Old, L. J., Knuth, A. (2003) *Proc Am Soc Clin Oncol*, p. 176 (abstr. 706).

II-restricted CD4 T cell responses, recombinant NY-ESO-1 protein in a saponin-based adjuvant (ISCOMATRIX) was used to immunize stage-III/IV melanoma patients without evidence of disease after tumor resection. NY-ESO-1 protein/ISCOMATRIX vaccines induced high-titered NY-ESO-1 antibody and CD4 and CD8 T cell responses in a high proportion of patients. Although not an endpoint of the study, it was noted that patients vaccinated with NY-ESO-1/ISCOMATRIX had a longer disease-free survival compared with patients treated with NY-ESO-1 protein alone or placebo (10, 18).

The present report presents clinical and immunological results after vaccination with rV/rF-NY-ESO-1. Four response categories were defined based on antibody status and CD4/CD8 T cell responses. Category-I patients ($n = 3$) failed to develop any demonstrable humoral or cellular NY-ESO-1 response after vaccination. Category-II patients ($n = 9$) remained sero-negative but developed CD4 and/or CD8 T cell responses. Category-III patients ($n = 6$) developed NY-ESO-1 antibody and CD4 and/or CD8 T cell responses. Category-IV patients ($n = 5$) who were sero-positive and CD4- and/or CD8-reactive before vaccination showed a broadening of T cell responses after vaccination. NY-ESO-1 seroconversion was less frequent with rV/rF-NY-ESO-1 vaccine than with NY-ESO-1 protein/ISCOMATRIX, whereas the frequencies of CD4 and CD8 T cell responses were similar, although the pattern of epitope recognition appeared to be distinctive. Spontaneous and vaccine-induced CD8 T cell responses demonstrable in this study were predominantly directed against NY-ESO-1 regions p81–110 and p151–170, whereas CD8 T cell epitopes recognized after vaccination with NY-ESO-1/ISCOMATRIX showed a broader range of response (10, 18). In addition to recognizing NY-ESO-1 peptide-pulsed target cells, CD8 T cells from patients with spontaneous NY-ESO-1 humoral and cellular immunity generally have the capacity to recognize naturally processed NY-ESO-1 presented by NY-ESO-1-positive tumor cells. In contrast, as mentioned above, CD8 T cells elicited by NY-ESO-1 peptide vaccines often failed to recognize naturally processed and presented NY-ESO-1 *in vitro* after standard presensitization, and this observation correlated with a lower affinity of these class I-restricted, peptide-induced CD8 T cells (17, 19, 20). This difference between spontaneous and peptide vaccine-induced T cell responses to NY-ESO-1 could have several explanations [e.g., (*i*) absence of CD4 T cell help leads to low-affinity CD8 T cell responses and/or (*ii*) high-affinity CD8 T cells are generated by peptide vaccines, but the presensitization conditions (generally involving peptide prestimulation) used to expand the CD8 T cell populations for assays select for low-affinity CD8 T cells]. Support for the latter hypothesis comes from the finding that presensitization of CD8 T cells from peptide-vaccinated patients with autologous tumor cells (in contrast to peptide-pulsed cells) favored outgrowth of CD8 T cells with tumor-recognizing capacity (E.J., unpublished work). In the present study, clonal analysis of the CD8 T cells induced in category-II (patient 22), category-III (patients 1, 19), and category-IV (patient 31) patients showed good recognition of naturally processed NY-ESO-1 in tumor cell lines, indicating that rV/rF-NY-ESO-1 vaccines can generate tumor-reactive T cells. In future NY-ESO-1 trials, much emphasis will be placed on broadening the characterization of CD8 T cell responses (e.g., affinity, T cell antigen receptor spectratyping, tumor recognition, cytokine secretion patterns, phenotype, and response to activation stimuli). Similar issues need to be addressed with regard to the CD4 T cell response and in particular with regard to the role of regulatory CD4 T cells in modulating CD8 T cell responses.

NY-ESO-1 immunological responses were the endpoints of this study. However, certain observations related to "clinical benefit" in rV/rF-NY-ESO-1-vaccinated patients should be noted. Extending overall survival needs to be the goal of all cancer therapeutics, but other endpoints such as tumor regression, stabilization of disease, and disease-free interval have been considered as possible predictors of overall clinical benefit. With regard to these parameters, no consensus as to what constitutes significant clinical benefit has been reached in the field of cancer vaccines. One opinion is that the appropriate criteria are those imbedded in RECIST (Response Evaluation Criteria in Solid Tumors) in characterizing chemotherapy responses (21, 22). However, there is a growing sense in the field of tumor immunology that RECIST criteria are inappropriate standards for immunotherapy, particularly at this early stage in the evolution of the field. Although stable disease has been termed a weak endpoint (23), we view stable disease as both a desirable and an achievable endpoint for cancer vaccines. From the standpoint of recent advances in our understanding of cancer immunosurveillance/immunoediting, vaccine-related stabilization would correspond to reversing the state of tumor escape back to a state of tumor equilibrium (24). Evaluating the significance of progressive disease, which is frequently the basis for discontinuing therapy, is another parameter that needs to be reconsidered from the immunological perspective. Progressive disease during vaccination may not warrant discontinuing the vaccine because vaccine-induced immune responses may have a delayed effect on tumor growth. In addition, heterogeneity of antigen expression in different metastases may lead to growth of antigen-negative metastases and regression or stabilization of antigen-positive metastases. Because cancer vaccinology is in its infancy, observation and experience is the key, not rules based on past experience with unrelated therapeutic modalities.

Because patients with NY-ESO-1 tumors of several histological types at different stages of disease were enrolled in this study, no statement can be made about the potential overall vaccine-induced benefit. However, in the patients with stage-III/IV melanoma, there was a strong impression that rV/rF-NY-ESO-1 vaccination altered the expected course of the disease. Of nine patients with progressive stage-III/IV disease at the onset of vaccination, seven have survived 17–63+ months. In contrast to other solid cancers, melanoma in advanced stages often shows a more homogeneous expression of cancer-associated antigens and MHC class I/II molecules along with brisk infiltrates of CD4 and CD8 T cells, a fact that may explain the apparent susceptibility of melanoma to immunological therapies (25). Therefore, tumor parameters of immunological relevance, such as homogeneity of target antigen, MHC class I/II expression, presence and location of CD4/CD8/Treg infiltrates, and expression of activating or inhibiting cytokines or receptors, need to be incorporated in the data set for each patient and evaluated for their contribution to the outcome of cancer vaccines.

Materials and Methods

Investigational Agents: rV-NY-ESO-1 and rF-NY-ESO-1. Recombinant rV-NY-ESO-1 and rF-NY-ESO-1 constructs were obtained from Therion Biologics Corporation (Cambridge, MA) (26).

Study Design. This study was a two-part, open-label cohort study of rV-NY-ESO-1, rF-NY-ESO-1, and rV-NY-ESO-1 followed by rF-NY-ESO-1 intended to evaluate the safety and the immunogenicity of the recombinant vaccines. Patients had advanced cancers expressing NY-ESO-1 as assessed by RT-PCR or immunohistochemistry. Patients were excluded if they had untreated CNS metastases, an allergy to eggs, immunodeficiency, or autoimmune disease. The protocol was approved by the Ethics Committee of the Landesärztekammer Hessen in Frankfurt. All patients gave written informed consent.

Patients were treated in five cohorts. Patients in cohorts 1–4 were HLA-A2-positive. Cohorts 1 ($n = 4$) and 3 ($n = 3$) received rV-NY-ESO-1 at two different dosages, 3.1×10^7 and 3.1×10^8 pfu, respectively. Cohorts 2 ($n = 4$) and 4 ($n = 4$) received rF-NY-ESO-1 at dosages of 7.41×10^7 and 7.41×10^8 pfu, respectively. Four vaccines were administered at 4-week intervals. Cohorts 1 and 2 were opened concurrently, and patients were recruited to each cohort in an alternating fashion. Cohorts 3 and 4 were opened for

patient entry after completion of cohorts 1 and 2. Patients were recruited to cohort 5 ($n = 20$) independent of their HLA status. Patients in cohort 5 received two vaccinations with rV-NY-ESO-1 at a dose of 3.1×10^7 pfu followed by two vaccinations with rV-NY-ESO-1 at a dose of 7.41×10^7 pfu at 4-week intervals. Patients in all cohorts were allowed to continue vaccinations at 4-week intervals after the initial fourth vaccination until tumor progression was observed. Toxicity end points were assessed according to the National Cancer Institute Common Toxicity Criteria Scale (version 2.0; April 30, 1999).

Monitoring of the Immune Response. Immunological end points of the study were NY-ESO-1-specific antibody titers and detectable NY-ESO-1-specific CD4 and CD8 T cells before and after vaccination.

NY-ESO-1 antibody. NY-ESO-1-specific antibodies were measured in the serum by Western blot and ELISA analysis on the day of each of the four vaccinations and 4 weeks after the last vaccination, as described previously (6, 8).

NY-ESO-1-Specific T Cells. Presensitization of CD8 and CD4 T cells. For testing in enzyme-linked immunospot (ELISPOT) and cytotoxicity assays, purified CD8 T cells were presensitized with peptide-pulsed (NY-ESO-1 p157–165 in HLA-A2-positive patients) or adenoviral NY-ESO-1 (Ad2/ESO)-infected irradiated autologous peripheral blood mononuclear cells depleted of CD4 and CD8 T cells (all patients) as described (16, 17). Ad2/ESO was prepared by Dr. S. Yla-Herttuala (A. I. Virtanen Institute, University of Kuopio, Kuopio, Finland) for the Cancer Vaccine Collaborative. Presensitized CD8 T cells were used as effector cells on day 6 for ELISPOT analysis or restimulated on day 7 for assessment of cytotoxicity against peptide-pulsed T2 cells or other target cells in ^{51}Cr release assays on day 12 (5).

Postvaccine CD8 T cell lines were cloned by limiting dilution using the respective peptide epitope recognized after initial presensitization with Ad2/ESO or NY-ESO-1 p157–165. T cell clones were assessed for recognition of endogenously presented NY-ESO-1 antigen in autologous antigen-presenting cells (APCs) infected with Ad2/ESO or were pulsed with NY-ESO-1 peptides or with NY-ESO-1-expressing tumor cell lines as targets.

CD4 T cells were presensitized with overlapping NY-ESO-1 18- to 20-mer peptides and tested against peptide-pulsed autologous APCs as described (15).

Functional T cell testing. CD8 T cell clones were tested for recognition of naturally processed NY-ESO-1 in Ad2/ESO-transfected autologous APCs or NY-ESO-1-expressing tumor cell lines in ELISPOT and cytotoxicity assays (26, 27).

ELISPOT assays. The frequency of NY-ESO-1-specific CD8 T cells in the peripheral blood of patients was assessed by ELISPOT as described previously (28). In HLA-A2-positive patients, T2 cells pulsed with NY-ESO-1 p157–167 and p157–165 were used as APCs. For the assessment of CD8 T cell responses in non-HLA-A2-positive patients and of CD4 T cell responses in all patients, synthetic overlapping 18- to 20-mer NY-ESO-1 peptides covering the entire NY-ESO-1 protein sequence were pulsed onto autologous APCs (EBV-transformed B cells, phytohemagglutinin blasts, dendritic cells) and used as target cells.

Cytotoxicity assay. Cytotoxicity against NY-ESO-1 peptide-pulsed T2 cells and melanoma cell lines was determined as described (5). Unlabeled K562 (40:1) was added to nonclonal T cell populations to block nonspecific cytotoxicity.

Disease Assessment. The assessment of tumor lesions was performed according to World Health Organization criteria on x-ray, computed tomography, and MRI scans before vaccination and after the second and fourth vaccinations; assessments continued every 8 weeks in patients receiving additional vaccinations.

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