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Xenovaccinotherapy for melanoma

The objectives of this phase I-II trial were to assess the toxicity, immunological and clinical responses induced in stage III/IV melanoma patients by the subcutaneous administration of xenogenic polyantigenic vaccine (XPV) prepared from disrupted murine melanoma (B16) and carcinoma (LLC) cells. An inducing course of vaccinotherapy consisted of ten immunizations (five at weekly and five at fortnight intervals). Twenty-four hours following each of the first five vaccinations, the patient was subcutaneously given a low dose of the recombinant interleukin-2 (IL-2). A consolidating course of the vaccinotherapy consisted of monthly vaccinations. Grade 3 or 4 toxicities, as well as laboratory and clinical signs of developing autoimmune disorders, were recorded in none of the 40 XPV-treated evaluable patients. A significant increase in delayed-type hypersensitivity (DTH) skin reaction to vaccinal B16, but not to LLC antigens (Ags), occurred in patients after inducing vaccinations. At the same time, those patients demonstrated a marked augmentation of blood lymphocyte proliferation responses not only to B16 but also to LLC Ags. Vaccinations also led to increased cell-mediated reactivity to murine non-tumor, spleen cell (SC)-associated Ags, which, however, was not as significant as that to tumor-associated antigens (TAAs). Of great importance was the fact that XPV administration resulted in increased blood lymphocyte proliferative reactivity of patients to human melanoma-associated Ags, while not affecting their reactivity to the control alloantigens. With immunotherapy, concentrations of both interferon-gamma (IFN- γ) and interleukin-4 (IL-4) were elevated in patients' sera, suggesting an intensification of the T helper1/ T helper 2-mediated responses in the XPV-treated patients. The average survival of the 32 stage IV melanoma XPV-treated evaluable patients was noticeably higher than that of the 32 clinically comparable control patients (13 vs. 5 months). The overall 3 year-survival rate in the XPV-treated group and the control group was 25% (8 patients) and 3% (1 patient), respectively. In general, the results suggest that xenogenic tumor cells may provide a novel feasible approach to constructing clinically effective vaccines.

Key words: xenogenic polyantigenic vaccine, immunotherapy, melanoma

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Malignant melanoma is a cancer with one of the most rapidly increasing incidence rates [1]. Surgical resection of the early-stage localized disease is the only curative treatment. Metastatic melanoma is usually resistant to the standard cytotoxic therapy, including highly toxic combinations. Hence, immunotherapy has become the mainstay of treatment in advanced melanoma. An active specific immunotherapy (vaccination) is a strategy using tumor-associated antigens (TAAs) for inducing an antitumor immune process. However, only few of the TAAs capable of evoking immune responses have been identified [2]. Moreover, there is an antigenic diversity, even in the same histological tumor of cancer patients [3]. Since whole tumor cells elicit a broad spectrum of immune

responses to different TAAs, they should be more applicable to constructing cancer vaccines, compared to a single, tumor-associated, antigenic peptide. Although both autologous and allogeneic cell vaccines are well studied, we favor a xenogenic vaccine. Evidence has been accumulated to demonstrate that the xenogenic TAAs can be much more effective in eliciting antitumor immune responses than their homological analogs [4-8]. It is reasonable to suggest that xenoantigens may potentially represent an "altered self", with sufficient differences from self-antigens to render them immunogenic, but with sufficient similarities to allow reactive T cells to maintain recognition of self. The vaccine under study is composed of disrupted murine B16 melanoma and LLC carcinoma cells. This xenogenic polyanti-

genic vaccine (XPV) is intended to stimulate responses against multiple targets expressed on cancer cells.

In this paper, we report for the first time, the toxic, immunological and clinical effects of administrating XPV in advanced melanoma patients.

Patients and methods

Patients

This study was performed in exact accordance with the protocol approved by the Scientific Council and Ethics Committee at the Institute of Clinical Immunology. Informed consent was obtained from every subject who was enrolled in this study.

Eligibility criteria included histologically proven, measurable disease, no prior immunosuppressive therapy for a minimum of four weeks, a good performance status (Karnofsky scale, 70% or more) and adequate marrow, renal and hepatic functions.

Vaccine preparation

B16 melanoma and LLC carcinoma cells, both of C57 BL/6 (B6; H-2^b) origin, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (all reagents were from Sigma). After being harvested and washed, the cells were stored at -20°C until use. One vaccinal dose contained 50×10^6 B16 and 25×10^6 LLC dead cells.

Preparation of the cell lysates for immunoreactivity assays

B16 and LLC cells were harvested, washed extensively and further stored at -20°C until use. A suspension of B6 spleen cells (SCs) was prepared with the aid of a glass homogenizer using gentle pressing of organ fragments in the cold (4°C) RPMI medium. After being washed the cells were stored at -20°C until use. The human tumor cells were prepared from histologically proven metastases, which had been surgically isolated from soft tissues of 3 melanoma patients. The cells were washed, pooled in equivalent quantities and further stored at -20°C until use. Peripheral blood mononuclear cells (PBMCs) were isolated from the same patients by centrifugation over Ficoll-Verografin in the standard way. Like melanoma cells, the PBMCs were washed, pooled and further stored at -20°C .

Abbreviations:

Ab	antibody
Ag	antigen
APC	antigen-presenting cell
DTH	delayed-type hypersensitivity
IFN	interferon
IL	interleukin
MRI	magnetic resonance imaging
PBMC	peripheral blood mononuclear cell
TAA	tumor-associated antigen
XPV	xenogenic polyantigenic vaccine

Preparation of soluble antigens (Ags) for immunoreactivity assays

B16 and LLC cells, as well as B6 SCs, were lysed by freeze-thaw procedures. Next, the solutions were separated from cell detritus by centrifugation, filter sterilized using a $0.22 \mu\text{m}$ filter unit (Sartorius), adjusted to a final protein concentration of 2.0 mg/mL, and stored at -20°C until use.

Treatment plan

An inducing vaccinal course consisted of 10 subcutaneous immunizations (five at weekly and five at fortnight intervals) and took about three months. Twenty-four hours following each of the first five vaccinations, each patient was given subcutaneously a low dose of a non-oxidated recombinant interleukin-2 (Ronkoleukin, 250000 U; Biotech, St. Petersburg, Russia) to potentiate vaccine-induced immune responses. Throughout the subsequent consolidating treatment each trial patient was vaccinated monthly.

Vaccine toxicity grading system

For systemic toxicities, the National Cancer Institute Common Toxicity scale was used. For local vaccine toxicities, the following scale was used: grade I, erythema and induration < 20 mm; grade II, erythema and induration 20 mm without ulceration; grade III, ulceration or painful adenopathy; and grade IV, permanent dysfunction related to local toxicity.

Delayed-type hypersensitivity (DTH) skin testing

Patients were given intradermal inoculations of antigenic solutions (2.0 mg/mL) in 0.1 – mL volumes of physiological solution. Erythemas were measured 24 hours later in two perpendicular diameters.

Proliferation assay

PBMSs were cultured at 2×10^5 per well with antigenic stimuli in RPMI 1640 medium supplemented with 5 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M mercaptoethanol, antibiotics (all reagents from Sigma) and 10% autologous plasma, in a 96-well round-bottom plate (BDSL, Ayrshire, UK) for five days. The lysates of either B16, LLC or spleen cells (each 5×10^4 /well) were added in cultures as murine Ags, whereas the lysates of human melanoma cells or PBMCs (each 12×10^3 /well) were used as human Ags. As a control the responding PBMSs were cultured in the medium alone. The cell proliferation was measured by a ^3H -thymidine assay in the standard way. The results are expressed as the mean of triplicates. A stimulation index was calculated for each triplicate by dividing the mean radioactivity (cpm) of stimulated cells by that of unstimulated cells.

Antibody (Ab) assay

The solutions with soluble antigens (2 mg/mL) prepared from cell lysates using the above procedure were placed in a 96-well ELISA plate (Costar) in a volume of 50 μL per well and left overnight. The next morning the plate was washed extensively before adding 4% casein solution (Vector-Best; Kolzovo, Russia) into all wells to block non-specific absorption sites. Plasma samples diluted 1:100 were incubated in the wells for 1 hour and Ig G bound to the absorbed antigens was detected with peroxidase-

conjugated rabbit anti-human Ig G monoclonal Ab (Vector-Best), using tetramethylbenzidine as an enzyme substrate. The reaction was quantified at 450 nm in an ELISA reader. The assay was performed in duplicate for each serum sample. The values were expressed in titer units, which were calculated according a 5-point curve of measuring Ab titer in the pooled positive sera obtained from 3 vaccinated patients.

Serum cytokine assay

Interferon-gamma (IFN- γ) and interleukin-4 (IL-4) were determined in the patient's sera, using commercially available ELISA-kits, according to the manufacture's instructions (Vector-Best; Kolzovo, Russia).

Statistics

The paired Student's test was used to determine the significance of the observed differences. The Kaplan-Meier method was employed to estimate overall survival.

Results

Toxicity

A total of 40 patients (14 with III and 26 with IV disease stage; 26 females and 14 males) aged from 18 to 71 years (mean age 50 years) completed an inducing course of vaccinotherapy consisting of 10 immunizations and had adequate follow-up to monitor toxicity and immune responses. No III-IV grade systemic toxicity associated with the vaccine administration was noted. During 24-to-48 h post vaccination 19 patients (47%) exhibited an influenza-like syndrome in the form of a body temperature rise up to 38 °C and musculoskeletal discomfort, which were usually self-limiting. The local I-II grade toxicity in the form of developing irritation at the injection site was noted in 29 (72%) of evaluable patients. Such local manifestations usually disappeared within 24-to-48 h post-vaccination. There were no treatment-related hospitalizations or mortalities. Cell blood parameters, as well as renal and hepatic functions, remained within the initial ranges throughout the observation period. There were no significant changes in subpopulation composition of PMBCs tested for expression of CD3, CD4, CD8, CD20, and CD16 surface markers. Serum Ig A, G, M also remained in the initial ranges (data not shown).

Serum concentrations of a rheumatoid factor, but also of Abs specific to DNA, cardiolipin, thyroglobulin, and microsomal fraction of thyrocytes were measured in sera before and after the inducing the vaccinal course. No statistically significant changes in these parameters were noted (data not shown). Consistent with these data, the XPV-treated patients exhibited no clinical evidence for developing any systemic autoimmune disorders.

Immunoreactivity

As a consequence of inducing vaccinations, a remarkable increase in skin immunoreactivity (by 5 mm or more) to B16 Ags was found in 28 (70%) of 40 evaluable patients (figure 1). The median quantitative increase in anti-melanoma reactivity of the patients was statistically significant ($P < 0.01$).

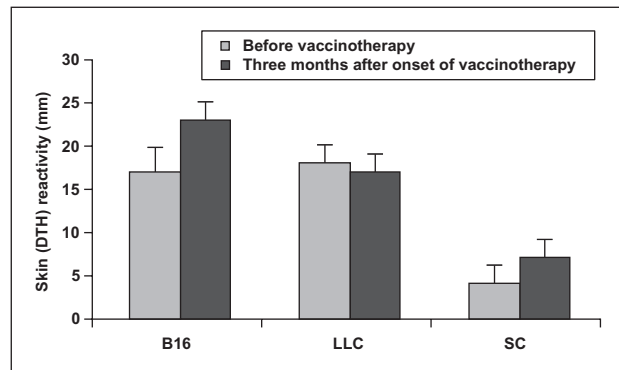


Figure 1. DTH skin reactions (M ± m) of the patients to B16, LLC and SC Ags before and after 10 vaccinations.

The majority (32 of 40, 80%) of assessable patients initially had a high (10 mm or more) skin reactivity to LLC Ags. As can be seen from figure 1, this reactivity did not undergo any significant XPV-related changes.

Before the treatment, skin reactivity to non-tumor SC antigens in most evaluable patients was 5 mm or less and its increase owing to vaccinations was moderate (figure 1).

To assess the reactivity of PBMCs to vaccinal Ags, a proliferative assay was used. As can be seen in figure 2, a statistically significant increase in reactivity to both tumor-associated and non-tumor xenogenic Ags occurred in the XPV-treated patients ($P < 0.02$). It should be noted, however, that with the vaccinations the B16 and LLC Ags became more effective stimulators of PBMC proliferation compared to the SC Ags.

An important aim of our study was to determine whether or not murine TAAs would be capable of contributing to the generation of immune responses specific to human melanoma-associated Ags. As shown in figure 3, inducing vaccinations gave rise to a marked augmentation of the proliferative responses of patients' PBMCs to the human melanoma-associated Ags, while not affecting their reactivity to the control alloantigens.

As shown in figure 4, the vaccinotherapy resulted in a noticeable elevation of the serum level of Ab binding to vaccinal B16 or LLC Ags ($p < 0.02$). It should, however, be noted that a similar elevation was also attributable to Abs binding SC Ag.

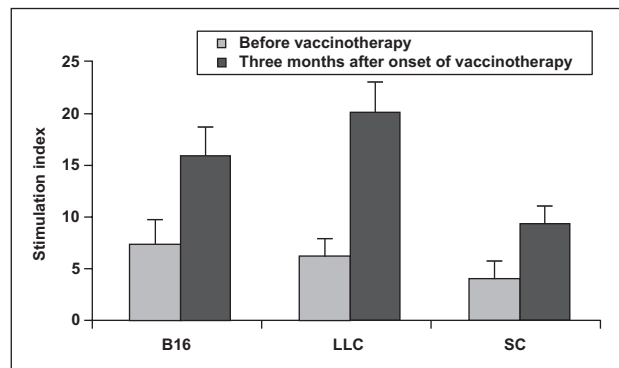


Figure 2. Proliferative PBMC responses (M ± m) of the patients to B16, LLC and SC Ags before and after 10 vaccinations.

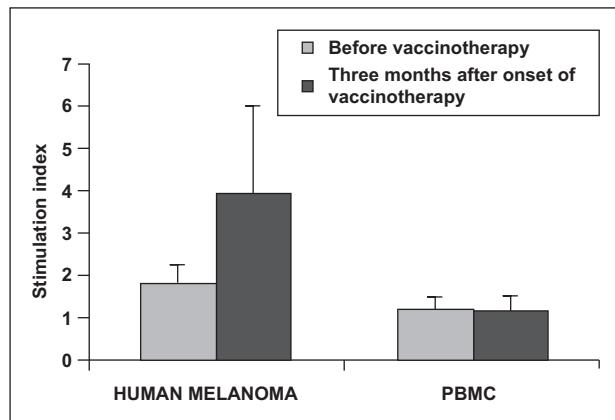


Figure 3. Proliferative PBMC responses ($M \pm m$) of the patients to human melanoma-associated and control (PBMC) Ags before and after 10 vaccinations.

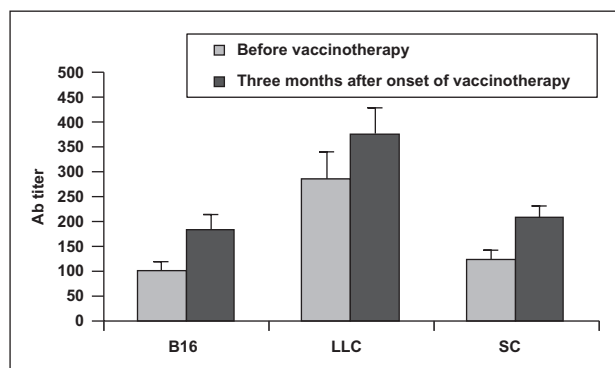


Figure 4. Serum concentrations of Abs with specificity for B16, LLC, or SC in the patients before and after 10 vaccinations.

Two types of T cells are categorized as T helper 1 and T helper 2 on the basis of their cytokine production. T helper 1 lymphocytes mainly produce interferon-gamma ($IFN-\gamma$) and mediate cellular immune response, whereas T helper 2 lymphocytes mainly produce IL-4 and mediate humoral responses. The data presented in *table 1* indicate that the inducing vaccinations led to a detectable increase in serum concentrations of both $IFN-\gamma$ and IL-4 ($P < 0.05$).

Overall survival

Although the primary end points of this trial were toxicity and immune-mediated responses to XPV, we also evaluated the overall 3-year survival in 32 XPV-treated patients with stage VI disease. Their characteristics are presented in *table 2*. All patients had measurable or evaluable disease.

Table 1. Serum cytokine concentrations (pg/ml, $M \pm m$) in the patients before and after 10 vaccinations

Cytokine	Before vaccinotherapy	Three months after onset of vaccinotherapy
$IFN-\gamma$	799 ± 210	1371 ± 196
IL-4	40 ± 15	55 ± 15

Table 2. Characteristics of the patients assessable for survival

Characteristic	Trial	Control
Number of patients	32	32
Males/females	10/22	10/22
Age, years (median, range)	48.8 (18–69)	48.2 (24–77)
Site of metastases:		
Lymph node, skin/soft tissue	23 (70%)	26 (81%)
Lung	10 (31%)	6 (19%)
Liver	7 (22%)	7 (22%)
Other organs	7 (21%)	8 (25%)
Prior treatment:		
surgery	17 (53%)	16 (50%)
surgery + chemotherapy	9 (28%)	10 (31%)
surgery + immunotherapy (IFN)	0 (0%)	1 (3%)
surgery + chemotherapy + immunotherapy (IFN)	2 (6%)	2 (6%)
surgery + chemotherapy + physiotherapy	2 (6%)	0 (0%)
surgery + chemotherapy + radiotherapy	1 (3%)	0 (0%)
no treatment	1 (3%)	3 (9%)

The control group was composed retrospectively of the patients who received conventional therapy. Each control patient was randomly selected to be a clinically comparable counterpart of a trial patient, so that control and trial groups were evenly balanced by both prognostic and clinical parameters. Throughout the follow-up period the trial patients received no other systemic therapy other than immunotherapy. If it was reasonable and possible, both trial and control patients underwent cytoreductive palliative surgery. As shown in *figure 5*, the median survival of the XPV-treated patients was significantly longer ($P < 0.05$) than that of the control patients (13.8 vs. 5.8 months). The longer survival of the XPV in our study appears to have been associated with a high DTH skin reactivity to the vaccinal B16 Ags. The median survival of the six patients, whose reactions after inducing vaccinations were < 10 mm, was 10 months, whereas that of the 26 remaining patients with high reactions (≥ 10 mm) was 20 months.

The overall 3-year survival rate in XPV-treated and control patients was 25% and 2%, respectively. A clinical effect of

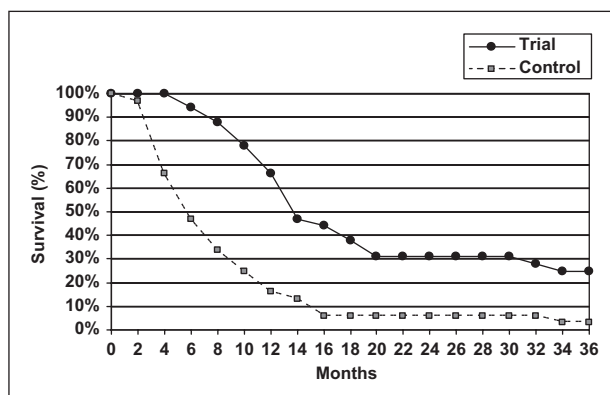


Figure 5. Survival of the XPV-treated and control patients.

various grades with a duration not shorter than 6 months was observed in 21 (66%) of the 32 trial patients: complete response, partial response and disease stabilization was achieved in 5 (16%), 2 (6%) and 14 (44%) patients, respectively. Thus, the results suggest that xenovaccinotherapy may significantly prolong the lifetime in a significant proportion of advanced melanoma patients.

Xenovaccinotherapy is potentially able to provide a regress of visceral metastases. The magnetic resonance imaging (MRI) scans shown in *figure 6* indicate the disappearance of liver metastatic lesions in a 25 year-old female patient 6

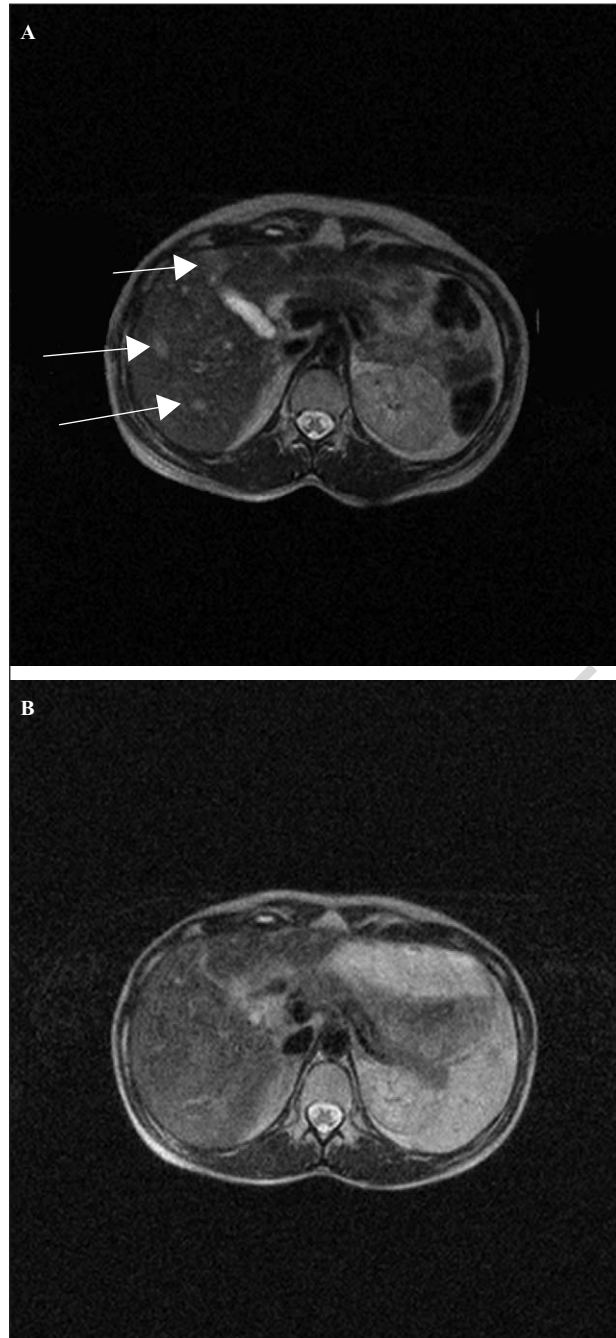


Figure 6. A MRI scan of patient P before (A) and at 6 months post immunotherapy initiation (B). The metastatic lesions are indicated by pointers.

months post immunotherapy initiation. This patient exhibited a long-term complete remission (the follow-up time was 3 years) and has had no evidence of further disease to date. Also of interest is that the development of connective tissue cysts in the sites of former visceral metastases was noted in several patients (data not shown).

Discussion

Immunizations with unmodified homologous (autologous or allogeneic) tumor cells have demonstrated only limited therapeutic success in cancer patients. There are two major reasons for the low immunogenicity of homologous tumor vaccines. Firstly, most of the homologous TAA represent self-Ags, which are not inherently immunogenic. T cells that recognize self-Ags with high avidity are believed to undergo a negative selection through clonal deletion in the thymus or anergy in the periphery [9]. Secondly, homologous tumor cells are not recognizable by antigen-presenting cells (APC) as cells that should be internalized and their antigens processed [10-12]. Presentation of tumor-associated peptides on the professional APC in association with classes I and II MHC molecules is a prerequisite for the activation of tumor-specific cytotoxic and helper T cells, respectively [10-12].

Because of their structural distinctions from homologous analogs, xenoantigens appear to be capable of effectively overcoming immune tolerance to self-Ags, including tumor-associated ones [4-8]. On the other hand, all humans possess natural (preexisting) Abs, which provide an acute rejection of any non-primate cells and function as a major barrier for the transplantation of animal organs to humans [13]. A significant part of these Abs represents the Ig G specific to the α -gal epitope that is expressed abundantly on glycoproteins and glycolipids of non-primate mammals and New World monkeys [13, 14]. By the opsonization of xenogenic tumor cells, the natural Abs could promote internalization of tumor material in APC via a Fc γ -receptor-mediated mechanism, and thereby enhance greatly the immunogenic presentation of tumor-associated Ags to tumor-specific T lymphocytes. This proposition is consistent with the data indicating a critical role of the Fc γ R-receptors in generating an effective antimelanoma immunity [15], as well as with the published results showing that the rejection of alphaGal positive melanoma cells can efficiently boost the immune response to other tumor-associated antigens present in alphaGal negative melanoma cells [16].

The majority of tumor-associated Ags are well known to be common. In other words, they can express on different types of tumors. To broaden the spectrum of immunizing targets, carcinoma (LLC)-associated Ags were included in XPV in addition to the B16 melanoma-associated Ags. Before vaccinations, the majority of melanoma patients demonstrated a relatively high (10 mm or more) and early (for the first several hours) skin response to the LLC Ags. In our view, by stimulating a local inflammation at the vaccination site, the LLC products might act as an adjuvant in developing the overall XPV-specific immune process.

Presumably, the positive role in generating antitumor responses by a xenovaccine might belong not only to the xenogenic Ags associated with tumor cell phenotype, but also to the xenoantigens that are not related with cell malignization. Consistent with this proposition are pub-

lished data indicating that in a model of active immunization, mouse gp75 expressed in insect cells may be significantly more effective in protecting mice from lung metastases, compared to gp75 expressed in homologous cells [4].

The results presented herein point out that the XPV-based therapy is safe for clinical use and has much less toxicity than current standard therapy for melanoma. Noteworthy is that the vaccine-treated patients exhibited no evidence of systemic autoimmune disorders, of which development could not be excluded initially because of the broad range of different Ags present in XPV.

It appears that the xenogenic Ags, both tumor-associated and inherent to normal cells, can be involved in XPV-induced immune responses. As evidenced by both DTH and PBMC proliferation, the immunoreactivity to B16 Ags observed in the XPV-treated patients was detectably greater than the immunoreactivity to SC Ags. This may imply that the cell-mediated, immune sensitization of the XPV-treated melanoma patients was due to melanoma-associated Ags more than to other xenogenic Ags present in normal murine cells. This, however, did not appear to be the case for Ab-mediated responses. No significant differences between the anti-B16 Ab titers and the anti-SC Ab-titers were found in the XPV-treated patients. These findings suggest that those antigenic determinants, which are present in not only malignant, but also non-malignant cells, may be preferentially responsible for XPV-generated Ab responses.

Of the utmost importance is that the XPV administration stimulated cell-mediated reactivity not only to murine, but also to human melanoma-associated Ags, thereby supporting the theoretical basis of applying xenovaccinotherapy in cancer patient management. One more matter of principle is whether or not the XPV is able to induce immune responses directed against the patient's own tumor. With vaccinations, an increase in T-cell mediated responses to self melanoma-associated Ags was observed by us in 2 out of 4 assessable patients. This increase was detectable in both proliferative and macrophage inhibitory factor production assays (data not shown). Unfortunately, we were unable to evaluate immune responses to self melanoma-associated Ags in other patients because autologous tumor material was not available.

As shown in this paper, the XPV administration resulted in serum level elevations of not only IFN- γ , but also of IL-4, suggesting intensification of both T helper 1- and T helper 2-mediated immune responses in XPV-treated patients. Similar results were obtained in colorectal cancer patients (data not shown). These findings are of great importance, in the light of previously reported data that indicate a critical role for cooperating cell- and Ab-mediated mechanisms in generating anti-melanoma cytotoxicity *in vivo* [15].

The results of the present study suggest that the xenovaccinotherapy is capable of significantly prolonging survival of advanced melanoma patients. The three-year survival rate of the XPV-treated patients was, however, inferior to the survival rate that had been previously reported for stage IV melanoma patients treated with allogeneic cell vaccines. One possible reason for this may be that a significant portion (66%) of the patients entered in our own study had initially unresectable metastases, whereas in the aforementioned trials all patients were vaccine-treated as early as possible following complete metastasis ablation [17].

According to our own experience, xenovaccinotherapy can result in eradicating visceral metastatic lesions in some melanoma patients. Nevertheless, stabilization of the disease appears to be the most common outcome of effective immunotherapy in advanced cancer patients. The XPV-based therapy is not an exception in this regard. Unlike the cytotoxic chemotherapy, tumor vaccine-based approaches may permit the host to reach a state of balance with the tumor, in which the net result of tumor growth and destruction is zero. That might lead to more significant survival benefits than a rapid destruction and rapid regrowth of the tumor following cytotoxic therapy.

In conclusion, this study represents the first demonstration of the safety, immunogenicity, and feasibility of administering a xenogeneic composite cell vaccine in humans, and outlines its relevance to overall anticancer therapy. Although the results are encouraging, they must be interpreted with caution because they are based on a small number of patients with very advanced disease. ■

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