Vaccination with Autologous Tumor-derived Heat-Shock Protein Gp96 after Liver Resection for Metastatic Colorectal Cancer

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ABSTRACT

Purpose: Heat shock proteins (HSP) from tumor cells contain the gp96 polypeptide associated with cancer-specific antigenic peptides. Mice that are immunized with HSP/peptide-complex (HSPPC) derived from cancer tissue reject tumor from which HSPs are purified. We tested in humans whether vaccination with HSPPC-gp96 (Oncophage) from autologous liver metastases of colorectal carcinoma induces cancer-specific T-cell responses in patients rendered disease free by surgery.

Experimental Design: Twenty-nine consecutive patients underwent radical resection of liver metastases [Memorial Sloan-Kettering Cancer Center (MSKCC) score 1–3 (good prognosis), 18 patients; score 4–5 (bad prognosis), 11 patients] and received autologous tumor-derived HSPPC-96. Two vaccine cycles were administered (four weekly injections followed by four biweekly injections after 8 weeks). Class-I HLA-restricted, anti-colon cancer lines T-cell response was measured by ELISPOT assay on peripheral blood mononuclear cells (PBMCs) obtained before and after vaccination. Feasibility, safety, and possible clinical benefits were also evaluated.

Results: Either a de novo induced or a significant increase of preexisting class I HLA-restricted T-cell-mediated anti-colon cancer response was observed in 15 (52%) of 29 patients. Frequency of CD3+, CD45RA+, and CCR7- T lymphocytes increased in immune responders. No relevant toxicity was observed. As expected, patients with good prognosis had a significantly better clinical outcome than those with poor prognosis (2-year overall survival (OS), 89 versus 64%, P = 0.001; disease-free survival (DFS), 46 versus 18%, P = 0.001). Patients with immune response had a statistically significant clinical advantage over nonresponding subjects (2-year OS, 100% versus 50%, P = 0.001; DFS, 51% versus 8%, P = 0.0001). Occurrence of immune response led to better tumor-free survival, whatever the predicted prognosis was (hazard ratio, 0.11–0.12 with/without stratification; P = 0.0012–0.0003).

Conclusions: HSPPC-96 vaccination after resection of colorectal liver metastases is safe and elicits a significant increase in CD8+ T-cell response against colon cancer. In this limited number of patients, two-year OS and DFS were significantly improved in subjects with postvaccination antitumor immune response, independently from other clinical prognostic factors.

INTRODUCTION

CRC,1 one the most common malignancies in humans, has frequent metastatic spread to the liver. Indeed, liver metastases are detectable in 15–25% of patients at the time of first diagnosis, progressing to about 60% of patients during their disease course (1, 2). Occurrence of liver metastases parallels the stage of primary CRC and represents the leading cause of death in advanced stages, with autopsy series showing liver metastases in more than 80% of patients who died from CRC (3).

Surgical resection remains the primary treatment, although complete tumor removal from the liver is achieved in only 20–40% of the patients (4) resulting in a 5-year OS of ~40% (1, 5) and tumor recurrence of 59–81% according to different stages at the time of surgery (4, 6). A large retrospective analysis has identified several independent determinants of

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The abbreviations used are: CRC, colorectal carcinoma; APC, antigen-presenting cell; CCR7, chemokine receptor 7; CEA, carcinoembryonic antigen; EP-CAM, epithelial cell adhesion molecule; DC, dendritic cell; DFS, disease-free survival; HSP, heat shock protein; HSPPC, HSP/peptide-complex; mAb, monoclonal antibody; NK, natural killer; OS, overall survival; PBMC, peripheral blood mononuclear cell; MSKCC, Memorial Sloan-Kettering Cancer Center; ELISPOT, enzyme-linked immunospot; ATCC, American Type Culture Collection.
prognosis after liver resection of metastatic CRC; the size and number of tumor nodules, the clearance of surgical margins, the status of liver hilum lymph nodes; and the CEA level are the most significant ones (7).

Adjuvant systemic or locoregional chemotherapy improves DFS and time-to-progression in some patient categories (1, 8). Subjects with CRC at high-risk of metastatic spread to the liver have also been treated with active nonspecific or specific immunotherapy (9–11). One randomized trial that used a vaccine made of irradiated autologous tumor cells and Bacillus Calmette-Guérin after resection of the primary colon cancer suggested a potential benefit for stage II but not for stage III patients (10).

T lymphocytes recognizing epitopes derived from CRC-associated proteins like CEA, Ep-CAM, Her2-Neu have been identified in PBMCs from CRC patients (12). Moreover, clinical and immunological responses have been observed in CRC patients vaccinated with DCs pulsed with a CEA-derived analogue (13).

Despite these results, antigens expressed by CRC cells and suitable for vaccine preparation remain to be identified. An alternative approach is offered by tumor-derived HSP, a group of glycoproteins that are the most common and abundant proteins in all forms of life. Previous work has demonstrated that purified homogeneous preparations of gp96, HSP-70, HSP-90, and other HSPs, are actually noncovalent complexes of HSP and peptides (14). The peptides derive from the proteins expressed in the cells from which the HSPs are purified and include normal self peptides as well as antigenic peptides. On immunization, the HSP bind to the CD91 HSP-receptors expressed by APCs, including DCs (15). The HSP-chaperoned peptides are then processed within the DCs and re-presented to T lymphocytes by MHC class-I molecules (15). This pathway has been demonstrated in murine (15) and human systems (16).

Studies with animal models indicate that vaccination with autologous tumor-derived HSP results in both prophylactic and therapeutic antitumor activity without the need to identify tumor-specific antigenic epitopes (17–19). The most potent antitumor activity of HSP was observed in animals rendered disease-free by surgery, but at high risk of recurrence of metastatic cancer (19). Moreover, vaccination with autologous tumor-derived HSP was found to be individually tumor-specific and not tumor histotype-specific, leading to the conclusion that the relevant tumor-antigenic, immunoprotective peptides are derived not from shared but from unique antigens that characterize each individual neoplasm (19, 20). In addition to these preclinical findings, the present study was predicated on a pilot human trial showing that vaccination with HSPPC-96, isolated from various types of autologous gastrointestinal, pancreatic, and renal cancers, was feasible and free of relevant toxicity (21). More recently, vaccination of metastatic melanoma patients with autologous HSPPC-96 resulted in 50% tumor-specific T-cell response and 18% clinical response (22).

In the present study, feasibility, safety, immunogenicity, and possible clinical benefits of an adjuvant vaccination with autologous tumor-derived HSPPC-96 were investigated in a consecutive series of patients rendered disease free from metastatic CRC through complete resections of liver metastases.

**PATIENTS AND METHODS**

**Eligibility, Patient Characteristics, and Surgical Procedure**

From February to December 1999, at the Hepatobiliary and Gastro-Pancreatic Unit of the Istituto Nazionale Tumori of Milan, a consecutive series of 29 patients (median age, 59 years; range, 37–74 years) with histologically confirmed diagnosis of liver metastases from CRC underwent potentially curative liver resection. All of the subjects underwent extensive preoperative work-up (total body helical-computed tomography, bone scan, colonoscopy, and positron emission tomography scan in case of multiple liver metastases) to rule out any detectable extrahepatic lesion. Different types of liver resections were performed, depending on tumor location and extension within the liver, with the goal of complete removal with clear margins of all neoplastic deposits. Intraoperative ultrasound of the liver was obtained at the beginning and at the end of each operation, to identify any possible metastases; hilar lymph node dissection was performed in all cases.

Eligibility criteria for vaccination included: (a) colon carcinoma metastases with resectability to yield an appropriate amount of nonnecrotic neoplastic tissue (at least 3 g for vaccine preparation plus 1 g for immunological assays); (b) a performance status (Zubrod) of 2 or less; (c) life expectancy of at least 16 weeks; (d) normal WBC and platelet count, hemoglobin level >10 g/liter; (e) bilirubin <1.5 times normal, ALT <4 times normal, and adequate renal function with serum creatinine of <2 times normal; (f) full recovery from prior anticancer therapy with at least a 4-week interval from the last administration of prior anticancer treatment; and, (g) positive response to common recall antigens as assessed by the Multitest Mérieux (Imtix, Milan, Italy) as a sign of sufficient immune function.

Patients were excluded from the study if they: (a) had active brain metastases; (b) had concomitant autoimmune or malignant diseases or were receiving concurrent anticancer or immunosuppressive drugs; (c) had primary or secondary immunodeficiencies; (d) had history of serious intercurrent medical illnesses. Women of child-bearing potential required negative serum pregnancy test before entry into the study and agreed to use an effective method of contraception while on treatment. All of the patients gave written informed consent to participate in the study.

Particular reference was made to the fact that no chemotherapy would be proposed after a complete tumor resection in the absence of cancer recurrence during the follow-up. The entire study was approved by the Ethics and Scientific Committees of the Istituto Nazionale Tumori of Milan.

**HSP Preparation**

Autologous HSPPC-96 vaccine was prepared from fresh tumor samples of each patient as described previously (23). Briefly, macroscopically nonnecrotic tumor tissue was obtained in sterile condition from the operating room, weighed, immediately frozen in liquid nitrogen, shipped to the Antigenics Inc. facility (Woburn, MA), and processed under good medical practice conditions as described previously (21). Preparations were considered to be of acceptable quality only if all of the following conditions were met: (a) the major protein band on SDS-PAGE was $M_\text{r}$ 96,000; (b) this band could be immuno-
Immunological Monitoring by ELISPOT

The effects of vaccination were measured in each patient with IFN-γ release ELISPOT assay using PBMCs obtained before visit 1 (V1), baseline and 4 (V5), 8 (V6), 12 (V7), 18 (V10), and 22 (V11) weeks after the onset of vaccination (see “Results” section). T-cell reactivity in PBMCs was evaluated in response either to autologous tumor cells (when available) or to class I HLA-matched allogeneic colon cancer cell lines. Blocking activity by HLA-class I or class II mAb was also evaluated to see whether antitumor T-cell reactivity was HLA restricted and T-cell mediated. The melanoma line 501mel was additionally used as negative control. All tests were performed at the Unit of Immunotherapy of Human Tumors of the Istituto Nazionale Tumori of Milan.

Patients’ immune response to vaccination was defined as a significant postvaccination increase in the number of PBMCs responding with IFN-γ release (as detected by ELISPOT assay, see below) to either autologous or allogeneic class I HLA-matched colon cancer cells as compared with prevaccination PBMCs. The time point of the assay was different in 6 of the 29 subjects because of the unavailability of enough PBMCs (see below). In some cases, PBMCs could be obtained at multiple time points and, therefore, a kinetics of the antitumor immune response could be determined.

Cell Samples for Immunological Monitoring

PBMCs. Eighty to 100 ml of heparinized blood for immunological assays were obtained by preparation of a buffy coat from each patient, before and after vaccine administration.

Table 1 Analysis of 20 clinicopathological characteristics and the MSKCC prognostic score (7) after curative liver resection for metastatic CRC in 29 consecutive patients

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>No. of patients</th>
<th>2-yr survival (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
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<tr>
<td>&gt;50</td>
<td>22</td>
<td>82</td>
<td>ns</td>
</tr>
<tr>
<td>≤50</td>
<td>7</td>
<td>71</td>
<td></td>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21</td>
<td>81</td>
<td>ns</td>
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<tr>
<td>Female</td>
<td>8</td>
<td>75</td>
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<tr>
<td>Dukes stage</td>
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<tr>
<td>B</td>
<td>6</td>
<td>100</td>
<td>ns</td>
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<tr>
<td>C</td>
<td>23</td>
<td>74</td>
<td></td>
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<tr>
<td>CEA level (preoperative)</td>
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<tr>
<td>≤10 ng/ml</td>
<td>12</td>
<td>75</td>
<td>ns</td>
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<tr>
<td>&gt;10 ng/ml</td>
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<td>82</td>
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<td>Liver involvement</td>
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<tr>
<td>&gt;25%</td>
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<td>Tumor distribution</td>
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<td></td>
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</tr>
<tr>
<td>Unilateral</td>
<td>19</td>
<td>84</td>
<td>ns</td>
</tr>
<tr>
<td>Bilateral</td>
<td>10</td>
<td>70</td>
<td></td>
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<tr>
<td>Extent of resection</td>
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<tr>
<td>Wedge-segmentectomy</td>
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<td>81</td>
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<td>Hepatectomy</td>
<td>8</td>
<td>75</td>
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<tr>
<td>Tumor size</td>
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<td>≤5 cm</td>
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<td>ns</td>
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<tr>
<td>&gt;5 cm</td>
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<td>70</td>
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<td>Resection margin</td>
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<tr>
<td>Poorly differentiated</td>
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<td>13</td>
<td>61</td>
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<tr>
<td>Total tumor volume</td>
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<td></td>
</tr>
<tr>
<td>≤34 cc</td>
<td>15</td>
<td>87</td>
<td>ns</td>
</tr>
<tr>
<td>&gt;34 cc</td>
<td>14</td>
<td>71</td>
<td></td>
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<td>MSKCC clinical risk score</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1–3</td>
<td>18</td>
<td>89</td>
<td>0.001</td>
</tr>
<tr>
<td>4–5</td>
<td>11</td>
<td>64</td>
<td></td>
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</tbody>
</table>

ns, not significant.

Factors considered in the MSKCC score.

Vaccination Schedule and Toxicity Assessment

Patients were treated with two cycles of intradermal vaccination courses at one of the three defined dose of HSPPC-96 (11 cases at 2.5 µg, 11 cases at 25 µg, and 7 cases at 100 µg) depending on tissue availability. The site of vaccination was rotated weekly and included: anterior deltoid region, median inguinal region and subclavicular regions. The first cycle lasted 1 month (four injections, once a week) and started 4–6 weeks after surgery, whereas the second course included four injections given at two-week intervals, starting 8 weeks after the end of the first cycle. Vaccine injection as well as clinical monitoring before and after each cycle were conducted in the Hepatobiliary Surgery outpatient clinic. At any patient’s visit, toxicity and possible relationship to vaccination were registered and graded according to the National Cancer Institute toxicity scale (24).

Clinical Monitoring and Prognostic Grouping

Physical examination, blood testing, liver ultrasound and/or computed tomograph scan and chest X-ray were performed monthly. In case of tumor recurrence, the vaccination was stopped, and the patient was assigned to other therapies, either re-resection or conventional chemotherapy for CRC, depending on tumor extension. In all cases, patients remained on permanent follow-up, and data on 20 clinical and pathological prognostic factors associated with prognosis after liver resection for CRC metastases were collected. Table 1 summarizes these factors and their relationship to patient prognosis. Patients were divided in two prognostic groups (A and B) based on our previous experience with over 400 liver resections for liver metastases and on the MSKCC score predictive of recurrence after hepatic resection for metastatic CRC (7). Briefly, Group A (good prognosis, i.e., with MSKCC score 1–3) included 18 patients with clear margins, ≤3 metastases, tumor-free hilar lymph nodes, and CEA ≤10 ng/ml; Group B included 11 patients with poor prognosis (i.e., with MSKCC score 4–5) and carrying any other risk factor not included in Group A.

The following prognostic factors are not listed because samples were too unbalanced: tumor site (colon versus rectum), TNM stage (II versus III), time of onset of metastases (metachronous versus synchronous), blood transfusions (yes versus no), number of liver metastases (1–3 versus >3), satellite nodules (yes versus no), vascular invasion (yes versus no), presence of invaded hilar lymph nodes (negative versus positive), DNA content of resected lesions (aneuploidy versus diploidy).

Eighty to 100 ml of heparinized blood for immunological assays were obtained by preparation of a buffy coat from each patient, before and after vaccine administration.
PBMCs were isolated by Ficoll (Pharmacia & Upjohn, Kalama-zoo, MI) gradient centrifugation, washed twice with PBS and cryopreserved, with the addition of RPMI 1640, plus 30% human serum and 10% DMSO (Fluka, Buchs, Switzerland) in liquid nitrogen. Before assaying, frozen cells were thawed, and functional analysis was performed after overnight resting in RPMI 1640 supplemented with 10% human serum. At the time of testing, PBMCs were at least 95% viable.

**HLA Typing.** Serological typing for HLA-A, -B, and -C was performed by the standard two-step NIH complement-dependent microcytotoxicity test. For molecular typing, genomic DNA was purified from protease-treated PBMCs with QIAamp DNA kit (Qiagen, Hilden, Germany), and HLA typing was performed by amplification with PCR with sequence-specific primers (PCR-SSRs; Dynal, Bromborough, United Kingdom).

**Tumor Cells.** Tumor cell suspensions were obtained by mechanical processing of liver metastases from colon carcinoma. Enzymatic digestion, with a pool of DNase (Sigma-Aldrich, Milan, Italy), collagenase (Sigma-Aldrich) and trypsin (Bio-Whittaker Europe, Verviers, Belgium) was additionally performed to achieve higher cell recovery. Tumor cells were then aliquoted, frozen, and stored in liquid nitrogen. Because of the poor viability of autologous tumor cells after thawing, such targets could be tested for PBMC recognition in ELISPOT assay only in two patients (patients 3 and 20). For the other patients, colon carcinoma cell lines matching at least one HLA-A or -B allele with each patient’s PBMCs were used as targets. These lines either were purchased from ATCC [Manassas, VA (SW480); HLA-A2, -24, -B7, and -B15; Col115; HLA-A1, -A3, -B35, and -B5; HT29; HLA-A1, -24, -B35, and -B44; SW1463: HLA-A1, -A2, -B8, and -B44; SW116: HLA-A2, -A23, -B44, and -B60]] or were kindly provided by Dr. C. Scheibenbogen (B. Franklin University, Berlin, Germany; Colo206, CX94, HCT116, all three typing as HLA-A2). In detail, 10 patients were tested with HLA-A2-matched (Colo206, HCT116, SW480), 5 with HLA-A24-matched (HT29, SW480), 4 with HLA-A1-(HCT116, Colo206, HT29), 4 with the HLA-A3-matched (Co115), 3 with HLA-B7-(SW480, Colo206), and 3 with HLA-B44-matched (SW1463, SW116) lines, respectively. In addition, the melanoma line 501mel (HLA-A2+, -A1+; Ref. 25) was used as an unrelated target.

**ELISPOT Analysis**

This assay allows direct testing of antigen recognition by patients’ T cells and has been used in melanoma patients vaccinated with different peptides (26, 27). As recently described (22, 27), sterile 96-well nitrocellulose plates (Millititer; Millipore, Bedford, MA) were coated with 50 μl/well anti-human IFN-γ mAbs (Mabtech, Nacka, Sweden) at a concentration of 15 μg/ml in sterile coating buffer) and were incubated overnight at 4°C. The following day, plates were extensively washed with PBS to remove unbound coating mAbs. PBMCs, previously thawed and incubated overnight at 37°C, were added to the plates at the concentration of 1.66 × 10^5/well in 100-μl well volume. A total of 10^6 PBMCs/patient were analyzed. After 30-min incubation at 37°C, target cells were added at the concentration of 1.6 × 10^4/well. To check for total IFN-γ release, cytokine release in response to pokeweed mitogen (1 μg/ml; Sigma, Munich, Germany) was included as a positive control. Blocking experiments were performed by incubating monocyte-depleted PBMCs with target cells preincubated for 30 min at 37°C with the anti-HLA class I mAb W6.32 (ATCC) or the anti-HLA class II mAb L243. To control the ELISPOT assay, the anti-Melan-A/MART-1_27,35 T-cell clone A42 (28), in the presence or absence of relevant targets (T2 lymphoma cells pulsed with Melan-A/MART-1_27,35 peptide and/or the Melan-A/MART-1- + melanoma line 501mel), was always included at the concentration of 400 cells/well in triplicate. Interassay variability, as evaluated by these two target controls was indeed low, producing 120 ± 18 and 297 ± 21 spots/well, respectively, in 56 assays considered. After an additional incubation for 20 h at 37°C and 5% CO2, plates were washed with PBS. Wells were then incubated for 2–4 h at room temperature with 50 μl/well biotinylated mouse anti-human IFN-γ mAbs (Mabtech, Nacka, Sweden) at the concentration of 1 μg/ml in PBS with 0.5% FCS. Wells were then washed and 100 μl/well streptavidin alkaline phosphatase (Mabtech, Nacka, Sweden), diluted 1 in 1000 in PBS-0.5% FCS, was then added. After 1-h incubation at room temperature, plates were washed and 100 μl/well substrate (Bio-Rad Laboratories, Hercules, CA) were added. Color development was stopped by washing in tap water when dark spots emerged (up to 30 min). Plates were then left to dry overnight at room temperature, and spots were counted by a computer-assisted ELISPOT reader (Bioline AID, Turin, Italy). Spot number per seeded PBMCs represents the average value of at least four replicates. To calculate the number of PBMCs responding to tumor cells by IFN-γ release, a background (i.e., the number of IFN-γ spots obtained with PBMCs alone: median, 6.5 spots/1.67 × 10^3 PBMCs; range, 0–36; n = 29 samples) was subtracted.

**Immunophenotyping of Effector Lymphocytes**

PBMCs were thawed and stained with the murine antihu-man CCR7 mAb (clone 2H4; BD Pharmingen, San José, CA) for 30 min at 4°C, were washed twice, and were incubated with the biotinylated rat antimouse IgM (BD Pharmingen) for 20 min at 4°C. After two washings, PBMCs were incubated with streptavidin-change in phycocytirhynch (Molecular Probe, Eugene, OR) and were washed three times. Cells were then stained with the anti-CD3-APC and the CD45RA-FITC mAbs (BD PharMingen). As negative controls, an isotype-matched (IgM) mAb followed by the biotinylated antirat IgM and isotype-matched (APC or FITC conjugated) mAb were used. Fluorescence was then analyzed by FACScalibur flow cytometer (Becton Dickinson, San José, CA) and CellQuest software (Becton Dickinson).

**Statistical Analysis**

The induction of detectable immune responses against CRC cells after vaccination with autologous tumor-derived HSPPC-96 in the absence of detrimental toxic effects was the primary end point of this cohort study. To evaluate this, pre- and postvaccine difference in the number of IFN-γ spots on colon cancer cells were compared in each patient by Student’s t test. There was no result adjustment of spot numbers, and the SDs were evaluated on three- or six-well replicates. T-cell responses were considered positive if: (a) a minimum of 10 tumor-induced
10 5 PBMCs were detected (after subtracting the spots of unstimulated PBMCs): (b) a 2-fold increase of tumor recognition in postimmunization samples as compared with preimmunization samples was achieved; and (c) the differences in the number of tumor-induced spots between PBMCs obtained before and after vaccination were statistically significant with a P of <0.05 using the Student’s t test.

OS and DFS were the secondary end point of the study. They were estimated according to the Kaplan-Meier method (29) and compared in univariate analysis with the use of the two-sided log-rank tests, with all survival intervals calculated starting from the date of liver resection. Follow-up was calculated up to 24 months. Because of the small sample size (29 consecutive cases), the analysis of prognostic factors was confined to the comparison of favorable versus unfavorable prognostic groups according to clinical MSKCC scores (7) and to the possible combined effect of patient immune response on recurrence-free survival. The Cox proportional hazard model was used, whether or not subjects were stratified for the prognostic score. Any variable, such as immune response to vaccine, with a hazard ratio of 1 with a P of <0.05 was considered to be significantly associated with a protective effect of the analyzed factor on prognosis (i.e., DFS).

Data on whether or not an immune response was achieved in each patient were acquired at least 1 year after the conclusion of the vaccination schedule. Thus, investigators were blinded with regard to the possible effects of immunization until the end of the study.

RESULTS

Immunological Results

Induction of Anti-Colon Carcinoma-specific T Lymphocytes after Vaccination with HSPPC-96. T-cell-dependent antitumor-specific immune reactivity in fresh PBMCs was used, whether or not subjects were stratified for the prognostic score. Any variable, such as immune response to vaccine, with a hazard ratio of <1 with a P of <0.05 was considered to be significantly associated with a protective effect of the analyzed factor on prognosis (i.e., DFS).

Fig. 1 Effect of HSPPC-96 vaccination on IFN-γ release by PBMCs in response to autologous/allogeneic class I HLA-matched colon carcinoma or to histologically unrelated tumor cells. The antitumor immune response before and after HSPPC-96 (Oncojope) vaccination was investigated by IFN-γ ELISPOT in PBMCs of 29 CRC patients with completely resected liver metastases. Postvaccine values refer to PBMCs collected at visit (V) 5 (22 cases), V6 (2 cases), V7 (3 cases), V9 (1 case), and V10 (1 case), i.e., weeks 8–22 after surgery. In 22 cases (exceptions are patients 2, 4, 7, 8, 15, 25, and 27), PBMCs were obtained also at time points V7 and V10, i.e., at the beginning and the end of the second cycle. In two subjects, antitumor reactivity was evaluated also at V11, i.e., 28 weeks after surgery. Black columns, prevaccine values. White columns, nonsignificant postvaccine increases. Gray columns, statistically significant postvaccine increases (P < 0.05, evaluated as described in “Patients and Methods”). Triplicate values were used for each patient and time point; error bars, SDs. A, recognition of autologous or allogeneic, class I HLA-matched CRC cells. As targets, allogeneic CRC lines (matching at least one HLA-A or -B allele with patient PBMCs, as described in detail in “Patients and Methods” section) were used. Recognition of autologous tumor cells is reported for patients 3 and 20 (gray boxed numbers). In 59% (17 of 29) of the patients, a significant increase in IFN-γ release against either class I HLA-matched or autologous CRC cell lines was detectable after vaccination. B, lack of recognition of histologically unrelated tumor cells. Over two-thirds of the patients (24 of 29) failed to display increased recognition of melanoma cell line (501mel), suggesting a specific recognition of colon cancer cells after vaccination of HSPPC-96. However, a significant increment in postvaccine PBMCs antitumor reaction was detectable in five patients (patients 1, 14, 15, 18, and 26).
nation was analyzed by IFN-γ ELISPOT assay. Because of the difficulty in establishing cell lines from fresh tumor samples, allogeneic HLA-A- or -B-matched (i.e., sharing at least one class I HLA allele with the patient) colon carcinoma lines available in our laboratory were additionally used as targets in the ELISPOT assay. The decision to use matching class I HLA was based on the evidence that most of the HSPPC-96-induced immune responses detected both in animals and in vaccinated patients were mediated by CD8+ T cells and, thus, were class I HLA-restricted (18, 19, 21, 22).

Postvaccine values refer to PBMCs collected at V5 (22 cases), V6 (2 cases), V7 (3 cases), V9 (one case), and V10 (one case), i.e., weeks 4–18 after the onset of vaccination. In 22 cases (exceptions are patients 2, 4, 7, 8, 15, 25, and 27), PBMCs were obtained also at time points V7 and V10, i.e., at the beginning and at the end of the second cycle of vaccination. In two subjects, antitumor reactivity was evaluated also at V11, i.e., 28 weeks from surgery. Overall, 17 (59%) of the 29 patients displayed a statistically significant increase in postvaccination frequency of PBMCs that released IFN-γ in response to either autologous (patients 3 and 20) or allogeneic HLA-matched colon carcinoma cells (Fig. 1A). In these 17 subjects, the postvaccination time refers to weeks 4 (V5), 8 (V6), 12 (V7), 16 (V9), and 18 (V10) from the onset of vaccination in 10, 2, 3, 1, and 1 cases, respectively. Thus, the vast majority of these subjects was tested at the same postvaccine time. When considered according to the dose of vaccine, the frequency of patients with an increased immune response was 4 of 7, 4 of 11, and 9 of 11 for subjects who received 100, 25, or 2.5 μg of vaccine, respectively. This may suggest, as reported in the murine system (30), that the lowest dose of HSPPC-96 was more immunogenic than the intermediate or high doses, although the number of cases tested was limited.

To evaluate whether such increased reactivity was specific for colon cancer cells, we also performed ELISPOT analysis in response to the histologically unrelated HLA-A2+/A1+ melanoma line 501mel. As shown in Fig. 1B, most patients failed to display any increase in the recognition of 501mel, suggesting that HSPPC-96 vaccine selectively boosted de novo generated T-cell responses specific for CRC. However, a significant increase in postvaccine anti-melanoma PBMC activity was detected in five patients (patients 1, 14, 15, 18, and 26), four of whom lack HLA-A, -B, -C alleles expressed by the control 501mel line, thus suggesting that the recognition was attributable to NK cells or to HLA-unrestricted T-cell activity. In one case (patient 1), randomly shared antigens between melanoma cell lines and colon cancers might have been involved because the two lines shared a HLA-A allele. The NK effect could have been caused by the ability of HSPPC-96 to activate nonspecific and innate components of the immune system (31). Blocking experiments with the anti-HLA-class I mAb W6.32 were performed on the PBMCs of patients who showed a postvaccination increase of anti-colon carcinoma cell activity. As shown in Fig. 2, significant inhibition of IFN-γ production by the anti-HLA-class I mAb was found in 15 of 17 patients, exceptions being subjects 9 and 11. No blocking activity was observed when an anti-HLA class II mAb was used (data not shown). PBMCs of patients 1 and 18 recognized both colon cancer line and the melanoma line, and in patient 1, such an activity could be inhibited on both targets by anti-HLA class I mAb (data not shown), thus suggesting the involvement of antigens recognized by CD8+ T cells and shared between the two different tumors.

These results indicate that treatment with autologous HSPPC-96 induced an in vivo expansion of CD8+ T-cells recognizing class I HLA-restricted colon cancer antigens in at least 15 subjects. In four patients, ELISPOT assays with PBMCs obtained throughout all of the vaccination schedule were performed to define the kinetics of the antitumor immune response after vaccination. In such cases, the immune response was already detectable at V5 and increased or remained sustained over the vaccination period (in two subjects peaking at V10), thus suggesting that a robust activation of the antitumor reaction occurred (data not shown).

To better characterize such CD8+ T-cell responses, we performed a phenotypic analysis of PBMCs, particularly for CCR7 expression, which, together with CD45RO and CD45RA, defines subsets of naive and memory CD8+ T lymphocytes with different functional activity (32, 33). PBMCs from patients showing significant enhancement of antitumor T-cell reactivity after vaccination, displayed an increased percentage of the CD45RA+/CCR7 subset (Table 2), which is known to define final cytotoxic effector T cells that are endowed with the ability to migrate into inflamed or neoplastic tissues (32, 33). On the contrary, no significant increase in such T-cell subsets was found in patients who showed no increase in antitumor T-cell responses (Table 2).

These data suggest that vaccination with autologous HSPPC-96 can induce the in vivo expansion of tumor-specific
CD8+ T-memory lymphocytes that bear a cytotoxic “effector” phenotype.

No reactions were observed in any patient at sites of HSPCC-96 vaccination.

Clinical Results

Vaccination-Related Side Effects. Twenty-two of the 29 patients completed the whole vaccination schedule, whereas 7 subjects (patients 2, 4, 7, 8, 15, 25, and 27) had only the first cycle (6 cases because of cancer recurrence and one case because of refusal). Neither severe toxicity nor serious adverse events were registered during vaccination, although there were 9 cases (31%) of grade 0 toxicity (3 hypertensions, 2 flu-like syndromes, 1 case each of fever, diarrhea, headache, and abdominal pain). In all instances, such toxicity was easily managed with common medications. Patients’ compliance with the vaccine schedule and follow-up visits was high, with no patient being lost or delayed during the study period.

Patients’ Outcome. All of the patients survived the liver resection procedure and left the hospital after a median of 7 days. Major postsurgical complications were not observed, and all subjects followed the vaccination protocol on schedule. After a median follow-up of 24 months, 23 patients (79%) are currently tumor free. Median time for recurrence was 7 months after liver resection (range, 2–24 months). Tumor locations of recurrences were: liver (11 cases), lung (5 cases), bone and pelvis (one case each). In 5 subjects, recurrence occurred during the second cycle of vaccine administration (<6 months from liver resection), whereas in the remaining 13 patients at least 6 months elapsed from the end of vaccine cycles and the evidence of relapsing disease. In 5 of 11 subjects with liver recurrence, repeated surgical resections were performed followed by conventional systemic chemotherapy, whereas in the remaining 6 cases, only chemotherapy was given. As of April 2002, 2 of 18 patients with early recurrences were still tumor free, and 10 patients were alive with disease; the remaining 6 patients died because of cancer progression. The actuarial 24 months OS of the entire study group is 79%, with a DFS of 33% (Fig. 3). When prognostic factors were considered according to MSKCC-score (7), 18 patients fell into Group A (good prognosis, score 1–3) and 11 in Group B (adverse prognosis, score 4–5) as indicated in the “Patients and Methods” section and in Table 1. As expected, Group A had a significantly better outcome than Group B. In fact, at 2 years, OS was 89% versus 64% (P = 0.001), and DFS was 46% versus 18% (P = 0.001) in group A versus B, respectively.

Association between Immunological and Clinical Response. The 18 subjects with good prognostic score (Group A) had a more frequent postvaccine increase in antitumor T-cell immune response (67%, or 12 of 18 patients) when compared with cases with adverse prognosis (Group B; 45%, or 5 of 11 patients).

However, as shown in Fig. 4, patients with a postvaccination immune response had a statistically significant survival advantage compared with nonresponding patients. This was both on OS (100 versus 50%; P = 0.001; Fig. 4A) and DFS (51 versus 8%; P = 0.0001; Fig. 4B) at 2 years. More specifically, when tumor recurrence rate was plotted in a factorial table, cross-classifying patients based on immune response (absent versus present) and clinical prognostic score (favorable versus unfavorable), the occurrence of postvaccine antitumor immune response always led to better tumor-free survival, whatever the predicted prognosis was.

A potentially interesting result, among those presented in Table 3, is that the rate of tumor recurrence dropped from 92% in the immunological nonresponding subjects to 41% in post-
vaccine immune responders. In particular, patients with a good prognosis had an 83% recurrence rate in the absence of immune response but only a 33% recurrence rate in the presence of a postvaccine immune response. Thus, the observed effect of postvaccine immune response on patients’ outcome was independent of the prognostic categories (favorable versus unfavorable), as confirmed by proportional hazard models applied with or without stratification. In examining whether there was a significant effect of antitumor immune response on prognosis (Table 4), the hazard ratio was always less than 1, whether patients were stratified by prognostic group ($P = 0.0012$) or not ($P = 0.0003$). Moreover, values were very much alike in both strata with a $P = 0.9896$ at the interaction test. Because of the small sample size, a multivariate analysis was not applied. Therefore, even though the prognostic categories of the MSKCC score were also significant predictors of patient outcome (hazard ratio, 3.725; 95% confidence interval, 1.131–10.585; $P = 0.0136$), a confounding influence of other variables cannot be excluded.

**DISCUSSION**

This study demonstrates the de novo induction or augmentation of patients’ antitumor-specific T-cell response against CRC cells on vaccination with autologous HSPPC-96 in an adjuvant setting, namely in cases with advanced disease (i.e., liver metastases) rendered tumor free by surgical resection. In fact, preclinical and clinical data suggest that the efficacy of such an approach against cancer is increased if minimal residual disease has to be targeted (34).

Currently, the clinical setting of curatively resected CRC liver metastases is commonly used for adjuvant locoregional and/or systemic chemotherapy (1, 5, 8). Such a model seems particularly suitable for our vaccination approach because: (a) enough material for vaccine preparation could be collected from each patient; (b) HSPPC-96 was extracted in all instances; (c) postsurgical vaccination was not associated with serious adverse events; and (d) full patients’ compliance was achieved. This clinical setting seems suitable for additional studies on adjuvant vaccination against CRC in humans. On the contrary, in meta-

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**Fig. 3** Survival of 29 consecutive patients treated with autologous HSPPC-96 vaccination after complete liver resection of metastatic CRC. OS and DFS at 2 years are 79 and 33%, respectively; the median follow-up is 24 months.

**Fig. 4** Different clinical outcome between immune responders and nonresponders vaccinated with HSPPC-96 after complete liver resection of metastatic CRC. Patients with a postvaccination immune response (17 cases) had a significant survival advantage at 24 months compared with that of nonresponding patients (12 cases) both on OS (A; 100 versus 50% at 2 years) and on DFS (B; 51 versus 8% at 2 years).
Unfortunately, the demonstration of in vivo induction of T-cell responses against individual antigens could not be obtained because of the poor viability of fresh tumor cell suspensions and the difficulty of establishing cell lines from colon cancer metastases by which one could compare the T-cell recognition of autologous versus allogeneic, HLA-compatible colon cancer cells. However, the availability of a large panel of allogeneic HLA-class I matched colon carcinoma cell lines and the use of class I HLA blocking antibodies allowed the evaluation of CD8+ T-cell-mediated recognition of common tumor antigens and its modulation by HSPPC-96 vaccine. This conclusion is also supported by experiments showing recognition of well-defined CRC T-cell epitopes (e.g., CEA, epithelial-cell adhesion molecule) by some of the HLA-A*0201 patients vaccinated with HSPPC-96.

A significant increase of class I HLA-restricted T-cell-mediated recognition of colon cancer cells was observed in 52% (15 of 29) of patients. In two additional cases, this increased reaction did not appear to be HLA-restricted and, thus, should be attributed to NK cells or to MHC-unrestricted T-cell activity (35). Such a measurable effect of HSPPC-96 vaccination has been shown to be related to the time course of the disease, because the activation of the immune response was significantly associated with prognosis, an effect that rarely occurs with other types of vaccines (34). As in animal models, the intensity of vaccine-elicted antitumor immune response could discriminate between control of tumor growth and control of tumor progression (18). This observation confirms results obtained in metastatic melanoma patients (22).

The present study also suggests that HSPPC-96 vaccination can expand the effector compartment of tumor-specific T lymphocytes, with special reference to CD8+ T cells recognizing CRC antigens in a HLA-class I restricted fashion. Corroborating this conclusion is an increase in the CD45RA+/CCR7− effector T lymphocytes that was observed in patients with enhanced immune response to tumor cells (32).

The number of patients studied is too small for any definite conclusion on the possible clinical benefit of adjuvant vaccination with tumor-derived HSPPC-96 administered after complete resection of CRC liver metastases. At first glance, the 29 consecutive patients whose liver metastases were resected had an OS and DFS after vaccination that was similar to those described in a much larger series of patients operated on with the same intent of cure (6, 7, 36, 37). Although this is a pilot study, the results obtained in vaccinated patients are of interest when compared with our own experience with over 400 patients who never showed a 2-year OS and DFS better than 75% and 35%, respectively, particularly when one considers the subset of patients who mounted an immune response after vaccination. In fact, more than 50% of our patients who were vaccinated with HSPPC-96 developed a detectable T-cell reaction against CRC cells and showed a significantly increased OS and DFS with respect to immunologically nonresponding subjects (Fig. 4). Indeed, regression analysis (Table 4) demonstrated that an immune response to vaccine is an independent factor predicting improved recurrence-free survival in all cases, whether or not the patients had a score, including performance status, predictive of good or bad prognosis. These data suggest that HSPPC-96 vaccine not only generated an immune response in more than one-half of our patients but may also have contributed to improved OS and DFS.

Such results must be interpreted cautiously because the small number of patients prevented a multivariate analysis on the possible influence of other prognostic factors. Also, an intrinsic patient selection cannot be excluded, although cases were collected consecutively. One factor that strengthens the results in favor of the relevance of the effect of vaccine immunization is the protective effect on cancer recurrence that was evaluated in a blinded fashion until the end of follow-up and that then was compared with patient prognosis generated at the beginning of the study using a validated prognostic scoring system.

By comparing our findings with recent results of adjuvant chemotherapy (8, 38), the data are not sufficient to recommend HSPPC-96 vaccination as an alternative to conventional chemotherapy after resection of CRC liver metastases. Nevertheless, the results of the combination of tumor resection plus vaccine could justify a prospective clinical trial, especially for patients with good prognosis, particularly in light of the lack of toxicity observed. The use of HSP-derived vaccines should be further investigated, either alone or in combination with chemotherapy, in prospective clinical trials.

In conclusion, HSPPC-96 vaccination represents a feasible and safe approach for adjuvant treatment after curative resection of CRC liver metastases. A statistically significant increase in postvaccination, class-I HLA-restricted T-cell response against CRC cells was detectable in more than 50% of the patients

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**Table 3** Correlation between postvaccination antitumor T-cell reaction and recurrence-free survival after liver resection for metastatic CRC: immune response to vaccine and patient prognosis

<table>
<thead>
<tr>
<th>Immune response to vaccine</th>
<th>Patients</th>
<th>Tumor recurrence</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>12</td>
<td>11</td>
<td>92</td>
</tr>
<tr>
<td>Favorable prognosis</td>
<td>6</td>
<td>5</td>
<td>83</td>
</tr>
<tr>
<td>Unfavorable prognosis</td>
<td>6</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Present</td>
<td>17</td>
<td>7</td>
<td>41</td>
</tr>
<tr>
<td>Favorable prognosis</td>
<td>12</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>Unfavorable prognosis</td>
<td>5</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>18</td>
<td>62</td>
</tr>
</tbody>
</table>

**Table 4** Correlation between postvaccination antitumor T-cell reaction and recurrence-free survival after liver resection for metastatic CRC: Cox regression analysis

<table>
<thead>
<tr>
<th>Cox model</th>
<th>Hazard ratio</th>
<th>Confidence interval 95%</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without stratification</td>
<td>0.127</td>
<td>0.041–0.391</td>
<td>0.0003</td>
</tr>
<tr>
<td>With stratification</td>
<td>0.113</td>
<td>0.030–0.421</td>
<td>0.0012</td>
</tr>
<tr>
<td>Favorable prognosis</td>
<td>0.114</td>
<td>0.022–0.598</td>
<td></td>
</tr>
<tr>
<td>Unfavorable prognosis</td>
<td>0.112</td>
<td>0.013–0.970</td>
<td></td>
</tr>
</tbody>
</table>

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4 L. Rivoltini and W. Hansjochen, unpublished observations.
studied for the first time in such a clinical setting. Moreover, such immunization effect involved effector CD8+ T lymphocytes, reinforcing the conclusion that these lymphocytes were instrumental in destroying CRC cells in vivo. Two-year OS and DFS were improved in subjects showing an antitumor immune response, and, although prognostic scores (based on preoperative tumor characteristics) correctly predicted survival, the immune response after vaccination exerted a significant impact on tumor-free survival.

Although the number of patients was small, the present prospective cohort study suggests a possible clinical benefit of HSPC-96 vaccination in a homogeneous series of patients at high risk of recurrence after complete surgical removal of CRC hepatic metastases. This vaccination approach, therefore, warrants additional studies with a larger number of subjects.

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