Induction of p53-specific Immune Responses in Colorectal Cancer Patients Receiving a Recombinant ALVAC-p53 Candidate Vaccine


ABSTRACT

Purpose: The tumor-associated auto-antigen p53 is commonly overexpressed in various types of human cancer, including colorectal cancer. Experiments in preclinical models have shown that it can serve as a target for T-cell-mediated tumor-eradication. The feasibility of a p53-specific therapeutic vaccination was investigated in cancer patients.

Experimental Design: A Phase I/II dose-escalation study was performed that evaluated the effect of a recombinant canarypoxvirus (ALVAC) vaccine encoding wild-type human p53 in 15 patients with advanced colorectal cancer. Each group of five patients received three i.v. doses of one-tenth of a dose, one-third of a dose, or 1 dose of the vaccine [1 dose = $1 \times 10^7.5$ cell culture infectious dosis (CCID)\textsubscript{50}].

Results: Potent T-cell and IgG antibody responses against the vector component of the ALVAC vaccine were induced in the majority of the patients. Enzyme-linked immunosorbent-spot assay (ELISPOT) analysis of vaccine-induced immunity revealed the presence of IFN-γ-secreting T cells against both ALVAC and p53, whereas no significant interleukin-4 responses were detected. Vaccine-mediated enhancement of p53-specific T-cell immunity was found in two patients in the highest-vaccine-dose group.

Conclusions: This study demonstrated the feasibility, even in patients with advanced cancer, to elicit immune responses against the ubiquitously expressed tumor-associated auto-antigen p53. Our results form the basis for additional studies that will explore the antitumor capacity of p53 containing multivalent vaccines in cancer patients with limited tumor burden.

INTRODUCTION

Tumor antigens such as CEA\textsuperscript{1} (1–3), epithelial cell adhesion molecule (Ep-CAM; Refs. 4 and 5), and p53 (6–8) represent potential targets for the immunotherapy of colorectal cancer. Mutations in the p53 tumor suppressor gene are found in a wide variety of tumors, including ~50% of colorectal cancers (9–11). Because p53 is not expressed at the cell surface, p53-specific antibodies are unlikely to exert therapeutic antitumor effects. In contrast, p53-specific T-cell immunity may be exploitable for immunotherapy of cancer because p53-peptides are processed by the proteasome and presented by MHC class I molecules to CTLs (12–15). Furthermore, accumulated p53, when released from dying tumor cells, can serve as a potent immunogen for Th-cells. Both p53-specific MHC class I-restricted CTLs and p53-specific MHC class II-restricted Th-cells have been shown to exert antitumor efficacy \textit{in vivo} in mouse tumor models (8, 13, 16, 17). Importantly, these experiments demonstrated that p53-specific immunity was not accompanied by overt signs of autoimmunity.

In humans, p53-specific antibodies have been found in patients suffering from a variety of tumors (18–23). The induction of anti-p53 antibodies generally reflects a high tumor load and is, therefore, associated with bad prognosis. wt.p53-specific CTLs (12, 24–26) and Th-cells (27) have been detected in human PBMC cultures \textit{in vitro}. In addition, wt.p53-specific proliferative responses were demonstrated in patients with breast cancer (28) and for several years after resection in the majority of patients with resected primary colorectal cancer (29).

Immunization with recombinant poxvirus carrying a transgene that encoded the tumor antigen of choice can result in strong T-cell immunity against this antigen (30). Given its host-cell specificity, the canarypoxvirus ALVAC can infect a wide array of mammalian cells, including human cells, although

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\textsuperscript{1}The abbreviations used are: CEA, carcinoembryonic antigen; APC, antigen-presenting cell; CCID, cell culture infectious dose; ELISPOT, enzyme-linked immunosorbent-spot assay; ILA, human leucocyte antigen; IL, interleukin; MRM, memory response mix; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; Th-cell, T-helper cell; wt.p53, wild-type p53 (protein); mEU, milli-ELISA unit(s); SI, stimulation index.

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failing to replicate in such cells. The safety profile of ALVAC-based recombinants has been established in numerous animal models (31). In addition, recombinant ALVAC has been administered to more than 2000 humans, including cancer patients, with no signs of pathological reactions, dissemination of virus into the environment, or viral replication (31). Furthermore, canarypox recombinants effectively prime both the humoral and cellular components of the immune system against the transgene-encoded antigens (32) and, thus, are attractive vehicles for therapeutic vaccination in cancer. Immunization with ALVAC-encoding murine wt.p53 as well as human wt.p53 (ALVAC-hup53, designated vCP207) protected BALB/c mice from a challenge with a highly tumorigenic mouse fibroblast tumor cell line expressing high levels of mutant p53 (33). Optimal triggering of p53-specific T-cell immunity was found when ALVAC was injected i.v., after which it was primarily localized in the lung, liver, and spleen (34). To study the effect of i.v. ALVAC-hup53 injection with respect to general safety and the induction of autoimmunity in nonhuman primates, rhesus macaques were given three i.v. injections of ALVAC-hup53 at proportional doses up to 10-fold higher than those proposed for humans. Repeated administration of the highest doses was well tolerated and despite the >95% amino-acid identity between human and rhesus p53, no abnormalities were detected in hematological or clinical chemistry parameters or tissue pathology that could point to autoimmune reactions. One of four monkeys injected with the maximal dose proposed for humans developed a p53-specific antibody response (35).

To assess the safety and immunogenicity of ALVAC-hup53 in humans, a Phase I/II dose-escalating study was initiated in which 15 end-stage colorectal cancer patients were vaccinated three times i.v. After vaccination, strong humoral and cellular immune responses to ALVAC were induced. Importantly, p53-specific T-cell immunity was induced in several of the patients receiving the highest vaccine dose.

PATIENTS AND METHODS

Clinical Protocol. Adult patients (ages >18 years) with histologically proven colorectal cancer and evidence by imaging techniques of irresectable disease were eligible for inclusion in this study. Patients with metastatic disease that was untreatable by conventional therapies or patients with metastatic disease that was potentially treatable, but who refused conventional therapy were also eligible for this study. The protocol used in this study was approved by the local and national medical ethics committee as well as by the biological safety committee and the Dutch Ministry of Health and Environment. Inclusion criteria were: use of effective contraception; aspartate aminotransferase/alanine aminotransferase levels within the normal range; alkaline phosphatase levels within five times the normal range; bilirubin levels and blood cell counts within 1.5 times the normal range; serum CEA level of ≤10 μg/liter; and a health status corresponding to the WHO performance status level of 0 or 1. Additionally, at least 30% of the primary tumor or metastases were to express HLA class I and p53 by immunohistochemistry. Exclusion criteria were: pregnancy; autoimmune disease; symptomatic viral or other infections; HIV seropositivity or refusal to hear the results of the HIV test; receipt of organ grafts; life expectancy of <3 months; a history of allergy; a history of severe neurological, cardiovascular, renal, hepatic, endocrine, respiratory, or bone marrow dysfunction; known family history of Li-Fraumeni syndrome; known allergy to egg proteins or neomycin; chemotherapy or radiation within the 4 weeks preceding enrollment; immunotherapy, chemotherapy using nitrosourea; hormonal treatment (other than contraception) within the previous 6 weeks; or a history of treatment with growth hormone extract.

Patients were divided into three groups of five individuals each. Patients received three i.v. injections of ALVAC p53 at three-week intervals. Group 1 received one-tenth of the total dose (10^6.5 CCID_{50}) of ALVAC hup53 at each injection, Group 2 received one-third of the total dose (10^7.5 CCID_{50}) at each injection, and Group 3 received the total dose (10^7.5 CCID_{50}) at each injection. Blood was obtained for biochemical, hematological, and immunological assays before each vaccination. Patient visits (V) were scheduled as follows: (a) preinclusion visit (PV) at a maximum of 2 weeks before the first vaccination; (b) V1, week 0, first vaccination; (c) V2, week 3, second vaccination; (d) V3, week 6, third vaccination; (e) V4, week 7; (f) V5, week 8; (g) V6, week 14; and (h) V7, week 20. PBMCs collected before vaccination (PV) and 2 weeks after completing the immunization scheme (V5) were isolated, cryopreserved using a computer-controlled freezing device, and stored in liquid nitrogen. Sera were isolated from blood collected at each visit and stored at -20°C.

PBMCs and sera of anonymous healthy blood donors were isolated and used as control PBMCs in ELISPOT and proliferation assays or as negative controls in p53 antibody subtype ELISA.

Recombinant ALVAC-p53 Vaccine. ALVAC-hup53 (vCP207) is a recombinant virus, based on the canarypoxvirus-based vector ALVAC and the wild-type p53 gene. The vCP207 recombinant was generated by cotransfection of ALVAC-infected primary chick-embryo fibroblasts with an insertion plasmid and noninfectious purified ALVAC genomic DNA, leading to the integration of the foreign gene expression cassette into the viral genome via homologous recombination. The clinical lot used in these studies was produced by Aventis Pasteur (Marcy l’Etoile, France) and was purified twice through a sucrose gradient.

Antigens. Twenty-four peptides spanning the wt.p53 protein were synthesized as 30-mers overlapping by 14 amino acids. These peptides were divided into three pools: pool 1, peptide 1 (p1) to p8 (covering residues 1–142); pool 2, p9-p16 (residues 129–270); and pool 3, p17-p24 (residues 257–393). Recombinant baculovirus-derived human wt.p53 and gp100 protein were produced at Virogenetics, (Troy, NY). Inactivated ALVAC virus was donated by Dr. C. Blondeau (Aventis Pasteur, Marcy l’Etoile, France). MRM, a mixture of tetanus toxoid (150 limus flocculentius/ml; National Institute of Public Health and the Environment, Bilthoven, the Netherlands), Mycobacterium tuberculosis sonicate (2.5 μg/ml; generously donated by Dr. P. Klatser, Royal Tropical Institute, the Netherlands) and Candida albicans (0.005% HAL Allergenen Lab, Haarlem, the Netherlands) was used to control the capacity of PBMCs to proliferate in response to typical recall antigens.
Antibody Titers. ALVAC-specific IgG antibodies were measured in a standard ELISA. Briefly, inactivated ALVAC virus particles were coated at a concentration of 1 μg/ml. After blocking with PBS/BSA 1%, serial dilutions (1:100 to 1:3200) of preimmune and postimmunization sera and a positive-control working standard serum titrated from 130 to 0.2 mEU/ml were applied in duplicate wells. After an incubation period of 2 h at 37°C, wells were washed and incubated with antihuman IgG-horseradish peroxidase for another 2 h at 37°C. Color was developed with ABTS [2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonate); Sigma Chemical Co.] + 0.0075% H2O2. The absorbance at 415 nm was measured. The anti-ALVAC antibody titer (mEU/ml) in each serum sample was calculated for each dilution using a linear regression curve obtained by plotting the working standard concentration versus the absorbance. The mean titer of all serum dilutions was calculated. Anti-ALVAC titers <1000 mEU/ml were considered negative.

p53-specific antibodies were measured as reported previously (29). Briefly, microtiter wells were coated overnight at 4°C with 100 μl of recombinant baculovirus-derived p53 or, as a control, BSA at a concentration of 2 μg/ml. Wells were washed and then incubated with PBS + 1% powdered milk (Protifar; Nutricia, the Netherlands). After 1 h of incubation at 37°C, the wells were washed, and patient-derived serum or a positive-control working standard serum titrated from 130 to 0.2 mEU/ml were applied in duplicate wells. After an incubation period of 2 h at 37°C, wells were washed and incubated with antihuman IgG-horseradish peroxidase for another 2 h at 37°C. Color was developed with ABTS [2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonate); Sigma Chemical Co.] + 0.0075% H2O2. The absorbance at 415 nm was measured. The anti-ALVAC antibody titer (mEU/ml) in each serum sample was calculated for each dilution using a linear regression curve obtained by plotting the working standard concentration versus the absorbance. The mean titer of all serum dilutions was calculated. Anti-ALVAC titers <1000 mEU/ml were considered negative.

Analysis of Antigen-specific T-Cells by ELISPOT. The ELISPOT assay was performed as reported previously (36). Pre- and postimmunization PBMCs were analyzed for the production of both IFNγ and IL-4. Briefly, PBMCs were seeded at a density of 2.5 \( \times 10^5 \) cells/well of a 12-well plate (Costar, Cambridge, MA) in 1 ml of ISCOVE’s medium (Life Technologies, Inc.) enriched with 10% FCS, in the presence or absence of indicated pools of p53 peptide (5 μg/peptide/ml), inactivated ALVAC (5 μg/ml), or MRM at a 1:50 dilution. After 4 days of incubation at 37°C, PBMCs were harvested, washed, and seeded in six replicate wells at a density of 10^5 cells/well of a Multiscreen 96-well plate (Millipore, Ettten-Leur, the Netherlands) coated with an IFNγ-catching antibody or an IL-4-catching antibody (Mabtech AB, Nacha, Sweden). Pre- and postimmunization PBMCs, stimulated with the same antigen, were seeded in adjacent wells. The ELISPOT was further performed according to the instructions of the manufacturer (Mabtech). The number of spots was analyzed with a fully automated computer-assisted video imaging analysis system (Carl Zeiss Vision). Specific spots were calculated by subtracting the mean number of spots + 2 \( \times \) SD of the control (medium) from the mean number of spots of experimental wells. Results were expressed as the number of specific spots above the lower detection limit of the assay (10 cells/10^6 PBMCs) per million PBMCs.

Statistical Analysis. To compare the proliferative responses either to common recall antigens (MRM) or to PHA of patient-derived PBMCs versus PBMCs derived from healthy blood donors, the mean SIs were calculated, log-transformed, and compared in a Welch-corrected unpaired t test. The non-parametric ANOVA Kruskal-Wallis test was used to compare different vaccination doses with the maximal anti-ALVAC antibody titer or the number of postimmunization ALVAC-specific T cells in the ELISPOT. The maximal anti-ALVAC antibody titer in patients was correlated with preexisting ALVAC-specific T-cell frequencies using Spearman rank correla-

#### Table 1: Patient characteristics

<table>
<thead>
<tr>
<th>Gender</th>
<th>All patients</th>
<th>Group 1 ( n = 5 )</th>
<th>Group 2 ( n = 5 )</th>
<th>Group 3 ( n = 6 )</th>
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<tbody>
<tr>
<td>Male</td>
<td>12</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>Mean 60</td>
<td>53</td>
<td>62</td>
<td>65</td>
</tr>
<tr>
<td>Range 42–71</td>
<td>46–66</td>
<td>46–69</td>
<td>57–71</td>
<td></td>
</tr>
<tr>
<td>Diagnosis (mo)</td>
<td>Mean 25</td>
<td>35</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>Range 6–66</td>
<td>17–66</td>
<td>7–21</td>
<td>6–56</td>
<td></td>
</tr>
<tr>
<td>Therapy</td>
<td>First line chemotherapy</td>
<td>15</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Second line chemotherapy</td>
<td>7</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
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<td>ILTP</td>
<td>8</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>8</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

*ILP, isolated liver perfusion.*
Recombinant ALVAC-p53 in Colorectal Cancer Patients

RESULTS

General Immune Status of Patients. Sixteen patients were enrolled in the trial (Table 1), 15 of whom completed the immunization scheme. These 15 patients were analyzed for preexisting and vaccine-induced immunity.

Vaccination of end-stage cancer patients may fail to elicit immunity attributable to the presence of large tumor burdens that can result in immunosuppression as reflected by loss of reactivity to common recall antigens (37). In view of these considerations, we tested this recall response before and after the vaccinations were given. With the exception of patient 14, all of the patients showed a response to the mixture of common bacterial antigens at the start of the study. When the vigor of proliferation to the recall antigens of patients was compared with that found in healthy controls (not shown), both the mean proliferative responses (mean SI, 19 and 86, respectively; \( P = 0.001 \), Welch corrected \( t \) test) and the number of IFN\( \gamma \)-producing T cells (mean, 695 and 1506, respectively; \( P = 0.04 \), Welch corrected \( t \) test) of the patients were significantly lower, indicating partially suppressed cellular immunity. At the end of the study, patients 6, 7, and 16 lost reactivity to the recall antigens (Table 2). PBMCs of all of the patients responded well to the polyclonal stimulus PHA (Table 2), and, although the mean proliferative responses of patients were lower compared with the responses noted in 13 random healthy controls (mean SI, 207 and 409, respectively), this difference was not significant (\( P = 0.2 \), Welch corrected \( t \) test).

Vaccine-induced Immunity against the ALVAC Vector. To monitor the impact of the vaccine on the immune system, the response to ALVAC was measured. Both antibody and T-cell responses against ALVAC were found to be strongly increased after vaccination in all but one patient (Table 3 and Fig. 1A). The T-cell response in patient 5, who also failed to generate an anti-ALVAC antibody response, was only marginal compared with the responses against ALVAC in the other patients. ALVAC-specific T cells mainly produced IFN\( \gamma \)-producing T cells. Figures indicate the number of antigen-specific cytokine-producing cells per 10\(^6\) PBMCs. —, the number of antigen-specific T cells is below the detection level of the assay.

Vaccine-induced p53-specific Antibodies. The presence of p53-specific IgG antibodies in the sera of all of the patients was tested by a quantitative p53-specific IgG ELISA. In 10 patients, p53-specific IgG antibodies were detected after vaccination, two of which were relatively high titered (patient 6, 7 units/ml; patient 9, 14 units/ml). In three of these patients (patients 4, 7, and 12) no p53-specific IgG antibodies were

Table 2 Evaluation of the immunological patient status

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>2-day proliferation(^a)</th>
<th>IFN( \gamma ) ELISPOT(^b)</th>
<th>IL-4 ELISPOT</th>
<th>3-day proliferation(^c)</th>
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</thead>
<tbody>
<tr>
<td>PV V5 PV V5 PV V5 PV V5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2(^d)</td>
<td>7 7 1540 1810 70 —</td>
<td>43 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>23 NT NT 1260 60 280 NT</td>
<td>237 NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>18 16 890 440 30 50</td>
<td>71 39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>12 40 230 560 10 30</td>
<td>70 417</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NT NT 420 — 170 20</td>
<td>NT NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7 — 250 — —</td>
<td>61 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>76 72 2340 3770 — —</td>
<td>439 212</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>44 78 200 290 — —</td>
<td>107 114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>14 24 1120 240 — 30</td>
<td>82 203</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>— 10 50 410 — —</td>
<td>130 205</td>
<td></td>
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</tr>
<tr>
<td>12</td>
<td>10 105 120 1600 90 180</td>
<td>751 470</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>— — — — — —</td>
<td>318 316</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>— — — — — —</td>
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</tr>
<tr>
<td>16</td>
<td>34 NT NT 200 120 20 NT</td>
<td>118 975</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>11 12 410 380 40 —</td>
<td>— —</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) PBMCs were stimulated with the recall antigens containing MRM in eight replicate wells for 7 days, after which \( ^{[3]} \)Hthymidine incorporation was measured. The numbers shown are the SIs of the sample taken before immunization (PV) and 2 weeks after the last immunization (V5). SI ≥ 4 are regarded positive; —, SI < 4.

\(^b\) PBMCs stimulated for 4 days with MRM were harvested, counted, and seeded in ELISPOT plates in order to quantify the number IFN\( \gamma \)-producing cells per 10\(^6\) PBMCs. —, the number of antigen-specific T cells is below the detection level of the assay.

\(^c\) PBMCs were cultured for 3 days in the presence of PHA, after which \( ^{[3]} \)Hthymidine incorporation was measured. The numbers shown are the SIs. SIs ≥ 4 are regarded positive; —, SI < 4.

\(^d\) Patients 1 and 13 were not completely vaccinated and were excluded from the analysis.

\(^e\) NT, not tested because of unavailable PBMCs.

\(^f\) High background proliferation of the medium control.

Analysis was performed using GraphPad InStat (GraphPad Software Inc.).
detected before vaccination, which implies that these responses were induced by the vaccine (Table 4). Not unexpectedly, the other seven patients with advanced colorectal cancer had demonstrable p53-specific IgG antibodies before vaccination. These responses were, however, not boosted by the vaccine (Table 4). In two patients (patients 4 and 10), it was noted that the p53-specific IgM level was increased after one or two immunizations (Fig. 1B, and data not shown). Thus, in a fraction of advanced colorectal cancer patients, vaccination with ALVAC-hup53 results in the induction of p53-specific antibodies.

Vaccine-induced p53-specific T-Cell Immunity. After vaccination with the one-tenth or one-third dose of ALVAC-hup53, no induction of p53-specific proliferation or p53-specific cytokine production (ELISPOT, detection limit of 10 cells per 10^6 PBMCs; Table 4) was detected. Importantly, ELISPOT analysis of the PBMC samples of patients injected with the full dose of ALVAC-hup53 showed an IFNγ-producing p53-peptide-specific response in four of five patients. No p53-specific IL-4 production was detected. Patient 12 responded to peptide pool p9-p16 and p17-p24, patients 15, 16, and 17 responded to p9-p16 with T-cell frequencies ranging from 60 to 190 per million PBMCs in the postimmunization samples. In patients 16 and 17, these responses were also detected before vaccination (Table 4). Furthermore, the p53-specific response of the latter patient was also detected in the less sensitive lymphocyte-stimulation proliferation assay. In conclusion, our data demonstrated that injection of end-stage colorectal cancer patients with 10^7.5 CCID_{50} (full dose) of ALVAC-hup53 can induce p53-specific T-cell immunity.

**DISCUSSION**

Immunotherapy approaches against cancer that target self-antigens aim at the induction of a beneficial autoimmune response. Although the observation that p53 is commonly overexpressed in many cancer types makes it an attractive target antigen, the feasibility of p53-specific immunotherapy is challenging because this unique antigen is ubiquitously expressed in normal tissues. As such, immunological tolerance as well as p53-specific immunity to normal cells could preclude the use of vaccines aiming at the induction of p53-specific T-cell immunity. Notably, experiments in mice have indicated that p53-

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**Table 3** Humoral and cellular reactivity against ALVAC

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Antibodies&lt;sup&gt;a&lt;/sup&gt;</th>
<th>7-day proliferation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IFNγ ELISPOT&lt;sup&gt;c&lt;/sup&gt;</th>
<th>IL-4 ELISPOT&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>PV</td>
<td>MAX</td>
<td>PV</td>
<td>V5</td>
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<tr>
<td>2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>305</td>
<td>2157 (3)</td>
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<td>4</td>
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<tr>
<td>3</td>
<td>285</td>
<td>&gt;30,000 (2)</td>
<td>6</td>
<td>NT&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>277</td>
<td>23464 (5)</td>
<td>4</td>
<td>15</td>
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<tr>
<td>5</td>
<td>583</td>
<td>907 (7)</td>
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<td>6</td>
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<td>7</td>
<td>977</td>
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<td>8</td>
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<td>11908 (6)</td>
<td>13</td>
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<td>17</td>
<td>73</td>
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</tbody>
</table>

<sup>a</sup>The pre-existing (PV) anti-ALVAC antibody titer as well as the maximum level (MAX; mEU/ml) is shown. The numbers in parentheses indicate at which visit number this maximum level was reached. An antibody titer <1000 mEU/ml is considered negative.

<sup>b</sup>Inactivated ALVAC at 5 μg/ml was used to stimulate PBMCs. See Table 2, footnote a, for additional explanation of 7-day proliferation assay.

<sup>c</sup>See Table 2, footnote b, for information on ELISPOT assays.

<sup>d</sup>Patients 1 and 13 were not completely vaccinated and were excluded from the analysis.

<sup>e</sup>NT, not tested because of unavailable PBMCs.

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Fig. 1 Longitudinal analysis of the anti-ALVAC IgG response (A) and p53-specific IgM (B) response of patient 4 before and after vaccination with the lowest dose of ALVAC-hup53. Vaccinations were given at weeks 0, 3, and 6. OD, absorbance.
specific immunotherapy may be feasible. In view of these findings, we have conducted a Phase I study involving p53-specific vaccination of end-stage colorectal cancer patients. Antibody responses were induced in some, but not all, of the vaccinees. Moreover, p53-specific IFN-γ-producing T-cell immunity was found to be induced in two of five colorectal cancer patients vaccinated with the highest dose of ALVAC-hup53. The present study demonstrates that even in a group of end-stage cancer patients, with a less than fully competent immune system, immunization with ALVAC-hup53 results in the induction of p53-specific B-cell and T-cell immunity. Furthermore, the administration of multiple injections was well tolerated, and no clinical signs of autoimmunity were observed.

At first sight, the frequency of the preexisting p53-specific IgG antibody responses in our patient group (47%) seems higher than generally reported in literature (25%; Ref. 18). The induction of p53-specific antibodies is dependent on a subset of mutations in p53 and overexpression of these mutated p53 proteins (reviewed in Ref. 38). Normally, about 50% of colorectal cancer patients display p53 overexpression (39), but, as part of the inclusion criteria, a likely explanation is that this natural response is caused by cross-reactivity with vaccinia virus, to which most of our patients have been exposed in the past. When the maximal anti-ALVAC IgG level was evaluated in relation to the magnitude of the preexisting ALVAC-specific T-cell response, a clear correlation was found suggesting that the presence of ALVAC-specific T cells provided help to boost the humoral response to ALVAC. There was no correlation between ALVAC-immunity and the induction of p53-specific immunity.

Both humoral and cellular anti-ALVAC responses were induced in all but one patient. No clear relationship between the vaccine dose and the anti-ALVAC IgG response ($P = 0.07$) or the number of postimmunization ALVAC-specific T cells as detected by IFN-γ ELISPOT ($P = 0.1$) was found. Furthermore, the number of postimmunization ALVAC-specific T cells did not correlate with the maximal ALVAC-IgG level. Interestingly, preexisting ALVAC-specific T-cell immunity was detected in both healthy donors (not shown) and colorectal cancer patients. A likely explanation is that this natural response is caused by cross-reactivity with vaccinia virus, to which most of our patients have been exposed in the past. When the maximal anti-ALVAC IgG level was evaluated in relation to the magnitude of the preexisting ALVAC-specific T-cell response, a clear correlation was found suggesting that the presence of ALVAC-specific T cells provided help to boost the humoral response to ALVAC. There was no correlation between ALVAC-immunity and the induction of p53-specific immunity.

Both humoral and cellular anti-ALVAC responses were induced in all but one patient. No clear relationship between the

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T-helper responses (29, 36). In this Phase I/II trial, four of five patients receiving the highest dose of ALVAC-hup53 displayed p53-specific Th-cells, which, on recognition of a p53 peptide, produced IFNγ but not IL-4. Although p53-specific immunity was induced in two patients receiving the highest dose of the vaccine, the other two patients displayed p53-specific Th-cells already before immunization. Previously, it has been shown that p53-specific Th-cells can arise during tumor growth in mice (40) and humans (28, 29). In fact, our study in patients treated for primary colorectal carcinoma by surgery showed that the majority of these patients had developed a p53-specific Th-response (29). Our current data suggest that p53-specific Th-immunity is less frequently detected in end-stage colorectal cancer patients who failed conventional treatment such as surgery and extensive chemotherapy treatments. Altogether, our data show that p53-specific Th-cells either can develop as part of the natural immune response in tumor-bearing patients or can be induced by p53-specific vaccination (e.g., patients 12 and 15). Not unexpectedly, p53-specific T-cell responses were not accompanied by significant clinical responses. The end-stage patients had large tumor burdens that were diagnosed 25 months, on average, before vaccination, and the tumor response was evaluated 14 weeks after completing vaccinations.

A key question concerns the antitumor efficacy of these p53-specific Th-cells and the necessity to boost these responses by a vaccine. Cumulative evidence has shown that tumor-specific CD4+ Th-cells are pivotal for the efficient eradication of solid tumors, although such tumors usually do not express MHC class II (reviewed in Ref. 6). In several murine tumor models, tumor-specific CD4+ Th-cells critically contributed to the development and efficacy of antitumor responses (41–46). In two cases, Th-cells were shown to exert their antitumor effect by stimulating tumoricidal macrophages and eosinophils (43, 44), whereas, in the other cases, tumor-specific Th-cells drove the CTL-dependent antitumor immunity. The efficacy of these Th-cells lies not only in the property of providing CTLs with essential growth stimuli, primarily IL-2, during the effector phase (47) but also in the ability to deliver essential activation signals to APCs needed for an optimal priming of tumor-specific CTLs (48–51). Notably, whereas the delivery of this type of T-cell help to CTLs requires the APCs to present both tumor-derived MHC class I-restricted EpCAM as a target for passive and active specific immunotherapies in patients with colorectal carcinoma. Ann. N. Y. Acad. Sci., 910: 254–261; discussion, 261–262, 2000.


Induction of p53-specific Immune Responses in Colorectal Cancer Patients Receiving a Recombinant ALVAC-p53 Candidate Vaccine


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