Immunization of Colorectal Carcinoma Patients with a Recombinant Canarypox Virus Expressing the Tumor Antigen Ep-CAM/KSA (ALVAC-KSA) and Granulocyte Macrophage Colony-stimulating Factor Induced a Tumor-specific Cellular Immune Response

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ABSTRACT

Purpose: Colorectal carcinoma cells express the tumor-associated antigen epithelial cellular adhesion molecule (Ep-CAM)/KSA. Passive immunotherapy with monoclonal antibodies using this antigen has shown promising results. Ep-CAM might also be a target for active specific immunotherapy. Expression of the tumor antigen in a viral vector may facilitate appropriate antigen presentation. The feasibility of an Ep-CAM/KSA-specific therapeutic vaccination was investigated in cancer patients.

Experimental Design: The full-length Ep-CAM gene was inserted into the avipox virus ALVAC (ALVAC-KSA). Twelve radically operated colorectal carcinoma patients without evidence of remaining macroscopic disease (stages I, II, and III) entered the study. The first 6 patients were immunized with three injections of ALVAC-KSA (10^7.09 CCID50 per immunization) alone in weeks 0, 3, and 6. The subsequent 6 patients received the same schedule of ALVAC-KSA together with the adjuvant cytokine granulocyte macrophage colony-stimulating factor (GM-CSF; 75 µg/day for 4 consecutive days).

Results: The adverse reactions to the vaccinations were mild except for local skin reactions. In the ALVAC-KSA group a weak T-cell response was induced in 2 of 6 patients. In the ALVAC-KSA/GM-CSF group a marked IFN-γ response (enzyme-linked immunospot) was induced in 5 of 6 patients. The T-cell response appeared late, 1 month after the last immunization, with a peak at 4–5 months after immunization. No IgG antibodies against Ep-CAM were detected. Before vaccination the majority of patients had a type 1 T-cell response (IFN-γ) against the vector, which was noted in healthy donors as well. All of the patients developed high titers of IgG antibodies against the vector, and the T-cell response was vigorously boosted.

Conclusions: ALVAC-KSA, in combination with low dose local administration of GM-CSF may induce a strong, IFN-γ T-cell response (type 1). ALVAC-KSA seems to be an interesting candidate as a cancer vaccine for future clinical development.

INTRODUCTION

Despite tremendous therapeutic efforts, the prognosis for CRC is still poor for stages II, III, and IV, with 5-year survival rates of 60–80%, 30–55%, and <3%, respectively (1). New treatment modalities are needed. Tumor specific immunotherapy as cancer vaccines or monoclonal antibodies might represent an alternative.

The TAA Ep-CAM (also termed CO17-1A, KSA, GA733-2, KS1-4, and EGP) is a cell surface-expressed glycoprotein present at high density on the majority of CRC cells (2). Treatment with monoclonal antibodies against this antigen may induce tumor regression (3) and prolong survival of stage III CRC patients (4). Vaccination of CRC patients with antiidiotype antibodies mimicking Ep-CAM (5, 6) or with the

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3 The abbreviations used are: CRC, colorectal carcinoma; Ep-CAM, epithelial cellular adhesion molecule; GM-CSF, granulocyte monocyte colony-stimulating factor; CEA, carcino-embryonic antigen; TAA, tumor-associated antigen; PBMC, peripheral blood mononuclear cell; BCP, baculovirus control protein; PHA, phytohemagglutinin; SI, stimulation index; ELISPOT, enzyme-linked immunospot; ΔCT, Δ threshold cycle; RT-PCR, reverse transcription-PCR.
recombinant Ep-CAM protein antigen (3, 7) induced both cellular and humoral immune responses. In mice, immunization with a recombinant adenovirus expressing the full-length gene of Ep-CAM induced a specific humoral as well as cellular response and specific regression of syngeneic CRC cells transfected with the human Ep-CAM (8).

Thus, the Ep-CAM antigen might be a suitable target antigen for therapeutic vaccination. The possible unresponsiveness to self-antigens, like Ep-CAM, calls for an optimal delivery of the antigen with repeated administrations and the use of strong adjuvants to break tolerance. The replicative-deficient recombinant avipoxvirus, ALVAC, readily infects mammalian cells and induces protein synthesis by the inserted gene. This protein may then be processed and presented to the immune system by antigen-presenting cells (9).

A number of ALVAC-based recombinant vaccines have been tested in >2000 humans, including cancer patients, and shown to be well tolerated (10).

A strong immune enhancing effect of the adjuvant cytokine GM-CSF on the humoral and T-cell responses was noted in CRC patients immunized with the protein antigen CEA indicating the importance of adding GM-CSF (11). The capacity of GM-CSF to augment an antitumor response is attributed to its role of activating antigen presenting dendritic cells but also in facilitating the use of the MHC class I antigen presentation pathway (12–14).

The aims of the present study were to document the safety of a recombinant ALVAC-KSA vaccine with or without the adjuvant cytokine GM-CSF and to analyze the anti-Ep-CAM-specific immune response in CRC patients during a 46 week period of follow-up.

MATERIALS AND METHODS

Patients. Twelve patients, 10 males and 2 females, who had been operated on for American Joint Committee on Cancer stage I (n = 3), II (n = 5), and III (n = 4) CRC with no remaining tumor entered the study. Five patients had colon cancer, whereas in 7 patients the primary tumor was located to the rectum. The median age was 66.5 years (range, 46–78 years). ALVAC-KSA (10^7.09 CCID50/ml per immunization; Aventis-Pasteur, Lyon, France) was administered intradermally/s.c. in weeks 0, 3, and 6. The first 6 patients received ALVAC-KSA alone (ALVAC-KSA group). The subsequent 6 patients were given recombinant human GM-CSF (Escherichia coli-derived; 75 μg/day; Leucomax, Schering-Plough/Novartis, Kenilworth, NJ) intradermally/s.c. once a day, on days –1, 0, 1, and 2 (4 days) at each immunization at the same site as the ALVAC-KSA injection (ALVAC-KSA/GM-CSF group). The Ethical Committee of the Karolinska Institute approved the study, and informed consent was obtained from each patient.

Clinical Examination and Follow-Up. Before immunization, a complete case history was obtained, and physical examination, laboratory tests [hemoglobin, WBC with a differential, platelet count, blood chemistry including creatinin, electrolytes, albumin, liver function tests, thyroid function test, and serum tumor markers (CEA, CA19-9, CA50)], and standard urine analysis were performed. The patients were monitored at regular intervals (see “Results”). All of the patients were fol-lowed for immune responses during 46 weeks from start of immunization and clinically for a median time of 18 months (14–25 months). Local and systemic adverse events were evaluated according to Common Toxicity Criteria—National Cancer Institute of Canada guidelines.4

Antigens for in Vitro Testing. For in vitro testing, the extracellular domain of the Ep-CAM protein was used, produced in a baculovirus expression system according to Strassburg et al. (15). Serum-free medium SP900II (Life Technologies, Inc., Paisley, Scotland) was used during the whole culture period, and detergent was omitted from the elution buffer in the purification step based on immunoaffinity chromatography. In patient no 13 (HLA-A2+), three HLA-A2 restricted 9–11 amino acid long Ep-CAM-derived peptides (263–271) (GLKAGVIAV), p184–193 (ILYENVITI), and p174–184 (YQLDPKFTISI; 10 μg/ml) were also used. The peptides were selected on binding affinity for the HLA-A2 molecule [IC50 (μM) 5, 5, and 2, respectively] and the capacity to form stable peptide-MHC-complexes [decay time 50% (h) > 4] according to Ras et al. (16). An HIV-reverse transcriptase-derived peptide (ILKEPVHG) was used as a negative control in the peptide experiment. The peptides were purchased from Thermo Hybaid GmbH (Ulm, Germany). The ALVAC vector without any inserted gene was also used as a control.

ALVAC-KSA Vaccine. ALVAC-KSA is a recombinant canarypox virus expressing the full-length Ep-CAM gene. The vaccine was manufactured under GMP conditions by Aventis Pasteur (Marcy, France). The vCP 325 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 (17). The vaccine was filled in vials containing 10^7.09 CCID50. Vaccine vials were kept between +2 and +8°C until the reconstitution. The material was diluted with sterile saline to a total volume of 1.0 ml before administration to the patient. A minimum of 0.3 ml was injected intradermally and the remaining volume s.c. The injections were given at the same site.

Isolation of PBMCs. PBMCs were obtained by centrifugation of heparinized venous blood on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient (density: 1.077 g/ml). The cells were washed three times in PBS (11). PBMCs were used freshly isolated or cryopreserved. For cryopreservation, 20 × 10^6 PBMC/ml in complete medium (RPMI 1640; Life Technologies, Inc., Paisley, Scotland) was used during the whole culture period, and detergent was omitted from the elution buffer in the purification step based on immunoaffinity chromatography. In patient no 13 (HLA-A2+), three HLA-A2 restricted 9–11 amino acid long Ep-CAM-derived peptides (263–271) (GLKAGVIAV), p184–193 (ILYENVITI), and p174–184 (YQLDPKFTISI; 10 μg/ml) were also used. The peptides were selected on binding affinity for the HLA-A2 molecule [IC50 (μM) 5, 5, and 2, respectively] and the capacity to form stable peptide-MHC-complexes [decay time 50% (h) > 4] according to Ras et al. (16). An HIV-reverse transcriptase-derived peptide (ILKEPVHG) was used as a negative control in the peptide experiment. The peptides were purchased from Thermo Hybaid GmbH (Ulm, Germany). The ALVAC vector without any inserted gene was also used as a control.

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Proliferation Assay. PBMCs (10^5 cells/well) suspended in complete medium (see above) supplemented with l-glutamine (2 mM) and antibiotics (100 IU penicillin and 100 μg streptomycin/ml) were cultured in 96-well round-bottomed microtiter plates (Göteborgs Termometerfabrik, Stockholm, Sweden) at 37°C in humidified air with 5% CO2 for 6 days. Ep-CAM and a BCP (11) were added at concentrations of 0.1 ng/ml to 100 ng/ml, respectively. PHA (10 μg/ml) and purified protein derivate (2.5 μg/ml) were used as positive controls. During the

last 18 h of incubation 1 μCi/well [3H]thymidine (specific activity 5 Ci/mmol; Amersham, Life Sciences, Amersham, United Kingdom) was used. An automatic cell harvester (Skatron, Lier, Norway) was used. Radioactivity was measured in a liquid scintillation counter (Wallac, Åbo, Finland). The results are expressed as the mean of triplicates. A SI was calculated for each triplicate by dividing the mean radioactivity (cpm) of stimulated cells by that of unstimulated cells. 100 ng/ml of Ep-CAM as well as of the control protein gave the highest SI value and was used.

The proliferative response of healthy control donors (n = 32) against Ep-CAM was 1.49 ± 1.46 (mean ± 2 SD). On the basis of these results a SI of ≥3.0 for Ep-CAM was considered a positive response. A cutoff level for the native ("empty") vector cannot be established, as healthy individuals might have a specific immune response against poxvirus-derived or cross-reacting proteins. Therefore, a cutoff level of ≥3.0 was also used for the native vector without the foreign gene. The proliferative response to PHA in patients was 99.9 ± 12 (mean ± SE) and in healthy controls (n = 18) 139 ± 28.3 (not statistically significant). The corresponding figures for PPD were 64.5 ± 8.6 and 316 ± 89.2, respectively (P < 0.0001). The results indicate that the immune system in this respect was rather well preserved in patients but somewhat impaired compared with healthy control donors. BCP was included as a negative control, and SI using this antigen was 0.93 ± 0.05. There were no significant changes over time in the response against PHA, PPD, and BCP, respectively (data not shown).

**ELISPOT Assay.** The ELISPOT assay was used for detection of IFN-γ producing cells. Multiscreen 96-well nitrocellulose membrane bottomed-plates (Millipore, Bedford, MA) were coated with 100 μl/well of mouse monoclonal antihuman IFN-γ (10 μg/ml; clone 1-D1K; Mabtech AB, Stockholm, Sweden) at 4 °C overnight. After three manual washes with PBS, 100 μl aliquots of freshly prepared PBMCs were plated at a concentration of 10^6 cells/well. Cells were stimulated with Ep-CAM and BCP (10 ng/ml-1000 ng/ml), respectively, and HLA-A2 restricted Ep-CAM derived peptides (10 μg/ml) for 20 h in humidified air with 5% CO_2 at 37 °C. Cells stimulated with PHA (10 μg/ml) or PPD (2.5 μg/ml) were used as positive controls. After six washes with PBS containing 0.05% Tween 20 by using an automated plate washer (Hettich Labinstruments AB, Stockholm, Sweden), wells were incubated with 100 μl/well of mouse antihuman IFN-γ (1 μg/ml; clone 7-B6-1; Mabtech) for 2 h at 37 °C. After six wash steps as above, 100 μl of streptavidin-alkaline phosphatase (1:1000; Mabtech) was added for 1 h at room temperature. After an additional six washings, 100 μl of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (BCIP/NBT; Sigma, St. Louis, MO) was added. Color development was stopped by washing under running tap water after 10 min. The number of spots corresponding to cells secreting IFN-γ was quantified using an automated computer assisted video imaging analysis system (Axioplan 2; Carl Zeiss Vision, Jena, Germany). Results are expressed as mean number of spots of duplicate wells after subtraction of the mean number of spots of unstimulated cells (background). In healthy control donors (n = 14) the highest number of IFN-γ-secreting cells at any concentration used in response to Ep-CAM was 0.5 ± 1.9/10^5 PBMCs (mean +2 SD). The corresponding values for BCP were 1.0 ± 2.7 spots/10^5 PBMC. On the basis of these results, the presence of ≥5 spots/10^5 PBMC was considered a cutoff level for Ep-CAM-reactive T cells. Ep-CAM as well as HIV-RT-derived peptides induced ≤5 spots/10^5 PBMC in all of the HLA-A2-positive healthy control donors (n = 6). Thus, ≥5 spots/10^5 PBMC was considered a positive response also in the peptide ELISPOT assay. A cutoff level against the vector cannot be established (see above) and, therefore, ≥5 spots/10^5 PBMC was also applied in this test. The response against PHA in patients was 375 ± 13 (mean ± SE) spots/10^5 PBMC and in healthy controls (n = 7) 326 ± 53 spots/10^5 PBMC (not statistically significant). The corresponding figures for PPD were 149 ± 18 spots/10^5 PBMC and 134 ± 28 spots/10^5 PBMC, respectively (not statistically significant). These results indicate that the immune competence of patients and controls in this respect was comparable. There were no significant changes over time in the response against PHA, PPD, and BCP, respectively (data not shown).

**Real-Time PCR for IFN-γ.** PCR assays were performed as described in detail elsewhere (18). PBMCs were stimulated with the Ep-CAM protein (100 ng/ml) or the native ALVAC vector (1 viral particle/cell) alone for 20 h. Total RNA was extracted using the guanidine thiocyanate phenol-chloroform extraction method. First-strand cDNA was synthesized using 1 μg of total RNA. The cytokine (IFN-γ) gene expression was quantified using ABI prism 7700 Sequence Detection system (PE Applied Biosystems) as described previously (19, 20). Primers and probes were designed to span exon junction to prevent amplification of possible contaminating genomic DNA (21). Using labeled probes, fluorescence signals were generated during each PCR cycle via the 5’-3’ endonuclease activity of Ampli Taque Gold DNA polymerase. The β-actin gene was used as the endogenous and positive control. PCR premixes containing all of the reagents except for the target template were used as the negative control.

The level of each target cytokine is considered 1 for unstimulated PBMCs. The relative quantitative expression of cytokine genes in unstimulated and stimulated cells was determined using the arithmetic equation 2^-ΔΔCT according to Perkin-Elmer instruction manual (User bulletin #2, 1997). The amount of target gene was normalized to an endogenous reference gene (β-actin) at each stage. The calculation of ΔΔCT involves subtraction of ΔCT in unstimulated from stimulated cells for each cytokine. The relative increase of a cytokine was 2^-ΔΔCT subjects − ΔCT controls). ΔCT was calculated by subtraction of the endogenous control CT and the target cytokine CT (Perkin-Elmer instruction manual, 1997 #108; Ref. 22). In healthy control donors (n = 10) the IFN-γ response to Ep-CAM was 3.27 ± 6.53 (mean ± 2 SD). A relative value of ≥10 was considered a positive anti-Ep-CAM response.

**Anti-Ep-CAM and Anti-ALVAC IgG Antibodies.** Antibodies against Ep-CAM and the native vector (ALVAC) were assayed using flat-bottomed polystyrene high binding (Costar, Cambridge, MA) and Falcon PRO-BIND (Becton Dickinson, Franklin Lakes, NJ) microtiter ELISA plates, respectively. Plates were coated at 4 °C overnight with 100 μl of Ep-CAM (2.5 μg/ml), BCP (2.5 μg/ml), or ALVAC (1 μg/ml) in 0.05 M carbonate buffer (Sigma). After three washes, the wells were
blocked with PBS containing 0.05% Tween 20 and 1% gelatin (Sigma) for 2 h at room temperature. Serum samples were diluted serially (starting dilution 1:50) in blocking buffer and incubated overnight at 4°C (100 μl/well). Wells were washed and incubated with peroxidase-conjugated rabbit anti-human IgG (1:10000; DAKO, Glostrup, Denmark) for 2 h at 37°C. Serum samples were blocked with PBS containing 0.05% Tween 20 and 1% gelatin (Sigma) and incubated at room temperature for 15 min. The enzymatic reaction was stopped by adding 50 μl 3% H2SO4. The absorbance was measured at 490 nm (test) and 630 nm (reference) wavelength using VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). Results were analyzed by using the SOFTmax PRO software program (Molecular Devices, Sunnyvale, CA).

The development was carried out by adding 100 μl O-phenylenediamine dihydrochloride (Sigma) and incubated at room temperature for 15 min. The enzymatic reaction was stopped by adding 50 μl 3% H2SO4. The absorbance was measured at 490 nm (test) and 630 nm (reference) wavelength using VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). Results were analyzed by using the SOFTmax PRO software program (Molecular Devices) and are expressed as arbitrary ELISA units/ml (EU/ml) in relation to the corresponding standard curves. Samples were tested against Ep-CAM in triplicate wells and duplicates for ALVAC. The working standards for Ep-CAM and ALVAC-coated plates were pooled human sera (concentrations ranged between 0.4–100 EU/ml and 0.12–30 mEU/ml, respectively). The IgG concentration for each dilution of a sample was calculated from the standard curve. The titer of a sample is expressed as mean titer of at least two dilutions where the concentration coefficient was <15%. The titers against the control protein BCP were 0.23 ± 0.02 (mean ± SE) in patients and 0.47 ± 0.10 in controls. There was no change in these titers over time (data not shown).

Criteria for an Ep-CAM-specific Cellular Response.
A patient was considered to have developed an Ep-CAM-specific cellular response if a proliferative response (SI ≥ 3.0) and/or a cytokine response (ELISPOT ≥ 5 spots/105 PBMC) was recorded at least two different time points. Healthy donors were used to determine the baseline (cutoff level) for an Ep-CAM-specific humoral and cellular response, because we have shown previously that healthy donors do not seem to have an auto-anti-EpCAM immune response (5, 23, 24).

Statistical and Kinetic Evaluation.
Differences between groups were assessed using Mann-Whitney U test (Graph Pad Instat version 3.0; Mountain View, CA). The concentration-time curves (for ELISPOT and IgG antibodies) were adjusted to the data sets for the patient groups via nonlinear iterative least square regression analysis. area under concentration-time curve and maximum antibody concentration were calculated using Winnonlin version 2 (Pharsight, CA).

RESULTS
Clinical Characteristics of the Patients and Adverse Reactions to Immunizations.
Clinical characteristics of the patients are shown in Table 1. The vaccinations were well tolerated. No autoimmune reactions were observed. However, in the ALVAC-KSA/GM-CSF group, adverse events were more frequent and pronounced. Injection site reactions were more common, with pain, redness, swelling, and tenderness. In the ALVAC-KSA group only grade 1–2 skin reactions were observed, whereas in the ALVAC-KSA/GM-CSF group, 3 of 6 patients had grade 3 skin reactions were observed, whereas in the ALVAC-KSA/GM-CSF group, 3 of 6 patients had grade 3 local injection site reactions. The frequency and the intensity of the local reactions increased after each immunization (Table 2). The skin reactions (induration/redness) could be noted up to 40 weeks after the last immunization. Induration disappeared at 12 weeks after the last immunization in the ALVAC-KSA group but remained in the ALVAC-KSA/GM-CSF group for 46 weeks.

Considering all of the measurements during the 46 weeks of observation, a greater area of redness with a median of 6.27 cm2 was observed in patients treated with ALVAC-KSA/GM-CSF compared with 2.2 cm2 in patients receiving ALVAC-KSA (P = 0.06). However, induration at the injection site was highly statistically significant when patients treated with ALVAC-KSA/GM-CSF or ALVAC-KSA alone were compared (5.54 cm2 versus 0.63 cm2; P = 0.002).

No serious adverse event related to the study medication was reported. Systemic adverse events were of minor clinical significance. There were 53 such events recorded as possibly, probably, or definitely related to the study medication in the ALVAC-KSA/GM-CSF group as compared with 24 events in the ALVAC-KSA group (P = 0.01). The majority of the side effects were of grade 1
Anti-Ep-CAM Immune Responses

**Proliferative T-Cell Response.** None of the patients had a positive response against Ep-CAM before vaccination. An anti-Ep-CAM proliferative response was not seen in any of the patients during the 46 weeks of follow-up, although at some time points, a positive response was occasionally recorded (Table 3).

**IFN-γ Cytokine Secreting T Cells (ELISPOT).** A weak detectable IFN-γ response against Ep-CAM was induced in two patients (#3 and #6) in the ALVAC-KSA group. The number of spots was low, just above cutoff level (Table 3). In the ALVAC-KSA/GM-CSF group a strong anti-Ep-CAM IFN-γ response was detected in 5 of the 6 patients (#8, #9, #11, #12, and #13). In the ALVAC-KSA/GM-CSF group compared with the ALVAC-KSA group, an overall significantly higher number of IFN-γ secreting T cells was noted over the 46 weeks observation period \( (P = 0.0078; \text{Fig. 1}) \). Interestingly, induction of a response was not clearly noted until 3 months after the last immunization.

Patient no 13 (HLA-A2\(^+\)) was also tested at week 18 for peptide response against three HLA-A2-restricted 9–11 amino acid long peptides in ELISPOT. A positive IFN-γ response, 10 spots/10\(^5\) PBMC, was noted against p\(_{174-184}\) (YQLDPKFITSI), whereas no response was detected against the 2 other Ep-CAM-derived peptides p\(_{263-271}\) and p\(_{184-193}\), as well as against the control HIV peptide.

**Real-Time PCR for IFN-γ.** An Ep-CAM-specific immune response in the ALVAC-KSA group during immunization and follow-up could either not be detected or at best was very weak when using the proliferation assay and ELISPOT. The more sensitive technique real-time RT-PCR for IFN-γ detection was also applied in this group to analyze biological samples collected during the first 34 weeks of follow-up. As can be seen in Table 4, a PCR-positive response was noted at a low level for

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### Table 2
Skin reaction at injection site in CRC patients vaccinated with ALVAC-KSA and ALVAC-KSA/GM-CSF, respectively

<table>
<thead>
<tr>
<th>Skin reaction</th>
<th>Patient group</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>14</th>
<th>18</th>
<th>26</th>
<th>46</th>
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<tr>
<td>Induration (cm(^2))</td>
<td>ALVAC-KSA (n = 6)</td>
<td>0(^{a})</td>
<td>0.6</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>ALVAC-KSA/GM-CSF (n = 6)</td>
<td>0.0 (0–0.6)(^{b})</td>
<td>0.6 (0–0.6)</td>
<td>0.8 (0–1.0)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Redness (cm(^2))</td>
<td>ALVAC-KSA (n = 6)</td>
<td>0.6</td>
<td>1.0</td>
<td>3.1</td>
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<td></td>
<td>ALVAC-KSA/GM-CSF (n = 6)</td>
<td>0.0 (0.3–10.5)(^{b})</td>
<td>0.8 (0–14)</td>
<td>0.4 (0–16)</td>
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\(^{a}\) Median value.
\(^{b}\) Range.

### Table 3
Anti-Ep-CAM cellular immune responses in CRC patients vaccinated with ALVAC-KSA and ALVAC-KSA/GM-CSF evaluated by proliferation assay and ELISPOT (IFN-γ)

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>Weeks from start of immunization</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>14</th>
<th>18</th>
<th>26</th>
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<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>1(^{a})/(^{b})</td>
<td>1/1</td>
<td>1/1</td>
<td>1/2</td>
<td>1/1</td>
<td>1/0</td>
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<tr>
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\(^{a}\) Proliferation assay.
\(^{b}\) ELISPOT shown as number of spots/10\(^5\) PBMCs. Bold characters represent an Ep-CAM specific response (SI ≥3 and number of spots/10\(^5\) PBMC ≥5).
\(^{c}\) ND, not determined.

(68 of 77) and the of remaining grade 2 (7 of 77) or of grade 3 (2 of 77; those two events, asthenia and malaise, occurred in the same patient). Most commonly, fever, malaise, myalgia, and asthenia were experienced by the patients. Hematological analyses as well as blood, kidney, and liver function tests remained within the normal ranges during the observation period. No abnormalities were found in urine specimens.

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Recombinant ALVAC-KSA in Colorectal Carcinoma Patients

The number of cells was 172 \(\times 10^5\) PBMC (mean \(\pm\) SE). In healthy donors (\(n = 6\)) the corresponding number of IFN-\(\gamma\)-secreting cells was 18.2 \(\pm\) 17.3/10^5 PBMC, but only 3 of the healthy donors had \(\geq\)5 spots. The IFN-\(\gamma\) response increased after repeated immunizations, and then gradually declined (Fig. 2B). There was no statistically significant difference in the number of antivector IFN-\(\gamma\)-secreting cells between the ALVAC-KSA group and the ALVAC-KSA/GM-CSF group.

**Real-Time PCR for IFN-\(\gamma\).** A vector-specific IFN-\(\gamma\) response before vaccination was confirmed at the gene level. Five CRC patients were tested before vaccination, and the relative level of gene expression was 902 \(\pm\) 603 (mean \(\pm\) SE). In 10 healthy donors the response against the ALVAC vector was 690 \(\pm\) 369 (mean \(\pm\) SE; not statistically significant).

**Anti-ALVAC Antibody Response.** Similar antibody titers were found against the ALVAC vector in healthy donors (\(n = 30\); 0.896 \(\pm\) 0.679 mEU/ml) and in patients (\(n = 12\)) before therapy (1.25 \(\pm\) 0.75 mEU/ml; mean \(\pm\) SD; not significant). A significant increase in anti-ALVAC titers was observed in all of the patients already after the first vaccination (14.66 \(\pm\) 11.29 mEU/ml). The highest anti-ALVAC titers were elicited after the third immunization (at week 10), which was followed by a gradual decrease (Fig. 3). A highly significantly greater area under concentration-time curve for anti-ALVAC IgG antibodies (3076 \(\pm\) 489 mEU/ml \(\times\) week) for ALVAC-KSA/GM-CSF-treated patients compared with patients treated with ALVAC-KSA (1978 \(\pm\) 321 mEU/ml \(\times\) week) was found (\(P = 0.0001\)). Patients treated with ALVAC-KSA/GM-CSF also showed a significantly higher antibody tier (201 mEU/ml) at week 10 compared with that observed in patients treated with ALVAC-KSA (136 mEU/ml; \(P = 0.004\)). A significant difference in antibody titers remained at the last testing time (week 46; \(P = 0.007\)). Reciprocal end point titers ranged between 100 and 24,300.

**DISCUSSION**

This is the first clinical trial documenting the safety and immunogenicity of an avipox viral vector expressing the tumor antigen Ep-CAM (ALVAC-KSA) in patients with CRC. The ALVAC vector was selected because of its well-documented favorable safety profile in humans, the capacity to accommodate large size inserts (30 kB), and a relative easiness to produce in large quantities. Because of the nonreplicative nature of the virus in human cells, the ALVAC vector also seems to elicit a lower antivector immune response when compared with a replicative attenuated vaccinia virus (25). Delivery of a tumor antigen (DNA) by a viral vector may ensure antigen presentation through the class I antigen presentation pathway and thereby a CTL response (26). After infection of cells with the ALVAC-KSA construct, efficient Ep-CAM surface expression was seen indicating that ALVAC-KSA is phenotypically stable.\(^5\)

We have shown previously that GM-CSF was mandatory to induce a strong humoral and cellular response against the protein antigen CEA (11). In the present study half of the patients received local administration of a low dose of the ad-

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\(^5\) P. Moingeon, unpublished observations, Aventis-Pasteur/Virogenetics Corp. File on site.
juvant cytokine GM-CSF. Both a proliferation assay and a
cytokine assay were applied to detect an Ep-CAM-specific
immune response. These assays may not overlap, as antigen-
specific cells may respond to antigen stimulation by prolifera-
tion and/or cytokine production dependent on involved T-cell
subsets and stages of maturation (27, 28). Combination of these
two assays should give satisfactory information with regard to
the presence of antigen-specific T cells.

Two of 6 patients (#3 and #6) in the ALVAC-KSA group
developed a weak Ep-CAM-specific T-cell response. The weak
response in this group is additionally corroborated by real-time
PCR, a technology that might be more sensitive than ELISPOT
(29). At a few testing times, ELISPOT was marginally positive
but real-time PCR negative, which may be explained by already
degraded mRNA, as the time-kinetics for cytokine gene expres-
sion is highly variable.6 Cytokines as IFN-
and interleukin 2
were shown to have a peak in the transcript level after
peptide stimulation, whereas other genes such as tumor necrosis
factor peaked later (30). We performed 20 h of protein
activation, and we have noted that IFN-
transcript levels meas-
ured by quantitative RT-PCR had a maximum fold increase at
3–4 h but remained at a high level after 20 h of protein
activation and even for a longer time period (data not shown). In
the ALVAC-KSA/GM-CSF group 5 of 6 patients (all but patient
#10) mounted an Ep-CAM-specific T-cell response, which was

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**Table 4** Anti-Ep-CAM cellular immune response in CRC patients vaccinated with ALVAC-KSA evaluated by ELISPOT and real-time RT-PCR for IFN-γ

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* ELISPOT, number of spots/10⁵ PBMC (see Table 3).
* RT-PCR, Relative expression (a value ≥10 was considered an Ep-CAM specific response). Bold characters represent an Ep-CAM specific response.
* ND, not determined.

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Fig. 2 Cellular immune response against the “empty” ALVAC vector in CRC patients vaccinated with ALVAC-KSA (■) or together with the adjuvant cytokine GM-CSF (◆). A, DNA synthesis (SI); B, IFN-γ secreting T cells (ELISPOT). Arrows indicate vaccination times; bars, ±SE.

Fig. 3 Kinetics of anti-ALVAC IgG antibodies in CRC patients vaccinated with ALVAC-KSA alone (□; n = 6) or together with the adjuvant cytokine GM-CSF (◆; n = 6). The areas under the curves were highly statistically significant different (P = 0.0001). Arrows indicate vaccination times; bars, ±SE.

Two of 6 patients (#3 and #6) in the ALVAC-KSA group developed a weak Ep-CAM-specific T-cell response. The weak response in this group is additionally corroborated by real-time PCR, a technology that might be more sensitive than ELISPOT (29). At a few testing times, ELISPOT was marginally positive but real-time PCR negative, which may be explained by already degraded mRNA, as the time-kinetics for cytokine gene expression is highly variable.6 Cytokines as IFN-γ and interleukin 2 were shown to have a peak in the transcript level after ∼3 h of peptide stimulation, whereas other genes such as tumor necrosis factor α peaked later (30). We performed 20 h of protein activation, and we have noted that IFN-γ transcript levels measured by quantitative RT-PCR had a maximum fold increase at 3–4 h but remained at a high level after 20 h of protein activation and even for a longer time period (data not shown). In the ALVAC-KSA/GM-CSF group 5 of 6 patients (all but patient #10) mounted an Ep-CAM-specific T-cell response, which was

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6 H. Mellstedt, unpublished observations.
strong based on ELISPOT. The T-cell response was of type 1 (IFN-γ), and no type 2 cells (interleukin 4) were induced (data not shown). Furthermore, in 1 tested HLA-A2+ patient, an 11-mer Ep-CAM-derived peptide fitting to the HLA-A2 molecule induced a IFN-γ response. No IgG antibody response against Ep-CAM was evoked in either group.

The reason for the weak response against Ep-CAM using the ALVAC construct alone, even when applying the sensitive quantitative RT-PCR as a read-out system, is not clear. However, it should be underlined that we did not use antigen re-stimulation but fresh activated PBMCs, which more accurately reflects the in vivo situation (30). An explanation might be that we immunized too few times. Furthermore, viral infection (ALVAC construct) might down-regulate MHC class I expression rendering antigen-presenting cells less efficient (31), and the addition of GM-CSF may counteract this effect and up-regulate MHC class I and II molecules (32). It is unlikely that the poor Ep-CAM response was linked to an overall status of a poor immune responsiveness often observed in cancer patients, as they mounted a strong response against the native vector. Using an ALVAC-CEA construct it has been shown that three immunizations did not induce a CEA-specific cellular response, and more immunizations were necessary. The best immune response was obtained when immunizations with ALVAC-CEA was combined with a vaccinia vector (33).

It is also clear from the present study, as has been shown previously using other antigens (34), that the addition of the adjuvant cytokine GM-CSF augmented the immune response significantly. However, this is in contrast to another pilot trial using the ALVAC-CEA B7.1 construct and GM-CSF, where the addition of GM-CSF impaired immunity induction against CEA (35). The reason for the discrepancy is not clear. However, in that study the dose of GM-CSF was much higher, the schedule different, and the patients had metastatic disease. Dose and schedule of GM-CSF have been shown in animal studies to be critical (36–38). We have also shown recently that immunization with the Ep-CAM protein together with GM-CSF induced a strong T-cell response as well as IgG antibodies (39). In that study, a positive IFN-γ response in HLA-A2+ patients was also seen against p174–184 as well as against p184–193 but not p263–271 (Ref. 39; data to be published). In a study by Trojan et al. (40) CTL could be generated from healthy HLA-A2+ donors after repeated stimulation in vitro against p174–184, p184–193, and p263–271. Nagorsen et al. (41) tested HLA-A2+ CRC patients for a natural occurring IFN-γ response only against p263–271 and could show the presence of such T cells in patients with metastatic disease but not with limited disease.

As expected when using a viral vector capable to express the antigen in the cytosol of antigen-presenting cells a type 1 T-cell response was induced (42). Interestingly, there is a significant body of experimental evidence that T-cell responses to tumor antigens by way of IFN-γ secretion appear to be associated with conditions that favor tumor regression (43–45). However, the time kinetics of the immune response was somewhat unexpected. The pattern was similar in all 7 of the patients. No response was practically detected before week 10 i.e., 1 month after the last immunization. In the majority of the patients the response peaked 4–5 months after the last immunization. However, this was not the case for the immune response against the viral vector, which peaked after the last immunization. Using other ALVAC constructs, this type of time kinetics for the immune response against the inserted antigen has not been reported (10, 33, 35, 46). The mechanisms behind the particular response pattern are not known and might be because of different levels of tolerance established against various TAAs (3), but clearly the immune response against the tumor antigen was different from that against the viral proteins.

None of the 12 patients mounted an IgG antibody response against Ep-CAM. In other studies, using ALVAC constructs expressing foreign genes, about 5–20% of the patients developed IgG antibodies (33, 47, 48). A poor antigen-specific antibody response against the foreign gene using the ALVAC construct as a delivery system is not only seen against TAAs but also against, e.g., Japanese encephalitis proteins (49). Thus, the ALVAC construct may predominantly induce a cellular response. To induce a high titer IgG antibody response, priming with the ALVAC vector followed by a boost with the protein might be necessary as shown in HIV vaccination protocols conducted in humans (50).

Systemic side effects were mild and more frequently noted in the ALVAC-KSA/GM-CSF group. However, local reactions were pronounced, especially in the ALVAC-KSA/GM-CSF group, and persisted for 9 months after the last immunization. This kind of local reaction has not been observed in our previous vaccination trials using protein tumor antigens (idiotype, CEA, and Ep-CAM) together with local administration of GM-CSF (3, 5, 11). Thus, the long-lasting skin reaction was obviously dependent on the ALVAC-KSA construct and potentiated by GM-CSF. No autoimmune phenomenon was observed, although Ep-CAM is expressed in a variety of tissues (2).

All of the patients studied, as well as healthy controls, had a cellular response spontaneously against the vector, predominantly an IFN-γ response, whereas the proliferative response seemed to be weak, which may indicate that the prevaccination antivector response was mainly MHC class I restricted (28). The reason for the significantly higher antivector IFN-γ T-cell response in CRC patients as compared with healthy individuals is not clear. There is only one previous report on the anti-ALVAC response in CRC patients. Low levels of proliferative and IFN-γ responses against the ALVAC vector before vaccination were noted both in patients and healthy controls (47). However, the patients in that study had metastatic disease and had received chemotherapy suggesting that they were immunosuppressed, which was not the case for our patients. Furthermore, the level of antivector T-cell response of healthy donors in that study was comparable with our study. A memory T-cell response is probably because of cross-reactivity with human poxvirus proteins (e.g., vaccinia). All of the patients mounted a strong humoral and cellular response against the vector. An anti-ALVAC IgG antibody response in ALVAC-CEA B7.1 vaccinated patients has been observed previously (46), but a cellular response has not been reported. The clinical significance of a humoral and cellular immune response against the vector is not yet known but it may hamper subsequent immunizations even if patients have been vaccinated up to 16 times with the ALVAC-vector
with apparently no impairment of the immune response against TAA.7

In summary, ALVAC-KSA in combination with low dose local administration of GM-CSF induced a strong anti-EpCAM-specific type 1 T-cell response. The results of this preliminary study support the additional development of ALVAC-KSA as a cancer vaccine. Improvement of the vaccine efficacy and immunogenicity might be done by increasing the number of immunizations and/or combining ALVAC-KSA with vaccinia-KSA. The prime-boost concept, priming with DNA and boosting with the protein, might represent another effective approach to induce CD4 and CD8 T cells, as well as antibodies.

ACKNOWLEDGMENTS

We thank Birgitta Hagström for skilful technical assistance, and Gerd Stühlenberg and Gunilla Burén for excellent secretarial help.

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Immunization of Colorectal Carcinoma Patients with a Recombinant Canarypox Virus Expressing the Tumor Antigen Ep-CAM/KSA (ALVAC-KSA) and Granulocyte Macrophage Colony-stimulating Factor Induced a Tumor-specific Cellular Immune Response

Gustav J. Ullenhag, Jan-Erik Frödin, Szilvia Mosolits, et al.