Dendritic Cell Vaccination in Medullary Thyroid Carcinoma

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ABSTRACT

Purpose: Prognosis and treatment effectiveness for medullary thyroid carcinoma (MTC) are strictly related to tumor stage. Palliative treatment options show no significant benefit. A promising treatment approach for human cancer is based on the vaccination of autologous dendritic cells (DCs).

Experimental Design: The objective of this study was to evaluate the effectiveness of DC vaccines in MTC patients. Therefore, we generated autologous tumor lysate-pulsed DCs from 10 patients suffering from advanced MTC for repeated vaccination. Mature DCs were derived from peripheral blood monocytes by using CD14 magnetic bead selection and subsequent culture in the presence of granulocyte macrophage colony-stimulating factor, interleukin 4, and tumor necrosis factor α with or without addition of IFN-γ. DCs were loaded with tumor lysate and further injected into a groin lymph node. Toxicity, tumor marker profile, immune response, and clinical response were determined.

Results: Vaccination was well tolerated and induced a positive immunological response in all of the tested patients as evaluated by in vivo delayed-type hypersensitivity reactivity or in vitro intracytoplasmic IFN-γ detection assay. Three patients had a partial response, 1 patient presented a minor response, and 2 patients showed stable disease. The remaining 4 patients had progressive disease.

Conclusions: These data provide strong evidence that vaccination with tumor-lysate pulsed DCs results in the induction of a specific immune response in patients suffering from MTC. Objective clinical responses could be observed even for far-advanced disease. Therefore, we suggest that MTC is particularly suited for DC-based immunotherapy.

INTRODUCTION

Medullary thyroid carcinoma (MTC) represents about 5–10% of all thyroid tumors (1, 2). It originates from the parafollicular C cells and occurs in about 70–80% in the sporadic form, whereas the remaining 20–30% are represented by three different familial forms: multiple endocrine neoplasia type 2A; multiple endocrine neoplasia type 2B; and familial MTC not associated with multiple endocrine neoplasia (3).

Calcitonin is the most specific circulating and immunohistochemical marker for MTC and is, therefore, widely used for diagnosis (4). Prognosis and treatment response are closely related to the tumor stage (5). To date, the primary and solely curative treatment of MTC is the total surgical removal of all neoplastic tissue. This requires aggressive and meticulous surgery. The prognosis of patients with unresectable and/or distant metastases of MTC, although poor, is somewhat better than the prognosis of patients for other types of malignancies (6). Palliative treatment options like external beam radiotherapy, radionuclide therapy, and chemotherapy have not been demonstrated to be of significant value in the treatment of MTC (2).

As a result, there has been an extensive search for alternative strategies to treat patients with advanced MTC. Thus far, promising preclinical and clinical results have been obtained using various experimental approaches such as monoclonal antibodies, suicide gene therapy, or immunogene therapy (7–9).

It is well established that the immune system is of critical importance in controlling and eradicating the growth of tumors (10, 11). One of the basic principles thereof is that most tumors are characterized by the expression of tumor-associated antigens. On the basis of these antigens, the tumors are recognized as being “abnormal” by the cellular components of the immune system and subsequently are destroyed (12–14). This phenomenon is largely attributed to cytotoxic T cells, which, given adequate antigen recognition, can be specifically directed against tumor cells. However, a primary T-cell response can only be triggered via interaction with professional antigen-presenting cells. Recent data provide more evidence for the crucial role of these antigen-presenting cells in the immunological antitumor defense and have contributed to the characterization of dendritic cells (DCs) in tumor immunology (15, 16). DCs are the most effective antigen-presenting cells of the immune system and, therefore, play a decisive role in triggering primary immune responses and in augmenting secondary ones. Their unique ability to take up, process, and present antigens, and, subsequently, to activate naïve CD4⁺ and CD8⁺ T cells makes them promising candidates for an experimental immunotherapeutic approach.

This kind of adoptive cellular immunotherapy has already proven effective in the treatment of selected carcinomas such as...
Patients were required to have an expected survival of >1.5 years of age with metastasized MTC were included in this trial. All of the individuals suffered from the sporadic form of MTC. PATIENTS AND METHODS Ten patients between 18 and 75 years of age with metastasized MTC were included in this trial. All of the individuals suffered from the sporadic form of MTC. Substantial responses were achieved with this therapeutic model for MTC using DCs, lymphocytes, and MTC cell lines in vitro. Furthermore, there is also limited evidence that patients suffering from MTC that are treated with carcinoembryonic antigen and calcitonin-loaded DCs show immunological and clinical responses (23). However, the extent to which the application of DC-based immunotherapy is effective in MTC has yet to be investigated more comprehensively. We have established recently an experimental tumor model for MTC using DCs, lymphocytes, and MTC cell lines in vitro under autologous conditions (24). We could demonstrate that mature, tumor lysate-pulsed DCs obtained from patients with MTC can prime a HLA class I-restricted antitumor T-cell response against autologous tumor cells. In the present study, we have translated these findings to the clinical setting. We show the induction of a specific immune response as well as promising clinical responses in patients suffering from advanced MTC.

Table 1  Patient characteristics, therapy and response

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Sites of disease</th>
<th>Outcome/time (mo.) to progression</th>
<th>No. of vaccinations</th>
<th>DTH&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Intracytoplasmic IFNγ assay</th>
<th>Survival (mo.)</th>
<th>Alive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31/M</td>
<td>LNs&lt;sup&gt;c&lt;/sup&gt; (cervical, mediastinal), liver</td>
<td>MR/30</td>
<td>30</td>
<td>pos</td>
<td>n.d.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 + 32</td>
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</tr>
<tr>
<td>2</td>
<td>69/M</td>
<td>Liver, lung, bone</td>
<td>PD</td>
<td>5</td>
<td>pos</td>
<td>n.d.</td>
<td>3 + 4</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>57/M</td>
<td>LNs (cervical, mediastinal), lung</td>
<td>PD</td>
<td>10</td>
<td>pos</td>
<td>n.d.</td>
<td>72 + 9</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>61/M</td>
<td>LNs (cervical, mediastinal), lung, bone</td>
<td>SD/24</td>
<td>20</td>
<td>pos</td>
<td>n.d.</td>
<td>16 + 29</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>26/F</td>
<td>LNs cervical</td>
<td>PR/29</td>
<td>11</td>
<td>pos</td>
<td>pos</td>
<td>2 + &gt;29</td>
<td>Yes</td>
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<tr>
<td>6</td>
<td>39/M</td>
<td>LNs mediastinal, liver, spleen</td>
<td>PR/30</td>
<td>10</td>
<td>n.d.</td>
<td>pos</td>
<td>140 + 30</td>
<td>Yes</td>
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<tr>
<td>7</td>
<td>68/M</td>
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<td>PD</td>
<td>3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>9 + 3</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>48/M</td>
<td>LNs mediastinal</td>
<td>SD/&gt;15</td>
<td>10</td>
<td>n.d.</td>
<td>pos</td>
<td>268 + &gt;15</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>59/M</td>
<td>LNs (cervical, mediastinal)</td>
<td>PR/12</td>
<td>10</td>
<td>n.d.</td>
<td>pos</td>
<td>64 + &gt;12</td>
<td>Yes</td>
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<tr>
<td>10</td>
<td>70/M</td>
<td>LNs (cervical, tracheal)</td>
<td>PD</td>
<td>6</td>
<td>n.d.</td>
<td>pos</td>
<td>1 + 6</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup> DTH, delayed-type hypersensitivity; LN, lymph node; MR, minor response; PD, progressive disease; SD, stable disease; PR, partial response; pos, positive; n.d., not done.

<sup>b</sup> DTH was performed after the 4<sup>th</sup> and after the 10<sup>th</sup> vaccination (if available) and defined as positive for erythemas of > 1.5 cm.

<sup>c</sup> Survival: months after diagnosis until therapy + months after therapy.

malignant melanoma, prostate carcinoma, and renal cell carcinoma (14, 17, 18). Substantial responses were achieved with this therapeutic modality in several clinical studies (19–22). Furthermore, there is also limited evidence that patients suffering from MTC that are treated with carcinoembryonic antigen and calcitonin-loaded DCs show immunological and clinical responses (23). However, the extent to which the application of DC-based immunotherapy is effective in MTC has yet to be investigated more comprehensively. We have established recently an experimental tumor model for MTC using DCs, lymphocytes, and MTC cell lines in vitro under autologous conditions (24). We could demonstrate that mature, tumor lysate-pulsed DCs obtained from patients with MTC can prime a HLA class I-restricted antitumor T-cell response against autologous tumor cells. In the present study, we have translated these findings to the clinical setting. We show the induction of a specific immune response as well as promising clinical responses in patients suffering from advanced MTC.

**PATIENTS AND METHODS**

**Patient Characteristics.** Ten patients between 18 and 75 years of age with metastasized MTC were included in this trial. All of the individuals suffered from the sporadic form of MTC. Patients were required to have an expected survival of ≥3 months; a Karnofsky index of ≥60%; normal or “near-to-normal” renal, hepatic, and hematopoietic function; and not to have received any chemotherapy, radiotherapy, or immunotherapy for at least 6 months before study enrollment. Patients with antibodies against HIV-1/2, human T-cell lymphotrophic virus-1/2, hepatitis B or hepatitis C virus, and patients with autoimmune disease were excluded. Premenopausal females were required not to be pregnant and to take effective oral contraception. All of the patients included in the study gave written informed consent. The protocol was approved by the Institutional Ethics Committee and was conducted at the Department of General Surgery at the University of Vienna. Table 1 provides a summary of patient characteristics.

**Generation of DCs from Peripheral Blood.** For preparation of DCs, peripheral blood mononuclear cells were isolated from 100 ml of EDTA-treated whole blood by standardized density gradient centrifugation using Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Thereafter, CD14-positive cells were separated by magnetic sorting using VarioMACS technique (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Briefly, peripheral blood mononuclear cells were incubated with magnetic MicroBeads conjugated with monoclonal mouse anti-human CD14 antibody. Twenty μl of antihuman CD14 antibody were added per 10⁷ peripheral blood mononuclear cells and incubated for 15 min. Cells were washed and centrifuged at 300 × g for 10 min, supernatant was removed, and the cell pellet was resuspended and mounted on a magnetic column. Labeled cells were, thus, positively enriched (95–97%) and subsequently eluted from the column by removal from the magnetic device. Isolated CD14<sup>+</sup> cells were cultured at a concentration of 1 × 10⁶ cells/ml in standard culture flasks (Costar, Cambridge, MA) for 5 days in RPMI 1640 containing 10% FCS and supplemented with 5 mg/ml of gentamicin at 37°C in a humidified 5% CO₂ atmosphere in the presence of 1000 units/ml of recombinant human (hr) granulocyte-macrophage colony stimulating factor (Leukomax; AESCA, Traiskirchen, Austria) and 1000 units/ml of hr interleukin-4 (Pharma Biotechnologie Hannover, Hannover, Germany). On day 2, hr granulocyte-macrophage colony stimulating factor and hr interleukin-4 were again added to the cultures at a concentration of 1000 units/ml. On day 5, immature DCs were meticulously washed to remove FCS and interleukin-4, and subsequently pulsed with autologous whole-tumor lysate in RPMI 1640. During the implementation of this trial, there was no licensed good manufacturing practice technology available for the enrichment of CD14<sup>+</sup> monocytes. However, all of the used cytokines and reagents were good manufacturing practice grade.

**Preparation of Tumor Lysate.** All of the patients underwent surgical exploration. Tumor samples from surgical resections were subjected to histological examinations and further processed to tumor lysate. Tumor samples (~1 cm³) were frozen in liquid nitrogen under aseptic conditions and were treated under laminar flow conditions by mincing with a scalpel and dissolving in 5 ml of PBS (Life Technologies, Inc.). The samples were then lysed by five freeze-thaw cycles. Enzymatic
digestion was not performed to avoid potential alteration of tumor peptides. Samples were centrifuged at 900 × g, and the supernatant was passed through filters of 0.7-μm pore size. The protein concentration was determined according to Bradford.

Antigen Pulsing, Maturation, and Application of DCs. On day 5, tumor-lysate was added to FCS-free DC cultures at a final concentration of 100 μg/ml for 12 h. Thereafter, the culture was washed and incubated for 36 h in RPMI 1640 containing 1000 units/ml of rh granulocyte-macrophage colony stimulating factor and 1000 ng/ml of tumor necrosis factor-α (kindly provided by Dr. Richard Alexander, NIH/National Cancer Institute, Bethesda, MD) to promote DC maturation. In 6 patients (patient no. 5–10, Table 1), 1000 units/ml of IFN-γ (Imukin; Boehringer Ingelheim, Vienna, Austria) were added 12 h before application of DCs. Cells were then gently washed three times and finally dissolved in 300 μl of PBS. An aliquot of the cell culture was tested for sterility (bacteriology and Gram-staining) 2 days before application. Vaccine release criteria included a negative bacterial culture, a negative Gram-staining, and a fully mature DC phenotype confirmed by flow cytometry analysis (see below). The cell suspension was injected into the groin lymph node of the patient under sterile conditions using small part ultrasound performed by a trained specialist. Cautious injection under direct sonographic visualization was carried out to avoid disintegration of the lymph node. Three to 30 DC vaccinations were given at 21-day intervals on an outpatient basis.

Evaluation of DC Phenotype. The phenotype of monocytes, and immature and mature DCs was determined by single or two-color fluorescence analysis. Cells (3 × 10^7) were resuspended in 50 μl of assay buffer (PBS, 2% FCS, and 1% sodium acid) and incubated for 30 min at 4°C with 10 μl of appropriate FITC or phycoerythrin-labeled monoclonal antibodies. After incubation, the cells were washed twice and resuspended in 500 μl of assay buffer. Cellular fluorescence was analyzed in an EPICS XL-MCL flow cytometer (Coulter, Miami, FL). Fifteen thousand events were acquired for each sample, and the percentage of positive cells was reported. Monoclonal antibodies specific for human CD1a, CD3, CD19, CD11c, CD14, CD40, CD80, CD86, and CD83 (Immunotech, Vienna, Austria), and HLA-DR, as well as control IgG1 and IgG2a (Becton Dickinson, San Jose, CA) were used to characterize DCs.

Delayed-Type Hypersensitivity (DTH) Test. The DTH skin test was performed with tumor lysate-pulsed DCs and unpulsed DCs for control. DCs were intradermally injected into the forearm before the first vaccination, after the fourth vaccination, and after the tenth vaccination, when applicable. A positive skin reaction was defined by >1.5 cm erythema and induration of the skin 48 h after intradermal injection.

Intracytoplasmic IFN-γ Detection Assay. Intracellular staining for IFN-γ production of lymphocytes was performed as described recently (25). In brief, 5 × 10^7 peripheral mononuclear cells depleted of the CD14+ cell fraction were obtained before (T0) and after the fourth vaccination (T4), and were cocultured with 1 × 10^6 mature, tumor-lysate pulsed DCs for 18 h in RPMI 1640 containing 10% FCS without any addition of cytokines in a total volume of 2 ml. Monensin (2.5 μM; Sigma) was added during the last 3 h to block protein secretion. T0 and T4 cells were used as controls. Cells were harvested, washed, and permeabilized with Intra-Prep (Immunotech) according to the manufacturer’s protocol. Cells were double stained with phycoerythrin-labeled anti-CD69 or anti-IFN-γ and FITC-labeled anti-CD3-specific antibody (Immunotech). Appropriately labeled IgG1 antibodies were used as isotype controls. Samples were analyzed in an EPICS XL-MCL flow cytometer. Lymphocytes were obtained during the monocyte isolation procedure and were freshly used. IFN-γ assays were defined as positive in case of the appearance or increase of IFN-γ-producing T cells compared with appropriate controls.

Clinical Monitoring. Adverse events were graded according to the WHO toxicity criteria. All of the patients underwent assessment of tumor status at baseline and 3, 6, 9, and 12 months after the first vaccination using computed tomography scan, magnetic resonance imaging, or ultrasound. Disease progression was defined as >25% increase in target lesions and/or the appearance of new lesions. We further used standard response criteria to determine the clinical response.

In all of the patients, tumor marker monitoring was performed at the beginning of the study and after each vaccination cycle. Autoantibodies were analyzed before, and at least twice during, the vaccination period. The following autoantibodies were determined: rheumatoid factors; antinuclear antibodies (ab); antihistone ab; anti-dsDNA ab; anti-Ro/SSA ab; anti-La/SSB ab; anti-U1-RNA ab; anti-sm ab; antithyreoglobulin ab; antineutrophil cytoplasmic ab; antithyroid ab; antismooth muscle ab; antiparietal cell ab; antmitochondrial ab; anti-insulin ab; and antipancreatic islet ab.

RESULTS

In accordance with the protocol, a total of 10 patients were enrolled in the study from April 1999 to April 2003. All of them were vaccinated with autologous, tumor lysate-pulsed, monocyte-derived DCs. Except for patient 8, who received IFN-α-2b (Intron A) 4 years before study entry, none of the patients had received any prior therapy. Patient characteristics, including sites of disease (metastases), duration of disease before study entry, and response criteria, are summarized in Table 1. A total of 115 vaccinations were performed (3–30 per patient).

DC Phenotype. Sufficient quantities of mature DCs could be generated for each vaccination (2 × 10^7–4 × 10^7). DCs were analyzed by flow cytometry for the presence of antigens shown to be characteristic for monocyte-derived mature DCs. DCs expressed high levels of CD1a, CD11c, CD40, CD80, CD86, and HLA-DR, as well as control IgG1 and IgG2a (Becton Dickinson, San Jose, CA) could be generated for each vaccination (2 × 10^7–4 × 10^7). DCs were analyzed by flow cytometry for the presence of antigens shown to be characteristic for monocyte-derived mature DCs. DCs expressed high levels of CD1a, CD11c, CD40, CD80, CD86, and HLA-DR, and were negative for CD14. We usually performed single staining of the above-mentioned surface markers that were labeled either with FITC or phycoerythrin monoclonal antibodies except for CD80/86, which were applied in a double-staining procedure (CD80 FITC/CD86 phycoerythrin). Cells were obtained at 95% viability and 95% purity (i.e., marker expression). Interestingly, the addition of IFN-γ (patient no. 5–10) led to a considerable increase of costimulatory molecule expression, as well as of CD1a, CD11c, CD83, and HLA-DR (Fig. 1).

Treatment and Toxicity. DCs were injected into a groin lymph node under ultrasound guidance. All of the injections were successful in terms of targeting and maintaining lymph node architecture as documented by small part ultrasound. We did not observe any side effects at the site of intranodal vacci-
Fig. 1 Phenotype of mature dendritic cells (DCs). Flow cytometric analysis of (A) DCs matured with tumor necrosis factor α, and (B) DCs matured with tumor necrosis factor α and IFN-γ for expression of DC surface markers. We performed single staining for all markers except CD80/86, which were double stained (i.e., detected concomitantly). PE, phycoerythrin.
Two patients developed elevated body temperature (>38°C), which lasted for 24–48 h after vaccination. Three patients developed antihistone antibodies, and 1 of them developed rheumatoid factors during the vaccination, which returned to normal values within 3 months after the end of therapy. But none of these patients developed any clinical symptoms of autoimmune disease.

**Immunological Response.** To determine in vivo DTH reactivity, tumor-lysate pulsed DCs and unpulsed DCs for control were injected intradermally into the forearm of patients 1–5. All 5 of the patients developed a strong, positive DTH skin test after the fourth vaccination (Table 1). In 3 patients who were vaccinated ≥10 times, a second DTH skin test was performed and was found to be positive for all of them. At the injection site

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**Table 2** Intracytoplasmic staining of IFN-γ and surface staining of CD69 in CD3⁺ cells after coculture with mature, pulsed DCs before the first vaccination (T0/DC) and after the fourth vaccination (T4/DC)

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>T0ᵇ</th>
<th>T0/DC</th>
<th>T4ᵇ</th>
<th>T4/DCimᶜ</th>
<th>T4/DCwoᵈ</th>
<th>T4/DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>CD69</td>
<td>19ᶜ</td>
<td>24</td>
<td>39</td>
<td>n.d.</td>
<td>40.3</td>
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<tr>
<td></td>
<td>IFN-γ</td>
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</tr>
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<td>CD69</td>
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<td>19.8</td>
<td>44</td>
<td>40.9</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
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<td>0.9</td>
<td>1.1</td>
<td>1</td>
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</tr>
<tr>
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<td>CD69</td>
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<td>5.2</td>
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<td>n.d.</td>
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<tr>
<td></td>
<td>IFN-γ</td>
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<td>0.4</td>
<td>0.8</td>
<td>n.d.</td>
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<td>CD69</td>
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<td>11.3</td>
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<td>n.d.</td>
<td>40</td>
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<tr>
<td></td>
<td>IFN-γ</td>
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<td>0.2</td>
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<td>n.d.</td>
</tr>
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<td>10</td>
<td>CD69</td>
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<td>15.8</td>
<td>23</td>
<td>25.2</td>
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<td></td>
<td>IFN-γ</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

ᵃ DC, dendritic cell; n.d., not done.
ᵇ T0 and T4, CD3⁺ cells without DC coculture (control).
ᶜ Coculture of T4 with immature DCs with tumor lysate (control).
ᵈ Coculture of T4 with mature DCs without tumor lysate (control).
ᵉ Values are given in percentage of positive cells.
of unpulsed control DCs, we could also observe an erythema and induration, but they never met our criteria for a positive DTH skin reaction (>1.5 cm).

For the remaining patients (with the exception of patient 7 who died shortly after the third vaccination), we performed an *in vitro* assay to determine the immunological response. IFN-γ production in peripheral CD3-positive lymphocytes was assessed by flow cytometry in patients 5, 6, 8, 9, and 10. The results of patient 9 are given as an example (Fig. 2). After coculture with mature, tumor-lysate pulsed DCs, the expression of CD69 and IFN-γ in CD3⁺ cells (which were not treated with any other stimulants) was significantly higher in cells obtained after the fourth vaccination (T4) than in cells obtained before the vaccination protocol was initiated (T0). T0 and T4 cells alone, T4 cells cocultured with immature but loaded DCs, and T4 cells cocultured with mature unloaded DCs were used as controls. They did not exhibit a strong expression of either CD69 or intracellular IFN-γ as we could observe for T4 cells cocultured with mature, lysate-pulsed DCs. The results of all of the performed assays are summarized in Table 2.

**Clinical Response.** Three patients developed an objective partial response, 1 patient demonstrated a minor response, and 2 patients showed stable disease, which was confirmed by computed tomography scan or ultrasound imaging (Figs. 4 and 5). Serum levels of calcitonin and carcinoembryonic antigen, tumor markers that are characteristic for MTC, are shown for all of the patients in Fig. 3. In 7 of 10 patients, a transient or a

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*Fig. 3* Serum tumor marker levels after each vaccination of patients 1–10. Calcitonin levels are depicted by ■, carcinoembryonic antigen levels by ◇, and relate to the left and right axis of each diagram, respectively. *CEA*, carcinoembryonic antigen. *Pat.*, patient.
prolonged tumor marker decline of at least one tumor marker could be observed.

In patient 1, the tumor was infiltrating the esophagus and the trachea at the time of initial surgical exploration. During the first 10 vaccinations, the patient exhibited a partial regression of the mediastinal tumor mass, which was confirmed by computed tomography scan. Therefore, we decided to perform a second surgical intervention to obtain more tumor tissue for lysate preparation and to continue DC therapy. He was vaccinated for another 20 times. In contrast to the initial 10 vaccinations, tumor markers increased continuously, but the patient remained free of symptoms and was without any radiological evidence of tumor progression for 30 months. Thereafter, he developed metastases in the femur and pubic bone, and died 2 months later. For patient 2, we observed a remarkable decrease of calcitonin, but his clinical status rapidly deteriorated involving heavy back pain and symptomatic drug therapy. He died after the fifth vaccination. In patient 3, the initial decrease of calcitonin also correlated with a partial decrease of the cervical tumor mass. But the disease of the lung progressed, and the patient died 9 months after starting DC therapy. In patient 4, the tumor marker levels remained constant for 1 year. Radiological restaging analysis of this patient revealed stable disease for 2 years. However, tumor marker levels increased gradually after completion of the first 10 vaccinations. Therefore, we continued DC therapy for another 10 vaccinations, but the increase was not reversible. Two years after the first vaccination the tumor progressed, and the patient died 29 months after the start of immunotherapy. Patient 5 received 11 vaccinations and experienced a slight decrease of tumor markers. During the therapy, cervical metastases disappeared completely as confirmed by ultrasound imaging (Fig. 4). The patient is still in excellent clinical condition without any symptoms or signs of tumor progression 29 months after initiating DC therapy. Patient 6 also showed a decrease in tumor marker levels after the first series of vaccinations. In addition, he had a partial regression of liver and spleen lesions (Fig. 5). Up to now, he is free of symptoms and without any evidence of tumor progression 30 months after the first vaccination. Patient 7 suffered from rapid progressive disease and died after the third vaccination. Patient 8 received 10 vaccinations and showed a stable tumor marker profile. He is also free of symptoms and had no radiological evidence of disease progression over the past 15 months. Patient 9 demonstrated a continuous decrease of calcitonin and carcinoembryonic antigen serum levels, and he is free of discomfort after 10 vaccinations. Palpable tumor lesions in both cervical regions also disappeared within the first 3 months of immunotherapy, whereas the mediastinal tumor mass remained constant during the last 12 months. In contrast, patient 10 developed progressive disease after an initial tumor marker decline and died 6 months after commencing DC therapy.

**DISCUSSION**

Current treatment strategies for metastasized advanced MTC offer little hope for cure. This study demonstrates that DCs loaded with autologous tumor lysate have a beneficial effect in patients with advanced MTC. The presented DC vaccination protocol was based on magnetic bead selection of
monocytes to obtain highly purified, in vitro generated DCs. Cells were differentiated in vitro to DCs using culture medium containing FCS. We are well aware of the fact that the use of FCS may be controversial, but it has been approved by the local ethics committee. Furthermore, in our hands, addition of FCS has been a crucial prerequisite for obtaining high quality mature DC populations. Although FCS can be considered as an additional source of heterologous antigens, it has never led to any adverse effects during our Phase I clinical trial of DC-based vaccinations (26). In addition, it is important to bear in mind that the use of autologous serum may indeed harbor several disadvantages. For example, in some tumor stage IV patients, we found very high serum levels of vascular endothelial growth factor, which is well known as an inhibitor of DC maturation (27, 28). We are currently looking into other possibilities to avoid the use of FCS in the culture medium for generating high quality mature DCs and have obtained some promising preclinical results.

In this study, tumor lysate-pulsed DCs were either treated with tumor necrosis factor α alone (patient no. 1-4) or with tumor necrosis factor α and IFN-γ (patient no. 5-10) to obtain mature DCs before vaccination. Mature DCs, with high expression of MHC class II and costimulatory molecules, are of critical importance for the induction of tumor-specific cytotoxic T cell-mediated immunity (24, 29-31). Furthermore, it has been shown that immature antigen-presenting DCs, which express low levels of costimulatory signal molecules, induce immunotolerance rather than immunity (32). Therefore, we added tumor necrosis factor α to tumor lysate-pulsed DCs for the final maturation, which resulted in a homogenous CD83+ DC population at the time of vaccination. On the basis of in vitro data obtained in our laboratories, IFN-γ was applied as a second maturation stimulus in 6 patients. We observed a remarkable increase of costimulatory molecule expression on DCs and a more pronounced T-cell response in an in vitro proliferation assay. Thus, this finding was translated to a modification of the clinical protocol. Because of the limited number of study participants, a decisive conclusion on the beneficial impact of IFN-γ treatment for DC maturation cannot be drawn.

Another crucial aspect of DC therapy is the source of antigens. In contrast to other studies in which selected tumor-associated antigens (peptides) were applied, we used crude tumor lysate for DC pulsing. It has been demonstrated previously that DCs loaded with whole protein may be more effective than DCs pulsed with MHC class I restricted peptides in eliciting antigen-specific immune responses (33). This may be related to the wider repertoire of different antigens present in the lysate. Another important aspect is the fact that for most solid tumors, defined tumor antigens are rare or yet undiscovered, and hence, the use of crude tumor lysate is inevitable (34).

For the route of administration, we used direct intranodal injection as first described by Nestle et al. (14). This approach is supported by growing evidence that intranodal injection may be favorable to other forms of application in terms of DC/T-cell localization and immunological interaction (35, 36). Using a small-part ultrasound set, it could be demonstrated that it is unproblematic to apply the vaccine directly into the lymph node. This form of application was routinely performed in an outpatient setting and was well tolerated by all of the patients.

The primary aim of this study was to assess the feasibility and toxicity of adoptive immunotherapy using mature, tumor lysate-pulsed DCs in patients with MTC. This clinical trial demonstrated that the administration of magnetic bead-selected monocyte-derived DCs leads to no major toxicity. There was no clinical evidence for the development of autoimmune disease; any rise in auto-antibodies was transient. Furthermore, the therapy was well tolerated and could be performed on an outpatient basis.

In addition, we evaluated the immunological and clinical response, and explored the therapeutic potential of this method in advanced medullary thyroid cancer patients. DTH reactivity is a well-established clinical test to assess the immunological response that was performed in 5 patients and found to be positive for all of them. To study the specific immune response in more detail, we introduced the intracytoplasmic IFN-γ detection assay. It allows the distinction between the involved lymphocyte subsets (i.e., CD4+, CD8+, and natural killer cells) and will be further applied in future studies to clarify to what extent the different T-cell subsets are of importance in eliciting a tumor-specific immune response. All of the patients tested gave a positive result in the in vitro IFN-γ assay. However, we observed that T cells cocultured with immature, antigen-loaded DCs only exhibited a slight immune response, which might be...
caused by the fact that immature DCs induce immunotolerance rather than immunity (32). Because of the limited number of treated patients and their far-advanced tumor disease, it was not possible to find any explicit correlation among the results of the intracytoplasmic IFN-γ assay, the clinical response, and the survival time.

In 7 of 10 patients, we observed at least a transient decrease of tumor marker levels, and in 4 of 10 patients, we found radiological or clinical reductions in measurable lesions. In 2 patients, we observed a prolonged stable disease. Patient 1 suffered from recurrent bronchitis and had problems swallowing. During the first 10 vaccinations, all of the clinical symptoms disappeared. In patient 5, there was no radiologically detectable disease after the treatment course, but the tumor markers did not completely return to normal values. Therefore, she did not fulfill the criteria for a complete response. Patient 9 presented with a left vocal chord paresis and raucousness at the beginning of the treatment. These symptoms disappeared during the first 3 months of therapy. The clinical and radiomorphological improvements are particularly remarkable, given the far-advanced clinical stage of the disease and the current lack of conventional treatment options for these patients. Thus, based on the in vitro and in vivo investigations, we suggest that MTC is particularly well suited for DC-based immunotherapy (23, 24). We believe that additional studies are justified to confirm and extend these highly promising results. We have initiated a study in patients with MTC with available autologous tumor cell lines. We intend to compare autologous and allogeneic tumor cell lysates for DC pulsing. We will subsequently perform in vitro cytotoxicity assays against autologous and allogeneic tumor cells to confirm cross-reactivity of in vivo primed T cells. This could offer new possibilities in cancer immunotherapy and a better understanding of the immunological antitumor response.

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