ープンMRＩ装置を配置した際に考えられうる問題点の内、①外来電磁波ノイズ対策、②前述の画像診断装置が発生する干渉電磁波ノイズ対策、③オープンMRＩの発生する漏洩磁場対策、④手術室の高耐床荷重対策、⑤高重量の画像機器の搬入方法、搬入経路などを検討し、設計に反映させた。

これらの検討結果を踏まえ、手術室構築のための設計を実施し、具体的な施工に繋げた。例えば、オープンMRＩは重量が15トン強あるので、国立がんセンター中央病院9階の手術室フロアの大梁に、設置するMRＩの3本の脚部の内2本を配置し、残りの脚部1本を、元の躯体の小梁を補強するために鉄骨を配置して荷重を耐えられるレベルとした。

また、自走式CT装置のレール部分の補強やFPD-DR装置のCアーム部の天井吊り部も、上部の補強鉄骨を配置し、地震等外力が働いても大丈夫な構造とした。

[II]内視鏡の画像は、画像周辺部に歪みが認められるが、今回開発した2段階補正処理により、画像周辺部の歪みがより高精度に補正されることを確認した。これにより内視鏡画像とCT画像、MRＩ画像とを重畳表示することが可能であることを示した。

具体的な各種手術の場面では、軟性内視鏡で、術野の中心部を確認しながら手術処置を実施するが、周辺部の状況確認には、CT、MRＩで術前に撮像した3次元画像などを軟性内視鏡画像と重畳することで、手術の安全性を高め、効果的な手術を実現することが可能である。このように画像支援により、従来難しかった深部、狭小部、臓器の背後など直視できない領域の手術を可能にすると思われる。

[III]手術用ロボット装置の根幹部である操作コンソールの概念を構築し、その基盤技術開発を実施した。手術プロトコルを想定し、主術者と補助術者のためのコンソールを用意することを想定して、その要素技術を開発した。ユーザビリティを想定し、グラフィカルユーザーインターフェイスを視認性の良いものとして構築すべく、その基礎的な画面や操作性などの検証を実施した。これにより、次年度より実施する開発をより効果的に進めることが出来ると思われる。

今後、マスタースレーブ方式の手術用具を開発し、それらと一体になった手術用ロボット装置を実現することが今後の課題となる。実際にマスター側とスレーブ側のインターフェイス、及びその制御プロトコルの統一化など実施すべき項目は多く、今後日立製作所機械研究所と密接なタイアップを実現し、効率よく研究開発を進められるように考えたい。

E．結論

[Ｉ]今回の手術室環境構築の研究において、手術室内に設置の手術支援を実施する画像診断支援機器相互の干渉問題を解決することが今回の重要課題となった。特に、①外来電磁波ノイズ遮蔽対策、②X線診断装置、自走式CT装置などが内部に装備する電子機器類から放射するRF電磁波などが引き起こす電磁波ノイズの相互干渉を緩和するための対策及び外部に漏洩させないための対策、特に低漏洩電磁波対策、③M

ＲＩの静磁場発生装置からの漏洩磁場、④手術室環境の高耐床荷重対策、⑤質量の大きな画像診断装置の搬入経路、搬入方法などを検討し、今年度から次年度を通して完成させる国立がんセンター中央病院９階手術室フロアにあらたな手術室構築のための具体的設計を実施し、施工を開始した。

今回の手術室構築の検討過程において、各種画像診断機器をＲＦ電磁波シールド室内に設置することが非常に困難であることが明らかとなった。特に、オープンＭＲＩ装置には、元来撮像室内に引き込む各種信号線や傾斜磁場電流などを、すべてラインフィルタを通して供給する方策が確立している。しかし、同じ室内にＸ線診断装置やＸ線ＣＴ装置を設置すると、これらの電力線、信号線、制御線もすべてラインフィルタを経由して手術室内に供給する必要がある。しかしながら、これらの電力線、信号線、制御線は数百本にも達するものであり、これらをすべて動作確認して、機能や性能、並びに安定性を保証することは気の遠くなるような作業を繰り返す必要があり、現実的でないと判断した。

そこで、今回はＸ線診断装置とＸ線ＣＴ装置が同一の場所に設置されるので、これらをＭＲＩ撮像のためのシールドルームの外部に設置することとし、ラインフィルタの設置を不要とした。ただし、ここで手術室の有効利用のため、ＭＲＩ室とＸ線診断装置およびＸ線ＣＴ装置とを同室に設置して使用することを想定するため、両者の間にＲＦシールドカーテンを用いて、ＭＲＩ撮像時はカーテンを引き、Ｘ線診断装置あるいはＸ線ＣＴ装置を使用する際はカーテンを開くことで手術室の有効利用を考えた。

[II]内視鏡画像の歪補正に関して下記の結論を得た。

手術用ロボット装置のナビゲーションとして、内視鏡が持つ歪み量を抽出する評価手段として共線性の条件とヒルベルト変換を用いて、内視鏡画像歪み補正方法を確立した。また、術前に撮像した３次元ＭＲＩ、ＣＴ画像などと重畳する際、歪補正後の内視鏡画像を重ね合わせる方法、およびＭＲＩ、ＣＴ画像を逆に内視鏡の歪に合わせるように逆補正を施し、内視鏡の画像に重ね合わせる方法の両方法を検討した。

[III]　手術用ロボット装置を開発するに際し、今回の開発の主要根幹部になる操作コンソールの基盤技術を開発した。グラフィカルユーザーインターフェイスを用いたユーザーの視認性の高い表示画面を開発した。

前節で述べた内視鏡歪補正技術を搭載し、術者が手術の全体像を把握しやすい操作コンソールを開発する予定である。3 次元画像処理として一般的な MPR(Multi-Planar Reconstruction)、Curved MPR、3 次元 Volume Rendering、カットプレーン、仮想内視鏡機能(Virtual Endoscopy)などを装備するとともに、軟性内視鏡の先端部の位置認識を取り入れた断面表示機能を将来的に実現していく予定である。

また、臨床的には脳神経外科、胸部外科、乳腺外科、腹部外科、泌尿器外科などのさまざまな外科の術式を取り入れた手術プロトコルに対応できる柔軟性を実現するように操作コンソールの基盤技術の開発を継続するつもりである。

G．研究発表

1．論文発表

本年度は該当なし。

2．学会発表

本年度は該当なし。

H．知的財産権の出願・登録状況

1．特許取得

本年度は該当なし。

2．実用新案登録

本年度は該当なし。

3．その他

本年度は該当なし。

研究成果の刊行に関する一覧表

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Phase I Study of Autologous Tumor Vaccines Transduced with the GM-CSF Gene in Four Patients with Stage IV Renal Cell Cancer in Japan: Clinical and Immunological Findings

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We produced lethally irradiated retrovirally GM-CSF-transduced autologous renal tumor cell vaccines (GVAX) from six Japanese patients with stage IV renal cell cancer (RCC). Four patients received GVAX ranging from 1.4×10^8 to 3.7×10^8 cells on 6–17 occasions. Throughout a total of 48 vaccinations, there were no severe adverse events. After vaccination, DTH skin tests became positive to autologous RCC (auto-RCC) in all patients. The vaccination sites showed significant infiltration by $CD4^+$ T cells, eosinophils, and HLA-DR-positive cells. The kinetic analyses of cellular immune responses using peripheral blood lymphocytes revealed an enhanced proliferative response against auto-RCC in four patients, and cytotoxicity against auto-RCC was augmented in three patients. T cell receptor β-chain analysis revealed oligoclonal expansion of T cells in the peripheral blood, skin biopsy specimens from DTH sites, and tumors. Western blot analysis demonstrated the induction of a humoral immune response against auto-RCC. Two of the four patients are currently alive 58 and 40 months after the initial vaccination with low-dose interleukin-2. Our results suggest that GVAX substantially enhanced the antitumor cellular and humoral

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immune responses, which might have contributed to the relatively long survival times of our patients in the present study.

Key Words: GM-CSF, renal cell cancer, CD4+ T cell, CD8+ T cell, T cell repertoire

INTRODUCTION

Each year, approximately 3000 people die of renal cell cancer (RCC) in Japan [1]. Conventional treatments, such as surgery, chemotherapy, radiotherapy, and cytokine therapies, have not been established for stage IV RCC. Approximately 25% of RCC patients have metastatic disease at the time of diagnosis, and RCC sufferers have a reported 2-year survival rate of less than 20% [2]. As RCC is considered an immunogenic tumor, various types of antitumor immunotherapy have been reported that use cytokines, such as interleukin-2 (IL-2) and interferon-α; cell therapy with LAK; or nonmyeloablative stem cell transplantation. As all of these therapies have their limitations, the introduction of more specific antitumor immunotherapy with less toxicity is required [2–8].

Granulocyte–macrophage colony-stimulating factor (GM-CSF)-secreting cancer cell vaccines, which are generated from cancer cells by *ex vivo* gene transfer, have been shown to elicit tumoricidal antitumor immune responses in a variety of animal models and in human clinical trials [9–11]. Irradiated GM-CSF-secreting cancer cell vaccines are thought to induce antitumor immune responses by recruiting antigen-presenting cells, such as dendritic cells (DCs), to the site of immunization. DCs, which are the most potent immunostimulatory antigen-presenting cells, are known to activate antigen-specific $CD4^+$ and $CD8^+$ T cells, by priming them with oligopeptides that are processed from the lethally irradiated dying cancer cells. The antitumor immune reaction induced by GM-CSF-transduced tumor cells has been reviewed previously [11].

Since the initial clinical report on the use of a GM-CSF gene-transduced tumor vaccine [10], there have been a number of clinical studies applying this technology to the treatment of melanoma, renal cell carcinoma, prostate cancer, pancreatic cancer, and non-small-cell lung cancer. All of these clinical studies were performed without any severe adverse events [12–22]. In a clinical study examining RCC, Simons *et al.* reported a randomized, double-blind dose-escalation study with equivalent doses of autologous, irradiated RCC vaccine cells, with or without *ex vivo* human GM-CSF gene transfer. GM-CSF gene-transduced vaccines were equivalent in toxicity to nontransduced vaccines up to the feasible limits of autologous tumor vaccine yield. There was no dose-limiting toxicity, no evidence of autoimmune disease, and no replication-competent retrovirus encountered in 18 patients receiving full follow-up care. This phase I study demonstrated the feasibility, safety, and bioactivity of autologous GM-CSF gene-transduced tumor vaccines for RCC patients. An objective partial response was observed in one of the three patients who received 1.2×10^8 GM-CSF gene-transduced cells and showed the largest delayed-type hypersensitivity (DTH) conversion [13,14]. However, the optimum number of GM-CSF-transduced autologous renal tumor cell vaccine (GVAX) cells for use in vaccination and boosting and the optimum frequency of cell administration remain to be determined.

To determine more precisely whether GM-CSF-secreting RCC vaccines can be used safely to induce antitumor immunity in advanced RCC patients, we conducted a clinical trial of this treatment strategy. Our clinical protocol consisted of tumor resection by nephrectomy, the establishment of primary RCC cultures, and *ex vivo* gene transfer, which was carried out in our own cell-processing facility [23]. The minimum dosage of the vaccine cells was set according to the previous report on RCC by Simons *et al.* [14], and the booster schedule was based on a previous report on non-small-cell lung cancer by Soiffer *et al.* [16]. This was the first clinical trial of human gene therapy for cancer patients approved by the Japanese government and performed in Japan. The results of the present study indicate that this novel RCC immunotherapeutic regimen, which features vaccination with GM-CSF-secreting, irradiated autologous RCC tumor cells, is feasible, safe, and capable of eliciting systemic immune responses against RCC tumor cells. Furthermore, these patients, some of whom also received systemic low-dose IL-2 therapy, have been followed up on an outpatient basis.

RESULTS

Case Presentations

Forty patients suffering from either primary RCC with or without metastases or postoperative relapsed RCC were evaluated at our hospital between July 1998 and March 2001. Of these, 6 preoperative patients with stage IV RCC (UICC classification 1997) with metastatic lesions were allowed to participate in the present clinical study by our ethics committee, based on clinical condition and eligibility criteria listed under Patients and Methods. As

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TABLE 1: Patient characteristics and clinical response to GVAX

	Patient			
	1	2	3	4
Age (years)/sex	60/male	71/male	57/female	50/male
Tumor site				
Primary	Right RCC	Right RCC	Left RCC	Left RCC
Metastases	Lung, liver	Sacral bone	Liver, lung	Lung
Previous therapy	None	Sacral irradiation	None	None
GM-CSF production[a] (ng/10^6 cells/24 h)	49	98	51	116
No. of GVAX treatments	10	17	15	6
Vaccinated total cell number	2.2×10^8	3.7×10^8	3.2×10^8	1.4×10^8
Adverse events				
Systemic	Low-grade fever	Low-grade fever	None	None
Local	Erythema, pruritis	Erythema, pruritis	Erythema, pruritis	Erythema, pruritis, blister
Eosinophil number[b] (/μl; mean ± SD)	718 ± 76	437 ± 306	226 ± 283	390 ± 150
Clinical response	PD	SD	PD	PD, MR
Survival (months from first vaccination)	7.5[c]	>62	45[c]	>44

PD, progressive disease; SD, stable disease; MR, mixed response.
[a] GM-CSF production rate from each autologous GM-CSF-transduced RCC.
[b] Eosinophil number was measured 48 h after vaccination.
[c] Patient passed away.

2 patients, a 48-year-old Japanese man having right RCC with multiple lung metastases and a 58-year-old Japanese man having right RCC with metastases to the right clavicle, bilateral lung, and liver, were excluded from this study because their GM-CSF-transduced RCC cells did not produce enough GM-CSF to satisfy the eligibility criteria as described under Autologous Vaccine Yield and Gene Transfer, 4 patients received GVAX.

The first patient (Case 1), a 60-year-old Japanese man, was diagnosed in August 1998 with RCC of the right kidney with multiple lung and liver metastases. His largest metastatic tumor, which was located in the right hilar region, was calculated volumetrically as 135 ml by computed tomography (CT) scan. The vaccine preparation used, his clinical course, and the autopsy findings have been reported previously [24]. Furthermore, he received a total of 2.2×10^8 GVAX cells over 10 subcutaneous injections. The adverse events he experienced during vaccination are summarized in Table 1. He received gamma knife irradiation for his brain metastases and was initiated with low-dose (700,000–140,000 IU) recombinant IL-2 (rIL-2; Imunace, 350,000 IU/vial; Shionogi, Osaka, Japan), which was administered intravenously according to the patient's request. One week after the start of the rIL-2 treatment, the patient's right hilar mass lesion became smaller and decreased by 30% of the total volume within 1 month (Fig. 1A). Unfortunately, this patient died of multiple RCC metastases on July 8, 1999, 10 months after nephrectomy and 7 months after the start of GVAX vaccination (Fig. 2A).

The second patient (Case 2), a 71-year-old Japanese man, was diagnosed in December 1998 with a sacral tumor that metastasized from RCC of the right kidney. He received a total dose of 30 Gy of irradiation of the sacral metastasis in February 1999 for severe pain, which was followed up with spinal anesthesia and oral morphine sulfate. The patient was nephrectomized on April 6, 1999, 43 days after the local irradiation, and pathology showed clear cell carcinoma. He received a total of 3.7×10^8 GVAX cells in 17 subcutaneous injections from June 3, 1999, to February 3, 2000. The adverse events he experienced during vaccination are summarized in Table 1. His pain at the sacral area disappeared completely after the 5th vaccination, and oral morphine sulfate was discontinued. He experienced mechanical ileus due to nephrectomy after the 13th vaccination, which resolved after a few days of iv fluid treatment. The ileus was not related to the vaccination, and no recurrence of the ileus was noted after 4 further vaccinations. During the course of vaccination, the growth rate of the sacral tumor was stable as assessed by CT scan. His clinical course with the change in tumor size is described in Fig. 2B. The serum level of the nonspecific tumor marker immunosuppressive acidic protein returned from double the normal level to normal after the 6th vaccination, and a thallium scan showed decreased uptake of thallium at the tumor site on completion of the vaccination protocol (data not shown). Eleven months after the start of vaccination, pathological examination of the biopsied sacral bone specimen showed no RCC. This patient had been doing well without any treatment, with a performance status of zero, until he experienced a dull pain in his right femoral area in late November of 2001, 29 months after the 1st vaccination. He was diagnosed as having a 1-cm lytic metastasis in the right femoral bone. He received local

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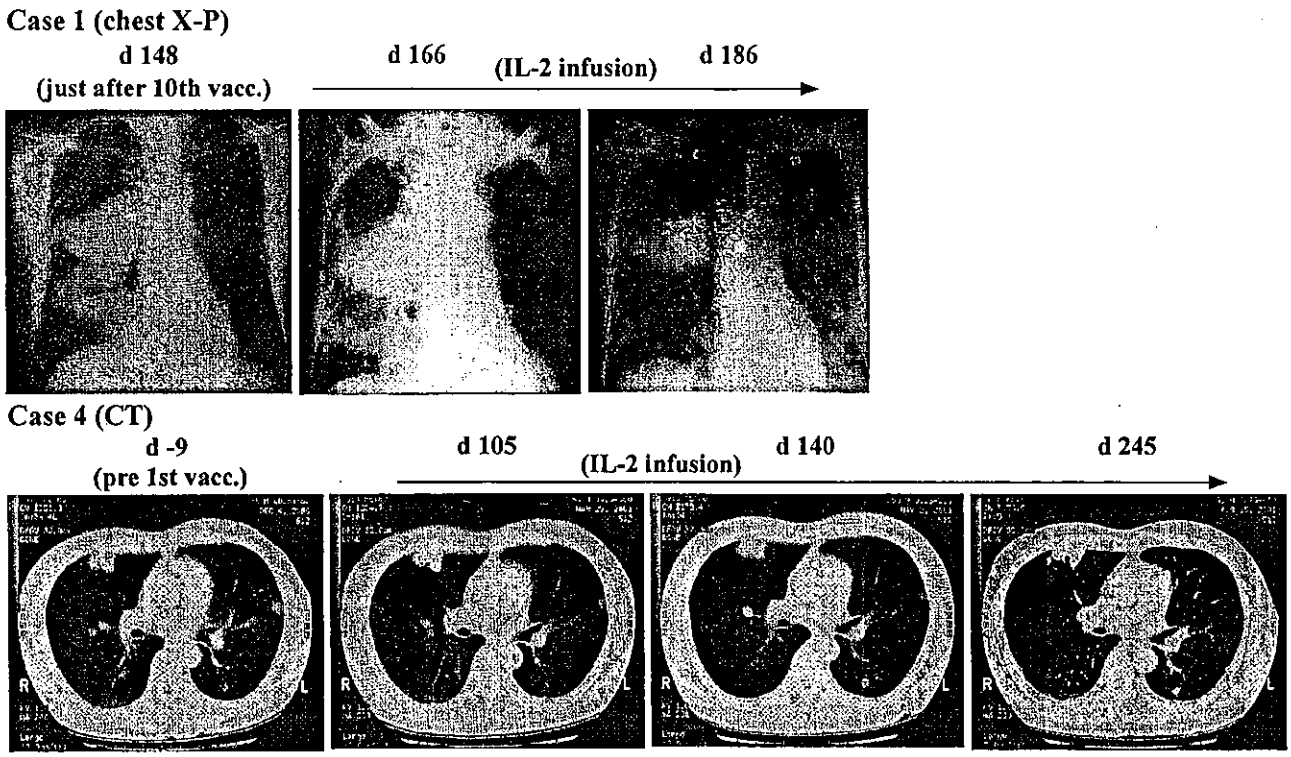

FIG. 1. Size change of the lung metastases in Cases 1 and 4 during the observation period after GVAX vaccination. (A) In Case 1, the size of the right hilar tumor, the largest metastatic lesion, became smaller after 1 week of low-dose IL-2 (d 166, day 166) and decreased by 30% after 1 month of low-dose IL-2 compared with the tumor just after the 10th vaccination. Chest X-ray films are presented. (B) In Case 4, the size of the left lung metastasis became smaller during vaccination as shown on two films for comparison between prevaccination and 105 days after the 1st vaccination. After the start of low-dose IL-2 between days 105 and 245, both the metastatic lesions in the left lung and those in the right lung became smaller. CT scan films are presented.

irradiation at a dose of 30 Gy to his femoral metastasis followed by daily low-dose rIL-2 (700,000–140,000 IU). His performance status at present, 58 months after the start of vaccination and with low-dose rIL-2 (350,000 IU) treatment, is zero.

The third patient (Case 3), a 57-year-old Japanese woman, was diagnosed in October of 1999 with RCC of the left kidney with multiple liver and lung metastases. She was nephrectomized on December 9, 1999, and the pathology showed clear cell carcinoma. She received a total of 3.2×10^8 GVAX cells in 15 subcutaneous injections, from February 22, 2000, to September 19, 2000. The adverse events she experienced during vaccination are summarized in Table 1. During the course of vaccination, the growth rate of the multiple liver tumors slowed, but the numbers and sizes of the masses did not decrease as assessed by CT scan (Fig. 2C). The sizes of the metastases in her right renal pelvis and lungs, observed on CT scan, were stable during vaccination. Her performance status was maintained at zero. After completion of the vaccination regimen, she requested systemic rIL-2 and interferon-α, but the cytokine treatments were discontinued due to the appearance of liver dysfunction, which resolved after discontinuation of cytokines. Thereafter, she received monthly LAK (lymphokine-activated killer cells) therapy, upon her request. However, her metastatic lesions gradually increased, and she ultimately died of multiple RCC metastases on November 3, 2003, 47 months after nephrectomy and 45 months after the start of GVAX vaccination.

The fourth patient (Case 4), a 50-year-old Japanese man, was diagnosed in July of 2000 with right RCC with multiple lung metastases. He was nephrectomized on September 20, 2000, and pathology showed clear cell carcinoma. He received a total of 1.4×10^8 GVAX cells in six subcutaneous injections from December 13, 2000, to February 20, 2001. The adverse events he experienced during vaccination are summarized in Table 1. During the course of vaccination, the growth rate of the largest lung tumor slowed, and several tumors disappeared or were reduced in size, i.e., a mixed response was obtained. However, the sum of all the masses was increased, as assessed by CT scan. After the sixth injection, he was found to have a metastatic brain lesion with a maximum diameter of 1 cm, and the vaccination was discontinued according to our eligibility criteria. He received gamma knife irradiation to his brain metastasis and low-dose rIL-2 (700,000–140,000 IU) was initiated, which was given

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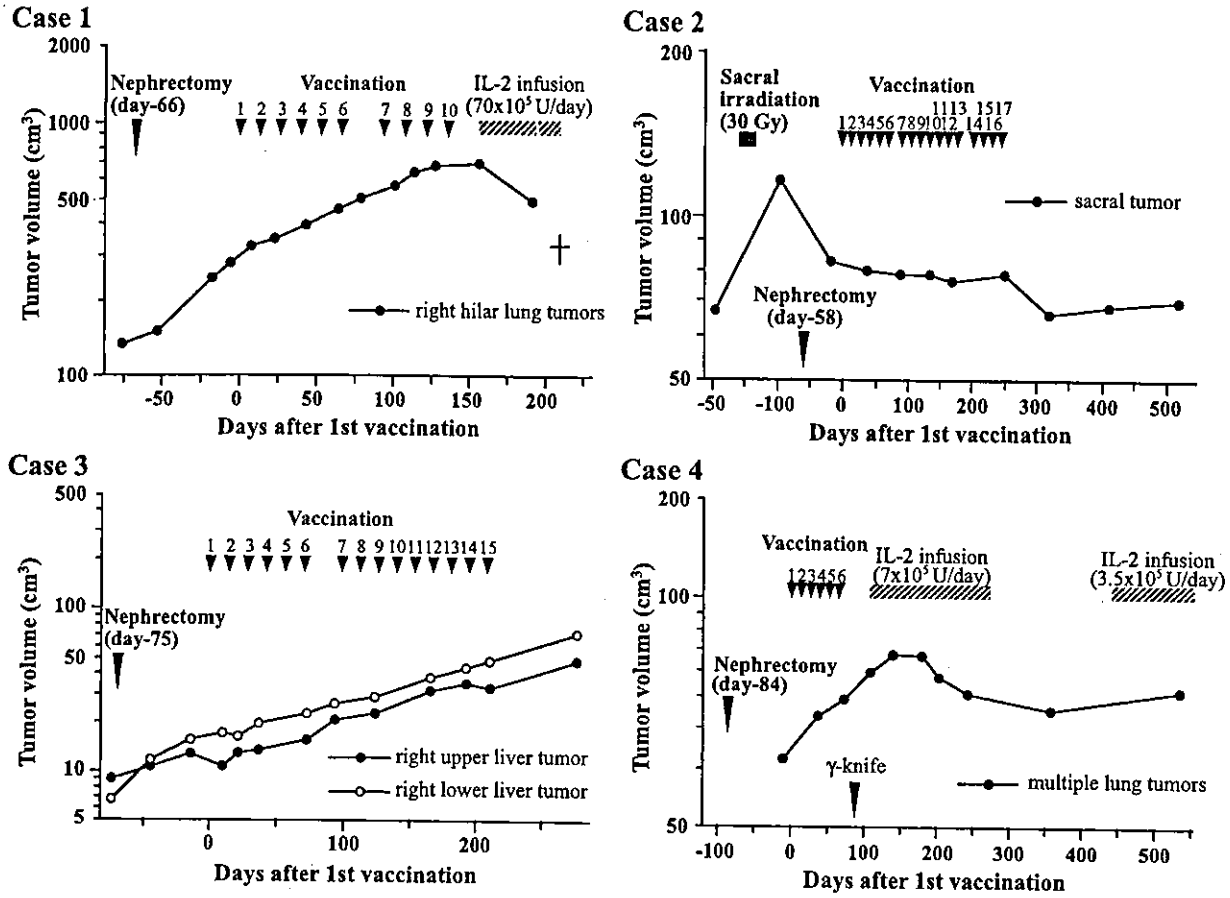

FIG. 2. Clinical summary of the four stage IV RCC patients (Cases 1 to 4) who received GVAX. The days of each vaccination are indicated by the short arrows, and the upper numbers signify the number of vaccinations. The long arrow indicates the time of nephrectomy of an RCC that involved the kidney. The tumor volume of each target metastatic lesion was measured periodically by CT scan or MRI. In Cases 1 and 4, low-dose IL-2 was administered after vaccination, upon request by the patients. The sacrum and brain were irradiated in Cases 2 and 4, respectively, to control sacral pain and brain edema.

subcutaneously, according to the patient's request. One month after the start of the rIL-2 treatment, the patient's total lung tumor volume was reduced and decreased to 30% of the peak volume over 3 months (Figs. 1B and 2D). His metastatic brain tumor was resected in January of 2002, and his performance status at present, 40 months after the start of vaccination and with low-dose rIL-2 (350,000 IU) treatment, is zero.

Autologous Vaccine Yield and Gene Transfer

In this trial, we generated the primary RCC cultures from large, advanced cancers with some areas of necrosis. The rate of successful vaccine cell expansion was 100% (6/6). In our preclinical models, the expression of paracrine GM-CSF by vaccine cells at levels higher than 40 ng/10^6 cells/24 h induced antitumor immunity, and thus, we excluded cases producing less than this level from the present study [13,14]. A single transduction with MFGS-GM-CSF generated GM-CSF secretion levels of >40 ng/10^6 cells/24 h in four of the six patients (66.6%) and their production levels are shown in Table 1. The level of GM-CSF secretion by nontransduced cells ranged from 0 to 19 ng/10^6 cells/24 h. We excluded two of the patients, who had GVAX production of only 20 and 12.4 ng/10^6 cells/24 h, respectively, from our study. Cells from these individuals incorporated fewer copies of the integrated GM-CSF cDNA, and the cell doubling times were approximately two times greater than those of the cells producing >40 ng/10^6 cells/24 h GM-CSF (data not shown). It is likely that the extended cell doubling times resulted in poor GM-CSF transduction efficiency in the two excluded cases.

Safety of Administration and Systemic Toxicities

All of the six patients' primary cultures met the vaccine cell yield specifications for at least six injections. Importantly, the tests for microbial contaminants gave negative results for all six GM-CSF-transduced products. All four patients, designated Cases 1, 2, 3, and 4, satisfied all of the eligibility criteria for this study and

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received vaccinations (Table 1). No surgical complications were encountered that would preclude subsequent vaccination, although Case 1 had mechanical ileus 30 days after nephrectomy, which was before vaccination, and Case 2 had ileus 219 days after nephrectomy, between the 12th and the 13th vaccine injections. These symptoms were resolved by intravenous fluid replacement for several days. In the latter case, ileus was thought to be a late adverse event related to nephrectomy, but not vaccination because reinitiation of vaccination did not cause any other ileus symptoms. When vaccine yield and clinical status permitted, we performed multiple vaccinations for analysis of cumulative side effects. Cases 1, 2, 3, and 4 received GVAX at 66, 58, 75, and 84 days after nephrectomy, respectively. They received 48 fully evaluable, 14-day treatment cycles. Finally, Cases 1, 2, 3, and 4 received total cell doses of 2.2×10^8, 3.7×10^8, 3.2×10^8, and 1.4×10^8, respectively. During the vaccinations, we observed no hepatic, renal, pulmonary, cardiac, neurological, or gastrointestinal toxicities in any of the patients other than mechanical ileus in Case 2 as stated above. We observed significant increases in the numbers of peripheral blood eosinophils, but not other leukocytes, after immunization, as shown in Table 1, and the peak eosinophil level gradually increased in each case after repetitive vaccinations (data not shown). We did not observe the two most concerning toxicities, vaccine site-specific ulceration and development of acute autoimmune disease and, specifically, nephritis in uninephric patients, except in Case 4, who experienced blister formation at the vaccination site following the 6th vaccination (Table 1). We detected no RCR (replication-competent retrovirus) during the postvaccination follow-up period in any of the four patients who received the vaccine cells. The apparent lack of acute, systemic toxicity in this trial was paralleled by the lack of plasma elevation of GM-CSF in pharmacokinetic studies following treatment (data not shown). Follow-up observations for long-term toxicity, including autoimmune disease, have been under way on our two surviving patients, Cases 2 and 4, and no vaccine-related long-term toxicity has been noted to date.

Phenotype of Cells at the Sites of Vaccination

Although there was some individual variation, we noted significant infiltration by $CD4^+$ T cells and eosinophils by day 30 (after the third vaccination); Case 3 had modest eosinophilic but intense mononuclear cell infiltration throughout the course of the vaccination protocol. Thereafter, these cell infiltrations were reduced in Cases 1 and 4, but increased in Cases 2 and 3. We could detect $CD68^+$ macrophages and CD20cy-positive B cells as minor populations, but their levels were unaltered during the course of the vaccination protocol. The level of HLA-DR expression by infiltrating cells was initially low, but increased by day 30. Intradermal $S100^+$ dendritic cells were occasionally observed in most cases. These findings were comparable with previous reports [14–20].

Delayed-Type Hypersensitivity Reactions

DTH tests using Multitest CMI showed that Cases 1 and 3 had anergic scores and Cases 2 and 4 had normal scores, i.e., within the range seen for normal volunteers or patients with localized cancer (data not shown). As shown in Table 2, we did not observe significant DTH reactions (>10 mm) to unpassaged, irradiated autologous RCC cells in any of the four patients prior to treatment. Following vaccination, we observed significant DTH reactions in all patients and they were strongest after the sixth vaccination. We also observed DTH reactions to normal renal cells (NRCs), but these reactions were almost always smaller than those to RCC cells.

We examined pathological phenotypes and numerical analysis of the DTH reactions. In all four cases, significant DTH reactions against RCC were induced by days 24–28 (following the second vaccination), compared with the day 0 controls (prevaccination). $CD4^+$ T cells were more dominant than $CD8^+$ T cells at the sites of DTH reaction, followed by $CD68^+$ macrophages and a few B cells, which was a common feature of DTH in all cases. We also observed various degrees of eosinophilic infiltration with degranulation. There were no significant differences between RCC and NRC with regard to the phenotypes of the cells in the DTH reaction, although more intense cell infiltration was observed against RCC than against NRC (data not shown). Although we detected significant DTH reactions until day 133 in Case 1, we observed a certain degree of attenuation in other cases.

Immunophenotypic Analysis of Tumor-Infiltrating Lymphocytes (TILs)

We performed immunophenotypic analysis of TILs for Case 1. Immunohistochemical analysis revealed that $CD4^+$ T cells were the predominant infiltrating cell type in pretherapy primary tumors, followed by B cells (data not shown). Fig. 3 shows TILs in perivascular areas (Fig. 3A), around the foci of tumor cell apoptosis (Figs. 3B and D), and in an unremarkable area (Fig. 3D), within biopsied skin metastatic RCC specimens that were obtained 5 months after initiation of therapy. Regardless of the area under observation, the T cells in this specimen were $CD8^+$, and we detected virtually no B cells. In addition, we observed increased numbers of $CD68^+$ macrophages, especially around the apoptotic foci. In contrast to the results of the DTH test, we did not observe eosinophilic infiltration of the tumors (data not shown). Interestingly, immunophenotypic analysis of the infiltrating cells at the sites of surgically resected renal cancer and normal renal tissue, autopsied normal liver, lung, and kidney showed a predominance of

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TABLE 2: Immunological findings in patients who received GVAX

	Patient 1	2	3	4
Response to DTH skin test (mm)[a]				
Prevaccination	7 × 7/6 × 4.5	0 × 0/4 × 2	3 × 6/2 × 6	0 × 0/2 × 2
Peak reaction	85 × 65/30 × 35 (6)[b]	15 × 15/10 × 10 (6)[b]	25 × 25/2 × 1 (9)[b]	17 × 11/22 × 18 (6)[b]
Lymphocyte proliferation (cpm)				
Prevaccination	5,513	5,385	1402	1550
Post third	11,637	16,486	2836	6084
Post sixth	15,845	40,578	2442	6445
Cytokine production (pg/ml)				
IFN-γ				
Prevaccination	2746	UD	50	102
Post third	4952	199	481	UD
Post sixth	3568	394	967	UD
IL-5				
Prevaccination	UD	UD	395	128
Post third	1124	UD	863	1331
Post sixth	2088	792	1850	3017
IL-10				
Prevaccination	170	UD	UD	UD
Post third	80	UD	43	254
Post sixth	235	155	130	297
Cytotoxicity assay (%)				
Prevaccination	62.8	0.2	16.0	13.0
Post third	51.4	17.0	52.9	21.0
Post sixth	45.3	27.3	36.8	27.5
TCR Vβ gene-segment repertoire analysis	PB: 9,14,15,17 TIL: 10,17,21 DTH: 10,17	PB: 1,7,10,11,21 DTH: 1	PB: 4,18 DTH and Vac: 4,7	PB: 21,23 Vac: 9 DTH: 21

UD, under the detection level; PB, peripheral blood; TIL, tumor infiltrating lymphocytes; DTH, delayed type hypersensitivity.

[a] DTH reactions were examined using cultured autologous RCC cells/normal renal cells as antigens.

[b] The numbers in parentheses show that peak DTH reactions were observed after sixth or ninth vaccination.

$CD4^+$ cells, whereas analysis at the sites of biopsied or autopsied tumor tissues obtained after vaccination or after vaccination followed by low-dose IL-2, respectively, showed a predominance of $CD8^+$ cells (data not shown).

Vaccination Enhances the Proliferative Responses and Cytokine Production Against Autologous Tumors

We assessed the cellular immune responses using the peripheral blood mononuclear cells (PBMC) of patients who received GVAX. PBMC proliferated well in response to autologous RCC cell stimulation at all times tested (Table 2). In Case 2, the proliferative response observed before vaccination was augmented after vaccination. In all cases, vaccination markedly enhanced the proliferative responses to autologous RCC in the presence of IL-2. Especially, in Cases 2 and 4, those with prolonged clinically stable disease, the proliferative responses against autologous tumor cells remained high until the end of the study (data not shown).

IFN-γ in cultures stimulated with autologous RCC was enhanced after the initial vaccinations in Cases 1, 2, and 3, but not in Case 4 (Table 2). Conversely, IL-5 and IL-10 production was enhanced after vaccination in all cases. The enhancement of IL-5 seemed to correlate with the eosinophilia observed after the sixth vaccination. We also measured IL-4 production, but the levels of this cytokine were all below the limits of detection (data not shown).

Vaccination Induces Cytotoxicity Against Autologous RCC, Allogeneic RCC, and Autologous NRC

Case 1 showed comparatively high cytotoxicity against autologous RCC before vaccination. This level was maintained until after the fifth vaccination, after which it decreased. This was consistent with the higher DTH responses against autologous RCC and NRC seen in this case (Table 2). In Cases 2, 3, and 4, vaccination increased and maintained cytotoxicity against autologous RCC (Table 2, Fig. 4). Moreover, the addition of F(ab′)2 anti-CD3 mAb efficiently inhibited cytotoxicity against autologous RCC, suggesting the involvement

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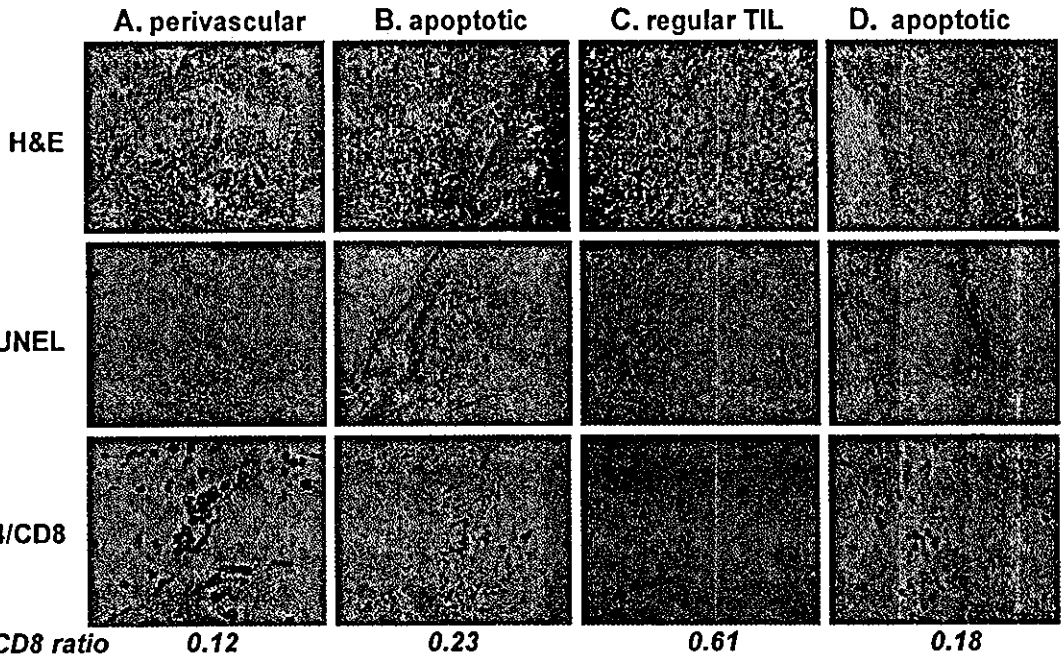
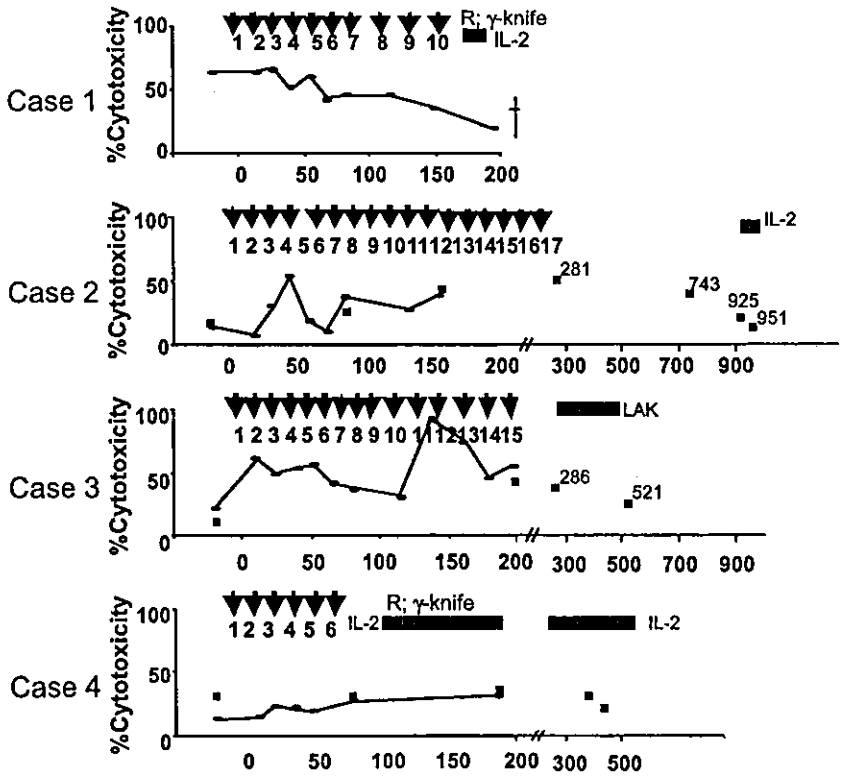


FIG. 3. Immunophenotypic analysis of tumor-infiltrating lymphocytes (TILs) in the RCC tumors of Case 1. TILs were observed in various areas, particularly (A) in the perivascular area, (B, D) around foci of tumor cell apoptosis, and (C) in unremarkable areas, within metastatic RCC that were obtained 7 months after the initiation of therapy. The T cell phenotype in this specimen had been converted to CD8 dominant, and few B cells were detected. To detect tumor apoptosis, the TUNEL method was applied as described under Patients and Methods.

FIG. 4. Cytotoxicity against autologous RCC in Cases 1 to 4. PBMC were cultured with irradiated GM-CSF-transduced autologous RCC in the presence of IL-2 for 7 days in 96-well microplates and ^{51}Cr-labeled target cells were added. After a 6-h incubation, cytotoxicity was measured as described under Patients and Methods. Asterisks in the panels for auto-RCC targets show the cytotoxicity, which was inhibited by more than 25% by the addition of F(ab')2 anti-CD3 mAb. Additional experimental values (squares) assessing the long-term results with additional treatment after the last vaccinations are also shown. IL-2, the rectangular bar represents the period of the administration of low-dose IL-2; R; γ-knife, gamma knife treatment was done for brain metastasis before the administration of IL-2.



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of MHC-restricted T cell receptor (TCR)-mediated cytotoxicity.

TCR Vβ Clonotypic Analysis in DTH, Lung Metastasis, and RCC

In Case 1, we analyzed TCR Vβ gene usage in peripheral blood lymphocytes (2 days before vaccination and after the 6th and 9th vaccinations) and in tumor-infiltrated lymphocytes collected from tissue samples from the original surgically resected tumor, DTH skin biopsy (biopsied after the 4th vaccination), a metastatic skin lesion (biopsied after the 10th vaccination), and autopsied right hilar main lung metastases. We estimated the variation in the signal intensity by comparing the ratio of each Vβ signal observed in the regressed metastasized lung lesion with that observed in the nonregressed lung lesion or at the biopsied RCC or NRC DTH sites. The Vβ repertoire, which was overexpressed in the former sample, but not in the latter, was considered to indicate strong candidate T cell clones that were specifically induced by GVAX. In Case 1, we observed oligoclonal expansion of T cells with Vβ 9, 14, 15, and 17 repertoires in peripheral lymphocytes after the 9th vaccination (Fig. 5A). T cells with Vβ 10, 17, and 21 repertoires infiltrated the regressed tumor to a greater extent than the nonregressed tumor. These cells were also observed in the metastatic skin lesion (Fig. 5B). Interestingly, after vaccination, T cells with Vβ 10, 17, and 21 repertoires expanded clonally in the peripheral blood of Case 1, and the amplified TCR exactly matched those of the amplified fragments in a tumor-specific manner, namely T cells with Vβ 10 expanded dominantly in original tumor and lung metastasis, T cells with Vβ 17 in lung metastasis and much less in original tumor, and T cells with Vβ 21 in arm and lung metastasis and less in original tumor (Fig. 5C).

In Case 2, a T cell clone with Vβ 1 was increased in the pre- and postvaccination peripheral blood, nephrectomized tumor, biopsied sacral tumor, and DTH sites (Table 2). We saw oligoclonal expansion of T cells with Vβ 7, 10, 11, and 21 in peripheral lymphocytes after the vaccinations. Among them, T cell clones with Vβ 10, 11, and 21 expanded gradually after the first vaccination, and those with Vβ 7, 11, and 21 were also found in the nephrectomized original tumor.

In Case 3, the number of peripheral blood T cells with Vβ 18 was increased after the 6th, 9th, and 11th vaccinations and those with Vβ 4 were increased only after the 1st vaccination. Interestingly, T cells with Vβ 4 and 7 were increased at the RCC DTH sites after vaccination. These cells were also found at the vaccination site. T cells with Vβ 4 also infiltrated the metastasized liver tumor and nephrectomized original tumor.

In Case 4, peripheral lymphocytes contained increased numbers of T cells with Vβ 23 after the vaccinations. T cells with Vβ 9 were expanded after the sixth vaccination at the vaccinated site. T cells with Vβ 21 were expanded at the RCC DTH sites. The results of the T cell repertoire analysis are summarized in Table 2.

Western Blotting

To examine whether the therapeutic regimen induced antitumor antibody responses in patients, we compared the serum antibody reactivity against autologous tumor cell lysates before and after initiating the therapy by immunoblot analysis. Using posttherapy serum as probes, high-molecular-weight proteins of around 250 kDa generated clear signals, whereas the pretherapy serum showed no or only weak signals at the same position (Fig. 6A), suggesting that the GVAX induced an antibody response in Cases 1, 2, and 4, while the results were less clear in Case 1. The signals appeared in similar positions in all patients, suggesting the presence of common antigens. These high-molecular-weight antigens were present in both tumor lysates and NRC lysates (Fig. 6A). In addition, human lip-derived fibroblasts might have identical antigens, while H69 lung cancer cells did not (Fig. 6B). Furthermore, the changes in the magnitude of the antibody immunoreactivity over time were analyzed using serum from Case 2. The strongest signal was observed in serum obtained 67 days after the initial vaccination, between the 5th and the 6th vaccinations. This response was maintained from day 67 until day 281, just after the 17th vaccination, and the immunoreactivity remained until the last time point examined at day 950 (Fig. 6C).

Clinical Outcomes

According to the standard clinical criteria, Case 2 was in stable disease, Case 4 was in mixed response, and Cases 1 and 3 were in progressive disease during the course of GVAX treatment (Table 1). As described under Case Presentations, Case 1 and Case 3 died of multiple metastases 7.5 and 45 months, respectively, after the first vaccination. Case 2 and Case 4 are alive 62 and 44 months, respectively, after the first vaccination in a stable condition with a performance status of zero. Interestingly, Case 1 with progressive disease and Case 4 with mixed response showed 30% decreases in their main or total lung metastatic lesions, respectively, 1 to 3 months after the start of low-dose IL-2 treatment (Figs. 2A, B, and 4).

DISCUSSION

Although the production of the GVAX from all six patients was successful, in two cases the levels of GM-CSF produced were not high enough for cell injection. Our production rate of 67% was compatible with previous reports [14–16]. The poor transduction efficiencies in our two patients were probably due to slowed proliferation of these RCC cells, as reflected by the extended doubling times. To overcome the heterogeneous transduction efficiency with retroviral vectors,

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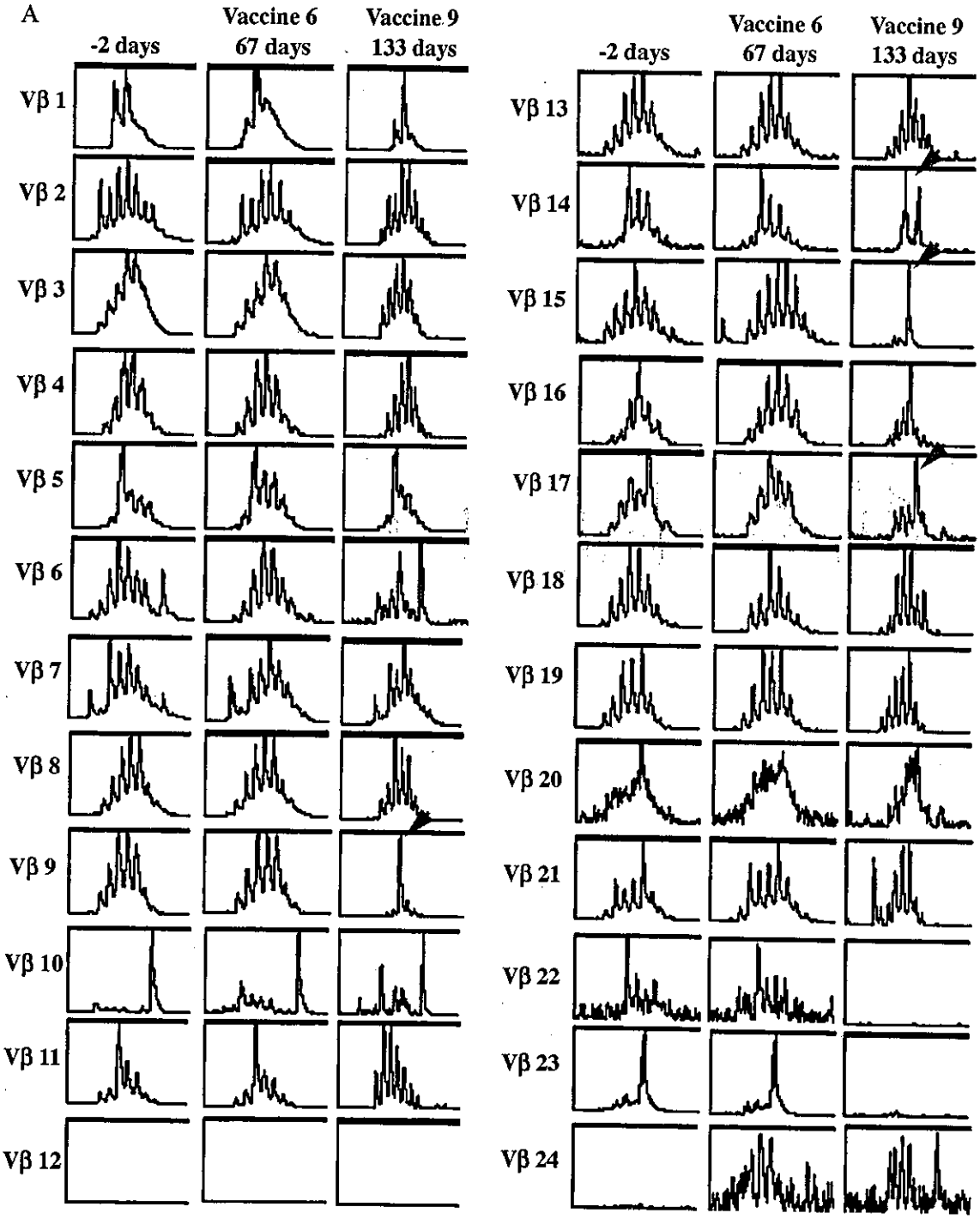

FIG. 5. TCR Vβ clonotypic analysis of the T cell infiltration in DTH, lung metastasis, and renal cell carcinoma in Case 1. (A) Oligoclonal expansion of T cell Vβ 9, 14, 15, and 17 repertoires was observed in peripheral lymphocytes after the ninth vaccination. (B) Larger numbers of T cells with Vβ 10, 17, and 21 repertoires infiltrated the regressed tumor than the nonregressed tumor. These cells were also observed in the skin metastasis designated Arm Meta. (C) After vaccination, T cells with Vβ 10, 17, and 21 repertoires were clonally expanded, and the amplified respective Vβ fragments exactly matched those of the amplified fragments in a tumor-specific manner.

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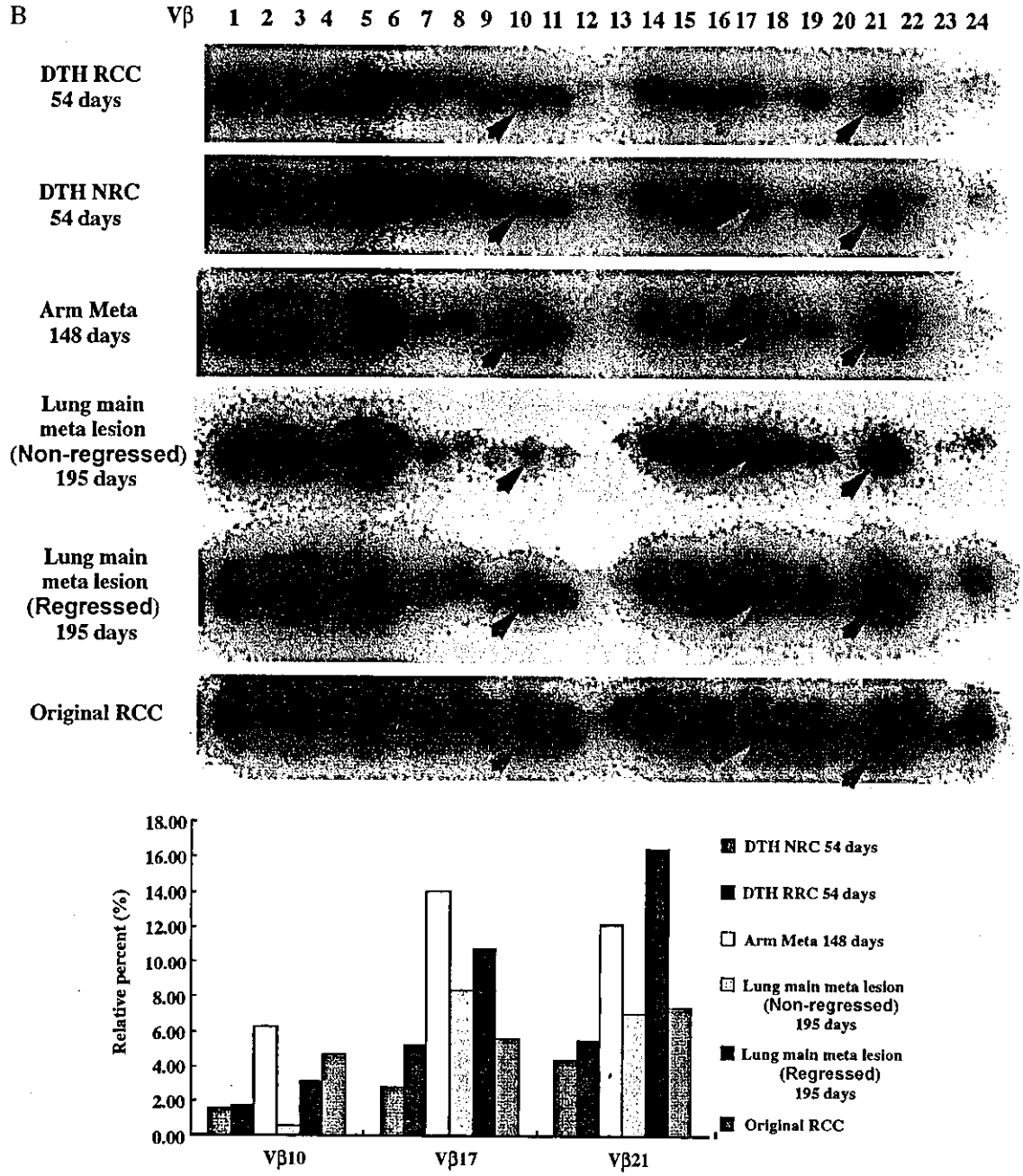

FIG. 5 (*continued*).

adoption of adenovirally transduced GM-CSF autologous tumor cells, allogeneic GM-CSF-transduced cell lines, or autologous tumor cell-based vaccines using GM-CSF-producing bystander cells should be examined as suggested previously [18–20,25–27].

We administered GVAX to four postnephrectomy patients with stage IV RCC without inducing severe vaccine-related adverse events. Furthermore, no remarkable long-term adverse events have been observed in three patients, including two living patients. Our histological findings at the vaccination sites also support the previous observations of triggering the antitumor immune response at these sites [12,14–20,28]. DTH responses in Cases 1, 2, and 3 tended to show stronger reactions to autologous RCC cells than autologous NRC and suggested that anti-RCC-specific immunity was

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