源停留位置は自動的に決定される。前提条件となるPTVやrisk臓器の輪郭の描出が重要な意味をもつため、入力に関しては細心の注意を要する。Risk臓器の位置関係を考慮して複雑な線源停留位置を設定する場合、線源停留位置の幾何学的形状のみから停留時間の比が定まるGO法はIP法に比較し客観的評価が困難であり、治療計画上のIP法の優位性は明らかである。IP法の小線源治療計画への応用に関しては治療計画に要する時間が懸念されたが、Lessardらは熟練した医師が45分以上かけてマニュアルで最適化する方法をとっており、それと同程度以上の計画がIP法を用いて1分以内で達成されたと報告している。今回の検討上IP法を用いた治療計画に要する時間は、許容できる範囲内であると考えられ、医師の輪郭入力作業の習熟によりさらなる時間短縮が可能と考えられる。

E.結論

肺癌をはじめとした小型腫瘍に対する小線源治療に必要な治療計画の標準化において重要な、最適化に関する客観的評価方法について検討を行った。小線源治療における、より適切な線量分布を取得するための最適化法として、IP法の本治療への応用が可能であった。今後、磁気を応用した診断・治療装置による悪性腫瘍の小線源治療への応用により、アプリケータをより適切に配置することが可能となれば、IP法による最適化された治療計画の実現により、高線量率組織内照射の特徴をより強調した効果の向上と副作用の軽減を実現する放射線治療が可能となることが期待される。

F.健康危険情報

小線源治療の効果および副作用に関し多くの報告がなされており、適切な対象の選択により一定の効果と副作用が予想される。

今後の臨床への応用においては、他のより適切な方法の有無を充分検討し、インフォームドコンセントを尽くして、実施することとする。

G.研究発表

1．論文発表

Sekine I, Noda K, Oshita F, Yamada K, Tanaka M, Yamashita K, Nokihara H, Yamamoto N, Kunitoh H, Ohe Y, Tamura T, Kodama T, Sumi M, Saijo N.: Phase I study of cisplatin, vinorelbine, and concurrent thoracic radiotherapy for unresectable stage III non-small cell lung cancer. Cancer Sci. 95: 691-695, 2004.

2．学会発表

The Patterns of Care Study for Non-small Cell Lung Cancer Treated with Radiation Therapy in Japan; Analyses of Age. Sumi M, Uno T, Ikeda H, Teshima T, Sawa Y. American Society for Therapeutic Radiology and Oncology 46 th Annual Meeting, October 3-7, 2004

H.知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tani K, Kakizoe T, et al.	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.	Mol Ther	10(4)	799-816	2004
Nakagawa T, Kanai Y, Ushijima S, Kitamura T, Kakizoe T, Hirohasi S	DNA hypomethylation on pericentromeric satellite regions significantly correlates with loss of heterozygosity on chromosome 9 in urothelial carcinomas.	J Urol	173	243-246	2005
Kobayashi T, Kakizoe T, et al.	Magnetic anchor for more effective endoscopic mucosal resection.	Jpn J Clin Oncol	34(3)	118-123	2004
Nomori H, Kobayashi T, et al.	Fluorine 18-tagged fluorodeoxyglucose positron emission tomographic scanning to predict lymph node metastasis, invasiveness, or both, in clinical T1 N0 M0 lung adenocarcinoma.	J Thorac Cardiovasc Surg	128	396-401	2004
Kakinuma R, Kobayashi T, et al.	Progression of focal ground-glass opacity detected by low-dose helical computed tomography screening for lung cancer.	J Comput Assist Tomogr	28	17-23	2004
Yamazaki A, Sendoh M, Ishiyama K, Arai K I, Kato R, Nakano M, Fukunaga H	Wireless micro swimming machine with magnetic thin film.	J Magn Magn Mater	272-276	e1741-e1742	2004
山崎彩、仙道雅彦、石山和志、荒井賢一	らせん型磁気マイクロマシンの泳動特性に対するらせん長の影響	日本応用磁気学会誌	28(4)	632-635	2004
Inaba Y, Kamata M, Arai Y, Matsueda K, Aramaki T, Takaki H.	Cervical oesophageal stent placement via a retrograde transgastric route.	Br J Radiol	77	787-789	2004
Tateishi U, Nestor L. Muller, Johkoh T, Onishi Y, Arai Y, Satake M, Matsuno Y, Tobinai K.	Primary mediastinal lymphoma. Characteristic features of the various histological subtypes on CT.	J Comput Assist Tomogr	28(6)	782-789	2004

Inaba Y, Arai Y.	Transcatheter arterial embolization for external iliacartery hemorrhage associated with infection in postoperative pelvic malignancy.	J Vas Intervent Radiol	15	283-287	2004
荒井保明、稲葉吉隆、他	緩和医療における狭窄対策	臨牀消化器内科	19(1)	81-89	2004
荒井保明	消化器癌肝転移に対する動注化学療法	臨牀消化器内科	20(2)	189-197	2005
荒井保明	大腸癌肝転移に対する肝動注化学療法の位置づけ	大腸疾患NOW		93-99	2005
Sugiura S, Satake M, et al.	Effect of interfacial tension on thedynamic behavior of droplet formation during microchannel emulsification.	J Colloid Interface Sci	269	178-185	2004
Sugiura S, Satake M, et al.	Preparation characteristics of water-in-Oil-in-water multiple emulsions using microchannel emulsification.	J Colloid Interface Sci	270	221-228	2004
Ishii H, Satake M, et al.	Hepatic arterial infusion of 5-fluorouracil and extrabeam radiotherapy for liver metastases from pancreatic carcinoma.	Hepato-Gastroenterol	51	1175-1178	2004
Tateishi U, Satake M, et al.	Synovial sarcoma of the soft tissues. Prognostic significance of imaging features.	J Comput Assist Tomogr	28(1)	140-148	2004
Tateishi U, Satake M, et al.	Prognostic significance of MRI findings in patients with myxoid-round cell liposarcoma.	AJR	182	725-731	2004
角美奈子、野村和弘	SRT (Stereotactic Radiotherapy)	先端医療シリーズ29 脳神経外科 脳神経外科の最新医療		173-178	2004
Shibamoto Y, Sumi M, et al.	Primary central nervous system lymphoma in Japan 1995-1999: Changes from the preceding 10 years.	J Cancer Res Clin Oncol	130(6)	351-356	2004
Sekine I, Sumi M, et al.	Phase I study of cisplatin, vinorelbine, and concurrent thoracic radiotherapy for unresectable stage III non-small cell lung cancer.	Cancer Sci	95(8)	691-695	2004

角美奈子	前立腺がん：メカニズム、診断、治療選択　放射線治療の新しい展開	BIO Clinica	19(3)	225-230	2004
角美奈子	固形腫瘍の新しい治療 小児固形腫瘍・脳腫瘍の放射線治療	小児科診療	67(4)	543-549	2004

doi:10.1016/j.ymthe.2004.07.001

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

Kenzaburo Tani,[1,2,*] Miyuki Azuma,[3] Yukoh Nakazaki,[1,2] Naoki Oyaizu,[1] Hidenori Hase,[1] Junko Ohata,[1] Keisuke Takahashi,[1] Maki OiwaMonna,[1] Kisaburo Hanazawa,[4] Yoshiaki Wakumoto,[4] Kouji Kawai,[5] Masayuki Noguchi,[5] Yasushi Soda,[1] Reiko Kunisaki,[1] Kiyoshi Watari,[1] Satoshi Takahashi,[1] Utako Machida,[1] Noriharu Satoh,[1] Arinobu Tojo,[1] Taira Maekawa,[1] Masazumi Eriguchi,[1] Shinji Tomikawa,[1] Hideaki Tahara,[1] Yusuke Inoue,[1] Hiroki Yoshikawa,[1] Yoshitsugu Yamada,[1] Aikichi Iwamoto,[1] Hirofumi Hamada,[6] Naohide Yamashita,[1] Koh Okumura,[7] Tadao Kakizoe,[8] Hideyuki Akaza,[5] Makoto Fujime,[4] Shirley Clift,[9] Dale Ando,[9] Richard Mulligan,[10] and Shigetaka Asano[1]

[1]*Advanced Clinical Research Center, The Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan*
[2]*Department of Advanced Molecular and Cell Therapy, Division of Molecular and Clinical Genetics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan*
[4]*Department of Urology* [7]*and Department of Immunology, Juntendo University School of Medicine, Tokyo 113-8421, Japan*
[5]*Department of Urology, University of Tsukuba School of Medicine, Ibaraki 305-8575, Japan*
[3]*Department of Molecular Immunology, Division of Oral Health Sciences, Graduate School, Tokyo Medical & Dental University, Tokyo 123-8519, Japan*
[6]*Department of Molecular Medicine, Sapporo Medical University, Sapporo 060-8556, Japan*
[8]*Department of Urology, National Cancer Center Hospital, Tokyo 104-0045, Japan*
[9]*Cell Genesys, Inc., South San Francisco, CA 94080, USA*
[10]*Division of Molecular Medicine, Children's Hospital, Department of Genetics, Harvard Medical School, Boston, MA 02115, USA*

**To whom correspondence and reprint requests should be addressed at the Department of Advanced Molecular and Cell Therapy, Division of Molecular and Clinical Genetics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1, Maidashi, Higashi-Ku, Fukuoka-shi, Fukuoka 812-8582, Japan. Fax: 81 92 642 6444. E-mail: taniken@biqreg.kyushu-u.ac.jp.*

Available online 19 August 2004

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

1525-0016/$30.00

doi:10.1016/j.ymthe.2004.07.001

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

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

doi:10.1016/j.ymthe.2004.07.001

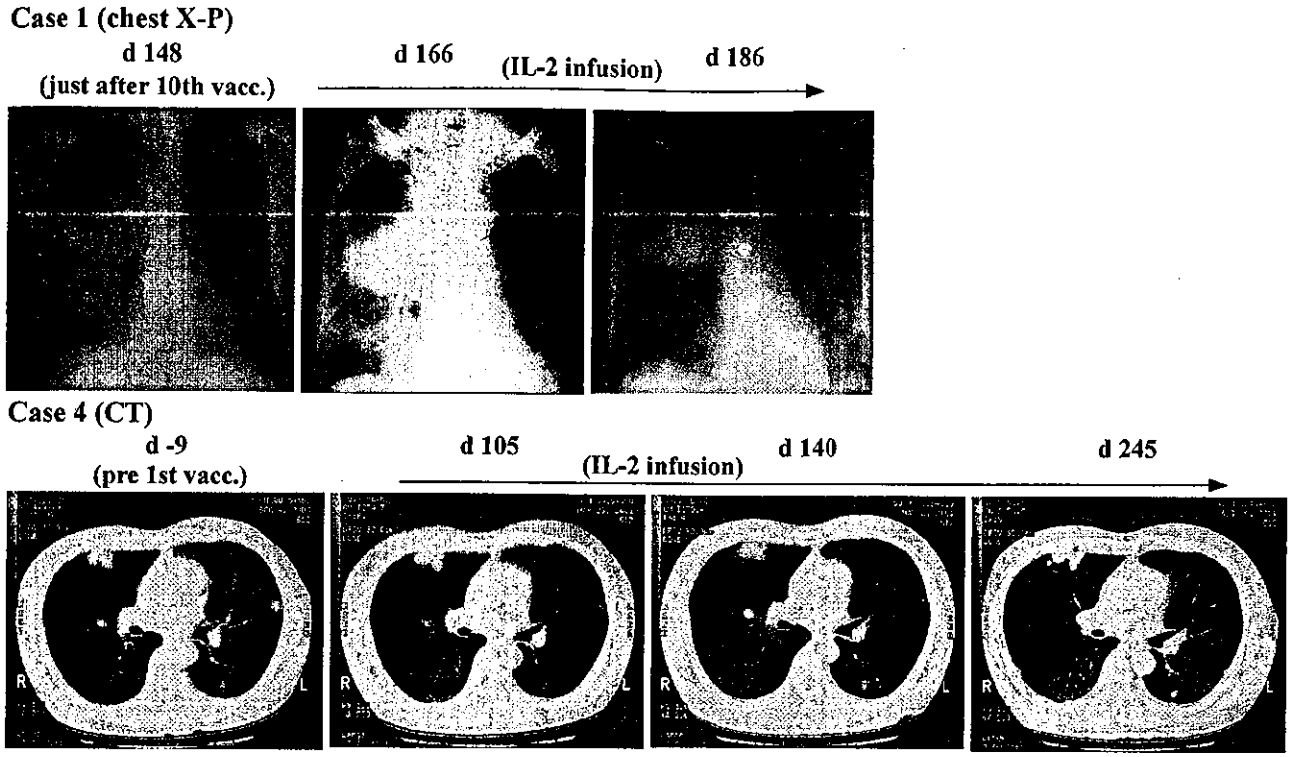


FIG. 1. Size change of the lung metastases in Cases 1 and 4 during the observation period after GVAX vaccination. (A) In Case 1, the size of the right hilar tumor, the largest metastatic lesion, became smaller after 1 week of low-dose IL-2 (d 166, day 166) and decreased by 30% after 1 month of low-dose 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

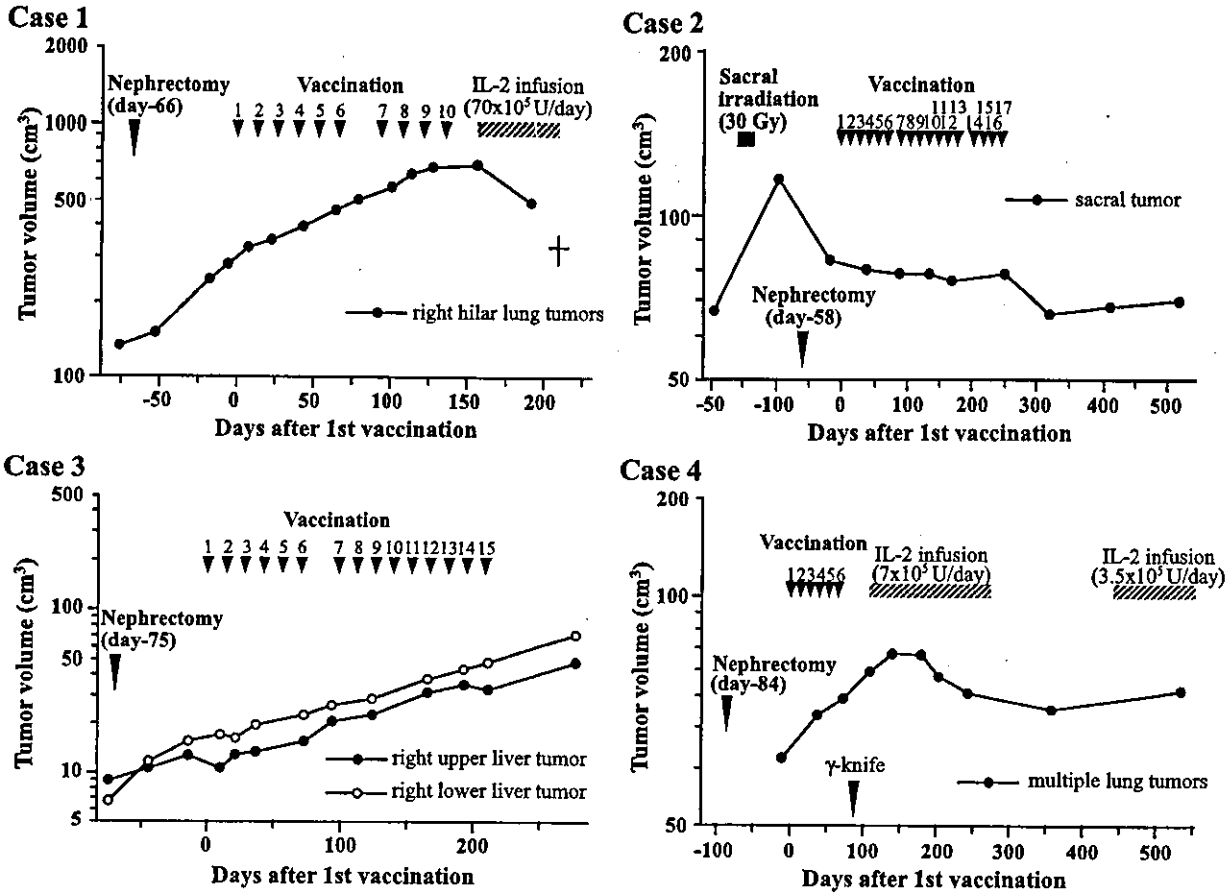


FIG. 2. Clinical summary of the four stage IV RCC patients (Cases 1 to 4) who received GVAX. The days of each vaccination are indicated by the short arrows, and the upper numbers signify the number of vaccinations. The long arrow indicates the time of nephrectomy of an RCC that involved the kidney. The 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

doi:10.1016/j.ymthe.2004.07.001

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

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

doi:10.1016/j.ymthe.2004.07.001

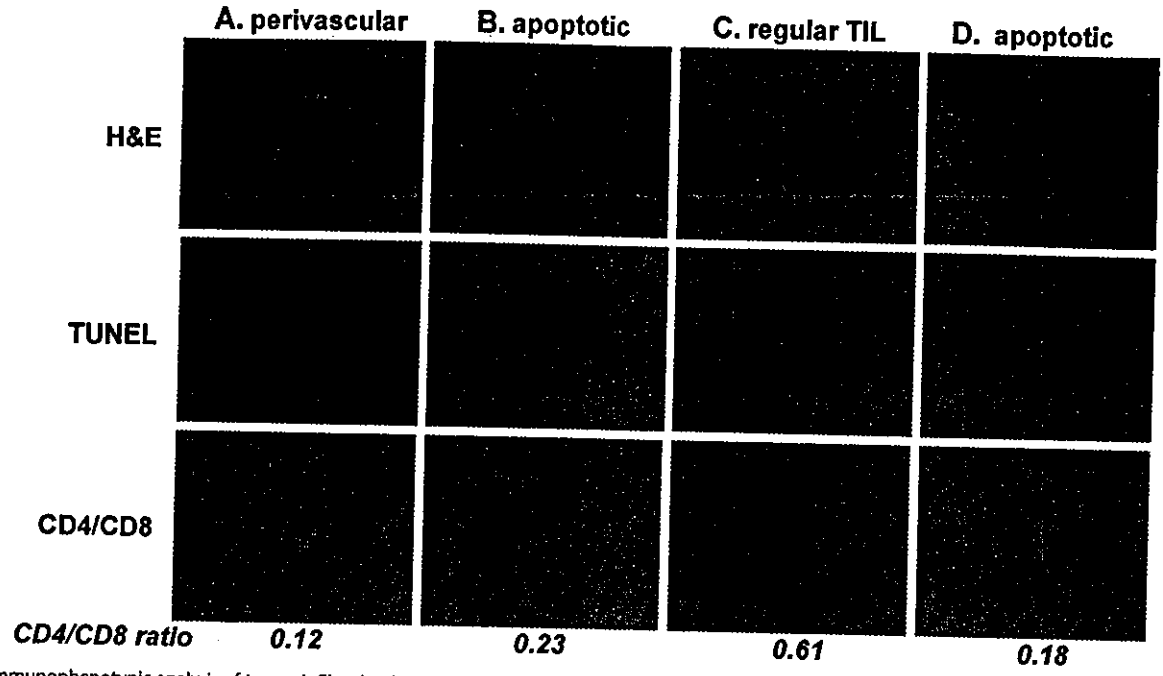
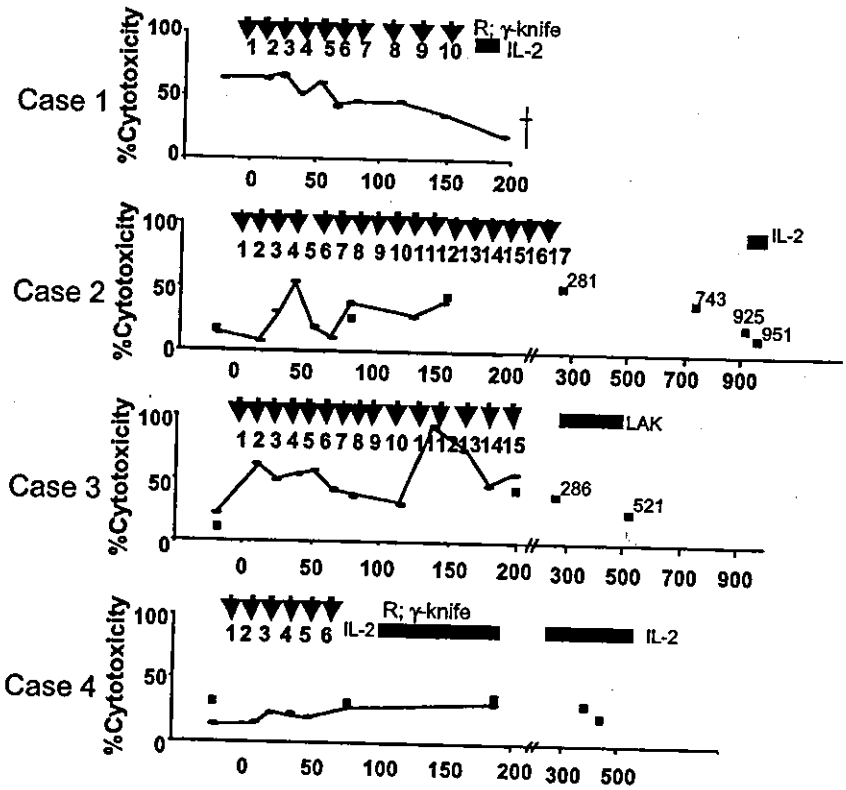


FIG. 3. Immunophenotypic analysis of tumor-infiltrating lymphocytes (TILs) in the RCC tumors of Case 1. TILs were observed in various areas, particularly (A) in the perivascular area, (B, D) around foci of tumor cell apoptosis, and (C) in unremarkable areas, within metastatic RCC that were obtained 7 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.



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,

doi:10.1016/j.ymthe.2004.07.001

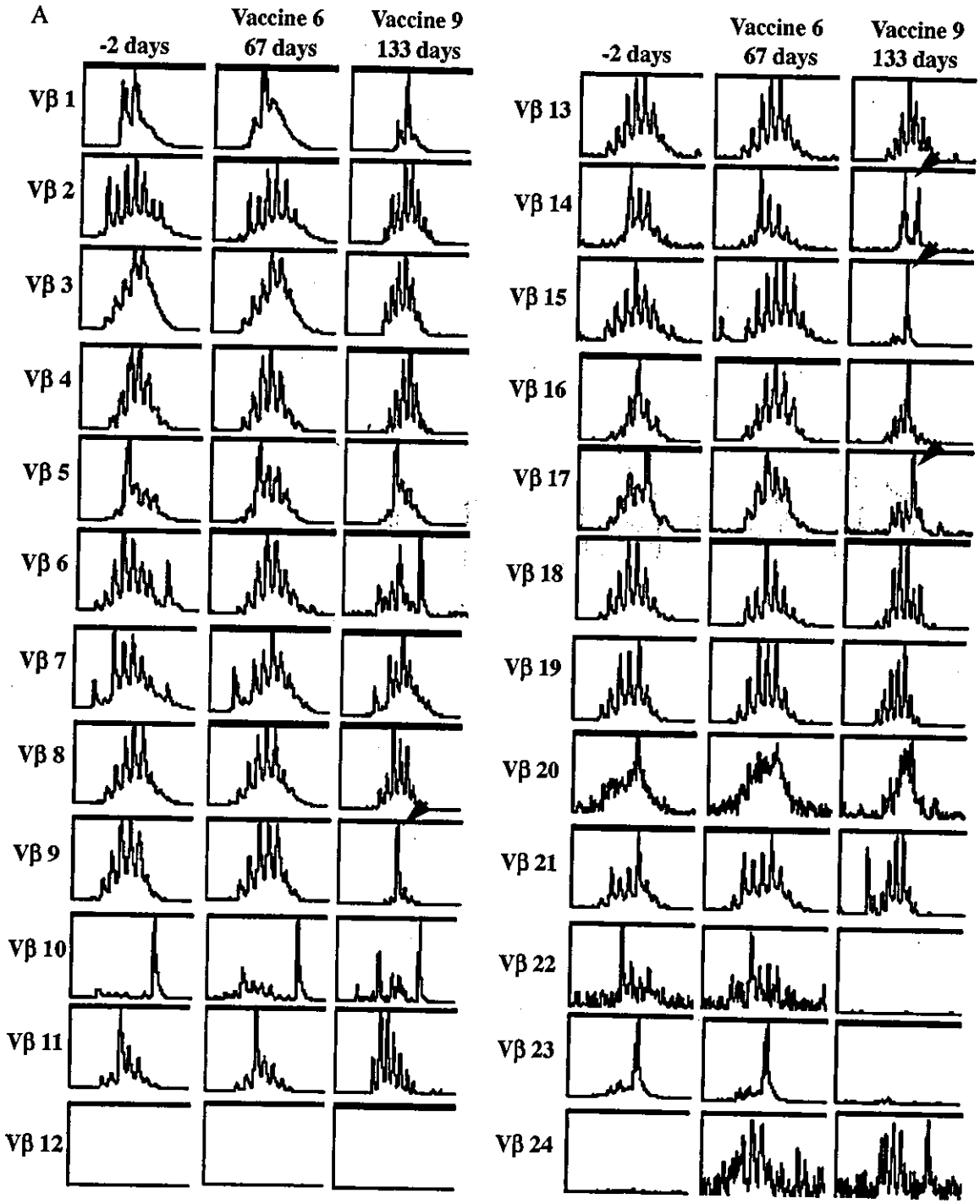


FIG. 5. TCR Vβ clonotypic analysis of the T cell infiltration in DTH, lung metastasis, and renal cell carcinoma in Case 1. (A) Oligoclonal expansion of T cell Vβ 9, 14, 15, and 17 repertoires was observed in peripheral lymphocytes after the ninth vaccination. (B) Larger numbers of T cells with Vβ 10, 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.

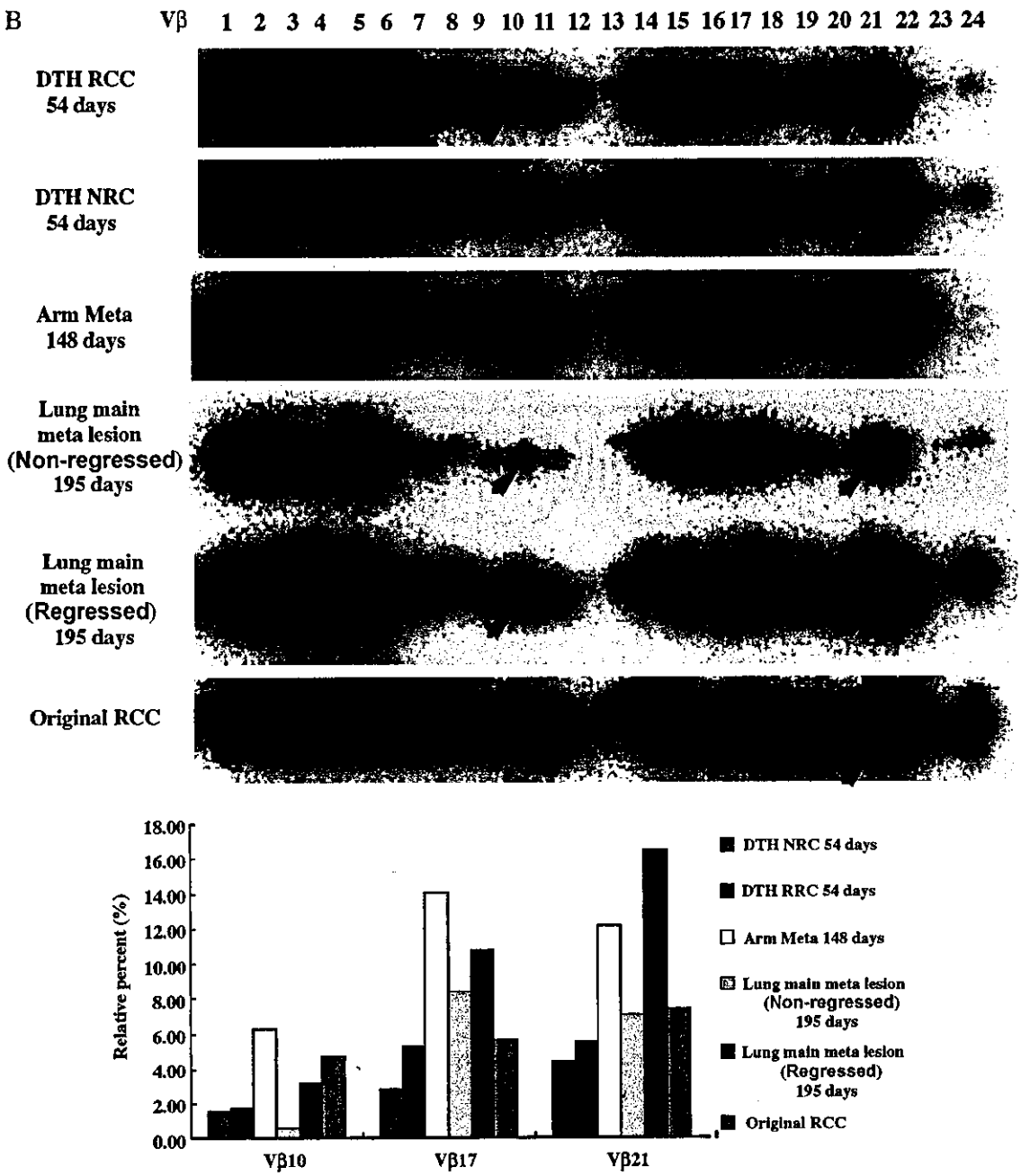


FIG. 5 (*continued*).

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

doi:10.1016/j.ymthe.2004.07.001

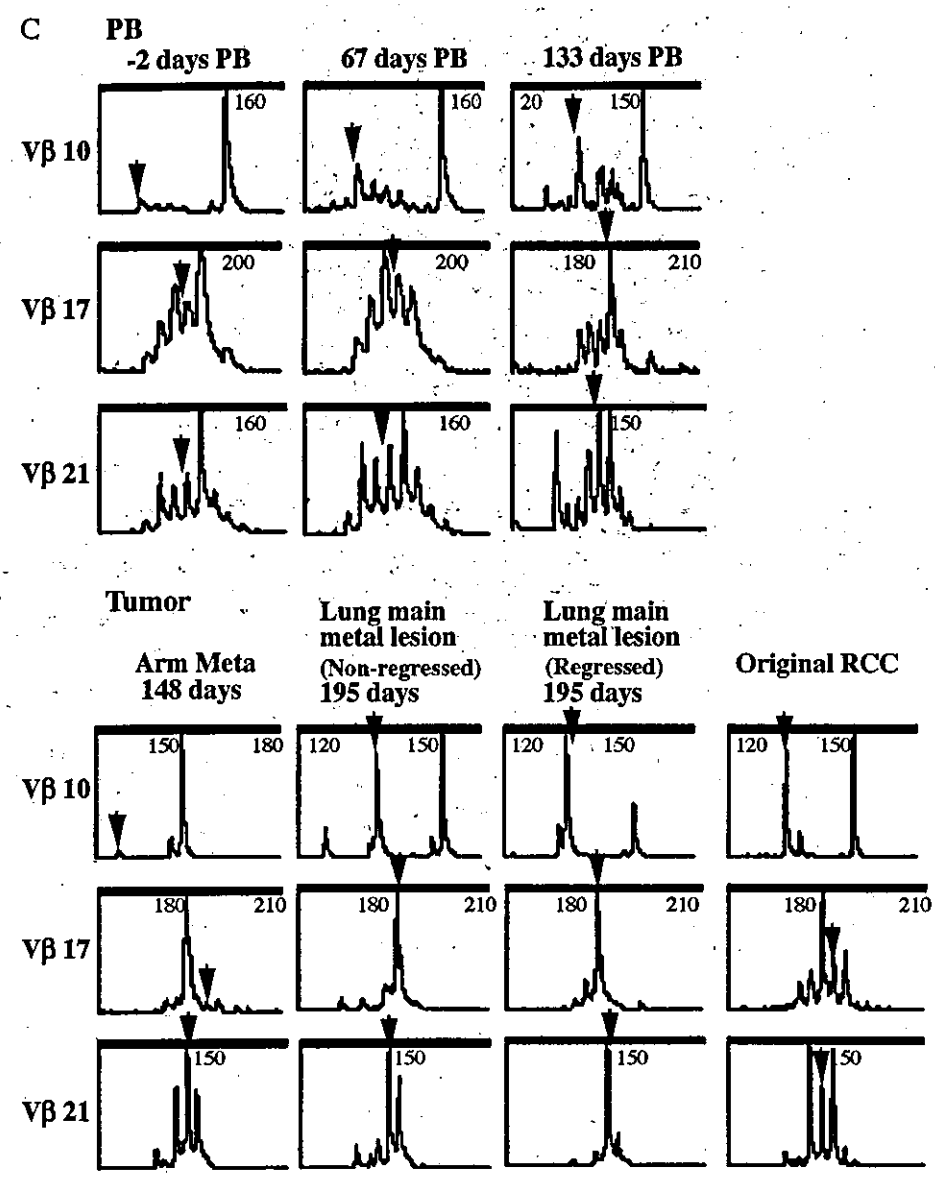


FIG. 5 (*continued*).

induced in these patients. Conversely, the reactions were also positive for NRC, most notably in Case 4, as judged by the skin reaction size (Table 2). The possible causes for this strong background DTH reaction include minimally residual xenoproteins of collagenase or trypsin in GVAX [14] and unknown common antigens existing between NRC and RCC, as the cells that infiltrated into the vaccination sites of RCC and NRC were phenotypically identical, although the number of the former cells was more prominent (data not shown). Thus, possible adverse events of autoimmune nephritis should be monitored carefully. Our follow-up observations over 3 years showed no remarkable renal dysfunction in Cases 2, 3, and 4. Also, Case 1 had no pathological changes associated with autoimmune nephritis in autopsy specimens 7 months after the vaccinations (data not shown). This long-term observation might support the safety of GVAX and its capability of inducing anti-RCC-specific immunity.

The significance of our effector-phase pathological analysis should be emphasized. We had a chance to

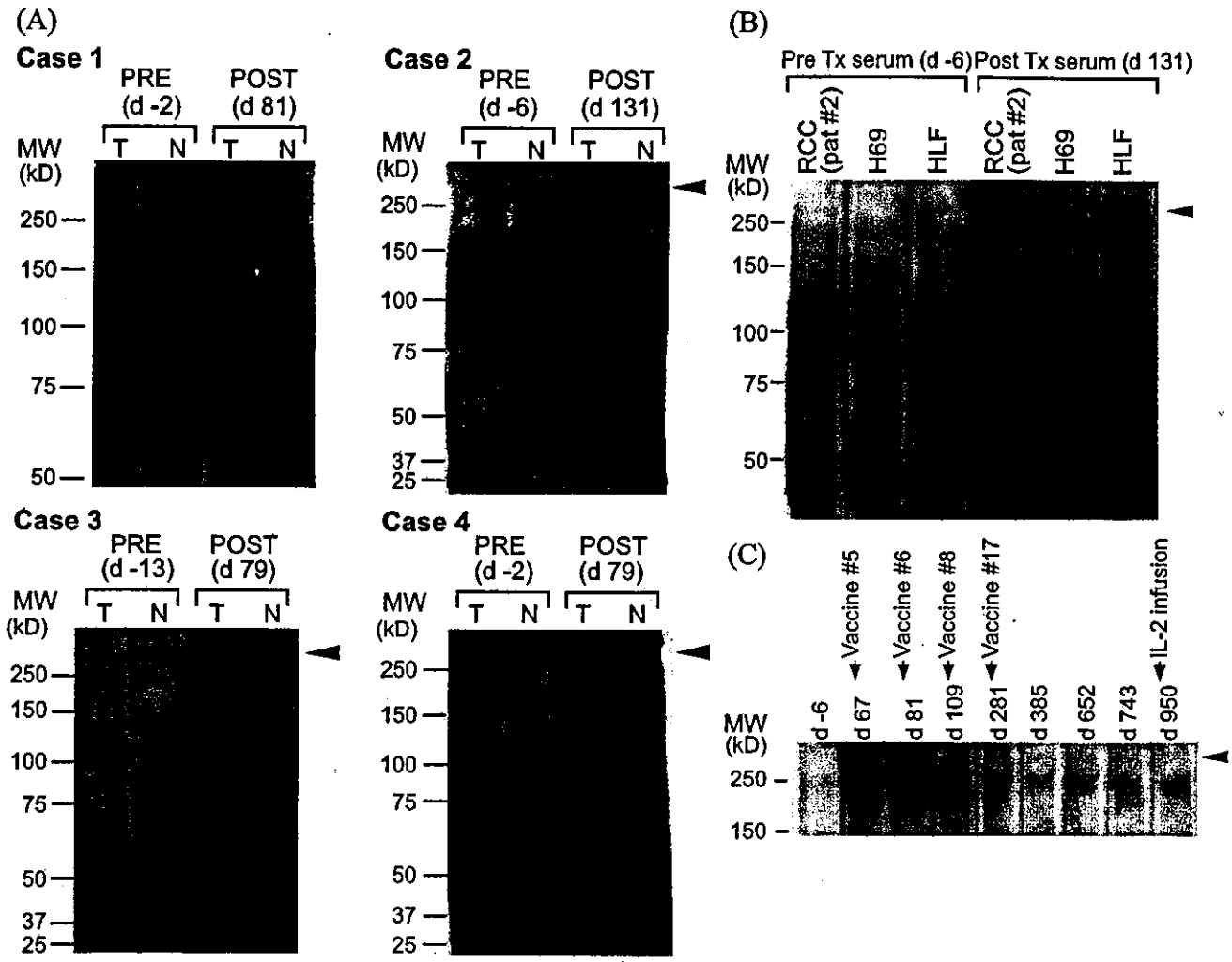


FIG. 6. Appearance of antitumor antibody responses in Cases 1 to 4, who received GVAX. (A) Comparisons of serum reactivity to autologous RCC proteins pre-GTx and post-GTx. Proteins extracted from cultured tumor cells (lane T) or normal kidney cells (lane N) were electrophoresed, transferred onto PVDF membranes, and detected with autologous patient sera (Cases 1 to 4). Sera were harvested before (PRE) and after (POST) the first vaccination (day 0). (B) Comparison of the serum reactivity to autologous RCC, H69 cells, and human lip fibroblasts (HLF). The reactivity to high-molecular-weight proteins was significant in autologous RCC and HLF, while it was weak in H69 cells. Autologous RCC from Case 2 (pat #2) was used. (C) Time course of changes in serum reactivity to high-molecular-weight proteins of approximately 250 kDa in Case 2. Immunoblotting of RCC proteins from Case 2 with autologous sera harvested at several postvaccination days (post-5th, 6th, 8th, and 17th vaccination and before the administration of low-dose IL-2).

investigate pathologically the RCC before and after vaccinations in Case 1 and have demonstrated the induction of tumor site-specific infiltration of predominantly $CD8^+$ T cells. This was associated with tumor apoptosis in postvaccinated biopsy and autopsy tumor specimens, whereas $CD4^+$ T cells predominated and tumor cell apoptosis was negligible in the original RCC (Fig. 3). Notably, these changes were demonstrated in the biopsy specimen that was obtained before IL-2 administration. These observations strongly suggest the induction of tumor-specific immunity by GVAX. Although GVAX could induce both the localization of $CD8^+$ cells within metastatic tumors and significant apoptosis, not all of the tumors showed regression. Thus, GVAX-induced antitumor immunity per se may not be sufficient for clinical efficacy.

We studied various parameters, as it is still unknown which immune factors can be used to predict the therapeutic efficacy of antitumor immune gene therapy. The results of our *in vitro* assessment of cytokine production were compatible to those reported by Soiffer *et al.* [13]. These cytokine profiles indicated the coordinate expression of gene products associated with both Th1 and Th2 cells and suggested that multiple lymphocyte effector mechanisms contribute to the potent antitumor immune response. The cytokines produced by

doi:10.1016/j.ymthe.2004.07.001

these $CD4^+$ T cells activate eosinophils, as well as macrophages that produce both superoxide and nitric oxide. Both of these cell types then collaborate at the site of the tumor challenge to cause its destruction [13]. Our observation suggested that this Th2-dominant immunological response was particularly enhanced, namely, an *in vivo* immune shift from Th1 dominance to Th2 dominance was induced after repeated vaccinations and maintained. Previous studies of GM-CSF immune gene therapy assayed cytotoxicity using PBMC or TIL [13–15]. Kusumoto *et al.* reported that vaccination with irradiated autologous GM-CSF-producing melanoma cells appeared to increase the cytotoxicity against autologous tumor cells in five patients, although repeated vaccination appeared to decrease the CTL activity in two of these cases. They suggested that vaccination of these patients with autologous melanoma cells caused T cell anergy or tolerance [14] without demonstrating the precise underlying immune mechanism involved. In the present study, Case 1, with large lung metastases, which might have contributed to the observed immunological suppression, showed similar results. Although cytotoxicity assayed using PBMC gradually decreased in Case 1, our pathological findings in the metastatic lesion showed the predominant infiltration by CD8 T cells. These findings might support the limited predictability of the *in vivo* antitumor reaction using only traditional immunoassays using PBMC.

Recently, T cell receptor β chain repertoire analysis methods were reported to facilitate the detection of clonal T cell expansion in various biological specimens. As RCC is thought to be a tumor whose growth may be controlled by the immune response, characterization of T lymphocytes found in RCC patients may demonstrate this important issue [16,17]. Using CDR3 length pattern analysis, Puisseux *et al.* demonstrated a selective localization of oligoclonal T cell populations in malignant tissues after comparisons to the T cell repertoire in the tumor and in the autologous peripheral blood lymphocytes or normal adjacent kidney [16]. Importantly, in our clinical studies, the induction of oligoclonal expansion of T cells with the selected TCR in the peripheral blood, skin biopsy specimens from DTH sites, and tumors was demonstrated after vaccination. The reasons for the observed different clonal T cell expansions in the different tissues in our studies may arise from either a polymorphic T cell response to the same antigen or a different immunogenic environment [17]. Hanada *et al.* recently demonstrated the important role of posttranslational protein splicing in the immune recognition of self and foreign peptides using human RCC antigens, and this phenomenon may explain our results [29]. Although we could not prove directly that these oligoclonally expanded T cells responded to RCC antigens, our findings of the generation of MHC-restricted and TCR-mediated cytotoxicity against autologous RCC and the predominant infiltration of CD8 T cells and apoptosis in metastatic lesions [1] supported this possibility.

In addition to the enhanced antitumor cellular immunity, GVAX is thought to induce antitumor humoral immunity. Simons *et al.* measured increased titers of antibodies recognizing prostate tumor antigens in sera from patients vaccinated with GM-CSF-transduced autologous prostate tumor cells. New antibodies recognizing polypeptides of 26, 31, and 150 kDa in extracts from LN CapPCA cells were observed in three of eight patients following the final vaccinations [18]. Soiffer *et al.* reported similar observations, with antibodies recognizing different polypeptides, in melanoma patients [13]. In the present study, Western blot analysis identified RCC-derived polypeptides of 65 and 250 kDa. We are currently screening RCC cDNA expression libraries with our patients' sera using the SEREX method to look for RCC-specific antigens other than RAGE and G250 [30,31]. We have already cloned several candidate cDNAs and are studying their RCC specificities and the possibility of their future application in anti-RCC immunotherapy.

Currently, several candidate strategies to enhance the systemic anti-RCC immunity of GVAX can be considered. These include the coadministration of IL-2 to enhance basal antitumor immunity [3,5–7,32–34], allogeneic stem cell transplantation including nonmyeloablative stem cell transplantation to introduce allogeneic immunity [27], IL-12 or CD80 cDNA-transduced autologous tumor cells for the direct activation of CTLs, the blockade of CTLA-4/B7 interactions with monoclonal antibody to activate costimulation signals, and the functional activation of dendritic cells using HSP gp91 [35–38]. The administration of low-dose IL-2 as an anti-cancer immunotherapy has recently been introduced to decrease both the side effects and the cost of treatment [39–42].Our experience with three patients who were given GVAX followed by low-dose IL-2 would cast new light on anti-cancer immunotherapy, possibly by inducing tumor-specific immunity by GVAX, followed by enhancement of the broad antitumor immunity with systemic low-dose IL-2. In the present study, *in vitro* CTL analysis in these two patients supported the hypothesis that the antitumor CTL activity was maintained after administration of IL-2. The optimal duration of treatment with low-dose IL-2 in combination with GVAX remains to be determined by closely monitoring antitumor immunity both *in vitro* and *in vivo*.

PATIENTS AND METHODS

Selection of Patients

The details of the study design and methods of vaccine production were essentially the same as those reported by Simons *et al.* [13,14], except for modifications that were implemented according to the regulations for

clinical gene therapy announced by the Japanese government between 1995 and 1997. Briefly, patients with stage IV RCC (Union Internationale Contre le Cancer classification of 1997) were eligible. Chemotherapy, radiotherapy, systemic IL-2- or interferon-α-based regimens, or other investigational agents were also offered as treatment options to these patients. The following eligibility criteria were used: primary RCC in place with evaluable metastasis after nephrectomy; Eastern Cooperative Oncology Group performance status of zero or one; appropriate surgical candidate and estimated life expectancy of at least 6 months; no major surgery, radiotherapy, chemotherapy, immunotherapy, or immunosuppressive medications within 1 month prior to enrollment; age >18 years; absence of active infection, i.e., WBC count <4000/μl, platelets <100,000/μl, total bilirubin <1.5 mg/dl, and creatinine <2.0 mg/dl; HIV seronegativity; and no history of autoimmune disease. The exclusion criteria included age <20 years; pregnant or lactating women; double malignant tumors; surgery; local or systemic treatment with corticosteroids; immunotherapy; irradiation or anti-cancer drugs 1 month before registration; leukocytosis of unknown origin; history of systemic lupus erythematosis, sarcoidosis, rheumatoid arthritis, autoimmune hemolytic anemia, autoimmune thyroiditis, glomerulonephritis, or vasculitis; apparent infection requiring treatment before second stage; apparent brain metastasis detected on CT scan or MRI; postnephrectomy deep vein thrombosis or pulmonary embolism that required treatment; and opium or alcohol abuse. The study was reviewed and approved by the Committee on Clinical Investigation and Institutional Gene Therapy Ethical Committee, The Institute of Medical Science, University of Tokyo, in April 1998, and by the Joint Committee of the BioScience Committees of the Ministry of Health, Labor, and Welfare and the Ministry of Education, Culture, Sports, Science, and Technology in August 1998.

Study Design

Patients were enrolled from September 1998 to May 2001. Eligible patients were nephrectomized after giving their initial informed consent. The second informed consent was obtained after safety confirmation tests, which included negative tests for microbial contaminants such as bacteria, fungi, mycoplasma, RCR, and endotoxin, and when sufficient production (>40 ng/10^6 cells/24 h) of GM-CSF was detected in the GM-CSF gene-transduced RCC. The vaccination schedule of GVAX, including additional vaccinations, is described precisely under Vaccine preparation and administration. Peripheral blood was obtained (as per NIH Recombinant DNA Advisory Committee and Food and Drug Administration guidelines) for detecting RCR before treatment, after vaccination, monthly for 3 months, every 3 months for the next 9months, and then yearly [14]. Long-term follow-up, including periodic evaluation for autoimmune disease and tumor progression, was performed.

Clinical Evaluation

The patients received daily physical examinations and periodic laboratory tests, which included hematological parameters and liver, renal, and immunological functions, prior to and after the vaccinations. The metastatic lesion volumes were measured using CT (lung, liver, bone, brain), MRI (liver, bone, brain), and thallium or technetium scintigraphy (whole body). Unenhanced helical CT images that covered each lesion were obtained during a single breath-hold. The thickness of the slices ranged from 3 to 10 mm, depending on the lesion size. The data were transferred to a workstation (Advantage Windows; General Electric Medical Systems, Milwaukee, WI, USA) to calculate the tumor volumes. Low-density areas, which represent lung parenchyma, were excluded at a threshold of −400 HU, and lesion sections were selected manually from the remaining areas of each slice. The lesion volume was calculated with a 3-D utility on the workstation for Cases 1, 2, and 3. The lesion in Case 4 was calculated as the sum of the perpendicular diameters of all lesions measured by CT scan, due to difficulties in measuring small multiple tumors volumetrically.

Vaccine Preparation and Administration

The methods used for autologous RCC vaccine preparation and MFGS-GM-CSF gene transfer at the Clinical Cell Processing Facility of the Institute of Medical Science Hospital at the University of Tokyo have been described previously [13]. The procedure complied with good manufacturing practices. Primary cultures were established and transduced at the first passage. Following *in vitro* expansion, the vaccine cells were irradiated at 150 Gy to prevent clonogenic survival *in vivo* after vaccination. GM-CSF production was determined using a GM-CSF ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Genomic integration of the GM-CSF cDNA into the patients' autologous RCC cells was determined by the standard Southern blotting method using MFG-GM-CSF plasmid DNA to determine the copy number, as described elsewhere [43]. The tests for microbial contaminants, i.e., bacteria, fungi, mycoplasma, RCR, and endotoxin, were all performed by BioReliance Corp. (Rockville, MD, USA). The vaccine cells were stored in liquid nitrogen until use. On the day of vaccination, 4×10^7 viable cells were administered intradermally in the first injection, and thereafter, 2×10^7 cells were administered at least five times at 2-week intervals, which was considered to be a superior vaccination schedule as described by Soiffer *et al.* [16]. Each patient was carefully screened for eligibility according to the inclusion criteria by the Institutional Review Board (IRB) of the Institute of Medical Science, University of Tokyo. The IRB permitted additional

doi:10.1016/j.ymthe.2004.07.001

administration of vaccine every 2 weeks when the yield of cells was higher than the 1.4×10^8 cells required for the six scheduled administrations and in cases in which the patient's physical condition was acceptable after further informed consent was obtained. The vaccinated sites were biopsied for microscopic examination at 3 and/or 7 days after every second vaccination.

Toxicity Assessment and Pharmacokinetic Analysis of Serum GM-CSF Levels

The levels of toxicity were graded using the National Cancer Institute's cancer common toxicity criteria for clinical trials. Toxicities were identified by medical history, physical examination, and review of the laboratory studies performed. Patients' sera were frozen in 1-ml aliquots at −80°C until the day of testing. The serum GM-CSF levels were determined for all collection time points by enzyme-linked immunosorbent assay using the Biotrak human GM-CSF ELISA system (Amersham International Plc., Amersham, UK) according to the manufacturer's protocol.

Histological Studies

Six-millimeter punch biopsies were removed from the intradermal injection sites on days 3 and/or 7 following the first vaccination. Prevaccination skin biopsies were obtained for comparison. Similarly, skin biopsies were also taken for evaluation of the DTH reaction 48 h after intradermal inoculation of RCC cells and NRC. Surgically removed and autopsy materials were used for the histological evaluation of tumors and tumor-infiltrating cells. Biopsy materials were fixed in 10% buffered formalin, embedded in paraffin, stained with H&E, and labeled with antibodies to CD3, BMP (rabbit antiserum to human myelin basic protein; DAKO Corp., Carpinteria, CA, USA), AE1/AE3 (pooled mAbs to human epithelial keratin, IgG1 subtype; Boehringer Mannheim, Indianapolis, IN, USA), S100 (rabbit anti-cow S100; DAKO), CD68 (anti-human macrophage CD68 mAb, IgG3-subtype; DAKO), HLA-DR (clone LN3, IgG2a subtype; Lab Vision Corp., Fremont, CA, USA), CD3 (clone PS1, mAb, IgG2a subtype; Novocastra Laboratories, Newcastle, UK), CD4 (clone 1F6, mAb, IgG1 subtype; Novocastra Laboratories), CD8 (clone 1A5, IgG1 subtype; Novocastra Laboratories), and CD20cy (B cell marker, clone L26, mAb, IgG2a subtype; Lab Vision Corp.). For the evaluation of tumor apoptosis, the TdT-mediated dUTP–biotin nick end-labeling (TUNEL) method was applied using an ApopTag Kit (Intergen Co., Purchase, NY, USA).

Delayed-Type Hypersensitivity Testing

To evaluate the cell-mediated immunity status of each patient before and after treatment, DTH testing was performed using seven common recall antigens (Multitest CMI; Connaught Laboratories, Swiftwater, PA, USA) according to the manufacturer's instructions. Reaction scoring was also performed according to the manufacturer's instructions. The patients were tested simultaneously for reactivity to autologous, irradiated cultured RCC cells and NRC. The autologous RCC cells and NRC for DTH testing were prepared and stored in liquid nitrogen according to the same procedure used for vaccine cell production omitting GM-CSF transduction. During storage, sterility testing for bacteria, fungi, mycoplasma, and endotoxin was carried out at the Department of Laboratory Medicine, Institute of Medical Science, University of Tokyo. PBMC were isolated using the standard Lymphoprep (d = 1.077; Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation method. These cells were washed three times with HBSS, counted, and injected intradermally at 10^6 cells/0.2 ml. DTH reactions were observed 48 h after each DTH injection, i.e., 1 week before the first vaccination and 1 week after the second, fourth, and sixth vaccinations in all four patients.

Tumor Tissues, Peripheral Blood, and Skin Biopsies from Patients

Single-cell suspensions of tumor tissues were obtained from biopsied or autopsied (Case 1) tumor specimens that were minced mechanically and treated with collagenase and DNase. RCC cells and TILs were separated by density gradient centrifugation, as described elsewhere [24]. Heparinized peripheral blood samples (20 ml) were drawn from patients every other week before vaccination. For follow-up, samples were also drawn when the patients permitted. Patients' sera were frozen at −80°C until use for Western blot analysis. PBMC were isolated as above. PBMC and TIL (5×10^6 cells/tube) were cryopreserved using a programmable freezer and stored in liquid nitrogen. In addition, the cell pellets were frozen in liquid nitrogen until used for RNA extraction. Skin biopsies obtained from the DTH reaction site (6 mm in diameter) were cut into pieces measuring approximately 1 × 1 mm and rapidly frozen in liquid nitrogen until used for RNA extraction.

Assessment of Lymphocyte Proliferation and Cytokine Production

On the day of the assay, the cryopreserved samples were thawed. PBMC (1×10^5 cells/well) were cultured in the presence of irradiated (150 Gy) GM-CSF-transduced autologous tumor cells (1×10^4 cells/well) plus IL-2 (40 U/ml), in 96-well flat-bottomed plates. RPMI 1640 medium with L-glutamine (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) and gentamicin was used as complete medium. On day 3 or 6, culture supernatants (100 μl/well) were collected from each well to determine the cytokine levels, and fresh medium was added. The cultures were then pulsed with [^{3}H]thymidine (0.5 μCi/well; DuPont-NEN, Boston, MA, USA) for a final 18 h and harvested on a Micro 96 harvester (Skatron, Lier, Norway), and the incorporated radioactivity was measured using a