

# Allogeneic Gene-Modified Tumour Cells in Metastatic Kidney Cancer. Preliminary Report.

( vaccine / gene-modified / immunotherapy / IL-2 / MRCC / renal cancer )

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**Abstract.** An allogeneic irradiated RCC cell line, engineered to produce IL-2 (ACHN-IL-2), admixed with autologous metastatic formalin-treated tumour cells, was used to vaccinate ten MRCC patients in progression of disease in spite of IL-2 immunotherapy. The cells were administered subcutaneously and/or intra-tumourally. Sixty-four MRCC patients in progressive disease, not treated by vaccination but receiving similar IL-2 immunotherapy, were considered as the control group.

Patients received 4–16 injections (mean  $9 \pm 4$ ), containing an average of  $10.6 \times 10^7 \pm 7.7 \times 10^7$  ACHN-IL-2-transfected cells (a minimum of  $4 \times 10^7$ , and a maximum of  $31 \times 10^7$ ). Four patients also received intra-tumour injections. Vaccination was administered during 30–418 days, and the follow-up continued for  $649 \pm 353$  days (190–1342). Throughout this period, the patients continued receiving the previously set immunotherapy treatment.

No adverse side effects related to the treatment were observed. One complete and one partial tumour response were observed, as well as two stable and one no-relapse disease. All but one patient died. Responding patients resumed progression in 4–11 months and died 18 and 36 months after beginning the vaccine therapy. In spite of the small number of treated patients, Wilcoxon's test showed a significant ( $P < 0.05$ ) improvement of the survival in the vaccinated group compared to that of the control. The described vaccination protocol seems safe, devoid of adverse side effects and promising. It warrants further investigation.

Patients suffering from metastatic renal cell cancer (MRCC) have a poor prognosis and 5 years after diagnosis, a survival rate less than 5% (Hrushsky and Murphy, 1977; Elson et al., 1988). In 4542 patients under chemotherapy, a 6% response rate was reported (Yagoda et al, 1995). Today it is accepted that the most promising systemic therapy for MRCC is immunotherapy, viz. i.v. interleukin (IL)-2 administration (Vogelzang and Stadler, 1998). Indeed, in 1411 MRCC patients thus treated, the overall response rate was 20% (Bukowski, 1997, 2000; Pizza et al., 2002). Similar results, but with significant less toxicity, were obtained using intralymphatic administration of IL-2 (Pizza et al., 2001). Although encouraging, these observations cannot be considered satisfactory and new therapeutic approaches are under study, tumour vaccination being one of them.

The concept of tumour vaccines is not new. For example, first-generation tumour vaccines were composed of whole inactivated cancer cells or tumour

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Abbreviations: ATC – autologous tumour cells, ATV – autologous tumour vaccine, CR – complete response, IL – interleukin, i.m. – intramuscularly, LAK – lymphokine-activated killer cells, LST – lymphocyte stimulation test, MRCC – metastatic renal cell cancer, PB – peripheral blood, PBL – peripheral blood lymphocytes, PHA – phytohaemagglutinin, PR – partial response, PROG – tumour progression, PS – performance status, pt(s) – patient(s), s.c. – subcutaneous, sd – standard deviation, STAB – stable disease, TAA – tumour-associated antigens, TNM – tumour, node, metastasis, TF – transfer factor, U – unit, y – year(s).

lysates administered together with immune adjuvants. However, advances in gene transfer technology, tumour immunology, molecular biology, and methods of monitoring the antitumour response have allowed novel, more specific vaccine approaches.

Although no definite proof is offered concerning the presence of tumour-specific antigens in kidney cancer, at least six tumour-associated antigens (TAA) have been described recently (Ueda et al., 1981), but their specificity for the renal cancer is still uncertain (Oosterwijk et al., 1986). It thus seems that until TAA shared by most of the renal tumours have been identified, the best approach for vaccine therapy remains to be the use of autologous tumour cells obtained from the primary tumour or from metastases. Hence, certain investigators established short-term cultures from autologous tumour cells capable of producing skin test conversions towards autologous tumour vaccine (ATV) in MRCC-treated patients (Dillman et al., 2001). Others, using ATV together with interferons both alpha and gamma, or BCG as adjuvants, observed a variable response with mild toxicity (Schwaab et al., 2000).

Because the activation of cellular immunity requires synergistic signals, including presentation of specific tumour antigens, co-stimulatory signals and propagation of the immune response via cytokine release (Antonia et al., 2000), certain investigators decided to insert pertinent cDNA, which governs the release of some of the most important interleukins, directly into the autologous tumour cells (ATC) to be used as ATV. However, transfection of ATC is difficult to carry out for each patient (Dillman et al., 2001). Consequently, another approach has been developed using established allogeneic renal tumour cell lines to produce, *inter alia*, IL-2 by transfection with pertinent cDNA (Belledgegrun et al., 1993). In these studies, the IL-2-producing cells increased the cytotoxic activity of peripheral blood lymphocytes (PBL). We chose a similar approach in engineering the ACHN renal cancer cell line (Pizza et al., 1999).

Since it is known that cytotoxic T lymphocytes infiltrating renal tumours are able to kill ATC *in vitro* (Koo et al., 1991; Finke et al., 1998) and that small amounts of IL-2 directly injected into the tumour can activate an immune response with complete and/or partial regression of the disease (Pizza et al., 1984, 1987), we decided to investigate whether the injection of allogeneic renal tumour cells engineered to secrete small amounts of IL-2 in metastatic RCC lesions were able to activate tumour regression. It was further hypothesized that such administration could act as a surrogate vaccination. In order to reinforce the effect, we also injected *s.c.* allogeneic tumour cells engineered to produce IL-2, admixed with formalin-treated ATC cells that were obtained from pathological bone fractures due to metastases or from curative resection of lung tumour nodules. As regards ATC, we used formalin treatment, since it

has the ability to preserve the antigenicity of tumour cells for several years, as assessed *in vitro* (Drake et al., 1972; Ross et al., 1975, 1983; Pizza et al., 1976, 1977; Levine et al., 1981) and *in vivo* (Corrado et al., 1984; De Vinci et al., 1987).

We report here the first results in ten MRCC patients in progression of disease in spite of IL-2-based immunotherapy, and who were vaccinated using autologous metastatic cancer cells (ATC), together with an allogeneic renal cancer cell line engineered to produce IL-2. The treatment was administered following a 3-year clinical experimental protocol authorized by the Ministry of Health and the members of the local Ethics Committee.

## Material and Methods

### *IL-2 expression vector*

A human IL-2 expression vector, pcDNA-I Neo-IL-2 (7683 bp length), was prepared by insertion of IL-2 cDNA (683 bp length) in *HindIII/BamHI* sites of the plasmid of the polylinker pcDNAINeo (InVitrogen S. R. L., San Donato Milanese, Italy). The human IL-2 cDNA (683 bp) was taken from the pBC12 CMV/hIL-2 plasmid (Cullen, 1986) and was obtained by double digestion with *BamHI/HindIII*. The fragment of 683 bp (cDNA IL-2) was then purified on 1% agarose gel and linked to the pcDNAINeo plasmid. The latter was prepared by digestion of *BamHI/HindIII* and dephosphorylation reaction. The pcDNAINeo is a eukaryotic expression vector containing the cytomegalovirus (CMV) promoter/enhancer conferring a high transcription level to the plasmid, and it also contains a polyadenylation signal consisting in a sequence of origin and a transcription terminus obtained from the SV40 (Simian virus 40). The pcDNAINeo also carries the neomycin gene under the control of the LTR of Rous sarcoma virus. The pcDNAINeo was grown in the bacteria host MC 1061/P3 (InVitrogen) in the presence of ampicillin and tetracycline.

### *Gene transfer*

The amount of  $10^7$  ACHN cells have been transfected with 10  $\mu\text{g}$  of pcDNA-I Neo-IL-2 using the  $\text{CaPO}_4$  technique (Graham and Vanderb, 1973; Cavallo et al., 1993). After transfection, the cells were incubated 24 h in medium with 5 mM sodium butyrate, then set in suspension using trypsin, and cultured at low density in complete medium containing Geneticin® at 400  $\mu\text{g}/\text{ml}$  (G-418, Gibco Laboratories, Grand Island, NY). Resistant clones were isolated and IL-2 production was evaluated using the CTLL-2 line (Gillis and Watson, 1981) or phytohaemagglutinin (PHA) (Difco, Detroit, MI) and IL-2-conditioned human blasts (Pizza et al., 1984). The IL-2-producing clones were assessed for IL-2 mRNA using the Northern blot technique.

### *Tumour cell line*

The ACHN line (ATCC, American Tissue-Type Culture Collection), established from a kidney cancer, was obtained from the Emilia-Romagna and Lombardia Regions Experimental Institute of Zoo-prophylaxis. The cell line is virus, bacteria and mycoplasma free. Before transfection, the spontaneous production of interleukins such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-8 (Endogen Inc., Woburn, MA) and transforming growth factor- $\beta$  (TGF- $\beta$ ), as assessed by commercial kits (DRG Diagnostics<sup>®</sup>, Marburg, Germany), was evaluated. The respective production in tissue culture for  $10^6$  cells/24 h was: 240, 500, 1650 and 650 pg. No production was observed for other cytokines, i.e. IL-1- $\beta$ , IL-2, IL-4, IL-10, IL-12 and interferon- $\gamma$  (Endogen Inc.). No production of HLA-G type was noticed.

### *Radiation treatment*

The transfected cell line was expanded in disposable plastic flasks (Falcon 3028) in RPMI 1640 (Gibco, San Giuliano Milanese, Italy) medium with 10% of heat-inactivated AB pooled human serum obtained from the hospital blood bank. When the cells appeared confluent, they were irradiated with a 60-cobalt bomb at 100 cGy/min to a total of 40–60 Gy. Immediately after irradiation, the cells were collected using trypsin, twice washed, and suspended in RPMI 1640-containing 20% AB serum and 10% dimethylsulphoxide (DMSO). They were stored in liquid nitrogen, in ampoules containing  $10^7$  cells. A few days later, and for each stock prepared, one ampoule was taken, quickly thawed, and the cells were cultured for testing sterility, viability, proliferation, duration of IL-2 production, and days of survival.

### *In vitro IL-2 production of irradiated cells*

The amount of  $5 \times 10^6$  transfected and irradiated cells were cultured in a 250 ml flask with 24 ml of standard medium. Every 72 h the medium was replaced. The culture medium was centrifuged at 400 g and the supernatant stored at  $-80^\circ\text{C}$ . Medium was collected until all the cells were dead, usually 25–36 days. The biological activity of IL-2 was assessed using the growth maintenance of PHA-activated human lymphocytes, according to the technique described by Gillis and Watson (1981) and modified by Pizza et al. (1984). Briefly, PBL from healthy human donors were collected on a Ficoll-Hypaque (F-H) (Amersham-Pharmacia, Uppsala, Sweden) gradient, washed 3 times with medium and stimulated for 24 h with 1  $\mu\text{g}/\text{ml}$  of PHA-P (Difco) in RPMI 1640 medium + 10% of foetal calf serum (FCS) at  $10^6$  cells/ml in a 5%  $\text{CO}_2$  humidified incubator. These PHA-activated blasts were used to test the IL-2 activity. The cells were washed twice and cultivated in RPMI 1640 + 10% FCS and 20% of MLA-144 (a gibbon lymphoid cell line spontaneously producing IL-2 *in vitro*)

supernatant for additional 5 days (Rabin et al., 1981). Triplicate cultures containing  $1 \times 10^5$  cells (Falcon 3040, BD, Milan, Italy) in 0.1 ml of RPMI 1640 + 10% FCS were incubated in the presence or absence of 0.1 ml of a 2-fold serially diluted supernatant from the ACHN-IL-2 irradiated cells. After 24 h incubation in a 5%  $\text{CO}_2$  humidified incubator, 1  $\mu\text{Ci}$  of methyl- $^3\text{H}$ -thymidine (spec. act. 2 mCi/mM, Amersham Biosciences, Buckinghamshire, UK) was added and the cells were incubated for an additional 24 h. The cultures were subsequently harvested on glass filter paper and the incorporated radioactivity was evaluated in a beta-counter and expressed as disintegrations per minute (dpm). A stimulation index (SI) was obtained by dividing the mean dpm of the triplicate cultures in the presence of IL-2 with the mean dpm of the cells cultivated without IL-2. The SIs were then plotted against Log2 of the IL-2 dilutions to give a dose-response curve. The reciprocal of the X-axis dilution coordinate crossing this curve at 1.5 Y-axis coordinate (corresponding to a 50% increase of the radioactivity incorporated into the cells in the presence of IL-2, compared to that of cells cultivated without) was defined as the number of IL-2 units/ml (Pizza et al., 1984).

### *Vitality and replication assay after irradiation*

After quickly thawing the frozen ampoules in a water bath at  $37^\circ\text{C}$ , the cells were washed three times in saline, and viability was assessed using the trypan blue dye (Gibco). Triplicate cultures were also carried out in a  $\text{CO}_2$  incubator with  $2 \times 10^5$  cells in each well and cultivated in 0.2 ml of complete medium for 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, and 36 days. One  $\mu\text{Ci}$  of  $^3\text{H}$ -methyl-thymidine was added 24 h before they were sacrificed, and the radioactivity incorporated into the cells was assessed as previously described (Pizza et al., 1984).

### *Tumorigenesis in nude mice*

The tumorigenicity of ACHN-IL-2 irradiated cells was tested using nude mice. Eight nu/nu CD1 strain animals (Charles River Italia, Calco, Lecco, Italy) were intraperitoneally and s.c. injected with  $50 \times 10^6$  ACHN-IL-2 cells, irradiated with 40, 50, or 60 Gy. The animals were sacrificed three months later. No tumour cells were detected at necropsy, either at the injected or at distant sites, neither was organ involvement observed.

### *Autologous metastatic tumour cells (ATC)*

ATC were obtained from patients' metastases during surgery carried out mainly because of pathologic bone fractures. Tumour samples were immediately processed under sterile conditions, washed 3–4 times with saline, the necrotic areas discarded, then passed through a metal mesh (49 G), gently washed again to obtain a tumour cell suspension, which was frozen at  $-80^\circ\text{C}$  in saline containing 10% DMSO. When necessary, red cells were eliminated by a mild hypotonic shock.

Subsequently, cell samples were thawed as needed, suspended in a formalin buffer (1v/25v) and left at room temperature overnight. They were further washed three times, suspended in saline at a concentration of  $5 \times 10^6$  cells/ml and stored in 1–2 ml aliquots at  $+4^\circ\text{C}$  (Pizza et al., 1980). A few days later, two samples underwent sterility tests, slides were prepared for histological examination and the cell types were counted.

### Patients' selection

Inclusion criteria were MRCC in progression of disease in spite of continuing immunotherapy (Pizza, et al., 2001), confirmed histological diagnosis and patient's written informed consent. The exclusion criteria were: age less than 18 years, life expectancy less than one month, Karnofsky index less than 40, presence of acute viral, bacterial and/or autoimmune diseases, serum creatinine  $> 0.2$  g/litre, cardiac infarction during the last 2 months, cardiac failure requiring medication. Patients who needed cortisone medication were also excluded.

Table 1. Age, sex, histology grading, anatomical site of metastasis, duration of immunotherapy and follow-up of treated patients and controls.

	No. treated pts	No. control pts
Patients	10	64
Sex F.	1 (10%)	14 (21.8%)
Sex M.	9 (90%)	50 (78.2%)
Age (years)	52-70 (63.3±6.6)	19-83 (61.8±13.4)
Pts with synchronous metastases	4 (40%)	24 (37.5%)
Pts with metachronous metastases	6 (60%)	40 (62.5%)
Months of appearance of metastases following nephrectomy	5-43 (29±6.2)	2-169 (60.5±52.9)
Stage IV	10	64
Histology grading:		
G1	0	0
G2	1 (10%)	11 (17.2%)
G3	7 (70%)	21 (32.8%)
G4	2 (20%)	4 (6.2%)
G?	0	28 (43.7%)
Site of metastases:		
Lung	8 (80%)	28 (43.7%)
Liver	-	4 (6.2%)
Bone	7 (70%)	12 (18.7%)
Lymph nodes	5 (50%)	13 (20.3%)
Kidney/ Muscle/Skin/Brain (1 pt respectively)	-	4 (6.2)
Pleura	1 (10%)	3 (4.7%)
Suprarenal gland	-	4 (6.2%)
Multiple organ involvement	9 (90%)	12 (18.7%)
Pts with solitary metastasis	1 (10%)	3 (4.7%)
Pts with > 1 metastasis	9 (90%)	61 (95%)
Months of concomitant immunotherapy		
	2-66.7	2-120
Months of follow-up		
	4.8-105.3	4-161

Ten nephrectomized stage IV MRCC patients in progression of disease in spite of the immunotherapy treatment entered the vaccine protocol. All patients were treated and monitored in our Institution. In four of them, the diagnosis was synchronous with that of the primitive kidney tumour, in six metachronous, made at various post-nephrectomy time intervals. Their sex, age, appearance of metastasis from nephrectomy and organ involvement is reported in Tables 1 and 2. Two showed a performance status (PS), according to Karnofsky, between 80 and 100, eight between 50 and 79 (Table 4). All pts underwent nephrectomy: two showed histology grade 4, seven grade 3, one grade 2. The stage at the time of nephrectomy is reported in Table 2.

### Historical controls

Sixty-four stage IV nephrectomized MRCC patients, treated with the same protocol of immunotherapy and in progression of disease, represent our historical controls. All patients were treated and monitored in our Institution. In 24 of them, the diagnosis was synchronous with that of the primitive kidney tumour, in 40 metachronous, made at various post-nephrectomy time intervals. Their sex, age, appearance of metastasis from nephrectomy and organ involvement are shown in Table 1. Twenty-four patients had a Karnofsky performance status (PS) between 80 and 100, forty between 50 and 79. All pts underwent nephrectomy: four showed histology grade 4, 21 grade 3, 11 grade 2. For 28 patients, we were unable to obtain the grading, but only the pathologist's report stating "carcinoma of the kidney"; nephrectomy was performed outside our Hospital several years earlier and slides were not available. At the time of nephrectomy, six pts showed stage I, 11 stage II, 14 stage III, and 12 stage IV. For 21 patients, and for similar reasons, we were also unable to obtain the staging.

### Vaccine treatment protocol

The protocol was a combination of intra-tumour and s.c. injections of  $10^7$  allogeneic transfected and irradiated ACHN-IL-2 cells every 10–14 days for 4–6 times, during

Table 2. Demographic data, histology, site of metastasis, site of surgical removal of metastasis for clinical reasons

Code	Pt. No.	Sex	Age	Syn/Met (*)	Stage <sup>§</sup>	Grade	Site of metastasis	Site AUT-TC	Clinical reason for surgical removal of metastasis
10625	1	M	65	36	P2N0M0	3	LUNG, MEDIASTINUM	LUNG	Curative tumourectomy for single lesion
66867	2	M	70	0	T4N2M+	4	LUNG, LFN		
10047	3	M	69	0	P2N0M1	3	BONE, LUNG		
66684	4	M	55	36	T2NXM0	3	LFN, BONE^^	BONE	Pathol. fracture: D10 corporectomy, "en bloc" substitution
66770	5	M	71	43	T4N0M0	3	LUNG, BONE		
66942	6	M	59	34	T2N0M0	4	BONE	BONE	Pathol. fracture: D12 tumourectomy, prosthesis right femur
66988	7	M	52	0	T2N0M+	2	LUNG, MEDIAST, BONE^	BONE	Pathol. fracture: fibula and right ulna tumourectomy
67152	8(#)	F	69	20	T3N0M0	3	LUNG, BONE^^^	BONE	Pathol. fracture: right femur prosthesis
67168	9	M	62	0	T4N2M1	3	LUNG, PLEURA, BONE, MUSCLE	BONE	Pathol. fracture: right humerus prosthesis
67201	10	M	61	5	T2N1M0	3	LUNG, MEDIAST		

(\*) – following appearance of metastases

§ – stage at time of nephrectomy

(#) – also „polycythaemic and thrombocythaemic chronic myeloproliferative syndrome“ on treatment with „Myleran“

Syn/Met – months from nephrectomy to appearance of metastases

Site AUT-TC – anatomical site of the metastatic lesions taken and used for vaccine therapy

^ – fibular and ulnar bone metastases were surgically removed and used for vaccine therapy

^^ – the metastatic lesion was removed by „en bloc“ corporectomy and used for vaccine therapy

^^^ – the proximal humeral metastatic lesion was surgically removed, substituted with a prosthesis and used for vaccine therapy

a 45–60 day cycle. When possible, and according to cell availability,  $5-10 \times 10^6$  ATC were admixed and injected s.c. The duration of a treatment cycle was initially intended for 45–60 days, with disease restaging at 1, 2, and 4 months from the beginning of the vaccine therapy. Persistence of disease or CR was followed by an additional cycle. In case of disease progression, the treatment was discontinued, unless the patient wanted to pursue it for an additional cycle. Treatment was administered mostly on an outpatient basis, except for the intra-tumour injection in profound sites. Patients received, 30 min before treatment, 10 mg of clorphenamine maleate i.m. (Trimeton®) and 150 mg ranitidine chlorhydrate orally.

### Concomitant immunotherapy

The previously administered immunotherapy (Pizza et al., 2001) remained as a concomitant treatment. It consisted in one monthly intra-lymphatic administration of IL-2 and LAK cells after three consecutive days of inhalation of IL-2. The patients also received i.m. injections of transfer factor (TF) monthly and alpha-interferon bi-weekly. The lymphatic vessels of the foot were localized according to the standard technique for lymphography, cannulated with a fine 27G butterfly-needle connected to a syringe containing 5–10 ml of saline, 250 IL-2 units and  $2-8 \times 10^7$  LAK. The syringe was connected to a mini-dose-injecting pump (Lymphoject™, Sias, Castenaso, Italy). IL-2 treatment by inhalation was given according to Huland's protocol (1992), slightly modified. Briefly, 250 IL-2 U in 5 ml of saline were administered four times a day for three consecutive days every month. The inhalation lasted 15–20 minutes, and standard aerosol inhaler equipment was

used. On the fourth day, the patients received intralymphatic treatment. One million units of commercially available recombinant interferon- $\alpha$ -2a were injected i.m. bi-weekly, and 3.5 units of TF were administered monthly. The latter was produced in our laboratories from mononuclear cells obtained from pools of 100 buffy-coats of the hospital's blood bank donors. TF was extracted using standard techniques (Lawrence, 1955) slightly modified; it was stored lyophilized, and solubilized in 5 ml of saline immediately before injection. One unit was obtained from  $1 \times 10^8$  cells.

### Evaluation of the clinical response

The clinical response was evaluated by considering both the overall survival rate (Kaplan-Meier curve) and the response rate at the level of measurable metastases according to conventional parameters. Consequently, a complete response (CR) is the complete disappearance for at least one month of all evidence of tumour presence in all sites where the disease may be detected, without appearance of new lesions. A partial response (PR) is defined as a decrease by 50% of the sum of the perpendicular diameters of all measured lesions, in the absence of detectable tumour progression in any other site or the appearance (observed by TC scan or chest X rays) of new lesions for one month. Progression (PROG) is a 50% increase of the sum of the perpendicular diameters of all measured lesions, measured over the smallest sum observed (or over the baseline status if no decrease occurs), or reappearance of a lesion which had disappeared, or worsening of detectable non-measurable disease, or appearance of new lesions. Stable disease or no response is decided when the disease status is not modified following the criteria of complete or

partial remission or disease progression. At the beginning of the treatment, the restaging of the patients was carried out every 4 weeks for the known metastatic sites using standard X rays (lung and bone) or ultrasonography (liver, kidney, lymph nodes). CT scan was carried out every eight weeks for unknown sites or very small lesions (less than 0.5 cm). Every six months, we performed total body scintigraphy for the detection of new bone lesions. The serum and urine biochemical parameters (i.e. Na<sup>+</sup>, K<sup>+</sup>, transaminases, bilirubin, creatinine, cholesterol) were evaluated monthly, electrocardiograms were carried out bi-monthly. Blood pressure was measured twice, immediately before and at the end of IL-2 and vaccine cell administration, if not required earlier for other reasons.

### Lymphocyte stimulation test (LST)

Some patients agreed to donate periodically 30 ml of PB for *in vitro* studies, i.e. lymphocyte stimulation in the presence of tumour antigens already described (Pizza et al., 1980). Briefly, PBL were collected on an F-H gradient and washed three times with RPMI 1640 culture medium (Gibco). The amount of  $5 \times 10^5$  lymphocytes were incubated in 5% CO<sub>2</sub> humidified atmosphere for 6 days in 0.2 ml of medium supplemented with 10% autologous heat-inactivated (30 min at 60°C) serum in the presence of ACHN formalin-treated tumour cells at four different ratios: 1 : 50, 1 : 10, 1 : 2, 1 : 1. Triplicate cultures were also prepared in the presence of 1 µg of PHA-P (Difco). The lymphocyte response was evaluated by methyl-<sup>3</sup>H-thymidine incorporation, which was added 24 h before harvesting (1 µCi/ml; spec. act. 20 µCi/mM) with a harvester cell system on paper glass filter disks. Cultures were made in triplicate (Falcon 3040). The results were expressed as stimulation index (SI = mean dpm of lymphocytes in the presence of tumour cells/mean dpm of lymphocytes alone). The stimulation was considered as positive when the statistical analysis using Student's t test between mean dpm of unstimulated lymphocytes and mean dpm of stimulated lymphocytes gave  $P < 0.01$  and the SI was  $> 2.00$  (Pizza et al., 1980).

### Statistical analysis of survival

The most important indicator considered was the right-censored survival curves and for that, we used the Kaplan-Meier method (Kaplan and Meier, 1985). The survival of treated patients was compared to that of 64 historical control patients. For comparison, Wilcoxon's test was used (Kalbfleisch and Prentice, 1980).

## Results

### ACHN-IL-2 cell line

When thawed, more than 90% of irradiated cells were viable and survived in culture for 15–42 days, i.e. 50%

for 13, 32% for 30, and 0–1% for 42 days. No methyl-<sup>3</sup>H-thymidine uptake was observed in the replication test, confirming the inability of irradiated cells to divide. The biological activity of the produced IL-2 was  $637 \pm 688$  U/day/ $10^6$  cells for a period of 30–42 days after irradiation with 50 Gy (Fig. 1). Since the amount needed is 3 U/ml/day (Hersey et al., 1981), the yield is sufficient to maintain the growth of IL-2-dependent-PHA-activated human PBL. When evaluated using ELISA, the *in vitro* IL-2 production of the irradiated cells appeared to continue for 32 days, with a mean concentration of 230 pg/day/ $10^6$  cells and a maximum production on day 3. The amount of 2.5 U of IL-2 thus determined (Pizza et al., 1984) corresponds to 1 pg of human IL-2 as assessed using the ELISA kit "Biotrak"® (Amersham, Life Science, England). Although in diminished amounts, the cells continued to secrete the cytokines observed before transfection, with the exception of GM-CSF: i.e. 30 and 40 pg of IL-6 and IL-8, respectively, were daily produced until day 7 and 14.

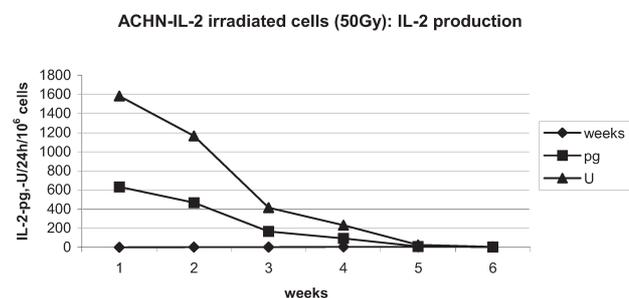


Fig. 1. Days and amount of IL-2 *in vitro* production by ACHN-IL-2 irradiated cells (50 Gy)

### In vitro studies

At the beginning of the vaccine treatment, eight patients accepted to donate 30 ml of peripheral blood for evaluating their cell-mediated immune reactivity against ACHN cells using mixed lymphocyte cultures (Table 5, Fig. 2). In three patients, a positive stimulation index (SI  $\geq 2$ ) was noticed: average in pts. No. 6 and No. 10 (SI = 3.01 and 3.04, respectively), but strong in pt. No. 4 (SI = 14.5). Fisher's exact test, although not significant, showed a trend of correlation between survival and positive SI ( $P < 0.07$ ). Interestingly, three out the four positive patients were still alive at the end of the study, i.e. 518, 514 and 550 days from the beginning of the vaccine treatment. However, in the fourth patient alive (No. 7), in whom a complete regression of lung and mediastinal metastases was observed, no PBL reactivity was noticed against the ACHN cells *in vitro*, both before and after vaccine therapy (Fig. 3). Regarding this patient, it is also worth noting that a strong and unusual replication of the non-stimulated lymphocytes (control culture) was observed in the tests performed both before and 15 months after the beginning of vaccination.

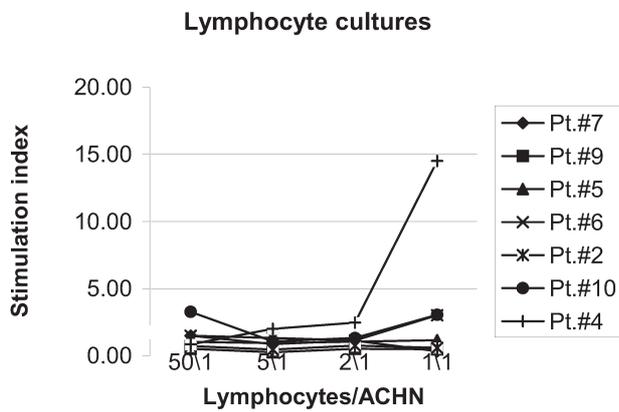


Fig. 2. LST of vaccinated patients before treatment

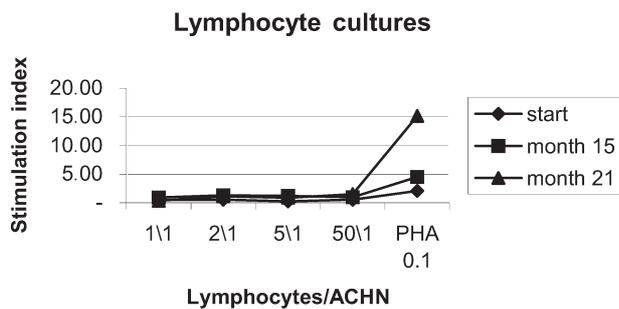


Fig. 3. LST Pt #7

**Treatment**

Ten MRCC patients were treated. Their sex, age, time of appearance of metastases from the date of

nephrectomy or of progression, organ involvement, site, and clinical reasons of the surgical removal, use of derived ATC, are reported in Tables 1–2. The mode of administration, number of injections and days of vaccine treatment are reported in Table 3. Ninety-nine injections were administered (Table 3) (4–16 for each patient with a mean of  $9 \pm 4$ ), containing  $987 \times 10^6$  ACHN-IL-2 transfected cells, with a mean of  $10.6 \times 10^7 \pm 7.7 \times 10^7$  per patient, and a minimum of  $4 \times 10^7$  and maximum  $31 \times 10^7$  each.

Only four patients received intra-tumour administrations (pts. Nos. 1, 2, 9, 10), but all received s.c. injections in the crural region. Six patients received s.c. transfected cells admixed with ATC (pts. Nos. 4, 6, 7, 8, 9 with cells from bone, and pt. No. 1 from lung lesions). The length of the administration period was 30–418 days, with a mean of  $151 \pm 135$  (Table 3). The entire follow-up was of  $630 \pm 355$  days (190–1342) (Table 4), during which patients continued the concomitant immunotherapy treatment previously administered during  $434 \pm 598$  days, with a follow-up of  $913 \pm 1037$  days (Table 3). Clinical results, type and site of clinical response, performance status according to Karnofsky and status of the patients, both at the end of the trial period and at the 18<sup>th</sup> month of follow-up are shown (Table 4).

**Clinical results**

Eight treated patients had a performance status in-between 50–79 and two in-between 80–100. In the same range, the number of control patients was respectively 40

Table 3. ACHN-IL-2 administration, days of immunotherapy and follow-up until June 2001

Pt. code	Pt. No.	No. ACHN	ACHN-INTR	ACHN s.c.	TC AUT-s.c.	DD-Immunoth.	FU-Immunoth.	DD-THER-ACHN	DD-FU-ACHN
10625	1	6(60)	4(40)	2(20)	1(6)	635	1501	39	190
66867	2	4(40)	3(30)	1(10)	0	240	300	30	30
10047	3	14(120)	0	14(120)	0	2037	3213	418	534
66684	4	7(70)	0	7(70)	1(3)	596	1875	69	1092
66770	5	16(110)	0	16(110)	0	278	1282	366	702
66942	6	9(90)	0	9(90)	6(59)	90	148	180	514
66988	7	8(80)	0	8(80)	5(24)	70	289	118	1098
67152	8	8(56)	0	8(56)	6(60)	148	149	68	560
67168	9	16(310)	6(240)	10(70)	7(90)	120	211	140	579
67201	10	11(130)	2(40)	9(90)	0	128	166	84	550
Mean		9(106)	4(87)	8(71)	4(40)	434	913	151	527
sd		4(77)	2(101)	4(11)	2(34)	598	1037	135	283
Total		99(987)	15(350)	84(716)	22(242)				

() – In parentheses numbers of ACHN-IL-2 and/or autologous tumour cells injected  $\times 10^6$

ACHN-INTR – number of intra-tumour injections

ACHN-s.c. – number of subcutaneous injections performed in the inguinal region

TC AUT-s.c. – number of subcutaneous injections performed in the inguinal region and, in parentheses, number of autologous tumour cells mixed to ACHN-IL-2 cells

No. ACHN – total number of injections, both intra-tumour and subcutaneous

DD-Immunoth. – number of days of immunotherapy before the beginning of vaccine therapy

FU-Immunoth. – days of the immunotherapy preceding the vaccine therapy and its period of follow-up

DD-THER-ACHN – days of the vaccine therapy period

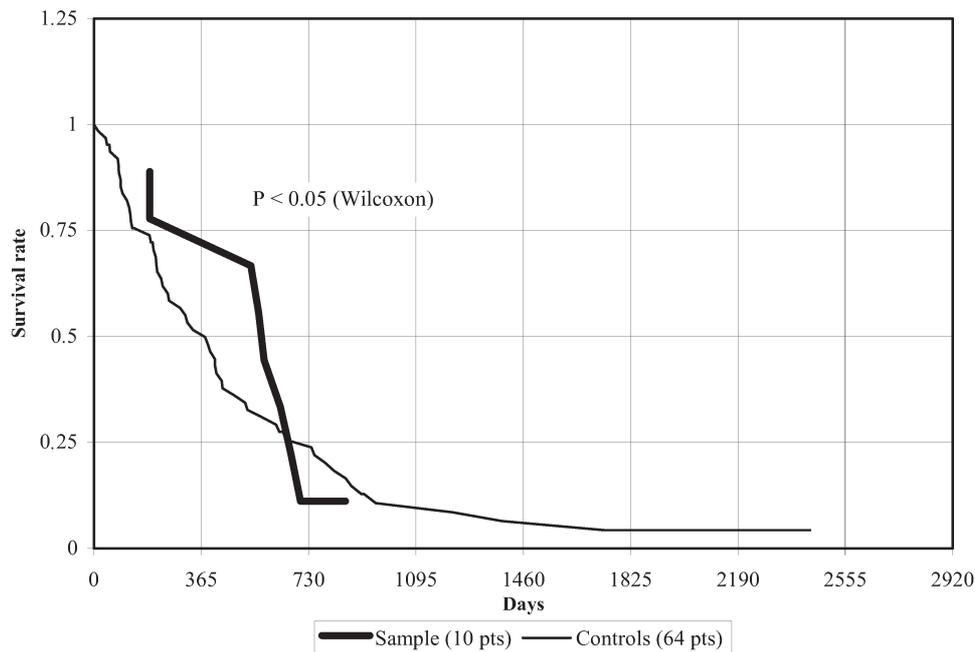
GG-FU-ACHN – days of follow-up including the vaccine therapy period and the subsequent concomitant therapy

No. – number; sd – standard deviation

and 24. Details of the treatment in the ten patients are summarized in Tables 1–4. In 36 months of surveillance one CR (pt. No. 66988), one PR (pt. No. 67152), two patients in stable disease condition (pts. No. 10047 and No. 66770) and one in no-relapse (pt. No. 66684) were observed, with duration respectively of 330, 120, 534, 330 and 1092 days (Table 4). In one patient, partial incomplete and transitory (3 weeks) regression of a bronchial metastasis after the first intra-tumour injection of  $10^7$  ACHN-IL-2 (pt. No. 10625) was also observed. No early or late adverse side effects were noticed. Five patients died in

60–579 days for causes linked to tumour progression, one for causes unrelated (cerebral stroke), and four were alive during an observation period of 514–1098 days after the beginning of the vaccine therapy (Table 4). In the following 18 months after the end of the trial, three patients died (Table 4).

The survival curve (according to Kaplan-Meier) of treated patients and controls is reported in Fig. 4. Survival time was measured from the beginning of the therapy to the last date the patient was known to be alive. To this date, of the ten treated patients, nine have



Group	Pts	Censored	Median (Days)	I Quartile (Days)	III Quartile (Days)
Treated patients	10	1	579	362	686
Controls	64	9	374	149	684

Fig. 4. Survival curve (Kaplan-Meier) of the patients vaccinated and controls

Table 4. Clinical results at the end of vaccine trial (June 2001) and follow-up until December 2002

Code	Pt. No.	PS	Site of metastasis	Clinical response	Site of clinical response	Duration of clinical response (d)	Anatomical site of progression	June 2001		December 2002	
								Follow-up (d)	A/D	Follow-up (d)	A/D
10625	1	70	L, M	PROG			L, M	190	D		
66867	2	70	L, LFN	PROG			L, M	190	D		
10047	3	60	B, L, LFN	STAB		534	NO	534	D*		
66684	4	100	NO	NO RELAPSE			NO	518	A	1092	A
66770	5	50	L, B	STAB	L	300	B	702	D		
66942	6	60	B	PROG			B	514	A	634	D
66988	7	80	L, M	CR	L, M	330	C	1098	A	1342	D
67152	8	50	L, B	PR	L	120	B	560	D		
67168	9	70	L, B, PL	PROG			L, B	579	D		
67201	10	70	L, M	PROG			L, M, C	550	A	670	D

A – alive, D – dead, L – lung, M – mediastinal, LFN – lymph nodes, C – brain, B – bone, PL – pleura, Follow-up – days from the beginning of vaccine therapy with ACHN cells, (d) – days, PS – performance status according to Karnofsky, Site of metastasis – anatomical site of lesions at the beginning of vaccine therapy with ACHN cells, \* – death by cerebral ictus not related to the tumour

Table 5. Survival and LST (June 2001)

Code	Pt. No.	LST SI	Clin. resp.	A/D
66867	2	0.72	PROG	D
10047	3	1.65	STAB	D
66684	4	<b>14.5</b>	STAB	<b>A</b>
66770	5	1.15	STAB, PROG	D
66942	6	<b>3.01</b>	PROG	<b>A</b>
66988	7	1.49	CR	<b>A</b>
67168	9	0.94	PROG	D
67201	10	<b>3.04</b>	PROG	<b>A</b>

Clin. resp. – clinical response

SI – stimulation index (positive if > 2)

(SI > 2 versus alive, P = 0.07, not significant;  
Fisher's exact test)

died. The patients had a mean age of 63 (min. 52, max. 71) (Table 2). Of the 64 controls (the survival was measured from the beginning of the progression), 55 died and nine had censored survival times. The 1 and 3 quartile of the patient's group are 11.8 and 22.5 months versus 4.8 and 22.4 months in the control group. From the beginning of the vaccine therapy, the median survival of the treated patients was 18.9 months versus 12.2 for the controls (P < 0.05, Wilcoxon's test for paired data). Despite the small number of treated patients, Wilcoxon's test for paired data showed a significant (P < 0.05) improvement in the survival in the vaccinated group compared to that of the control.

### Case report

*Patient No. 1 (# 10625).* Male, he underwent nephrectomy at 63y (histology: RCC clear cell type, stage T2N0M0, grade 3). Three lung metastases appeared 3y later (diameter 0.7–1 cm). The lesions were surgically removed, immersed immediately in liquid nitrogen, and stored at –80°C for future use. Relapses appeared again 6 months later: 3 lung and 2 mediastinal. He then started intralymphatic IL-2 and LAK cell administration together with IL-2 inhalations. Transfer factor was given intramuscularly. Sixteen months after the beginning of the immunotherapy, a complete regression of lung metastases was seen, with stabilization of mediastinal lesions. The disease resumed progression 2 years later, with appearance of new lung lesions (4–6, diameters 1–4 cm), increase of the mediastinal metastases, which infiltrated the tracheal carina with appearance of 9 intra-bronchial lesions. PS was 70. In the next 39 days, the patient underwent a laser removal of 7 intra-bronchial lesions, and weekly infiltration with 10<sup>7</sup> ACHN-IL-2 cells of the remaining 2 lesions that were not possible to eradicate by laser therapy. Twice the patient received an equal amount of vaccine cells s.c., and once admixed with 6 x 10<sup>6</sup> ATC from a lung metastasis. A transitory regression of the 2 infiltrated lesions was observed during 3 weeks, but subsequently progression was resumed. The patient died 6 months later. He had undergone

immunotherapy for 49 months. No adverse early or late side effects were noticed.

*Patient No. 2 (# 66867).* Male, he underwent nephrectomy at 70y (histology: RCC clear cell type, stage T4N2M0, grade 4). He was in progression of disease with appearance of multiple lung and lymph node metastases: mediastinal, retro peritoneal, left paracaval and in between aorta and cava vessels, extending from the splenic vein until common iliac vein. Bronchoscopy showed a normal bronchial tree on the left, but on the right side appeared numerous bleeding metastases at the level of the intermediate lobar bronchus, with extension to the door of main lobar bronchus. PS was 70. The major lesion was infiltrated with 10<sup>7</sup> ACHN-IL-2 cells, and 3 weeks later a relevant ischemia was noticed, with clear margin around the implanting base. In spite of two new injections, 3 weeks later the lesion became again florid and occupied almost entirely the bronchial lumen. No effect was noticed on the non-infiltrated lesions. In conclusion, only a transitory ischemia and a partial necrosis of the infiltrated lesion without effects on the distant lesions were observed. No early or late side effects were noticed.

*Patient No. 3 (# 10047)* was a male who underwent nephrectomy at 70y (histology: RCC 90% clear cell type, stage T2N0M1, grade 3) after osteo-synthesis of a pathological fracture of the left humerus because of a metastasis (diameter 3 cm) that was only partially removed. He underwent immunotherapy receiving intra-lymphatic injections of IL-2 and LAK cells, and i.m. injections of TF and interferon. In the following 10 years, only partial regressions were observed, followed by progression of disease. When vaccine therapy was started, the patient had had an almost complete substitution of the left humerus and presented multiple bilateral lung metastases of 3–5 cm in diameter, a solitary lesion in the middle of the remaining kidney, and multiple metastases in between aorta and cava lymph nodes. Metastatic lymph nodes were also found behind the peritoneum and in the confluence of splenic and portal veins. PS was 60. In 5 months, the patient received 14 s.c. injections of 10<sup>7</sup> ACHN-IL-2 cells, each one in both inguinal regions, obtaining stabilization of the disease during 17 months. He also suffered from hypertension and compensated heart failure and had an ictus symptomatology. A CT brain scan documented a large haemorrhage in the frontal-parietal right brain region. He died the fourth day following the ictus occurrence. No adverse side effects of the vaccine therapy were noticed. Apparently, the vaccine therapy resulted in disease stabilization for 17 months.

*Patient No. 4 (#66684).* A male who underwent nephrectomy at 52y (histology: RCC 90% clear cell type, stage T2NxM0, grade 3). Three years later, he suffered from metastatic lymph-node lesions. CR was induced by immunotherapy (intra-lymphatic IL-2 and LAK, inhalation of IL-2, i.m. injections of interferon

and TF). Sixty-one months later, he resumed progression and a new lesion was observed in the 10<sup>th</sup> vertebral body that was surgically removed. He started s.c. vaccine therapy, and in 2 months he received 7 s.c. injections of  $70 \times 10^6$  allogeneic and  $6 \times 10^6$  ATC. The patient is still tumour-free in 912 day follow-up period. No side effects were noticed.

**Patient No. 5 (# 66770).** Male, who underwent nephrectomy at the age of 67 (histology: RCC 100% clear type, stage T4N0M0, grade 3). Forty-three months later, he suffered from multiple lung bilateral lesions (diameter 0.5–1 cm). Immunotherapy stabilized the disease for 2 years, but afterwards progression and appearance of bone lesions with a pathological fracture at the proximal left femur were noticed. At the age of 71, and in the presence of multiple lung lesions (max. diameter 6 cm) and a PS of 50, the patient underwent vaccine therapy. Sixteen s.c. injections of  $10 \times 10^6$  of ACHN-IL-2 each, with a total of  $110 \times 10^6$ , were administered over a 12-month period and were followed by stabilization of the disease for 11 months, both at lung and bone levels. However, progression resumed later, but only at the bone level. A total body CT scan confirmed stabilization of the lung lesions, with the remaining organs being disease-free. The patient died 23 months after the beginning of the vaccine therapy. No early or late adverse side effects were noticed.

**Patient No. 6 (# 66942).** Male who underwent left nephrectomy at 52y (histology: RCC eosinophilic cell type, stage T2N0M0, grade 3–4). Three years later he suffered from bone metastases (vertebral body D4–D6, D12, left ileo-ischio-pubic branch and left tibia). He underwent immunotherapy according to our protocol. Because of severe pain caused from metastasis in D12, he also underwent tumourectomy with palliative intent thanks to the decompression of the vertebral spinal marrow. The autologous tumour was stored for vaccination purposes. Since progression was noticed at the bone level (left ankle and femur), the patient underwent again tumourectomy with prosthesis insertion, and the cells obtained were added to the previous batch and used for vaccine therapy. He received 9 s.c. injections with  $90 \times 10^6$  ACHN-IL-2 and  $59 \times 10^6$  autologous cells during 6 months; his PS was 50. Nonetheless, progression of disease was noticed again at the bone level. He died on day 634 after the beginning of the vaccine treatment. No adverse side effects were noticed.

**Patient No. 7 (# 66988).** Male. He underwent nephrectomy at 53y (histology: RCC, clear cell type, grade 2, stage T2N0M1). At the time he was also suffering from a metastasis in the right fibula that was surgically removed. Eighteen months later, the patient suffered multiple mediastinal and lung metastatic lesions that progressed reaching the number of 19, with diameters ranging from 1 to 3.5 cm (Fig. 5), in spite of immunotherapy with IL-2, LAK cells, TF, and interferon. PS was 80. A very painful distal ulnar metastasis

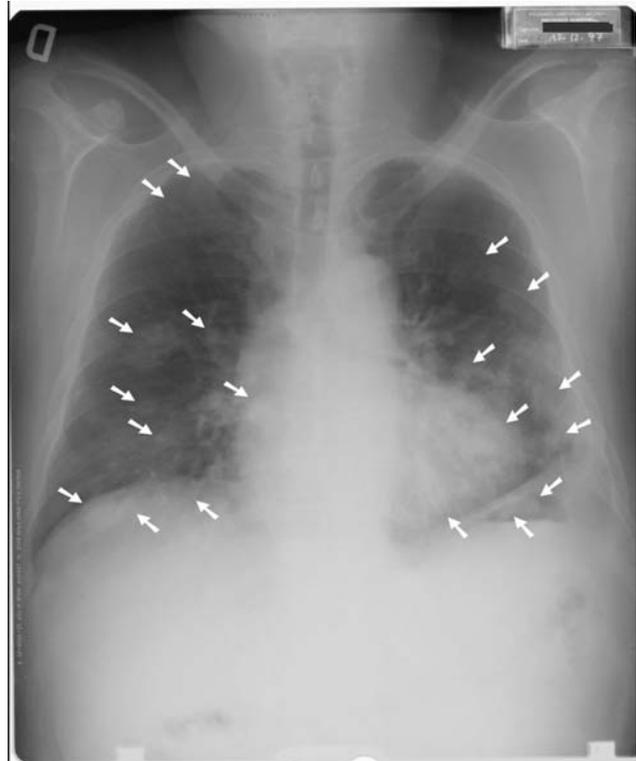


Fig. 5. Multiple lung and mediastinal metastases (arrows) of patient No. 7 before vaccine therapy



Fig. 6. Complete regression of all mediastinal and lung lesions observed 18 months after the first vaccine administration confirmed 4 months later

was surgically removed and the tumour cells were stored. Ten months after the beginning of the immunotherapy protocol, vaccine therapy was started. During 4 months, six s.c. injections in the inguinal region were performed, with a total of  $60 \times 10^6$  ACHN-IL-2 cells, and all the  $24 \times 10^6$  autologous tumour cells obtained from the ulnar lesion. After an initial partial regression, 18 months following the first vaccine administration, a complete regression of all mediastinal and lung lesions was observed, and it was confirmed 4 months later (Fig. 6). Ten months after CR, a brain metastasis was noticed, without any lung or mediastinal relapse. At the end of the vaccination trial, i.e. the 36<sup>th</sup> month from the beginning of the vaccine therapy, the patient was alive without detectable lung or mediastinal metastases, but he died, without relapsing at the lung or mediastinal level, eight months later, on the 1342<sup>nd</sup> day because of progression at the mediastinal level. No adverse clinical side effects were noticed.

*Patient No. 8 (# 67152).* A female, suffering from polycythaemia, treated by chemotherapy. She underwent right nephrectomy at 67y (histology: RCC, 100% clear cell type, stage T3N0M0, grade 3). Multiple bone and lung metastases appeared 20 months later. The PS was 50. Vaccine therapy was undertaken 5 months after the end of the immunotherapy, which failed to control the disease. Autologous metastatic tumour cells were obtained from a humeral metastasis, removed because of a pathological fracture. After 8 s.c. injections of a total of  $56 \times 10^6$  ACHN-IL-2 and  $60 \times 10^6$  ATC over a 6-month period, a partial regression of lung metastases and a stabilization of the remaining bone lesions were observed. Four months later, the patient resumed progression at the bone level, with lesions appearing at the left femur and the iliac wing. The patient died 18 months after the beginning of vaccine therapy without lung progression. No side effects were noticed.

*Patient No. 9 (# 67168).* Male. He underwent nephrectomy at 58y (histology: RCC, clear cell type, stage T4N2M1, grade 3). He suffered from a synchronous metastasis in the 6<sup>th</sup> rib that was removed. He immediately started s.c. immunotherapy with recombinant IL-2 in another Institution, subsequently followed by intralymphatic IL-2 and LAK cell administration, i.m. injections of interferon and TF, as well as IL-2 inhalations in our Institution. Immunotherapy failed and multiple new bone lesions appeared (lung, scapula, L-1 vertebral body, pleura and right pilaster diaphragm muscle). PS was 70. During 5 months, under CT scan control, the patient received 6 intra-tumour administrations of a total of  $240 \times 10^6$  ACHN-IL-2 cells in the major lung lesion of 6 cm of diameter. At the same time, 10 injections were administered in the inguinal region, with a total of  $70 \times 10^6$  ACHN-IL-2. On 7 occasions, the cells were admixed with a total of  $90 \times 10^6$  autologous formalin-fixed tumour cells obtained from a humeral proximal metastasis that was removed

because of a pathological fracture. No adverse side effects were observed, both from s.c. or intra-tumour injections, but the disease progressed and 19 months after the beginning of the vaccine therapy the patient died.

*Patient No. 10 (# 67201).* Male. He underwent nephrectomy at 60y (histology: RCC, clear cell type poorly differentiated, stage T2N1M0, grade 3–4). Five months later, multiple bilateral lung and mediastinal metastases appeared. He was immediately treated in another Institution with s.c. IL-2, and subsequently with intralymphatic IL-2 and LAK cells in our Institution. Intramuscular injections of interferon and TF, and IL-2 inhalations were added. Since the lesions increased in both number and volume, vaccine therapy was started. In 7 months, a total of  $90 \times 10^6$  ACHN-IL-2 cells were administered by 9 s.c. injections. No ATC were available. The disease progressed and some lesions infiltrated the superior right lobar bronchus, as discovered after a bronchial bleeding episode observed 18 months after the beginning of the vaccine therapy. Following that episode, a laser vaporization of the lesion occluding the bronchial lumen was performed under anaesthesia and 2 infiltrations of the site were carried out with injection of  $90 \times 10^6$  ACHN-IL-2 cells, using a bronchoscope. The patient was still alive 18 months after the beginning of the vaccine therapy. He died 4 months later from tumour progression, on day 670 from the beginning of the treatment.

## Discussion

One must be aware of the complexity of the vaccine therapy and of the heterogeneity of the antigenic sites expressed on the surface of tumour cells. The problem is better explained by the work of Brouwenstijn et al. (1998), whose cellular immunity studies in RCC corroborated the notion that renal carcinoma cells are immunogenic because of a broadly distributed antigenic structure that may serve as target to cytotoxic T cells, and may thus be a potential candidate for tumour vaccine development. However, the authors confirmed that the recognized antigenic determinants are neither unique nor specific for the RCC.

The limitations of our study and hence of the conclusions that may be drawn are obvious. For instance, we ignore whether tumour regression was mediated by the use of allogeneic or autologous antigens or by the synergy of both. And we cannot assert that the regression was in correlation with some known immune responses, e.g. LST in PBL. In addition, the role of the various cytokines produced by the ACHN line is not yet clearly understood, and the survival observed, because of the variability of response in MRCC patients, could be different in a larger sample of patients. However, it is worth underlining that the patients underwent vaccine therapy following the failure of the IL-2 treatment,

which represents one of the best protocols that can be offered to MRCC patients in progression of disease. Under this treatment, the median survival reported in the literature ranges between 9 and 17 months (Bukowski, 1997, 2000). Furthermore, a recent review describes some of the various vaccine therapy approaches of the last ten years in 126 MRCC patients with a response rate of 11% (Pizza et al., 2002).

Although these results are not very different from our own, in some cases, the techniques used were far more costly and complex than ours. Recently, using similar techniques, Chang and coworkers (2003) observed a better response rate, but with high toxicity (Chang et al., 2003). Tumour regression without a particular cell-mediated immune response of PBL has already been reported (Coulie et al., 2002). The absence of correlation with the *in vitro* cytokine production of cells obtained from vaccine-primed tumour-draining lymph nodes supports this contention (Chang et al., 2003).

To our knowledge, this is the first clinical trial using whole formalin-fixed ATC, admixed with allogeneic RCC engineered to secrete interleukins in MRCC patients in progression of disease, following immunotherapy with IL-2. A previous study was carried out using irradiated allogeneic RCC engineered to produce IL-2, but the cells were used alone and the authors only observed an increased susceptibility to tumour cell killing by PBL (Belldgrun et al., 1993). Similar attempts were carried out in melanoma patients (Arienti et al., 1994).

The rationale for choosing formalin-treated cells in our study needs further comment. It is well known that formalin treatment preserves the antigenicity of tumour cells for a long time (Drake et al., 1972; Ross et al., 1975, 1983). Furthermore, it was already shown that this treatment is able to significantly increase the exposure of the cell-surface antigenic determinants as assessed by indirect immunofluorescence antibody reaction (Pizza and De Vinci, 1987). Tests of cell-mediated immunity used to evaluate the presence of TAA over time are leukocyte migration inhibition and lymphocyte stimulation, i.e. lymphocyte cultures mixed with tumour cells (Pizza et al., 1976, 1977, 1980; Levine et al., 1981). These tests are still positive 40 months after the preparation of formalin-fixed tumour cells (Pizza et al., 1977). Lastly, similar antigenic preparations were able to immunize guinea pigs (Corrado et al., 1984; De Vinci et al., 1987) and their surface antigens were detected 14 years later by immunofluorescence (Pizza and De Vinci, unpublished). Thus, patients' ATC can be easily stored and used for a long period of time at very low cost.

In conclusion, the clinical results reported here, the high compliance of the patients to the protocol and the low cost of the proposed techniques are encouraging and warrant continuation of this investigation.

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