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Ruggero Ridolfi · Emanuela Flamini · Angela Riccobon
Franca De Paola · Roberta Maltoni · Andrea Gardini
Laura Ridolfi · Laura Medri · Giovanni Poletti
Dino Amadori

Adjuvant adoptive immunotherapy with tumour-infiltrating lymphocytes and modulated doses of interleukin-2 in 22 patients with melanoma, colorectal and renal cancer, after radical metastasectomy, and in 12 advanced patients

Abstract Adoptive tumour infiltrating lymphocytes (TIL) in combination with a modulated dosage of interleukin-2 (IL-2) can be used with acceptable toxicity in the treatment of immunogenic tumours. Following an experience of reinfusion in advanced melanoma, colorectal and renal cancer patients, treatment was given to disease-free patients after metastasectomy. The high risk of relapse and favourable ratio between reinfused TIL and possible microscopic residual disease determined this choice of adjuvant treatment. A group of 12 patients with advanced disease (7 melanoma, 4 colorectal carcinoma, 1 kidney carcinoma) were treated with TIL (median 5.8×10^{10} cells) and IL-2 (West's schedule) modulated towards a lower dosage (from 12 to 6 MIU/day) in order to maintain an acceptable level of toxicity. As treatment was well tolerated, it was offered to another 22 patients in an adjuvant setting after metastasectomy (11 melanoma, 10 colorectal carcinoma, 1 renal cancer), the median dose of TIL reinfused being 4.95×10^{10} cells. No objective response was observed in advanced patients: all patients progressed after a median of 1.5 months (0–8 months) and median survival was 8 months (3–22+ months). Thirteen patients from the second group are still disease-free after a median of 23+ months (9+–47+ months). The remaining 9 patients relapsed after a median of 5 months (3–18 months). Toxicity was moderate as clinical and hepatic/renal function parameters were used to assess the need for dose reductions. Consequently, there was great diversity in IL-2 dosages administered. In par-

ticular, there seemed to be a difference in IL-2 doses administered between disease-free cases and those who progressed (17.5 MIU/day versus 7 MIU/day in melanoma patients; 11.2 MIU/day versus 7.1 MIU/day in colorectal cancer patients). By contrast, no differences were observed between number of TIL reinfused and clinical response. Phenotypical characteristics of reinfused TIL were similar to those reported in the literature: 97% were CD3 and 92% were CD8. Aspecific cytolytic activity was evaluated on 12 cases whereas, in 2 melanoma cases, autologous tumour tissue was available for the specific cytotoxicity test. Perforin levels in TIL measured at the end of culture were generally high or very high. Cytokine levels were measured on the supernatant at the end of culture, with an extreme variability in results. Finally, ζ chain and p56^{lck} were histologically assessed on the resected tissue from which TIL were cultivated. There were virtually none of the former and a complete absence of the latter, which concurs with data reported in the literature. The same immunocytochemical analysis was carried out on TIL at the end of culture. This time an almost complete restoration of both functions was seen, especially in melanoma patients, who are still free from disease. The study is on-going and it has been decided to focus on disease-free patients after metastasectomy in order to increase the number and possibility of clinical and histological correlations.

Key words Adoptive immunotherapy · Interleukin-2 · Tumour-infiltrating lymphocytes · ζ chain-p56^{lck}

R. Ridolfi (✉) · E. Flamini · A. Riccobon · R. Maltoni · D. Amadori
Medical Oncology Department, Morgagni-Pierantoni Hospital,
viale Forlanini, I-47100 Forlì, Italy
Fax: +39 543 731736

F. De Paola · A. Gardini · L. Ridolfi
Istituto Oncologico Romagnolo, Forlì, Italy

L. Medri
Pathology Department, Morgagni-Pierantoni Hospital, Forlì, Italy

G. Poletti
Laboratory Medicine Department, Morgagni-Pierantoni Hospital,
Forlì, Italy

Introduction

Tumour-infiltrating lymphocytes (TIL) have always been regarded as an expression of immunoreaction by the host [44]. On the basis of preclinical data concerning in vitro TIL activation against the autologous tumour, clinical reinfusion trials were conducted using TIL plus interleukin-2 (IL-2) [43, 2]. The role of these lymphoid effectors when they come into contact with neoplastic cells in vivo

Table 1 Adoptive immunotherapy with tumour-infiltrating lymphocytes (TIL) + interleukin-2 (IL-2) in solid tumours. CR complete response, PR partial response, NSCLC non-small-cell carcinoma

Disease	Patients	CR	PR	Objective response (%)	Duration of CR (months)	Reference
Advanced disease						
Melanoma	21	1	5	24	4	[7]
	86	5	24	34	20, 21+, 23, 38, 46+	[38]
	16	3	–	19	6, 15, 33+	[14]
	9	–	5	55	–	[34]
Kidney cancer	62	5	15	34.4	14 (0.8+, 64+)	[9]
	16	1	4	31	–	[26]
Gastric cancer	23	3	5	44.7	–	[48]
Ovarian cancer	8	–	4	50	Ascites regression and Ca125 reduction value	[11]
Adjuvant treatment						
Ovarian cancer	13 vs 11 ^a				3-year survival: 100% vs 67.5%	[12]
NSCLC (stages II, IIIa, IIIb)	57 vs 56 ^a				Survival for 22.4 months vs 14.1 months (<i>P</i> < 0.05)	[33]

^a Treated group versus control

has yet to be fully understood [46]. However, it is clear that they are not capable of eliminating the tumour from which they were extracted, even though it has been proved that they recognize the antigenic characteristics of the tumour [42, 47]. Even less is known about their therapeutic effectiveness once reinfused, as TIL studies are sporadic and generally based on small patient populations (Table 1) [7, 9, 38, 11, 30, 26, 15, 48, 14, 34, 33, 12].

We know that there is a positive correlation with clinical response as regards the extent of lymphocyte infiltration after reinfusion and the consequent tumour necrosis [1, 6]. Similar correlations exist between positive clinical response, the brevity of time in culture and the capacity of TIL to secrete granulocyte/macrophage-colony-stimulating factor (GM-CSF) [39]. It has been proven that TIL position themselves in the tumour site in more than 80% of cases and that the longevity of their memory *in vivo* can reach up to 260 days [31, 22]. However, the above data give a somewhat fragmentary picture of TIL activity and research has yet to identify the complex mechanism that transforms TIL from simple cells capable of recognizing several non-self antigens into effective cytotoxic agents *in vivo*. Several authors have reported defects in the activation of TIL when examined in the tumour site; in particular, an expression defect has been identified in the T-cell receptor ζ chain and in the posttranscriptional enzyme p56^{lck} [10, 25, 21, 32, 41, 49]. There have been numerous reports in the literature of various mechanisms of immunosuppression caused by the tumour in its own environment; in this context, TIL would appear to operate *in situ*. What happens once they have been isolated, activated, expanded *in vitro* with IL-2 and reinfused into the patient has yet to be understood.

Another important question to be clarified in adoptive immunotherapy trials is IL-2 dosage. The administration of IL-2 is, in itself, effective in the treatment of certain tumours (melanoma, renal carcinoma) [36, 28]. However, it is not yet clear whether “the highest tolerated doses”, as suggested by some authors, are really necessary or whether lower doses could be as effective, with consequently less

toxicity and greater drug tolerance [8, 3]. Nevertheless, it is certain that TIL reinfusion must be accompanied by a dosage of IL-2 that guarantees a continuation of therapeutic activation *in vivo* [36, 16]. Unfortunately, the most effective dosage of IL-2 in this type of therapy is also not known. For this reason, one of the first aims of this study was to investigate IL-2 administration in continuous infusion using West’s scheme in order to limit toxicity (grade IV) and avoid the need for treatment suspension [45]. Doses of IL-2 thus started at 12 000 000 IU/day (12 MIU/day), and were decreased to a minimum of 6 MIU/day on the basis of clinical and haematochemical evaluation parameters. In this way there were few difficulties in reinfusing a series of patients with their own TIL cultivated from surgically removed metastases. It was decided to use this treatment mainly as adjuvant therapy in patients who had undergone radical metastasectomy. This is because there is a high probability of microscopic residual disease in these cases, creating a situation wherein TIL should theoretically be at their most effective. Small tumour masses are less organized and immunosuppressive than larger ones, thus enabling TIL to reach and attack them more easily. Finally, if we consider Gansbacher’s theory that ten lymphoid effectors are needed to kill one tumour cell *in vitro*, the cases in this study should have an ideal numerical ratio [13]. A study based on TIL reinfusion with modulated doses of IL-2 in metastasectomized patients was begun in February 1993 and has enabled us to carry out a series of biological evaluations that can be correlated with clinical data.

Materials and methods

From February 1993 to December 1996, 34 patients who had previously undergone metastasectomy (18 melanoma, 14 colorectal cancer and 2 renal cancer) were treated in the Department of Medical Oncology in Forlì [35]. Full details of patient characteristics are given in Table 2.

Table 2 Patient characteristics

Patient number	34		
Male	24		
Female	10		
Median age (years)	58		
Range	17–72		
	Patient status		
Type of disease	Advanced	Disease-free	Total
Melanoma	7	11	18
Colorectal cancer	4	10	14
Renal cancer	1	1	2
Total	12	22	34
Metastases removed			
Melanoma	14 lymph node; 4 skin		
Colorectal cancer	9 liver; 3 lung; 2 peritoneum		
Renal cancer	1 lung; 1 lymph node		

TIL culture and expansion

Tissue obtained from metastatic lesions were mechanically fragmented and incubated for 2–16 h in a solution of enzymes containing collagenase (type IV 0.1%), hyaluronidase (type V 0.01%) and DNase (type I). Mononucleate cells were separated according to density gradients. The cell suspension obtained was cultured at a concentration of 1×10^6 cells/ml in multiwells using AIM-V medium (Gibco) supplemented with 6000 IU/ml recombinant IL-2. Cells were diluted to a concentration of 1×10^6 /ml every 3–4 days. Cell culture was transferred to culture bags (Baxter-Fenwal) once the total number of cells had exceeded $(0.5-1) \times 10^9$. Phenotyping and immunohistochemical analysis were carried out to check for the presence of residual tumour cells. An aliquot of the cell suspension was cultured without IL-2 to allow tumour cell growth. Suspensions of tumour cells and TIL were cryopreserved in liquid nitrogen in 10% dimethylsulphoxide and 90% fetal calf serum for subsequent studies. When cell numbers had exceeded 1×10^{10} , the lymphocytes were concentrated using a CS 3000 Baxter cell separator and reinfused with IL-2 into the patient over 2–3 h. Screening for contaminating micro-organisms was performed on the cell suspension 48 h before reinfusion.

Phenotype analysis

TIL were phenotyped with monoclonal antibodies against CD3, CD4, CD8, CD16, CD56, CD19, CD25, CD71, HLA-DR before and after *in vitro* expansion. Lymphocytes were resuspended in Hanks' balanced salts solution (without phenol red), incubated with each monoclonal antibody for 20 min in the dark and then washed. Flow-cytometric analysis was performed on a Cyturon Absolute Ortho and the percentage of positive cells was then calculated.

Immunohistochemical and immunocytochemical staining

TIL immunostaining was performed at the moment of reinfusion on both cytocentrifuged slides and paraffin-embedded tissues derived from the tumours treated to obtain TIL expansion. Staining was performed according to the manufacturer's instructions using the StrAvigen Super Sensitive kit (Bio Genex Laboratories). The monoclonal antibodies used were anti-T (T cell receptor ζ) (Santa Cruz Biotechnology) used at a 1:120 dilution, anti-p56^{lck} (Santa Cruz Biotechnology) used at a 1:100 dilution and anti-CD43 (Dako Co.) used at a 1:30 dilution. Immunostaining was developed using New fuchsin (Bio Genex Laboratories). The slides were counterstained with haematoxylin. Positive controls consisted of tonsillar frozen sections stained with the monoclonal antibodies and negative controls included the use of equivalently diluted non-immune mouse IgG. The slides were evaluated with conventional light microscopy using the following

scoring: 0, no positive cells; +, 0–10% positive cells; ++, 10%–40% positive cells; +++, 40%–80% positive cells; +++++, $\geq 80\%$ positive cells.

Cytotoxicity assay

Cytotoxicity assays were performed using the ^{51}Cr -release assay just before patient reinfusion. Target cells included fresh (or cryopreserved) autologous or allogenic tumour cells, K562, Daudi and M 14 cell lines. TIL cytolytic activity was determined by plating 1000 target cells labelled with ^{51}Cr in V-bottom microtitre plates. Effector cells were then added at various concentrations to achieve effector:target ratios of 100:1, 50:1, 25:1 and 12:1. Microtitre plates were incubated in a humidified incubator with 5% CO_2 for 4 h, after which 100 μl /well of supernatant was removed and counted in a scintillator.

Percentage lysis was calculated according to the following formula:

$$\text{Specific lysis (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous}} \times 100$$

Cytokine analysis

TIL were tested for the production of IL-4, IL-6, IL-10, IL-12, IFN γ , TNF α , TGF β 1, GM-CSF. Culture supernatant was harvested from the final TIL cultures just before reinfusion into patients and approximately 5 days after the last feeding of IL-2. All cytokines were tested by enzyme-linked immunosorbent assay (ELISA): IL-4, IL-10, IL-12 (Medical Systems), TGF β 1 (Genzyme), GM-CSF, interferon γ (IFN γ), tumour necrosis factor α (TNF α), IL-6 (Medgenix). Minimum detection levels were as follows: TGF β 1, 0.05 ng/ml; IL-4, 13 pg/ml; IL-10, 1 pg/ml; IL-12, 5 pg/ml; GM-CSF, 3 pg/ml; IFN γ , 0.03 IU/ml; IL-6, 2 pg/ml; TNF α , 3 pg/ml.

Identification and expression of perforin

Perforin expression was determined just before the cytotoxicity assay was carried out. Lymphocytes (1×10^6) were fixed with cold 2% paraformaldehyde solution (Sigma) and resuspended in phosphate-buffered saline containing 0.2% Tween-20 to permeabilize cells. Lymphocytes were washed and incubated with δ G9 monoclonal antibody anti-(human perforin) conjugated with fluorescein isothiocyanate (FITC), resuspended in 1% paraformaldehyde and analysed. Negative control cells were only incubated with FITC-conjugated goat anti-(mouse Ig). Flow-cytometry analysis was performed with a FACStar Plus (Becton Dickinson, USA) flow cytometer, calibrated for fluorescence intensity measurements using FITC-labelled microbead standards (fluorescence-activated cell sorting) with assigned MESF values (molecules of equivalent soluble fluorochrome). Lymphocytes were selected using polygonal windows that excluded dead cells and debris, and 5×10^3 cells were analysed for each sample.

Treatment

Concentrated TIL were placed in a transfusion bag containing 5×10^5 – 1.5×10^6 IU IL-2 and reinfused into the patient on day 1 of a 5-day continuous IL-2 infusion according to West's schedule [45]. According to this regimen, therapy was repeated for 4.5 days after an interval of 1 week. If the number of TIL in culture was particularly high, a second reinfusion on the first day of the second week of therapy was given. IL-2 doses began at 12×10^6 IU/day but were decreased to 9×10^6 IU/day and 6×10^6 IU/day on the basis of the following parameters: alveolar pressure < 12 kPa; heart rate > 140 min^{-1} ; diuresis < 150 ml; creatinine > 2.5 mg/dl; bilirubinaemia > 4 mg/dl; transaminase > 90 U/l. If the above values persisted despite dose modulation, treatment was suspended. All but the first six cases treated received a maintenance therapy of 3×10^6 IU subcutaneous IL-2 for 5 consecutive days each month over a period of 6 months (treatment was suspended if

Table 3 Characteristics of patients with advanced disease, numbers of TIL reinfused, IL-2 doses and response to treatment. *Lymph* lymph nodes, *PS* performance status, *TTP* time to progression, *OS* overall survival, *P* progression, *SD* stable disease

Disease	Patient no.	Sex/age (years)	PS	Metastases removed	Residual disease	$10^{-10} \times$ TIL reinfused	IL-2 doses (MIU)	Response	TTP (months)	OS (months)
Melanoma										
	36	M/38	2	Skin	Inguinal lymph + skin	0.043	99	P	–	3
	62	M/58	0	Lymph	Lymph	10.8	47.5	SD	2	22+
	54	F/36	1	Skin	Lung + peritoneum	8.1	120	P	–	12
	90	M/34	1	Lymph	Liver + lung	3.42	60	SD	4	10
	92	M/47	1	Lymph	Lung	7.5	30	SD	2	3
	89	M/55	1	Lymph	Liver	1.63	84	P	–	3
	93	M/51	2	Lymph	Lung	39.5	15	P	–	6
Colorectal ca.										
	20	M/60	1	Peritoneum	Liver	4.2	108	SD	8	14
	24	M/67	1	Peritoneum	Liver	9	63	SD	5	10
	40	F/51	2	Liver	Liver	0.12	72	SD	1	5
	81	F/56	1	Liver	Abdominal lymph	19	108	SD	7	12
Renal ca.										
	70	M/68	2	Lung	Lung + bone	1.5	18	P	–	22+

progression occurred). Evaluation of response and toxicity was performed on the basis of WHO criteria [24]. All patients gave their informed written consent to receive treatment. We declare that this study was examined by the Ethics Committee of our Local Health and Social Services (Azienda USL - Forlì) and was therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Results

Clinical data

Treatment was administered to 12 patients with advanced disease (7 melanoma, 4 colorectal cancer, 1 renal cancer; Table 2). No objective responses but only stabilizations of short duration (1–8 months) were observed, mainly in colorectal cancer patients. No differences could be seen in overall IL-2 dosages or in the number of TIL reinfused when cases of stable disease and disease progression were compared (Table 3). Analysis of the data on disease-free patients (Table 4), shows that, of the 11 melanoma patients, 6 are still disease-free (54.5%) with a median duration of 36.5+ months (14+–47+ months). Five progressed after a median of 5 months (3–7 months), with a median survival of 11 months (9–22+ months). Observing the IL-2 dosages given within the melanoma group, an interesting difference can be seen: patients who are still disease-free had received an average IL-2 dose of 157 MIU (approximately 17.5 MIU/day) whereas patients who progressed had received an average dose of 61 MIU (approx. 7 MIU/day). By contrast, there seems to be no difference between the two subgroups as regards number of TIL reinfused. Ten disease-free colorectal patients were treated: 6 (60%) are still without evidence of disease after a median of 20.5+ months (9+–43+ months), whereas 4 progressed after a median of 8.5 months (range 4–18 months), with a median survival of 24.5 months (7–43+ months). As in melanoma patients, no difference is seen in the number of TIL reinfused in the two colorectal cancer subgroups (disease-free and progressing)

whereas a less evident increase can be observed as regards IL-2 dosage: the average dose was 101 MIU for patients who are still disease-free (11.2 MIU/day) compared to an average of 63.8 MIU for patients who progressed (7.1 MIU/day). The patient with renal cancer is still free from disease after 21+ months. He received a total of 120 MIU IL-2 (average daily dose of 13.3 MIU). Despite the relatively small number of patients in this study, certain factors such as sex, age, performance status and site of disease do not appear to be of great significance.

Toxicity

Specific parameters of moderate cardiological, hepatic and renal toxicity were used to down-regulate IL-2 dosage automatically. Consequently, there were no cases in which toxicity was severe enough to warrant immediate and/or definitive suspension of treatment.

Grade 4 toxicity was observed in only 2 patients: the first (patient 73) had a mild epileptic fit during the last day of therapy, probably caused by hyperpyrexia, which was rapidly resolved. The second case of severe toxicity occurred in patient 70 who interrupted IL-2 infusion after 3 days because of a benign perforated postanastomotic ulcer (the patient had previously undergone gastric resection), which was probably a consequence of the use of antipyretics. Three cases of autoimmune parotitis/thyroiditis were observed. Although the acute phase of this side-effect spontaneously regressed, patients subsequently needed thyroid hormone treatment (Eutirox). Most of the toxicity observed was related to medium to high IL-2 dosages, as has been described in the literature by numerous authors [36, 29]. Table 5 presents toxicity data in relation to severity and frequency.

Table 4 Characteristics of disease-free patients, numbers of TIL reinfused, IL-2 doses and response to treatment. DFS disease-free survival, OS overall survival, Lymph lymph nodes

Disease	Patient no.	Sex/age (years)	PS	Metastases removed	$10^{-10} \times$ TIL reinfused	IL-2 doses (MIU)	DFS (months)	OS (months)
Melanoma	25	F/55	0	Inguinal lymph	1.5	180	47+	47+
	33	F/56	0	Inguinal lymph	0.27	162	42+	42+
	43	F/50	0	Cervical lymph	20	72	38+	38+
	49	M/25	0	Axillary lymph	2.7	282	35+	35+
	52	M/58	0	Cervical lymph	7	62	4	11
	66	M/68	0	Axillary lymph	10.9	120	24+	24+
	72	F/55	0	Inguinal lymph	1.92	48	5	9
	75	M/57	0	Skin	7.5	54	6	22+
	77	F/49	0	Inguinal lymph	9.18	108	3	11
	79	F/48	0	Axillary lymph	15.6	36	7	11
	87	M/57	0	Skin	9.94	126	14+	14+
Colorectal ca.	28	M/69	0	Lung	0.54	81	43+	43+
	27	M/69	0	Liver	7	54	18	43+
	32	M/58	1	Lung	3.5	90	5	31
	59	M/71	0	Lung	16.2	54	12	18
	69	F/40	1	Liver	1.28	114	23+	23+
	73	M/72	0	Liver	7.036	48	22+	22+
	80	M/64	0	Liver	3.96	120	19+	19+
	98	M/66	0	Liver	3.02	132	10+	10+
	97	M/52	1	Liver	3.47	57	4	9+
	101	M/59	0	Liver	7.28	114	9+	9+
	Renal ca.	76	M/17	0	Supraclavicular and abdominal lymph	2.9	120	21+

Biological data

Tissue was obtained from metastatic deposits. The median weight of specimens processed was 5.9 g (range 1–69). After enzymatic digestion, a single-cell suspension containing both tumour-infiltrating lymphocytes and tumour cells was cultured in the presence of IL-2. The percentage of lymphocytes determined by immunocytochemical methods ranged from 1% to 90% (median 10%). TIL were expanded in vitro over a period of 33–71 days (median 44 days). Culture growth was characterized by the gradual decrease in tumour cells and the gradual increase in lymphocytes. There were no visually detectable tumour cells in the

growing cultures after the 3rd week of culture with IL-2. The median expansion index was 3×10^3 ($22\text{--}3.6 \times 10^6$). The number of TIL infused ranged from 0.04×10^{10} to 20×10^{10} cells (median 4.08×10^{10}). Because of low cell yields, phenotypic analysis of lymphocytes at the beginning of expansion was possible in only a few cases and for a limited number of antigens. The median level of CD3 was 68% (51%–94%), that of CD4, 57% (44%–82%) and that of CD8, 12% (0%–23%). A more complete analysis was performed according to routine at the end of the culture period: the median level of CD3 was 97% (73%–100%), CD4, 2% (0%–96%), CD8, 92% (1%–99%), CD16, 6% (0%–30%), CD25, 8% (1%–97%), HLA-DR, 47% (13%–

Table 5 Toxicity

Toxicity	Grade				No. patients	Percentage (%)
	1	2	3	4		
Fever	–	20	14	–	34	100
Oliguria	14	14	4	–	32	94.1
Hypotension	7	14	5	–	26	76.4
Itching	8	13	4	–	25	73.5
Asthenia	1	18	6	–	25	73.5
Renal dysfunction	3	19	3	–	25	73.5
Hepatic dysfunction	6	11	1	–	18	52.9
Tachycardia/arrhythmia	6	5	2	–	13	38.2
Mucositis	6	6	–	–	12	35.2
Nausea/vomiting	2	6	1	–	9	26.4
Dyspnoea	2	1	–	–	3	8.8
Autoimmune parotitis/thyroiditis	2	1	–	–	3	8.8
Central nervous system dysfunction	–	–	–	1	1	2.9
Peptic ulcer perforation	–	–	–	1	1	2.9

Table 6 Evaluation of signal transmission. *White area* disease-free patients, *light-gray area* patients with progressive disease, *dark-gray area* patients with advanced disease

TIL n°	Melanoma TIL					
	Immunohistochemical			Immunocytochemical		
	CD43	TCR ζ	p56lck	CD43	TCR ζ	p56lck
25	++++	–	–			
33	++++	–	–			
43	++++	–	–	++++	+++	+++
49	++++	+	–			
66I				+++	++	++
66II				++++	++++	+++
52				++++	++++	++++
72				+++	+++	–
75	++++	–	–	+++	+++	–
77				++++	–	+++
79I				+++	+++	++
79II				+++	++	+
62				+++	++	–
90	++++	–	–	++++	+	–
93	++++	+	–			
36	+++	–	–			
Colorectal carcinoma TIL						
98	++++	–	–			
32	++++	–	–	++++	++++	++
59I	++++	–	–	++++	++++	++
59II				++++	++++	++
97	–	–	–	++++	++	+
20				++++	++++	++++
24				++++	++++	++++
81				++++	+++	+++

99%). Most cultures had a large predominance of T lymphocytes with an inversion in CD4 and CD8 content. Two cases (patients 93 and 96) showed the constant dominance of CD4 and a minimal presence of CD8. The majority of cells expressed the class II histocompatibility antigen HLA-DR whereas the expression of the CD25 antigen varied but in general decreased with the ageing of cultures. Cytotoxicity tests were carried out on 12 cases. Specific cytotoxicity was evaluated in only 2 cases (melanoma patients 66 and 79) in which autologous tumour growth occurred. Although an extremely high efficacy was not observed in these 2 cases, there did seem to be a more consistent cytolytic activity than that shown by the same TIL against allogenic lines (Allo-Mel or M-14). There also appeared to be a slightly higher aspecific cytotoxicity (against K562) in relapsed or advanced melanoma cases than in disease-free ones. Unfortunately the impossibility of evaluating cytotoxicity on autologous tumour tissue prevents us from correlating TIL specific activity and clinical results. However, our data do seem to indicate that TIL has an appreciable aspecific cytotoxicity and are quite effective against allogenic tumours. In 18 cases (7 melanoma, 11 colorectal cancer), perforin levels were measured by immunocytometry at the end of the culture period. High quantities of perforin were produced in all TIL tested. No particular differences in the synthesis of the molecule were noted in either disease-free or relapsed cases. The quantity

of cytokines produced in TIL culture supernatant before reinfusion was measured in relation to TNF α , TGF β 1 and IL-4, IL-6, IL-10, IL-12, IFN γ and GM-CSF. The data collected refer to 14 cases and are summarized in Table 6. The small number of patients involved in the study means that it is impossible to draw any conclusions, especially as the cases are subdivided according to the clinical course of disease. There would, however, appear to be a moderate increase in TNF α mainly in the TIL secretion of advanced patients. The “immunosuppressive” cytokines (IL-6; IL-10; TGF β 1) showed an extreme variability: complete absence in some cases and very high levels in others.

When possible, immunohistochemical evaluation was performed on the paraffin-embedded histological sample of the surgically removed metastasis. Immunocytochemical evaluation was also carried out on lymphocytes at the end of the culture period and before reinfusion. Both tests were done to determine the expression of the TCR ζ chain and the phosphotyrosine kinase p56^{lck} in the lymphocytes that infiltrated the tumour site as well as in those activated, cultured and expanded with IL-2. There was little or no expression of the TCR ζ chain or p56^{lck} in most of the lymphocytes infiltrating the tumour at the moment of surgical removal. However, the addition of IL-2 to the culture generally resulted in a substantial restoration of the above-mentioned elements, which are essential for the transmission of the lymphocyte activation signal (Table 6).

Discussion and conclusion

Solid tumours generally present a poor prognosis when they metastasize, and surgical metastasectomy is therapeutic in only very few cases. Melanoma, colorectal cancer and renal cancer are no exception to this rule.

The median survival of melanoma patients after the first radical metastasectomy is estimated to be approximately 17 months, with a 21% probability of survival at 2 years [27]. Similar data exist for colorectal cancer after radical hepatic or lung metastasectomy: median survival, 24.7 months; mean disease-free survival, 16.7 months; survival at 5 years, 29.6%; disease-free survival, 13.9% [17]. Analogous data can also be found in the literature regarding the prognosis of renal cancer following radicalization of a distant metastasis (23% survival probability at 5 years) [19]. Moreover, adjuvant chemotherapy after metastasectomy does not appear to achieve particularly good results in these types of tumours [4]. There are no data whatsoever in the literature regarding adjuvant adoptive immunotherapy. This type of treatment, first with lymphokine-activated killer cells and then with TIL in association with IL-2, has mainly been used in advanced melanoma and renal carcinoma. Results obtained have not been completely satisfactory, even though Rosenberg has reported on some cases of long-term complete response, which would seem to indicate that treatment has been successful [37, 38]. To date, TIL treatment in clinical trials has generally been reserved for patients with advanced disease, with the exception of two studies by Fuja and Ratto, in which therapy was given in an adjuvant setting (Table 1) [12, 33]. As Gansbacher affirms that the best ratio for *in vitro* cytolysis is ten lymphoid effectors to one tumour cell [13], and since the number of TIL that can be reinfused is 10^{10} – 10^{11} , we can only expect to treat minimal residual disease successfully. For this reason, our study gradually began to concentrate on patients whose distant metastases had been radically resected, as there is obviously an extremely high risk of microscopic residual disease. It is clear that there are not sufficient data in this work to validate an "adjuvant" treatment. A much larger case series is needed before any comparison can be made with current literature data on survival. A more accurate figure would involve at least twice the number of patients reported in the literature who are still disease-free 2 years after treatment, and at least 40 patients treated for each pathology. Nevertheless, the percentage of patients who are still disease-free is in line with our aims: 13/21 patients (after a median follow-up of 23 months, range 9–47 months).

The study also focused on the problem of trying to find the best IL-2 dosage to support TIL reinfusion. It is known that high-dose IL-2 causes severe toxicity and it was therefore decided to administer treatment intravenously according to West's scheme in an attempt to maximize efficacy and minimize toxicity. Reduction in IL-2 dosage was dependent on the alteration of certain toxicity parameters and on how well the drug was tolerated. In this way it was possible to treat all 34 patients, albeit with extreme

variations in IL-2 doses and in the number of TIL reinfused. Preliminary observation would seem to show a clear difference in the amount of IL-2 administered to patients who are still disease-free and in that given to those who progressed. In fact, disease-free patients received a much higher average dose of IL-2 than relapsed patients; this difference is more evident in melanoma patients than in colorectal cancer ones. By contrast, both the number of TIL reinfused and TIL phenotype characteristics do not seem to vary a great deal. This observation confirms Karp and Atkins' final comment regarding Ratto's study on non-small-cell lung carcinoma [18, 33]. If this dose phenomenon continues as the study progresses, it will be necessary to consider (when possible) maintaining higher doses of IL-2 even in the event of moderate to low toxicity parameters. Our result was certainly an unexpected one, as recently some authors seemed to have challenged Rosenberg's concept that "more is better", maintaining that IL-2 is effective at low doses as well [3, 5, 23]. Obviously further investigation is warranted. Additional research is also needed to clarify the role of IL-2 in the "fight" between the contrasting forces of tumour immunosuppression and lymphocyte activity. According to certain articles in the literature, the efficacy of IL-2 in opposing immunosuppression caused by the tumour can be seen mainly when the neoplastic cells are at the beginning of their expansion [16]. In our study, TIL and IL-2 may therefore be effective against micrometastatic diffusion. There is no doubt that the key to the success or failure of current immunotherapy lies in the delicate relationship between tumour host and tumour immunosuppression activity of the infiltrating lymphocytes. Increasing numbers of studies on TIL are now reporting immunosuppression mechanisms, such as functional blocks with molecular alterations, lymphocyte anergy determination and production of cytokines, which block or favour different lymphocyte specializations [32, 49]. The tumour has a negative effect on the lymphocytes which, after recognizing tumour antigens, arrive *in situ* to destroy it. In particular, there is evidence to suggest that the bigger the tumour, the greater its immunosuppressive activity [21, 41]. This was another reason why our work focused on trying to block the growth of micrometastases and it was decided to collect a series of biological data that could later be used to correlate clinical data. Cytotoxicity characterization is insufficient in this series as autologous tumour samples were available for specific cytolytic testing in only 2 cases. On the other hand, it is known that TIL specific cytolysis has a relative value. Nevertheless, it was attempted to compensate for this lack of data by measuring levels of perforin, an enzyme that is essential for cytolytic activity, at the end of the culture period [20]. Obviously the fact that perforin was present in great quantity in the TIL tested is no guarantee of specific cytolytic activity. For the future, however, the determination of markers that characterize the induction of apoptosis (Fas) may prove to be of further help if associated with perforin quantification [40]. A quantitative evaluation of cytokines present in the culture supernatant before TIL reinfusion was made to assess the secretory capacity of TIL. The numerical data obtained do

not permit any judgement to be made on whether there was an increase or decrease in TIL clinical efficacy on the basis of their secretory potential. This is because the small amount of data we have is further subdivided in relation to clinical results. Schwartzentrüber reported a correlation between good clinical response and TIL production of GM-CSF [39]. Goedegebuure has observed clinical correlations between TIL cytokine production and the clinical course of disease [14]. In our experience there would seem to be a greater production of TNF α in the TIL of patients with advanced disease. It is not yet understood whether this is a coincidence or a characteristic that TIL maintain and/or potentiate from the start of culture. One hypothesis to explain the latter phenomenon might be that the tumour, in an advanced stage, exerts an influence on the infiltrating lymphocytes. On the other hand, the influence of the tumour on TIL is evident from determination of the TCR ζ chain and from the presence of p56^{lck} in the lymphocytes infiltrating the tissue sample from which the culture was begun. ζ chain expression is virtually absent in several cases and p56^{lck} is not completely expressed in the majority of specimens. On the other hand, subsequent cytochemical determination at the end of culture shows that IL-2 had the effect of greatly improving these functions in most patients.

Moreover, there would seem to be a complete restoration of these two parameters, which correlates with patients who are still disease-free. By contrast, an incomplete restoration was observed in most progressed and advanced melanoma patients who did not respond to treatment. If these data continue to correlate with clinical results as the patient population increases, it could be an important finding in favour of the potential efficacy of this type of treatment. In conclusion, we are still a long way from being able even to suggest that TIL+IL-2 treatment is an effective adjuvant therapy after metastasectomy in the three types of solid tumour we considered. However, our findings to date have prompted us to continue with the present study. Further decisions may subsequently be made about IL-2 dosages in an attempt to balance drug efficacy and toxicity in patients.

Moreover, the evaluation of biological indicators determined on TIL confirm what has already been reported in the literature. With a larger case series, however, useful correlations could be made to promote a deeper understanding of the relationship between tumours and the human immune system.

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