minal adjacent peptide does not induce bone resorption. *Calcif Tissue* 1983, 35, 654.
24. Deftos LJ, Weisman MH, Williams GW, *et al.* Influence of age and sex on plasma calcitonin in human beings. *N Engl J Med* 1980, 302, 1351.
25. Body JJ, Heath III H. Estimates of circulating monomeric calcitonin: Physiological studies in normal and thyroidectomized man. *J Clin Endocrinol* 1983, 57, 897.
26. Tobler PH, Tschopp FA, Dambacher MA, Fischer JA. Salmon and human calcitonin-like peptides in man. *Clin Endocrinol* 1984, 20, 253.
27. Ittner J, Dambacher MA, Born W, *et al.* Diagnostic evaluation of measurements of carboxyl-terminal flanking peptide (PDN-21) of the human calcitonin gene in human serum. *J Clin Endocrinol* 1985, 6, 1133.

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Phenotypic and Functional Analysis of Tumour-infiltrating Lymphocytes from Patients with Melanoma and Other Metastatic Cancers

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Thirty tumour specimens, among which were 17 melanomas, were cultured with recombinant interleukin-2 (IL-2) in order to produce tumour-infiltrating lymphocytes (TIL). In the melanomas, three categories of TIL were characterised. The first, containing mostly CD3+ and CD8+ cells, lysed only autologous tumour cells; the second, containing mostly CD3+ and CD4+ cells, lysed both autologous tumour cells and allogeneic cells lines; the third, with mixed phenotype although cytotoxic for K562 targets, did not kill melanoma cells. The optimal conditions for a good development of TIL were established: we found that the lymph node or cutaneous origin of the tumour was unimportant, a 2 h enzymatic treatment was optimum and that TIL grew well in AIM V serum free medium. Therefore the easiness and the reproductibility of the TIL cultures from melanoma tumour samples allows the rapid development of therapeutic trials in metastatic melanoma.
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INTRODUCTION

ADOPTIVE IMMUNOTHERAPY consists of the transfer of immunologically competent cells, such as natural killer cells (NK), T lymphocytes or monocytes, to cancer patients in order to induce an antitumour response either by a direct cytotoxic effect or by the release of immunostimulatory or cytotoxic factors. This procedure was initiated by Rosenberg *et al.* who, after demonstrating the cytotoxic activity of lymphoid cells, previously stimulated with interleukin-2 (IL-2), against fresh murine [1] and human [2] tumours, reinjected them into tumour-bearing hosts. Three different cells have since been used: lymphokine-activated killer cells (LAK), interferon-activated monocytes, and tumour-infiltrating lymphocytes (TIL).

LAK cells are derived from peripheral blood mononuclear cells of patients previously stimulated *in vivo* by IL-2. The cells are then activated *in vitro* with IL-2 for 4 days, which results in a reinforcement of their cytotoxic activity and an increase in their number. The cells are reinjected into the patients together with IL-2. LAK cells are cytotoxic against fresh autologous and allogeneic tumour cells. This approach has been shown to be effective in inducing tumour regressions both in murine models and in humans with metastatic cancers of various origins (kidney, colon, melanoma) [3]. However, the cytotoxicity is not tumour-specific and the adverse effects of the treatment are considerable, as a large number of cells need to be reinjected and as IL2 associated with the reinjection must be administered in high doses.

Monocytes also play a role in antitumour defences; the isolation and culture of circulating blood monocytes gives rise

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to more mature and more cytotoxic macrophages. Phase I trials with these cells have shown their safety when they were reinjected intravenously or intraperitoneally but no antitumour effect has been yet documented [4].

Tumour infiltration by lymphocytes has been known for a long time and, according to some authors [5, 6], has a prognostic significance and a local antitumour role. TIL expanded in the presence of IL-2 from various tumours exert a potent cytotoxic activity, 50–100 times greater than that exerted by LAK cells [7, 8]. A large number of these lymphokine-activated TIL do not differ from LAK in terms of their specificity, particularly those developed from renal, colonic, lung and head and neck tumours, although a cytotoxicity specific for the autologous tumour has been more frequently reported with TIL obtained from melanomas. Although only a few experiments with TIL have been reported so far, preliminary data suggest that TIL may have promising applications especially for the treatment of metastatic melanoma.

In this paper, we report our experience with TIL cultures from 30 tumour specimens. This study was designed to optimise the culture conditions in view of reinjection to the patients and to assess the phenotypic and functional aspects of the lymphocytes. We conclude that TIL can develop from lymph node or skin melanoma tumours indistinctely; a 2 h enzymatic treatment is optimum and does not seem to disrupt the cellular interactions required for initiation of lymphocyte proliferation. In addition, TIL grow in AIM V medium as well as in RMPI plus HAB serum. Phenotypic and cytotoxic studies characterise three categories of TIL: the first, mostly composed of CD3+CD8+ cells, lyses autologous tumours targets, the second, mostly composed of CD3+CD4+, lyses both autologous and allogeneic targets and the third with a mixed phenotype is cytotoxic for K562 target cells, and not for melanoma cells.

MATERIALS AND METHODS

Tumours

17 melanomas were cultured: seven lymph node metastases, eight skin nodules, measuring 5 mm to 20 cm, and two abdominal tumours. 13 other tumours were also cultured: eight breast adenocarcinomas, consisting of four metastatic lymph nodes and four primary tumours, three primary renal tumours and two miscellaneous tumours, corresponding with regional lymph nodes from a seminoma and a head and neck tumour.

Culture medium

The medium originally described by Rosenberg, consisting of RPMI 1640 (Gibco BRL), 20% allogeneic LAK supernatant, 10% pooled and decomplemented human AB serum (HAB) (CNTS, Les Ulis, France), 2 mmol/l of L-glutamine, 2% of pyruvate, 100 μl/ml of penicillin and 100 μg/ml of streptomycin, was used initially.

We subsequently tested the serum-free AIM V medium (Gibco BRL) with LAK supernatant. The LAK supernatant was regularly produced by peripheral blood leucocytes (PBL) of healthy donors which, after isolation and washing, were cultured at a concentration of 3×10^6/ml in AIM V medium. After 4 days of culture, the supernatant was collected, tested bacteriologically and stored at −20°C.

Interleukin-2 (IL-2)

Human recombinant interleukin-2 (RU 49637) was supplied by Roussel Uclaf (Romainville, France). Its specific activity, measured by monitoring the proliferative activity on CTLL2 cells, was 10^7 BRMP U/mg of protein. The cells were cultured with 1000 U/ml of IL-2.

Targets

The following cells were used as targets in the cytotoxicity tests: K562, an erythroleukaemia cell line; Raji, a Burkitt's lymphoma cell line; and autologous tumour cells thawed on the day of the test. HT144, a human melanoma cell line, was also used in several cases.

Monitoring of the culture

Tumours were harvested aseptically, minced into 3–5 mm pieces, and stirred in 40 ml saline containing 3600 U of desoxyribonuclease, 50 mg of collagenase and 125 U hyaluronidase (Sigma) for 1–2 h at 37°C. The resulting cell suspension was filtered through Nitex mesh, washed twice and suspended in AIM V containing human recombinant IL-2 at 1000 U/ml. The cell suspension was cultured at $3–5\times10^5$ cells/ml successively in 75 then 175 cm^2 bottles (Becton Dickinson). When the concentration rose, the cells were transferred into 3 l bags (Baxter–Fenwall). The cultures were incubated at 37°C in 5% CO_2. The recovery was performed on a Stericell machine (Dupont de Nemours, France) which recovered the cells from the bags, concentrated and washed them. The cells were returned to the bags at a concentration of 5×10^5/ml by a Solution Transfer Pump (Fenwall). Specimens for bacteriological analysis were regularly collected in each bag of culture. During reinjection, the cells were concentrated in 300 ml bag in human albumin (CNTS, Orsay, France).

Immunophenotyping

The TIL were labelled by direct immunofluorescence with monoclonal antibodies directly linked either to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The following antibodies were used: anti-CD3 (pan T), anti-CD4 (helper/inducer T cells), anti-CD8 (cytotoxic suppressor T cells), anti-CD56 (NK cells) and anti-CD25 (receptor for the α chain of IL2), all purchased from Becton Dickinson. 5×10^5 cells/tube were incubated with the antibody for 20 min at room temperature, then washed twice with PBS–1% formaldehyde. The analyses were performed on a FACSCAN 440 (Becton Dickinson); 5×10^3 cells were analysed.

Cell-mediated cytotoxic assay

The tumour target cells were labelled in RPMI 1640 (Gibco) with 7.4 MBq of $^{51}NaCrO_4$ (5.8 GBq, Compagnie Oris Industrie, Gif-sur-Yvette, France) for 1 h at 37°C. They were washed twice in RPMI 1640, resuspended in standard culture medium and kept at room temperature for 30–60 min. They were then washed once, resuspended in culture medium and plated at 10^4 cells/well in 96-well (flat-bottomed) microtitre plates (CML, Nemours, France), in which the effectors had been previously plated at different concentrations. The assay was performed in triplicate at effector-to-target ratios (E/T) ranging from 50 to 1.5. Controls included labelled target cells incubated in culture medium alone (spontaneous release) and labelled target cells incubated with HCl 1% (maximum release). After a 4 h incubation in a 5% CO_2 atmosphere at 37°C, the plates were centrifuged for 5 min at 2000 rpm, 100 μl samples were harvested from each well and counted in a gamma counter (LKB, Les Ulis, France). The percentage of specific chromium release was calculated as follows: (experimental release − spontaneous release × 100 ÷ (maximum release − spontaneous release). Results are expressed

Table 1. Characteristics of the melanoma-derived TIL

Sample	Sex/age	Site	IL-2	Initial lymphocytes (%)	ET (h)	Lymphocytes 10^8	Day of max. growth	Arrest Day	Arrest Cause
1	F/87	LN	−	70	16	17	30	70	Death
2	F/45	LN	+	1	16	0.2	20		Insufficient growth
3	M/45	Nod.	−	30	16	ND	−	30	No growth
4	F/30	Nod.	+	19	16	ND	−	30	No growth
5	F/57	LN	−	3	16	ND	−	−	No growth
6	F/49	LN	−	1	0	300	35	35	Freezing
7	M/20	Nod.	+	10	2	120	45	45	Reinjection
8	M/52	Nod.	+	10	2	620	45	45	Reinjection
9	F/34	Nod.	+	3	2	1500	45	45	Reinjection
10	M/52	LN	−	90	2	ND	ND	22	Freezing
11	M/28	Nod.	+	90	2	ND	ND	30	Freezing
12	F/57	AT	−	23	2	ND	−	28	No growth
13	F/37	LN	−	90	2	ND	ND	20	Freezing
14	F/26	LN	+	13	2	866	45	45	Reinjection
15	F/49	Nod.	−	1	2	ND	−	30	No growth
16	F/50	AT	+	10	2	2.25	46	55	Insufficient growth
17	M/70	Nod.	−	16	2	ND	ND	17	Freezing

LN = lymph node, Nod. = nodule, AT = abdominal tumour, ND = not determined, Lymphocytes = maximum lymphocytes number, IL-2 = patients previously treated with IL-2, ET = enzymatic treatment.

as a percentage of specific chromium release (cpm) at different E/T ratios.

RESULTS

TIL Cultures

From melanomas. 17 cultures were started from fresh melanoma tumours; their characteristics are summarised in Table 1. 12 of the 17 samples developed TIL; they appeared, on average, on the 18th day (between the 15th and 21st day, depending on the sample) after which time the growth was regular (by a factor of 10 per week). The causes for arrest of the culture were either freezing (six cases) or reinjection to the patients (four cases) or arrest due to a fall in the number of cells over time (two cases); part of one culture was prolonged for 70 days (no. 1) and was then stopped because of cell death. No difference in the time of appearance of TIL was observed according to the origin of the sample (lymph node or subcutaneous nodule).

TIL did not develop from five tumours (nos. 3, 4, 5, 12, 15): samples 5 and 12 were obtained from the same patient at different times; sample 4 was obtained from another patient during treatment with IL-2, a second sample was collected subsequently, from which TIL were able grow (no. 9); sample 15 was obtained from a patient in whom a previous sample (no. 6) had grown TIL and sample 3 was very small. Four of these five cases were non-lymph node samples. Seven of the 12 cases in which TIL were grown corresponded with patients previously treated with IL-2. Four of the five failures had not been previously treated with IL-2.

From other tumours. The origin and course of these tumours are summarised in Table 2. Six of the eight breast cancers failed to grow; four of these six samples were derived from primary tumours; three of the four lymph node samples developed TIL,

Table 2. Characteristics of the non-melanoma tumour-derived TIL

Sample	Sex/age	Site	Initial lymphocytes (%)	Arrest Day	Arrest Cause
18	F/50	Breast	ND*	35	No growth
19	F/33	LN breast	ND	25	No growth
20	F/60	Breast	ND	15	No growth
21	F/50	LN breast	50	25	Growth freezing
22	F/66	LN breast	40	20	No growth
23	F/34	LN breast	90	25	Growth freezing
24	F/59	Breast	ND	25	No growth
25	F/51	Breast	0	15	No growth
26	F/75	Kidney	ND	25	No growth
27	M/58	Kidney	ND	25	No growth
28	F/39	Kidney	ND	30	Growth freezing
29	M/58	LN seminoma	26	20	No growth
30	M/58	LN cavum	ND	25	No growth

LN = lymph node, ND = not determined.

some only in small quantities (nos 22 and 23) and one (no. 21) developed abundant TIL. Out of the three cases of renal cancers, TIL could not be grown in two cases (nos 26 and 27); in both cases, the tumour was very necrotic. Two other tumours (nos 29 and 30) were cultured without the production of TIL: a metastatic lymph node from a seminoma and a regional lymph node from a tumour of the cavum.

Role of enzymatic treatment in the growth of TIL. Four tumours were cultured without preliminary enzymatic treatment (nos 6, 7, 8, 13): although TIL were produced, the proliferation appeared to be more efficient after enzymatic treatment, i.e. the clones appeared earlier. Two different durations of contact with the enzymes, 16 h and 2 h, were studied, but no difference was observed in either the date of appearance of the first clones or in their cytotoxic activity (data not shown). Thus, since melanoma tumour specimens are often friable, containing little connective tissue, a short period of enzymatic digestion suffices to obtain large numbers of TIL.

Time-course analysis of TIL cultures

Phenotypic analysis. Phenotypic tests were performed at different times during culture, 14 of the 17 melanomas were subject to phenotypic monitoring. Figure 1 shows two illustra-

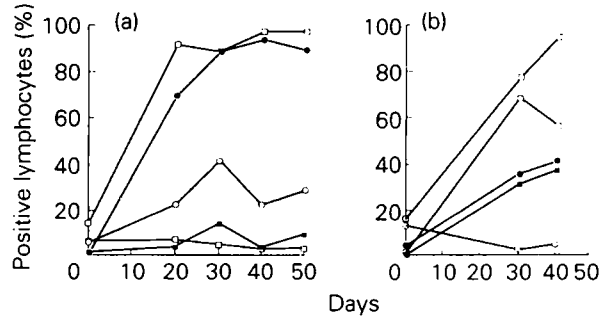

Fig. 1. Time-course of the phenotype of TIL (a) 9 and (b) 14. The percentage of positive lymphocytes was determined by enumerating the per cent of labeled cells in the lymphocyte gate as described previously [16]. -□- = CD3, -◆- = CD8, -□- = CD4, -◇- = CD56, -■- = CD25.

Table 3. Course of the phenotypes of TIL grown from melanomas

Patients	Day 0 CD3/CD4/CD8	Day 20 CD3/CD4/CD8	Day 30 CD3/CD4/CD8	Day 40 CD3/CD4/CD8
1	14/13/3	98/8/89	98/5/94	ND
2	NE	99/ND/90	99/ND/72	ND
3	NE	99/2/90	90/2/72	ND
4	67/16/47	73/22/ND	ND	ND
6	NE	99/16/91	99/1/99	ND
7	NE	ND	80/7/73	99/2/98
8	NE	95/21/79	98/8/92	99/7/90
9	15/6/1	92/7/70	89/5/90	98/4/94
10	61/37/26	95/21/70	94/37/80	ND
11	NE	84/50/20	95/70/14	ND
12	NE	14/0/16	ND	ND
13	25/5/18	84/24/60	ND	ND
14	29/3/14	ND	81/54/30	87/56/50
16	NE	ND	ND	83/64/65
17	NE	100/16/99	97/20/93	ND

NE = not enough lymphocytes, ND = not done.

Table 5. Comparison of phenotype and cytotoxicity

Sample	Day	Cytotoxicity* K562	Raji	Auto.	HT144	Phenotype† CD3	CD8	CD4	CD56
MHC-restricted									
7	40	16	1	59	4	99	98	2	5
9	20	25	3	77	12	92	70	7	23
6	30	1	ND*	19	9	99	99	1	56
14	30	8	2	27	ND	81	38	72	4
Non MHC-restricted									
1	15	77	37	41	ND	93	78	16	14
8	25	70	18	26	9	95	82	12	14
10	20	80	87	77	ND	94	80	37	23
11	15	60	13	40	ND	90	14	73	ND
16	46	82	75	30	ND	83	61	22	50
Non-antimelanoma									
13	20	45	30	10	ND	94	76	51	19
17	15	17	20	13	ND	97	93	11	20

*% of lysis at E/T 25/1.
†% of positive cells.
ND = not done.

tive patients: TIL 9 had an increase of CD3+ and CD8+ cells reaching a plateau at day 25, with CD4+ cells remaining low while TIL 14 had a mixed phenotype, essentially CD4+ and CD3+ cells accompanied by a lower but significant increase of CD8+ cells. The phenotypic profile of the melanoma-derived TIL is given in Table 3. In all cases, the number of CD3 positive cells increased by the 15th–20th days, reaching a plateau on day 30. The predominant populations were CD3+ and CD8+ cells for six cultures (nos 1, 2, 6, 7, 8, 9) and CD3+ and CD4+ cells for two other cultures (nos 11, 14). Two tumours (nos 1, 6) developed CD3+, CD56+ cells with a maximum of 50% on day 30 and 5 of the 15 tumours had a mixed phenotype (nos 10, 13, 14, 16 and 17).

The number of cells expressing CD25 increased in all cases with maximum values varying from 2% to 55% reaching a peak between days 20 and 30, after which the percentage often decreased over time (data not shown).

In TIL derived from tumours other than melanoma, mostly CD3+CD8+ with 30% CD56 cells developed from one sample (no. 21) while the other (no. 23) gave rise essentially to CD3+CD4+ lymphocytes.

Course of cytotoxicity. The cytotoxicity exerted by melanoma-derived TIL against the various targets (K562, Raji, autologous and allogeneic melanoma tumours) varied from one sample to another and over time. Table 4 represents the time course of cytotoxicity of tumour no. 1: at the E/T ratio of 25/1, the percentage of specific chromium release was low at the begining of the culture, but subsequently increased against K562 and Raji with a peak between days 15 and 20, followed by a decline, coinciding with the development of cytotoxicity directed against the autologous target cells. When the cytotoxicity against the various targets was compared with the phenotypic evolution at the same time, the TIL could be classified into three groups, as shown in Table 5: MHC-restricted TIL lysing the autologous tumour but not allogeneic cell lines: their phenotypic profile was CD3+, CD8+ in three out of four cases, while the fourth case (no. 14) was more heterogeneous; non-MHC-restricted TIL lysing autologous and allogeneic melanoma cell lines indifferently: their phenotypic profile was essentially CD3+ CD4+ in two out of four cases (nos 11 and 16) and CD3+ CD8+ for the other two cases (nos 1 and 10); and "non-antimelanoma" TIL lysing the K562 and Raji cell lines but not the melanoma cells; their phenotypic profile was mixed CD3+ CD8+ CD4+ in two cases (nos 13 and 17).

Table 4. Course of cytotoxicity of TIL 1

Days	K562*	Raji*	Autologous*
2	30	10	5
7	75	45	10
12	75	35	40
20	75	45	30
27	30	5	35

*% lysis at the E/T 25/1.

When tests were performed in the other tumours (three cases), the TIL were cytotoxic against K562, but the autologous tumour was not available for testing (data not shown).

Role of the culture medium

The "standard" TIL medium requires a large quantity of human HAB that introduces the risk of the presence of infectious agents, especially of viral origin. We consequently tested the serum-free AIM V medium. The clones grown in AIM V developed as rapidly as in HAB medium (results not shown) and no difference was observed in terms of their cytotoxicity and their phenotype as shown in Fig. 2.

Injections to patients

Four of the 17 melanoma-derived TIL were reinjected to the patients from whom the tumours came (nos 7, 8, 9, 14). The number of cells injected was 1.3×10^{10}, 6×10^{10}, 1.5×10^{11} and 9×10^{10}, respectively. The reinjections were performed by two 1.5 h infusion over 48 h in 3 patients and only a 1.5 h infusion in the fourth patient. The tolerance of the cells was excellent apart from a few episodes of chills; the only significant adverse

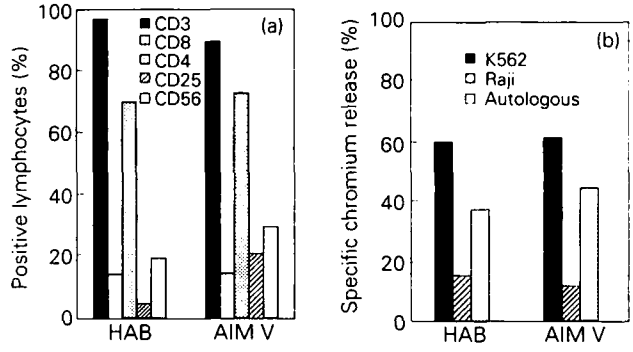

Fig. 2. (a) Phenotype and (b) cytotoxicity on day 20 of the TIL cultured in the serum-containing medium (HAB) and the serum-deprived medium (AIM V). Cytotoxicity was expressed as percentage of specific chromium release at 25/1 E/T and the phenotype expressed as percentage of positive cells.

effects were related to IL-2 injected after the cells, i.e. fever, anorexia and cutaneous toxicity.

DISCUSSION

Our results in 17 tumours confirm the development of TIL from melanoma tumours; no difference was observed according to the origin of the sample (lymph nodes or subcutaneous nodules) or according to the size of the sample. We have not systematically compared the various methods, but 11 tumours were enzymatically treated during 2 h, five for 16 h, five were cultured in AIM V medium alone, 10 in HAB-supplemented medium and two were cultured by the two ways (AIM V and HAB). From the data obtained, we concluded that the optimal culture conditions include 2 h of enzymatic treatment and culture in serum-free medium AIM V in the presence of IL-2 and LAK supernatant.

The poor development of TIL from non-melanoma tumours raised several problems, possibly related to the origin of the samples, which were essentially primary tumours, some of which were fibrotic while other were necrotic. The less constant development of TIL from non-melanoma tumours has already been reported in an extensive study by Yanelli [9]. Since in our series, TIL grew better from melanoma patients previously treated with IL-2, one cannot exclude that part of the difference between melanomas and other tumours in terms of *in vitro* TIL cultures is due to the fact that patients with non-melanoma tumours were not enrolled in therapeutic trials with IL-2. More studies, with higher number of cases needed to be establish if there is a correlation between *in vivo* IL-2 treatment and *in vitro* TIL development and therefore to propose that IL-2 infusion modifies the local immunological environment of the tumours.

Our functional and phenotypic results confirm that the development of autologous cytotoxicity appears to be correlated with the presence of an essentially CD8 phenotype. Rosenberg [9, 10] reported similar phenotype-function relations with a weaker autologous cytotoxicity in TIL with a mixed CD3+ CD4+ CD8+ phenotype. The relationship between autologous cytotoxicity and the CD8+ phenotype was confirmed by positive immunoselection of the TIL population: the "purified" CD8+ cells demonstrated a constant autologous cytotoxicity, while the CD4+ cells were devoid of such activity [11].

Although the CD3+ CD8+ phenotype can be correlated with *in vitro* autologous cytotoxicity, this phenotype cannot be correlated, at the present time, with therapeutic efficacy [12], the effector cells active *in vitro* being not necessarily active *in vivo*.

The therapeutic efficacy of tumour-specific cytotoxic TIL versus non-tumour specific cytotoxic or non-cytotoxic TIL needs therefore to be evaluated. Animals models in which a cDNA coding for IL-4 [13] or IL-2 [14] have been introduced into tumours suggest that a local activation of the immune system could have potent antitumour effects. Thus CD4+ or CD56+ TIL, not cytotoxic towards the patient's tumour, but producing cytokines such as IL-2, IL-4 or IFN-γ may turn out to be as effective as CD8+ tumour-specific cytotoxic lymphocytes.

The monitoring of the cultured TIL before reinfusion to the patients should allow to resolve the question of the class of TIL efficient in antitumour effect.

The reproducibility of the development of TIL from melanoma tumours samples allows to design therapeutic trials to address this question in metastatic melanoma, the use of serum-free medium eliminating a major risk for viral infection by human serum. Finally, the easiness and reproducibility of the TIL cultures will also allow further development of the novel therapeutic approach, using these cells as vectors for exogenous genes, coding for potentially antitumour substances [15].

1. Yvron I, Wood TA, Spiess PJ, Rosenberg SA. *In vitro* growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors. *J Immunol* 1980, **125**, 238–250.
2. Grimm EA, Robb RJ, Roth JA, *et al.* Lymphokine-activated killer cell phenomenon. III evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells. *J Exp Med* 1983, **158**, 1356–1361.
3. Rosenberg SA, Lotze MT, Muul LM, *et al.* A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high dose of interleukin-2 alone. *N Engl J Med* 1987, **316**, 889–897.
4. Andreesen R, Scheibenbogen C, Brugger W, *et al.* Adoptive transfer of tumour cytotoxic macrophages generated *in vitro* from circulating blood monocytes: A new approach to cancer immunotherapy. *Cancer Res* 1990, **50**, 7450–7456.
5. Vose BM. Quantitation of proliferative and cytotoxic precursor cells directed against human tumors: limiting dilution analysis in peripheral blood and at the tumour site. *Int J Cancer* 1982, **30**, 135–142.
6. Whiteside TL, Miescher S, Hurlimann J, Moretta L, Von Fliedner V. Separation, phenotyping and limiting dilution analysis of T-lymphocytes inflitrating human solid tumours *Int J Cancer* 1986, **37**, 803–811.
7. Topalian SL, Muul LM, Solomon D, Rosenberg SA. Expansion of human tumour infiltrating lymphocytes for use in immunotherapy trials. *J Immunol Meth* 1987, **102**, 127–141.
8. Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumour-infiltrating lymphocytes. *Science* 1986, **233**, 1318–1321.
9. Maleckar JR, Friddell CS, Sferruzza A, *et al.* Activation and expansion of tumour-derived activated cells for therapeutic use. *J Natl Cancer Inst* 1989, **81**, 1655–1660.
10. Topalian SL, Solomon D, Rosenberg SA. Tumour-specific cytolysis by lymphocytes infiltrating human melanomas. *J Immunol* 1989, **142**, 3714–3725.
11. Morecki S, Topalian SL, Myers WW, Okrongly D, Okarma TB, Rosenberg SA. Separation and growth of human CD4+ and CD8+ tumour-infiltrating lymphocytes and peripheral blood mononuclear cells by direct positive panning on covalently attached monoclonal antibody-coated flasks. *J Biol Response Mod* 1990, **9**, 463–474.
12. Topalian SL, Solomon D, Avis FP, *et al.* Immunotherapy of patients with advanced cancer using tumour-infiltrating lymphocytes and recombinant interleukin-2: a pilot study. *J Clin Oncol* 1988, **6**, 839–853.
13. Tepper RI, Pattengale PK, Leder P. Murine interleukin-4 displays potent anti-tumour activity *in vivo*. *Cell* 1989, **57**, 503–512.
14. Ley V, Langlade-Demoyen P, Kourilsky P, Larsson-Sciard EL.

Interleukin 2-dependent activation of tumour-specific cytotoxic T lymphocytes *in vivo*. *Eur J Immunol* 1991, 21, 851–854.
15. Rosenberg SA, Aebersold P, Cornetta K, *et al.* Gene transfer into humans – Immunotherapy of patients with advanced melanoma, using tumour-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* 1990, 323, 570–578.
16. Farace F, Mathiot C, Brandely M, *et al.* Phase I trial with recombinant interleukin-2 (r-IL2) alone or following pretreatment with recombinant interferon-gamma. *Clin Exp Immunol* 1990, 82, 194–199.

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Flow Cytometric Analysis of Tumour-draining Lymph Nodes in Breast Cancer Patients

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The phenotype and activation status of lymphocytes from the peripheral blood and axillary lymph nodes of 40 patients with breast cancer were analysed using flow cytometry and compared with lymphocytes from the blood and lymph nodes of 7 control subjects. There was little difference in the overall proportions of T and B lymphocytes but there was a much larger population of B cells bearing surface IgG and a greater number of CD4+ helper T cells, particularly in the regional nodes, in the breast cancer patients. Many more T cells in the cancer patients were found to be carrying the HLA DR and Tac antigens. The axillary lymph nodes were the major site of B cells and CD4+ T cells whilst the primary tumour was the source of the CD8+ suppressor/cytotoxic T cells. Any immune response appeared to be largely loco-regional and may therefore destroyed by conventional surgery or radiotherapy.
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INTRODUCTION

THE DEBATE about the surgical management of patients with breast carcinoma began in the 1960s, when the use of the Halsted radical mastectomy was first challenged. The major controversy at that time was over the treatment of the axillary nodes with the suggestion that the removal of these might be detrimental to those patients who were mounting a regional immune response against their tumours [1]. The emphasis of that debate has shifted, in recent years, to the surgical management of the primary lesion and the axillary nodes are excised in the majority of patients. The effect of this on the immune system is not clear. In an attempt to clarify the contribution of the regional lymph nodes to host defences, a large multicentre trial of patients with clinical stage I breast cancer was performed [2, 3]. 40% of the group in which axillary dissection was performed proved, on histological examination, to have stage II disease and as it was a randomised trial it can be assumed that a similar proportion of those undergoing simple mastectomy also had microscopic nodal metastases. While there was no overall survival difference between the two groups it was notable that only 15% of the simple mastectomy group ever presented with clinical disease in the axillary nodes while the other 25% remained clinically well after more than 10 years of follow up. This suggests that some patients remain well in the presence of occult disease and it is possible that this is due to some host antitumour immune response.

The development of monoclonal antibodies to markers on the cell membrane allows identification of the cell phenotype and activation status of the lymphocytes within any population. While these antibodies have been applied by many groups to the immunohistochemical study of the tumour infiltrating lymphocytes (TILs) [4–6] few studies have yet applied flow cytometry to this analysis [7, 8] and only two studies of the axillary lymph node lymphocytes have been performed using this method.

Morton *et al.* [9], comparing the peripheral blood and lymph node lymphocytes, found an increase in the proportion of B cells and CD4+ helper T cells in the nodal population and a greater proportion of cells bearing HLA DR. As this study included no control subjects, it was not possible to tell whether these differences related to the presence of breast carcinoma or were simply due to the lymphocyte source.

Mantovani *et al.* [10], comparing the lymph node lymphocytes (LNLs) of breast cancer patients with those from normal controls, found no major differences in the phenotypic proportions but did find a larger CD4+ T cell population in nodes invaded by cancer. The latter also studied the HLA DR expression and found no significant difference between patients with breast cancer and normal subjects.

No studies have compared the expression of interleukin 2 or transferrin receptors nor the proportion of IgG bearing B cells

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