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Detection of oligoclonal T lymphocytes in lymph nodes draining from advanced non-small-cell lung cancer

Abstract Despite the combined use of surgery and chemo-radiotherapy, the poor prognosis of advanced non-small-cell lung cancer (NSCLC) requires the definition of new therapeutic approaches. The presence of T lymphocytes, with peculiar phenotypic, functional and molecular characteristics within the tumour, suggested the possible use of these cells, expanded in vitro, in protocols of adoptive immunotherapy. We have described how a population of oligoclonal T lymphocytes, derived from advanced NSCLC, can be expanded in vitro and has the capability of lysing autologous cancer cells. What is more important, we observed that patients with advanced NSCLC, treated with TIL expanded in vitro and recombinant interleukin-2, seemed to have a disease-free period longer than that of patients treated with conventional chemoradiotherapy. In an attempt to find new sources of specific lymphocytes for immunotherapy, we describe the analysis of the phenotypic, functional and molecular characteristics of T lymphocytes, derived from lymph nodes draining advanced NSCLC. In this paper we show that these cells, have restriction patterns of T cell receptor β chain similar to those detectable in the population of infiltrating T lymphocytes. This finding suggests that T cells derived from draining lymph nodes of advanced NSCLC have peculiar characteristics and can be a suitable source of effector cells for protocols of adoptive immunotherapy in lung cancer treatment.

Key words Non-small-cell lung carcinoma · Tumour-infiltrating lymphocytes · T cell receptor · Restriction patterns · In vitro expansion

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Introduction

Advanced non-small-cell lung carcinoma (NSCLC) has a poor prognosis. The median survival of patients with stage IIIa or stage IIIb NSCLC treated by intensive means (chemo-radiotherapy) is 12 and 8 months respectively [2, 5]. For this reason, the evaluation of other treatment protocols, including trials that provide a non-conventional approach, is necessary. In the past decade, Rosenberg and co-workers have described the clinical efficacy of tumour-infiltrating lymphocytes (TIL) expanded in vitro, in the control of metastatic melanomas [7]. Interestingly, a significant reduction of pulmonary metastases has been achieved. We have recently described how T lymphocytes, isolated from advanced NSCLC, activated in vitro and expanded in the presence of recombinant interleukin-2 (rIL-2), have the capability of efficiently lysing autologous cancer cells [3]. In particular, Yoshino et al. [11] and we [3] have demonstrated that the population of TIL, detectable in advanced NSCLC, is oligoclonal, thus suggesting the presence of some unknown mechanism mediating the enrichment of a specific subset of T lymphocytes in the lung cancer. The presence of a population pre-selected in vivo allows the in vitro expansion of advanced NSCLC TIL for use in protocols of adoptive immunotherapy [3]. Using this approach, we recently carried out a pilot study demonstrating that adoptive immunotherapy with TIL and rIL-2 may be applied safely in more than 80% of patients with stage IIIa or IIIb NSCLC. This pilot study also suggested that adoptive immunotherapy could be effective, notably in patients with locally advanced disease [6]. The relevance of the dose(number of expanded TIL infused)/effect(response rate) ratio has been clearly demonstrated in studies of experimental adoptive immunotherapy in animal models [7]. Following this approach, large amounts of in vitro expanded TIL were inoculated during the course of clinical trials [8]. We have previously described that a number of TIL ranging from 4×10^9 to 72×10^9 can be obtained from cultures of advanced NSCLC [3]. Although these numbers seem to be suitable for the treatment of patients with

advanced NSCLC following surgery [6], these numbers are tenfold less than the previous study carried out for melanoma [8]. Therefore, in an attempt to find other sources of lymphocytes specific for advanced NSCLC cells, we analysed the phenotypic and molecular characteristics of lymphocytes detectable in draining (and/or metastatic) lymph nodes. In this paper we show that, in the majority of cases, the population of tumour-associated lymphocytes (TAL) detectable in advanced NSCLC draining lymph nodes has molecular and, to a lesser extent, functional characteristics similar to those of TIL isolated from cancer. This finding allows the use of draining lymph nodes, in patients with advanced NSCLC, to be a suitable source of cancer-cell-specific lymphocytes for further protocols of adoptive immunotherapy.

Material and methods

Tissue samples

Thirteen tissue samples were obtained using a sterile technique, from patients surgically resected for advanced NSCLC. Samples of the primitive tumour represented about one-third of the surgically resected neoplastic and perineoplastic masses. Hilar and mediastinal nodes, along the main directions of lymphatic drainage, were also collected. Sample preparation and dissection were performed by the pathologist in the operating room. All the material that was not necessary to perform a correct staging of the disease was sent to the immunology laboratory. Ten samples were obtained from men and three from women, whose median age was 56 years (ranging from 46 to 66). All samples were squamous-cell carcinomas. Eleven samples were from stage IIIb and two from stage IIIa. The median sample weight was 45 g (ranging from 18 g to 120 g). Between four and ten lymph nodes (corresponding to 50% of the resected nodes) were obtained and processed from all patients. Specimens were placed in sterile bags as described [3] and immediately processed. Draining lymph nodes were placed in other bags filled with the same transport medium. Patients enrolled in the study gave specific consent to the experimentation.

In vitro expansion of lymphocytes derived from cancer and draining lymph nodes

Following removal of necrotic and apparently normal tissues, neoplastic, perineoplastic and lymphoid tissues were dissociated as described [3]. Partially purified lymphocytes were extensively washed in RPMI-1640 medium (Celbio, Milano, Italy) and plated at a concentration of 2.5×10^6 cells/ml in RPMI-1640 medium with 10% fetal bovine serum (Technogenetics, Milano, Italy). Phytohaemagglutinin (PHA; Difco, Milano, Italy, 1% v/v) was added to half of the flasks in order to induce the activation and proliferation of any viable lymphocytes [4]. The remaining cultures were expanded in vitro without the pre-activation step (i.e. in the absence of PHA) and therefore incubated in the presence of only 10 IU rIL-2 for 7 days. These culture conditions allow the in vitro expansion of lymphocytes expressing the high-affinity IL-2 receptor (CD25), detectable on lymphocytes activated in vivo. When cells reached the concentration of 5×10^6 cells/ml, cultures were divided in the same medium and their expansion continued. Cancer cells as well as other "non-lymphoid" cells disappeared in 20–30 days and proliferating lymphocytes were then used for phenotypic, functional and molecular studies. Cells derived from lymphatic tissues were cultured using the same conditions in separated flasks.

Surface phenotype of cultured lymphocytes

The surface molecules on lymphocytes expanded in vitro were evaluated using anti-CD3, anti-CD4, anti-CD8, anti-CD16, anti-CD19, anti-CD25, anti-HLA-DR monoclonal antibodies (mAb). All reagents were directly labelled with fluorescein isothiocyanate and obtained from a commercial source (Coulter Sci, Milano, Italy). For the test, 0.05 ml lymphocyte cultures (containing 1×10^5–2×10^5 cells) was incubated with a pretitred dilution of mAb for 30 min at 4 °C, then extensively washed and analysed on an EPICS Elite flow cytometer, gated to exclude non-viable cells. The percentages of positive cells were calculated after subtraction of the negative control.

Cytolytic activities

Cytolytic activities, i.e. the capability of efficiently lysing cancer cells, were studied on different targets. Natural killer (NK) activity was evaluated on K562, a human erythroleukaemic cell line. Lymphokine-activated killer cell activity was evaluated on the NK-resistant Daudi cell line. Finally, in a selected number of cases, "specific" lysis was evaluated on autologous and allogeneic cells from primary cultures of NSCLC. Cells were labelled with $Na_2{}^{51}CrO_4$ (NEN, Milano, Italy, 100 µl/10^6 cells) for 1 h at 37 °C, then extensively washed as described [3]. Different effector/target ratios (ranging from 50:1 to 1.5:1) were incubated for 4 h at 37 °C, and specific lysis was calculated as described [3].

Analysis of restriction maps of T cell receptor (TCR) β chain

Rearrangements of the TCR β chain were evaluated on TIL activated in vivo (expanded in the presence of rIL-2 alone) as well as on PHA-activated, rIL-2-expanded TIL. The same procedure was applied to lymphocytes derived from draining lymph nodes. Finally, for each patient, a sample of peripheral blood lymphocytes, collected during the operation, was used as a control. DNA samples were extracted from 10×10^6 lymphocytes, using phenol as described [9], then digested with the restriction endonucleases EcoRI and HindIII (Biolabs, Milano, Italy). Digested DNA was run into a 0.6% agarose gel, then transferred to nylon filters (Genescreen plus, NEN) and hybridised with a TCR-β-chain-constant-region probe [10], labelled with ^{32}P. Hybridization was carried out in the presence of 10% dextran sulphate, 0.75% NaCl, 0.5% sodium dodecyl sulphate in 0.25 mM phosphate buffer at 65 °C, for 10 min, then overnight at 42 °C. After extensive washings at high stringency, autoradiography was performed. The presence of germ-line bands was considered to derive from a polyclonal population of T lymphocytes. On the other hand, detectable rearranged bands, in positions different from those of the germ line, were considered indicative of an oligoclonal population of lymphocytes [1]. Finally, the deletion of a single germ-line band was considered to be derived from a virtually monoclonal population of T cells.

Results

Proliferative capabilities

Samples collected from 13 patients were used for this study. In all patients, both the primitive tumour and draining lymph nodes were collected, dissociated and cultured in vitro. Table 1 summarises the results obtained. As shown in samples from 6 patients (BE, CO, ME, MI, RI and TR), the expansion of TIL and TAL, cultured both in the presence and in the absence of PHA, was obtained. In a single patient (ME) PHA-cultured TIL resulted in a very small number of lymphocytes, thus indicating the presence of cells with a damaged proliferative potential within the tumour mass.
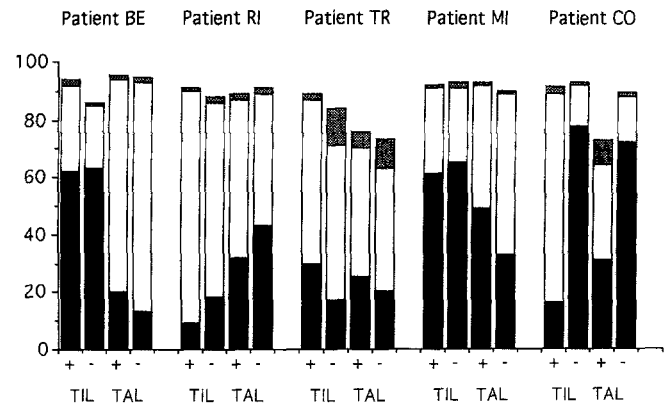
Table 1 Proliferative capabilities of tumour-infiltrating lymphocytes (TIL) and tumour-associated lymphocytes (TAL). Proliferative capabilities are shown as numbers of lymphocytes. TAL were lymphocytes derived from draining lymph node(s). +PHA culture activated in vitro in the presence of phytohaemagglutinin, then expanded using recombinant interleukin-2 (rIL-2), −PHA culture expanded in the presence of rIL-2 alone, NE culture not expanded in vitro

	$10^{-9} \times$ Proliferative capability (cells/ml)			
Patient	TIL + PHA	TIL − PHA	TAL + PHA	TAL − PHA
AN	10	NE	44	NE
BE	4	4	6	2
BU	8	NE	15	4
CA	3	NE	19	2
CO	10	5	10	1
MA	11	12	10	10
ME	0.1	0.01	13	2
ME	20	NE	NE	NE
MI	20	1	5	0.4
RI	0.2	0.1	16	2
TR	3	3	0.2	0.12
VI	16	NE	0.5	2
ZU	36	4	NE	NE

Fig. 1 Phenotypic analyses of five representative in vitro expanded cultures. TIL tumour-infiltrating lymphocytes, TAL lymphocytes expanded from draining lymph node(s), + cultured in the presence of phytohaemagglutinin (PHA), − cultured without PHA (see text). Black rectangles CD4+ "helper" cells, white rectangles CD8+ cytolytic cells, grey rectangles CD16+ (NK) cells

Finally, in the last 6 patients, PHA-activated TIL were efficiently expanded, whereas lymphocytes, derived from draining lymph nodes, grew heterogeneously.

Phenotypic analysis

The phenotypic analysis showed that virtually all cells expanded in vitro expressed the CD3 surface molecule and thus belonged to the mature T lymphocyte lineage. B and NK cells were absent. Within these CD3+ lymphocytes,

a heterogeneous pattern of phenotypes was detected. These included the same percentage of CD4 and CD8 cells in both TIL and TAL, or different percentages of CD4 and CD8 cells in the two different samples, as well as different subsets of cells in PHA-activated, rIL-2-expanded and solely rIL-2-expanded populations. Five representative cultures are shown in Fig. 1. Interestingly, "activation" antigens, such as CD25 and HLA-DR cells, were detected in high percentages (ranging from 38% to 75% and 59% to 96% respectively) both in PHA-activated, rIL-2-expanded and in solely rIL-2-activated cells.

Fig. 2 Cytolytic properties of TIL and TAL derived from patient BE, cultured in the presence (+ PHA) and absence (−PHA) of PHA. Vertical axes the percentage of lysis calculated according to [3]. Horizontal axes the different effector/target ratios used in the experiments. ■ Cytolytic activity against Daudi cells, □ cytolytic activity against K562 cells
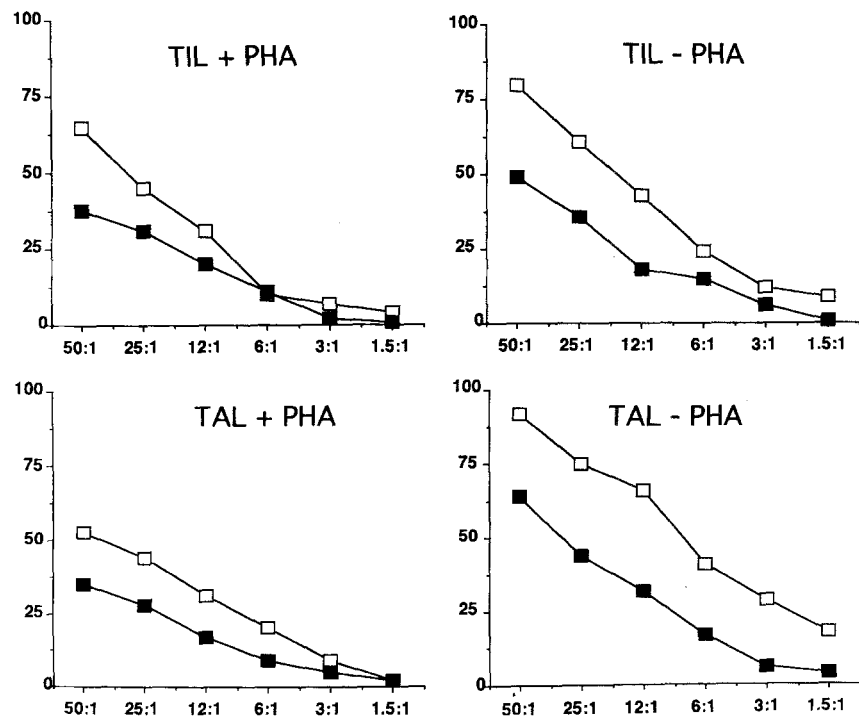
Table 2 Result of the analysis of T cell receptor restriction patterns. *NE* not expanded in vitro, *Oligo* oligoclonal population of T lymphocytes, *Poly* polyclonal population of T lymphocytes, *NT* not tested, *PBL* peripheral blood lymphocytes see also Table 1

Patient	TIL + PHA	TIL – PHA	TAL + PHA	TAL – PHA	PBL
AN	NT	NE	Oligo	NE	Poly
BE	Oligo	Oligo	Oligo	Oligo	Poly
BU	Oligo	NE	Oligo	Oligo	Poly
CA	Oligo	NE	NE	Oligo	Poly
CO	Oligo	Oligo	Poly	Poly	Poly
MA	Oligo	Oligo	Oligo	NE	Poly
MO	Oligo	Oligo	Oligo	Oligo	Poly
MN	Oligo	NE	NE	NE	Poly
MI	Poly	Poly	Poly	Poly	Poly
RI	Oligo	Oligo	Poly	Poly	Poly
TR	Oligo	Oligo	Oligo	Oligo	Poly
VI	Oligo	NE	NT	Poly	Poly
ZU	Oligo	Oligo	NE	NE	Poly

Cytolytic capabilities

An analysis of the cytolytic capability of TIL and TAL against autologous cancer cells was performed in two different samples (cultures of autologous cancer cells were obtained only in these two samples: RI and MI), whereas cytotoxic activity against established NK and LAK targets was determined in the majority of samples (9 out of 13). Analysis of cytolytic properties of lymphocytes expanded from the tumour site and from draining lymph nodes demonstrated that a strong "non-specific" cytolytic activity was detectable against both K562 and Daudi cells (Fig. 2). Cytolytic activity against autologous cancer cells was detectable in one out the two samples assayed.

T cell receptor β chain restriction patterns

The analysis of the constant (C) region of the TCR β chain was performed in all expanded cultures (Table 2). In 6 patients, the proliferation of TIL and TAL, cultured both in the presence and in the absence of PHA, was achieved. In these patients, different combinations of restriction maps (oligoclonal band lengths are shown in Table 3) were observed. In another 2 patients, only TIL grew and were oligoclonal, whereas TAL were unable to proliferate. In 1 patient, both TIL and TAL, expanded with or without PHA, became polyclonal, as demonstrated by the presence of germ-line-like patterns in all samples. By contrast, in 11 patients, TIL were oligoclonal. Cells of 7 of these were expanded in vitro, using PHA and rIL-2 or rIL-2 alone resulting in the same restriction patterns (Table 3). Of the

Table 3 Patterns of T cell receptor rearrangements

H oligoclonal population detectable in samples digested with the *Hind*III restriction enzyme, *E* oligoclonal population detectable in samples digested with the *Eco*RI restriction enzyme. Numbers indicate the length in kb of oligoclonal fragments in restriction maps. See also Table 1

Patient	TIL + PHA	TIL – PHA	TAL + PHA	TAL – PHA
ME	H (21, 11.5, 7.5, 6.5, 3.2)	H (21, 11.5, 7.5, 6.5, 3.2)	Polyclonal	H (21, 11.5, 7.5, 6.5, 3.2)
RI	H (21, 11.5, 7.5, 6.5, 3.2)	H (21, 11.5, 7.5, 6.5, 3.2)	Polyclonal	Polyclonal
MI	E (11.5, 9.5, 4)	E (11.5, 9.5, 4)	E (11.5, 9.5, 4)	E (11.5, 9.5, 4)
CO	H (21, 11.5, 7.5, 6.5, 3.2) E (11.5, 9.5, 4)	H (21, 11.5, 7.5, 6.5, 3.2, 1.8) E (11.5, 4)	H (21, 11.5, 7.5, 6.5, 3.2) E (11.5, 9.5, 7, 4)	H (21, 11.5, 7.5, 6.5, 3.2, 1.8) E (11.5, 9.5, 7, 4)
TR	H (21, 11.5, 7.5, 6.5, 3.2) E (11.5, 9.5, 4)	H (21, 11.5, 7.5, 6.5, 3.2) E (11.5, 9.5, 4)	H (21, 11.5, 7.5, 6.5, 3.2) E (11.5, 9.5, 4)	H (21, 11.5, 7.5, 6.5, 3.2) E (11.5, 9.5, 4)
BE	H (11.5, 10, 7.5, 6.5, 3.2)	H (11.5, 7.5, 6.5, 3.2)	Polyclonal	Polyclonal

Fig. 3 Restriction maps of TIL and TAL. *Left panel* restriction patterns after *Hind*III digestion. *Right panel* restriction patterns after *Eco*RI digestion. *Lanes 1–5* cells from patient BE: *1* PBL, *2* TIL+PHA, *3* TIL–PHA, *4* TAL+PHA, *5* TAL–PHA. *Lanes 6–10* cells from patient TR: *6* PBL, *7* TIL+PHA, *8* TIL–PHA, *9* TAL+PHA, *10* TAL–PHA. *Lane 11* germ line. *+PHA* cultures in the presence of PHA, *–PHA* cultures in the absence of PHA. *Arrows* germ-line restriction fragments (kb)
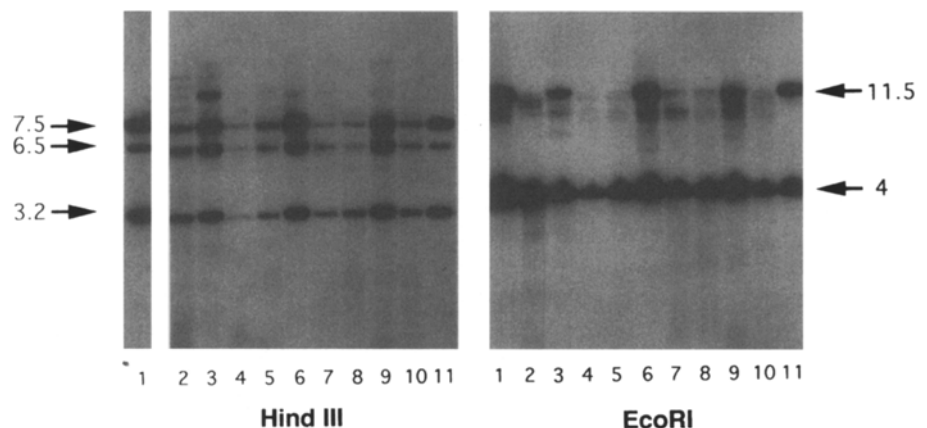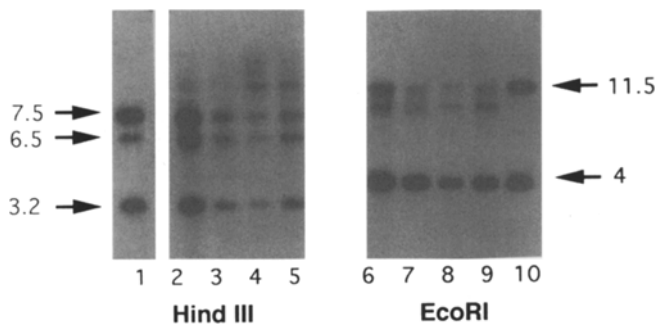
Fig. 4 Restriction maps of TIL and TAL from patient ML. *Left panel* restriction patterns after *Hind*III digestion. *Right panel* restriction patterns after *Eco*RI digestion. *Lanes 1, 10* germ line; *lanes 2, 3, 6, 7* derived from TAL; *lanes 4, 5, 8, 9* derived from TIL; *lanes 3, 5, 7, 9* cultures in the presence of PHA; *lanes 2, 4, 6, 8* cultures in the absence of PHA. *Arrows* germ-line restriction fragments (kb)

TAL samples, 4 were polyclonal, 2 were unable to grow (ME and ZU) and 7 were oligoclonal. Figures 3 and 4 show the restriction maps of three representative samples. From a genetic point of view, the virtually constant presence of the 4-kb band, associated with barely detectable or rearranged 11.5-kb bands, in *Hind*III-digested DNA, demonstrated that the Cβ1 region of TCR is more frequently rearranged than the Cβ2 region.

Discussion

TIL, obtained from advanced NSCLC and expanded in vitro, were compared with TAL derived from draining lymph nodes. The analysis of phenotypic characteristics of the two populations showed that, within certain limits, a clear heterogeneity of the two compartments was detectable. For example, even if present at the beginning of the culture in lymph nodes (not shown), B lymphocytes and NK cells completely disappeared during long-term cultures. For this, the population of in vitro expanded lymphocytes was represented by virtually pure T cells. Within this subset, a variable proportion of both CD4+ and CD8+ was detectable. Interestingly, similar percentages of these two subsets could be observed in TIL cultured in the presence and in the absence of PHA. Furthermore, similar percentages were detectable in TIL and TAL cultured in the same conditions. Whatever the case, no precise rule could predict the distribution of T cell subsets in the cancer and in draining lymph nodes. From a functional point of view, the difficulty of culturing NSCLC cells allowed the evaluation of cytolytic capability against autologous cancer cells in only two cases. In one, this capability was present, whereas in the other it was absent. On the other hand, almost all expanded samples had a strong cytolytic "non-specific" activity directed against the K562 and Daudi cell targets. Even if this property cannot have a central role in the control of cancer progression, some recent data, obtained in other neoplasms, such as melanoma, clearly indicated that "non-specific" effectors may be efficient surrogates compensating for the absence of "specific" cytolytic cells. Along this line, we have described that in TIL, derived from NSCLC, specific activity is always strictly linked to non-specific activity [3].

Although phenotypic and functional analyses did not result in clear differences between TIL and TAL, more significant results were obtained by the analysis of TCR restriction patterns. It is well known that peripheral blood lymphocytes (PBL), in patients without haematological malignancies, are polyclonal: this is due to the presence of virtually all TCR structures within the population of T cells. The presence of a polyclonal population is indicated by the evidence of germ-line TCR rearrangement patterns, as well as by the presence of a smeared pattern of rearrangement. These two situations are caused by the presence of both unrearranged alleles and a very large number of restriction bands in a polyclonal population. By contrast, the evidence of a single band in a position different from those of the germ-line, indicates that the same restriction fragment is present in a significant number of T cells within a given population. According to this, a barely detectable restriction band represents at least 10%–20% of the total amount of lymphocytes in the culture, thus indicating the presence of a selected population with a given TCR within the expanded culture.

Data obtained from our experiments confirm that virtually all T cell cultures, derived from TIL, were oligoclonal. In addition, 7 out of 11 proliferating samples, collected from draining lymph nodes, were oligoclonal, whereas 4 were polyclonal. Finally, all PBL samples were polyclonal. This finding indicated that, independently from the phenotypic and functional characteristics, most lymphocytes, derived from draining lymph nodes were a selected population of effector cells. More interestingly, restriction patterns observed in TIL, in cultures performed both in the presence of PHA and without PHA, were similar to those observed in TAL (not shown), thus indicating that similar recombinations of TCR were achieved in the two situations. The presence of the same TCR pattern suggested that a similar antigen could be recognized by lymphocytes derived from the two sources. Even if the presence of a putative antigen(s), specific for lung cancer cells, is only suspected in humans, the evidence of similar restriction patterns indicates that this antigen may be present. However, the nature of this unknown antigen is under evaluation; Yoshino et al. suggested a possible role for heat-shock proteins, whereas other data, including ours, indicate the presence of a tumour-associated antigen strictly related to NSCLC. Further studies will be necessary to define the specificity of these TCR. Nevertheless, the clear evidence that T lymphocytes derived from draining lymph nodes of advanced NSCLC have restriction patterns comparable to those of TIL indicates that these populations could be used as a source of effector lymphocytes in protocols of adoptive immunotherapy of lung cancer.

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