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Isolation and *In vitro* Expansion of Lymphocytes Infiltrating Non-small Cell Lung Carcinoma: Functional and Molecular Characterisation for Their Use in Adoptive Immunotherapy

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Tumour infiltrating lymphocytes (TIL) have the capability of recognising and lysing autologous cancer cells, both *in vitro* and *in vivo*. Advanced non-small cell lung carcinoma (NSCLC) is partially insensitive to chemo radiotherapy and has a poor prognosis: thus, for this, an immunotherapeutic approach could be attempted. We expanded *in vitro* 46 out of 70 samples of TIL derived from NSCLC. From proliferating TILS, a number varying from 10 to 50 × 10^9 cells was obtained. These lymphocytes belonged to the T cell lineage, had the capability of growing for 45–60 days and lysed autologous better than allogeneic cancer cells. In addition, analysis of the restriction maps of T cell receptor (TRC)-β, demonstrated that an oligoclonal population of T cells was preselected *in vivo*, near the tumour site, and might be expanded *in vivo*, using phytohaemagglutin and interleukin 2 while maintaining the same characteristics of the original population. These results give a clear rationale for the use of *in vitro* expanded TIL from NSCLC in protocols of adoptive immunotherapy in patients with residual disease following surgery.
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INTRODUCTION

IT HAS been shown recently that tumour infiltrating lymphocytes (TIL) are enriched in T lymphocytes capable of recognising the tumour-associated antigen(s) expressed on the surface of autologous cancer cells [1]. In addition, it has also been shown that a cytolytic subset of TIL efficiently lyses autologous neoplastic cells upon *in vitro* expansion in the presence of recombinant interleukin-2 (rIL-2) (Proleukin, Eurocetus, Amsterdam, The Netherlands) [2]. More recently, studies in animal models [3] and in humans have shown that tumour regression may be achieved *in vivo* following intravenous (i.v.) infusion of large numbers of *in vitro* expanded TIL. In particular, tumour regression has been observed in patients with advanced melanoma [4] and ovarian carcinoma [5]. This therapeutic approach has been termed "adoptive immunotherapy of cancer".

Non-small cell lung carcinoma (NSCLC) is well known to be partially insensitive to chemotherapy [6]. Following ablative surgery, despite adjuvant treatment with chemo radiotherapies, patients with NSCLC belonging to stage IIIb have a short (2 years in 20% of patients) expected survival period [7]. For these reasons, patients with advanced NSCLC could be candidates for adoptive immunotherapeutic protocols, using autologous TIL derived from surgical samples. Along this line, some authors have demonstrated no specific killing of tumour cells mediated by freshly isolated, as well as by rIL-2-treated TIL [8–11]. Nevertheless, it has been shown recently that TIL isolated from NSCLC contain an oligoclonal population of lymphocytes, suggesting *in vivo* selection of cells with distinct characteristics, and confirming the potential specificity of infiltrating cells [12]. Furthermore, the actual proliferative capabilities of TIL from NSCLC still remain partially uninvestigated. Using a minor modification of the technique described by Rosenberg *et al.* [2], we were able to obtain large numbers of functionally active TILs from surgical specimens of NSCLC. In this study, we have analysed the specificities and the proliferative capabilities of NSCLC infiltrating lymphocytes, the rate of *in vitro* proliferation, the index of expansion and their phenotypic, functional and molecular characteristics, in the light of possible uses in adoptive immunotherapy protocols.

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MATERIALS AND METHODS

Tissue samples

Seventy tissue samples were obtained by sterile techniques from NSCLC. The samples represented more than 50% of the surgically-resected neoplastic and perineoplastic masses. Sixty samples were obtained from males and 10 from females. The median age was 61 years (range 43–73). Forty-seven samples were squamous cell carcinomas and 23 were adenocarcinomas. Two samples were obtained from patients at stage 1, seven from stage II, 20 from stage IIIa, 37 from stage IIIb and four from stage IV. Median sample weight was 21 g (range 5–240 g). Specimens were placed in sterile bags partially filled with RPMI 1640 containing antibiotics (penicillin and gentamycin) and amphotericic-B, and immediately processed.

Single cell suspension

Following removal of necrotic and apparently normal tissues, neoplastic and perineoplastic tissues were dissociated mechanically, then incubated in RPMI 1640 medium containing DNAse (Sigma, St. Louis, Missouri, U.S.A. 0.002%, 100 l/mg), collagenase type IV (Sigma, final dilution 200 l/ml), hyaluronidase (Sigma, 1500 U/ml) and gentamicin (16 mg/100 ml), in a magnetic stirrer for 4 h at 37°C. Isolated cells were collected, filtered through a coarse wire grid in order to exclude undigested fragments, washed and then immediately cultured. In some cases, erythrocytes, debris and dead cells were eliminated using a Ficoll-Hypaque discontinuous gradient as described previously [2]. The cells found at the gradient interface (such as lymphocytes, cancer cells and alveolar macrophages) were extensively washed in RPMI 1640 with 10 × antibiotics concentrations, and plated at 5×10^5 cells/ml in complete medium (CM). CM contained RPMI 1640 with 10% human AB serum (or autologous serum when collected), and 500 U/ml of rIL-2. Phytohaemagglutinin PHA (Difco, Detroit, Michigan, U.S.A., 1% v/v) was added to half of the flasks in order to induce activation and proliferation of any viable TIL [13]. Cultures were then incubated in 175-cm^2 plastic flasks (Falcon, Becton Dickinson, Milan, Italy) for 3–5 days in 5% CO_2 at 37°C.

In vitro *expansion and mass culture*

In vitro cultured cells were washed extensively and living mononuclear cells were isolated from any remaining contaminants (such as red blood cells, granulocytes, cancer cells, dead cells and debris) using a discontinuous gradient of Ficoll-Hypaque. Mononuclear cells, which included both lymphocytes and cancer cells in different proportions, were then washed twice and incubated at $1–2 \times 10^6$ cells/ml in RPMI 1640 containing glutamine, gentamicin, 10% human AB (or autologous) serum in plastic flasks in the presence of 500 U/ml of rIL-2. When cell concentrations reached $3–4 \times 10^6$ cells/ml, cultures were divided 1:2 in the same medium and their expansion was continued. In "positive" cultures, cancer cells as well as other "non-lymphoid" cells disappeared in 2–3 weeks and a pure population of proliferating lymphocytes was obtained.

Culture of cancer cells

Single cancer cell suspensions were obtained by mechanical and enzymatic dissociation of neoplastic tissues derived from 36 samples of NSCLC (29 squamous cell carcinomas and 7 adenocarcinomas). Briefly, mechanically dissociated tissue fragments were incubated in the presence of collagenase (500 U/ml), hyaluronidase (30 U/ml) and DNAse (10 U/ml), for 4 h at 37°C, then filtered through polyester screens. Single-cell suspensions were seeded on a feeder layer of lethally irradiated 3T3J2 mouse fibroblasts, and cultured in Dulbecco's modified Eagle's and Ham's media (2:1 mixture) as described previously [14]. After 2 days of culture, epidermal cell growth factor (EGF, 10 ng/ml) was added. Cancer cell colonies were scored visually on the feeder layer, and the presence of neoplastic cells was assured by cytological controls and/or immunofluorescent assay, using an anti-cytokeratin monoclonal antibody (CAM 5.2, Becton Dickinson). Neoplastic cells were frozen in liquid nitrogen for further studies and/or labelled with ^{51}Cr for cytotoxicity tests.

Surface phenotype of cultured lymphocytes

Indirect immunofluorescence and flow cytometry were employed for immunophenotyping the cultures. The following monoclonal antibodies (MAbs) were used: CD3 (Leu-4, Becton Dickinson), CD4, CD8, CD19, CD25, HLA-DR (T4, T8 and B4, Coulter Science, Hialeah, Florida, U.S.A.), CD16 (KD1, produced in our laboratory [15]) and fluorescein isothiocyanate (FITC)-labelled anti-human IgG (Southern Biol. Ass, Birmingham, Alabama, U.S.A.). Briefly, 0.05 ml of lymphocyte cultures were incubated with a pretitred dilution of MAbs for 30 min at 4°C, then washed and incubated with 0.05 ml of FITC-labelled anti-mouse IgG (Southern Biol. Ass.) for 30 min at 4°C. Cells were then extensively washed, analysed on an EPICS Elite flow cytometer and the percentages of positive cells were calculated.

Cytolytic activities

Cytolytic activities were studied on different targets. Natural killer (NK) activity was evaluated on K562, a human erythroleukaemic cell line. Lymphokine-activated killer (LAK) cell activity was evaluated on the NK-resistant Daudi cell line. Finally, "specific" lysis was evaluated on autologous and allogeneic cells from primary cultures of NSCLC. Cells were labelled with $[^{51}Cr]Na_2O_4$ (100 μl/10^6 cells) as described previously [15]. Different effector/target dilutions (range 50:1 – 1.5:1) were incubated for 4 h at 37°C, and specific lysis was calculated as described previously [15]. The specificity of the lysis of autologous cancer cells was evalutated by adding both a 1:20 000 dilution of anti-CD3 (OKT3, Orthoclone, Ortho, Milan, Italy) and cold target cells, i.e. different numbers (range 625–5000) of unlabelled autologous and allogeneic cancer cells to the cytolytic assay as described previously [16].

Analysis of restriction maps of T cell receptors (TCR)

TCR rearrangements were evaluated on *in vivo* activated TIL (expanded in the presence of rIL-2 alone) as well as on PHA activated, rIL-2 expanded TIL. DNA samples were extracted from lymphocytes derived from bulk cultures as described previously [12], then digested with the restriction endonucleases *Eco*RI, *Bam*HI and *Hind*III (Biolabs, Milan, Italy). Digested DNA was run on a 0.6% agarose gel, then transferred to nylon filters (Genescreen plus, NEN), as described previously [17] and hybridised with a TCR-β chain constant region probe [18], labelled with ^{32}P [17]. Hybridisation was carried out in the presence of 10% dextran sulphate, 0.75 mol/l NaCl, 0.5% sodium dodecyl sulphate (SDS), 50% deionised formamide, 1x Denhart solution and 0.2 mg/ml salmon sperm carrier DNA in 0.25 mmol/l phosphate buffer at 65°C for 10 min, then overnight at 42°C. After extensive washings at high stringency (0.1x SSC), autoradiography was performed.

RESULTS

The total cell yield from the 70 different NSCLC samples ranged between 40 and 3200 million cells. May–Grunwald–Giemsa and Papanicolaou staining showed large proportions of cancer cells in virtually all analysed samples. The percentages of lymphocytes ranged from 0.01 to 3% of total cells.

Cultures of cancer cells

Twenty-four out of 36 attempted primary cultures of NSCLC cancer cells were obtained. Of these, 19 were derived from squamous cell carcinomas and 5 from adenocarcinomas. Cytological and immunofluorescence (anti-cytokeratin CAM 5.2 monoclonal antibody) tests, performed on growing cultures, showed that a variable (but in general > 80%) proportion were neoplastic epithelial cells. Cells were cultured for 2 weeks and then used for cytotoxicity tests or stored frozen for further studies.

Bulk cultures of lymphocytes

Large numbers of activated lymphocytes, expanded from TIL of NSCLC, were obtained in bulk cultures in the presence of rIL-2. Forty-six cultures expanded well *in vitro*, and the ratio of lymphocytes in the original samples to lymphocytes obtained ranged from 50- to 2000-fold. In particular, PHA-pretreated cultures were expanded by 260- to 2000-fold, whereas the PHA-untreated ones expanded only by 50- to 440-fold. These proliferative capabilities were in no way related to sex, age or histological type. In addition, the earlier stages (I and II) of NSCLC were infiltrated by a greater number of lymphocytes/g of tissue, than that achieved in advanced stages (III and IV). However, the larger the sample, the larger the number of proliferating lymphocytes gained. Only 24 out of 70 TIL cultures were unsuccessful: in particular, four cultures derived from heavily infected samples were contaminated by fungi after 2 days of cultures. Three other cultures were contaminated in the laboratory. Seven cultures (with positive cytological examination for lymphocytes) were negative during the first period of the study, probably due to inapproprate culture technique. Ten cultures were negative (i.e. no lymphocytes were expanded *in vitro*): in these cases, cytological examination of the original cell population showed that TILs were virtually absent in the cultured specimen. Finally, nine cultures proliferated *in vitro*, but the number of expanded TILs was $< 1 \times 10^9$.

Surface phenotype of in vitro *expanded TIL*

Immunophenotyping was carried out on all *in vitro* expanded samples: virtually all TIL undergoing *in vitro* expansion belonged to the CD3+ mature T cell lineage, whereas variable proportions of CD4+ (ranging between 18 and 88%) and CD8+ (ranging between 14 and 82%) were observed (Fig. 1). Interestingly, as expected, cultures obtained from PHA pretreated lymphocytes differed from untreated samples (Fig. 2). Finally, B and NK cells were absent in virtually all expanded TIL cultures.

Cytolytic activities

The large majority of TIL from NSCLC, expanded *in vitro* in the presence of rIL-2, had potent cytolytic activity against the NK-sensitive K562 cell line (Fig. 3). It is also worth noting that the highest NK activities were obtained from PHA-untreated cultures. A clear cytolytic activity against the NK-resistant Daudi cell line target was oberved in PHA-untreated lymphocytes, whereas PHA-pretreated cultures showed a lower LAK activity (Fig. 3). Cytolytic activity against autologous cancer cells (AUCC) was evaluated in a selected number of samples: 18/24 cultures had a significant cytolytic activity against AUCC and, in general, a lower cytolytic activity against allogeneic cancer cells was observed (Fig. 3). Interestingly, with AUCC no differences between PHA-preactivated and PHA-untreated cells were observed, thus showing that the cytolytic potential against AUCC is not restricted to the CD25+ *in vivo* activated TIL. When the anti-CD3 and cold autologous and allogeneic target cells were added to the cytolytic assay, a clear reduction of percentage of ^{51}Cr release was observed, except when allogeneic cells were used (data not shown). In particular, anti-CD3 completely abrogated the lysis of autologous cells, whereas it only in part (less than 10% of reduction) and inconsistantly inhibited the lysis of allogenic targets. The addition of cold autologous cancer cells resulted in a clear reduction of ^{51}Cr release (range from 8 to 55% at different numbers of cold targets) in cytolytic tests, whereas allogeneic cancer cells did not inhibit

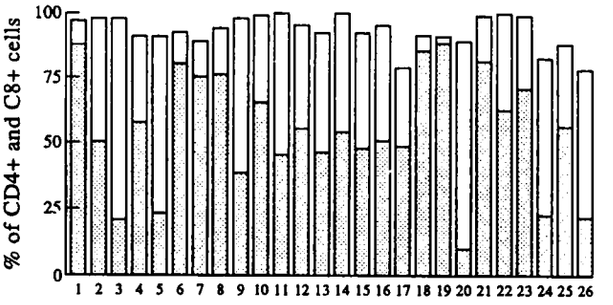

Fig. 1. Phenotypic analysis of 26 representative cultures of TIL derived from NSCLC. Immunophenotyping was carried out 3–4 weeks after the onset of the culture in PHA-activated NSCLC TILs. Virtually all expanded cells belonged to the T cell lineage, as shown by the sum of CD4+ and CD8+ lymphocytes (grey and white rectangles, respectively).

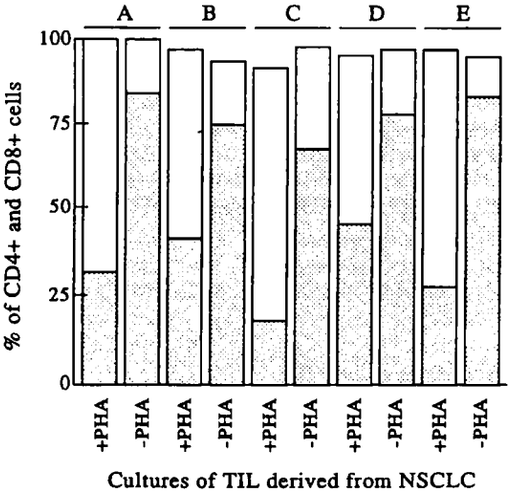

Fig. 2. Phenotypic analysis of five representative cultures of TIL (A, B, C, D, E), expanded from NSCLC both in the presence (+PHA) and in the absence (-PHA) of a polyclonal T cell activator. A clear expansion of the CD4+ population (grey) was observed in PHA-untreated samples, whereas the presence of PHA resulted in the expansion of the cytolytic CD8+ subset (white).

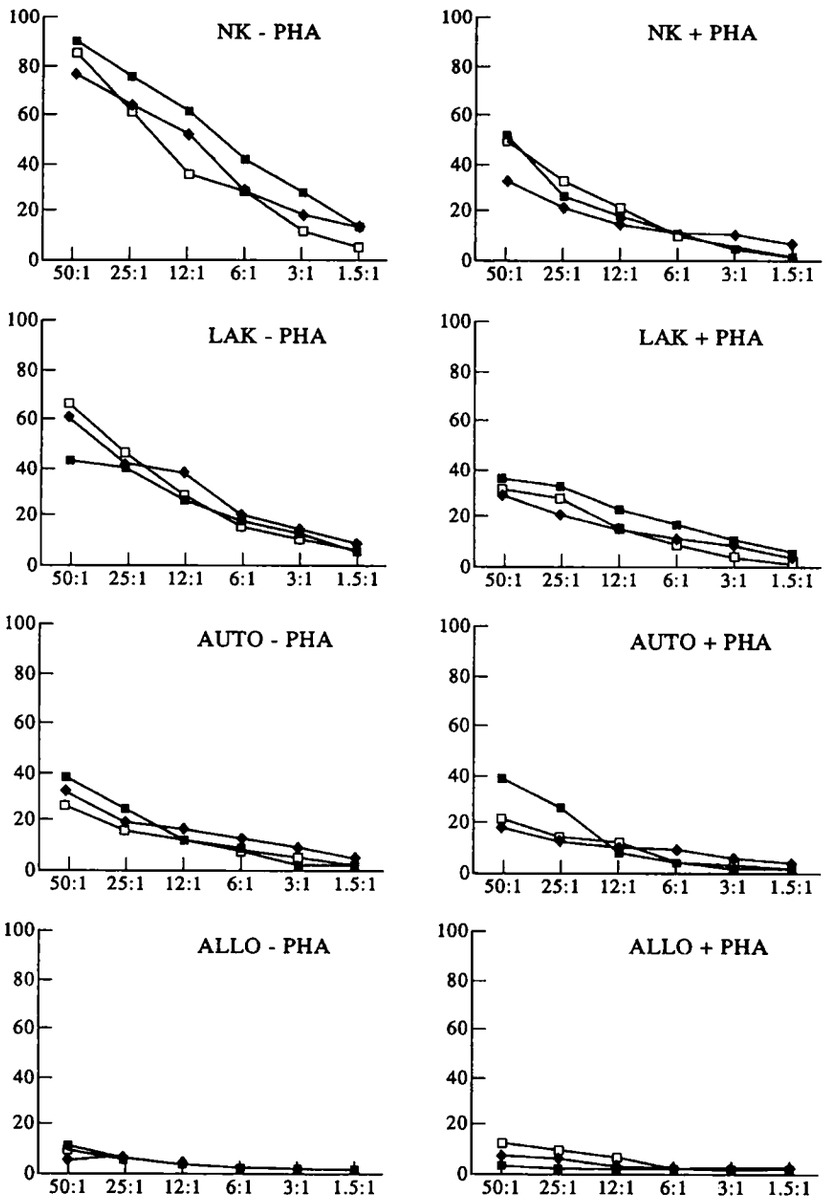

Fig. 3. Cytolytic activities of three representative cultures of TIL derived from NSCLC both in the presence (+PHA) and in the absence (-PHA) of phytohaemagglutinin. NK, natural killer activity against the K562 cell line. LAK, lymphokine-activated killer cell activity, evaluated on the NK-resistant Daudi cell line. AUTO, cytolytic activity against autologous cultured cancer cells. ALLO, cytolytic activity against allogeneic cultured cancer cells. 50:1, 25:1, 12:1, 6:1, 3:1 and 1.5:1 are the different effector/target ratios used in the assays. On the vertical scale, the percentage of lysis is shown.

the lysis of autologous nor allogeneic-labelled targets (data not shown).

TCR β restriction patterns

Analysis of the TCRβ restriction was performed in eight samples to evaluate the different clonality (monoclonality, oligoclonality and polyclonality) of *in vitro* expanded populations. As shown in Fig. 4, the absence of significant differences between cultures pretreated with PHA, in comparison with untreated cultures, indicates that an *in vivo* preselected TIL population was present. In particular, the restriction patterns of cells derived from different culture conditions, indicate that an oligoclonal population of lymphocytes was present in the original samples, and that this oligoclonality was maintained in long-term cultures. Figure 4 also shows that patient A, analysed using the *Eco*RI restriction enzyme, has only a 4-Kb band in the blot, and that the 11.5-Kb band is lost: the complete absence of this region strongly suggests that a monoclonal or, perhaps, an oligoclonal population of TIL was detected. Similar patterns can be observed in restriction maps obtained using other restriction enzymes, such as *Hind* III and *Bam*HI. Patient A (lanes 2 and 3) has similar oligoclonal bands (indicated by the arrows) in both gels. In contrast, patient C has partially smeared germ-line bands, thus suggesting that a polyclonal population was present in the sample: however, lane 5 in *Eco*RI-treated samples, shows clearly a small oligoclonal band (4 Kb), thus suggesting that in

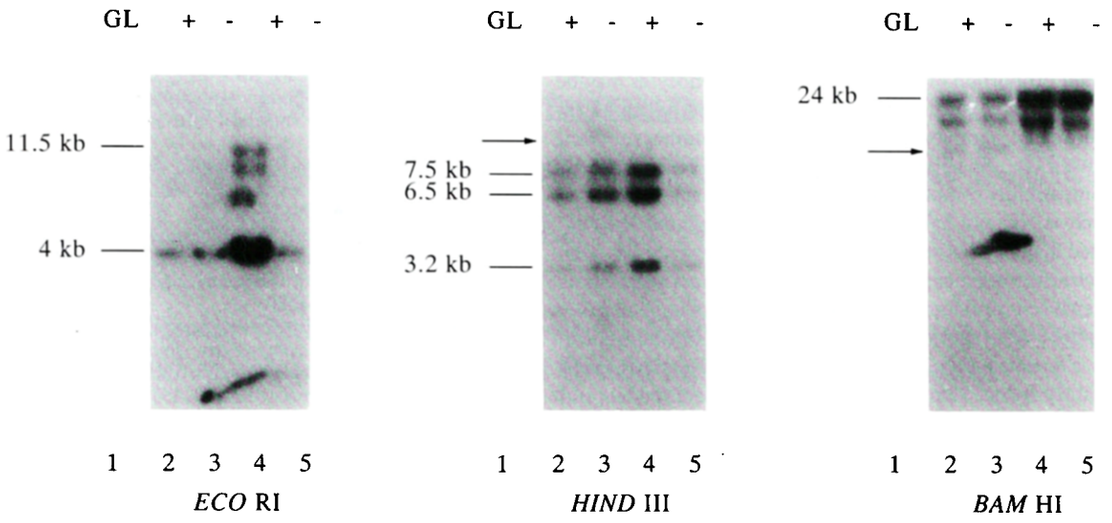

Fig. 4. Restriction map for TCRβ constant region. Lane 1, (GL) indicates the position and the size of germline restriction fragment(s). Two representative samples (lanes 2 and 3, patient A, lanes 4 and 5, patient C), derived from PHA-treated (lanes 2 and 4, indicated as +) and PHA-untreated (lanes 3 and 5, indicated as −) cultures, were hybridised with a C region-specific probe. No differences between the two culture conditions may be observed within the same patient. Cβ2 region is more frequently used, as indicated by the presence of a 4-Kb band in *Eco*RI-digested DNA. GL, position of the germ line (human B lymphoblastoid Raji cell line) bands.

this sample, only *in vivo* activated lymphocytes (obtained by expansion of TIL in the presence of rIL-2 alone) were oligo- or monoclonal. In addition, the presence of small fragments of hybridised DNA suggests that the Cβ2 region of the TCR sequence was predominantly used. Interestingly, patient F (Fig. 5, lanes 2 and 3), for which no lymphocytes were obtained at the end of the culture, in the absence of PHA (indicated as "-" at the top, lane 3), had two different patterns of restriction: germ-line bands in the absence of PHA and two bands indicating the use of the Cβ2 region of the TCR in the culture expanded in the presence of PHA. The cytological analysis of cells in the lymphocyte-negative sample showed that only normal and neoplastic non-lymphoid cells were present, thus explaining the presence of germ-line bands in the blot. This indicates that an oligoclonal population of lymphoid cells (as shown in lane 2) is sometimes present in the original population, but does not express the high affinity IL-2 receptor. This also implies that the treatment with rIL-2 alone (in the absence of PHA) cannot always induce the expansion of an *in vivo* preselected population. Also in Fig. 5, lanes 4 and 5 (patient B) clearly show another sample where an oligoclonal population (as indicated by the very thin 11.5-Kb bands in both culture conditions) was present and expanded using both rIL-2 alone and the association of PHA and rIL-2. This finding demonstrates that the activated population (IL-2 receptor-positive TIL) is only an *in vivo* activated subset of the whole infiltrating population but has the same molecular characteristics, thus suggesting that there was only one population, part at rest and partly activated *in vivo*. As a control, it is of note that cultures obtained from both PHA-treated and PHA-untreated peripheral blood lymphocytes, derived from the same patients, were characterised by the absence of oligoclonal bands: i.e. germ-line bands as well as smears of DNA (clear sign of a polyclonality) were always observed (data not shown).

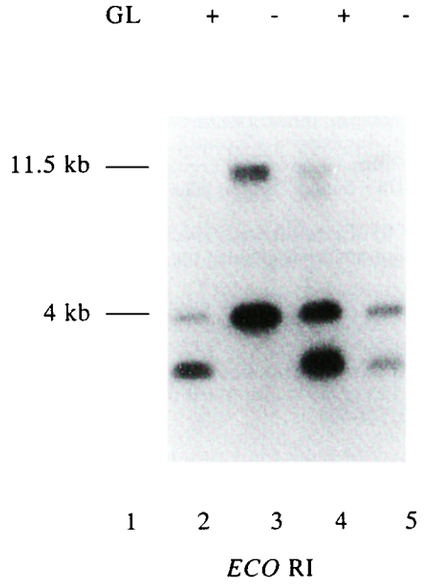

Fig. 5. Restriction map for the C region of the TCRβ in two DNA samples (digested with *Eco*RI) or TIL derived from NSCLC. Lane 1 indicates the position and the size of germ line bands. Patient F, lane 2 derived from TIL expanded in the presence of PHA; lane 3, restriction patterns of non-rearranged cancer cells. In this culture, only germ line bands are present because no proliferation of lymphocytes was observed in the culture in the absence of PHA. Unrearranged germ line bands were detected in the DNA extracted from cancer cells not lysed by *in vitro* expanded TIL, due to the absence of such cells. As a control, in lanes 4 and 5, patient B has the same rearrangement patterns in the two different experimental conditions.

DISCUSSION

Many reasons could explain the partial lack of experimental data on TIL derived from NSCLC: lungs are highly vascularised and the isolation of TIL from contaminating peripheral blood lymphocytes should be difficult. In addition, lung and bronchial tissues are not sterile (bacteria and fungi can be easily detected in lung biopsies). Finally, several lung tumours are necrotic. All these features make long-term cultures of TIL, derived from NSCLC, difficult. However, our data indicated that 46 out of 70 cultures of TIL isolated from NSCLC expanded *in vitro*. These

were either in the presence or absence of a polyclonal lymphoid cell activator PHA. More importantly, our data indicate that a significant proportion of cytolytic (CD8+) lymphocytes may be expanded *in vitro* from PHA-activated cultures, whereas, in PHA-untreated samples, there are a large number of non-cytolytic CD4+. In apparent contrast with this finding, major histocompatibility complex (MHC)-unrestricted cytolytic activities (such as NK and LAK activity) were more potent in PHA-untreated than in PHA-activated cultures. However, it has been shown clearly in other experimental models, such as in melanoma [4] or renal cell carcinoma [2], that TIL contain a large number of lymphoid cells specific for autologous (and not allogeneic) tumour-associated antigen(s), and this "specific" activity could significantly reduce tumour masses both in animal models and in humans. In this context, the higher, MHC-unrestricted, cytolytic activity observed in TIL, derived from PHA-untreated samples, seems to be irrelevant. On the contrary, it is of interest that both PHA-activated and PHA-untreated cells have the same cytolytic activity against autologous cancer cells. This evidence is in apparent contrast with what has been observed by other authors: in particular, no specific cytolytic activity against autologous cancer cells was described either in resting or in rIL-2-treated TIL derived from NSCLC [9, 10]. All these data were obtained using tumour cells directly obtained from the neoplastic masses. However, it is a common belief that a population of uncultured neoplastic cells derived from NSCLC contains cancer cells in variable proportions, as well as normal epithelial cells, alveolar macrophages, fibroblasts, white and red blood cells. This heterogeneity does not allow the use of this unpurified population as a target. In contrast, we cultured the cells derived from the neoplastic masses for 14 days, and obtained a highly pure population of cancer cells as shown by the reactivity against the anti-cytokeratin MAb as well as by cytological examination. The use of a purified population as target cells allowed us to observe that TIL derived from NSCLC were able to recognise and lyse efficiently autologous better than allogeneic purified cancer cells. Moreover, it is of note that TIL expanded in the presence of PHA have the same specific cytolytic activity of TIL derived from culture in the presence of rIL-2 alone. This finding is strongly supported by the same restriction maps for TCR-β observed both in PHA-treated and in PHA-untreated cells, and indicates that the *in vitro* expansion of *in vivo*-activated cells (CD25+) alone, as suggested in other experimental models [2], does not result in a more specific culture against autologous cancer cells. On the contrary, the growth rate and the final number of cells obtained are significantly lower in PHA-untreated than those in PHA-activated cultures. This, therefore, suggests that in protocols of adoptive immunotherapy of surgically resected and advanced NSCLC with macroscopic or microscopic residual disease, large numbers of PHA-activated TIL should be used.

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