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Activation and expansion of tumour-infiltrating lymphocytes by anti-CD3 and anti-CD28 monoclonal antibodies

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Summary. Cytotoxic T lymphocytes from healthy donors can be expanded to high numbers from the peripheral blood using combinations of anti-CD3 and anti-CD28 monoclonal antibodies (mAb). We investigated whether these antibodies could also be used to induce outgrowth of tumour-infiltrating lymphocytes (TIL) from tumour tissue. In the initiation phase of TIL culture immobilized anti-CD3 antibodies together with anti-CD28 mAb and low-dose interleukin-2 induced a rapid expansion of T cells from various human tumour tissues. The cultured cells showed high levels of cytotoxic T lymphocyte activity, but low levels of lymphokine-activated killer cell activity were generated. This study shows that TIL can be efficiently expanded from tumour tissue by combinations of anti-CD3 and anti-CD28 antibodies. This protocol for cell expansion in vitro may substantially reduce the time required to reach sufficient numbers of TIL for re-infusion to the patient.

Introduction

In adoptive immunotherapy of cancer, autologous lymphocytes are activated in vitro to attain anti-tumour activity and are subsequently transferred to the patient together with interleukin-2 (IL-2) to mediate tumour regression. Lymphocytes can be isolated either from the patients' peripheral blood or from the tumour site itself. The latter cells have been designated tumour-infiltrating lymphocytes (TIL). In vitro, IL-2-activated TIL have been shown to be both more specific and efficient in tumour cell lysis than IL-2-activated peripheral blood lymphocytes or lymphokine-activated killer cells [21].

Since only a limited number of the TIL activated in vitro will eventually reach the tumour site(s) [1], vast numbers of cells are needed for re-infusion, i.e. 3×10^{10}–3×10^{11} per patient [19]. The expansion of TIL consists of two phases. The first or initiation phase is the period during which lymphoid cells are grown from the tumour suspension. The total number of cells usually drops during this phase [2]. The second phase represents the period during which the cells expand in culture after tumour cells have been eliminated. Outgrowth of TIL is most frequently accomplished by culturing cells with high doses of IL-2, with or without the addition of autologous tumour cells [2, 14, 17, 20]. The expansion rates as well as the phenotypes of expanded TIL populations show considerable variation. Such variations may contribute to the differences observed with regard to the cytotoxic specificity and therapeutic potency of TIL.

Monoclonal antibodies (mAb) to the T cell receptor/CD3 complex have been widely used to simulate antigen-specific proliferation and differentiation of human T lymphocytes in vitro. We and others have recently demonstrated that plastic-immobilized anti-CD3 mAb can provide a powerful, accessory-cell-independent, stimulus for resting T cells [6, 24]. Not only strong T lymphocyte proliferation, but also the outgrowth of cytolytic T lymphocytes (CTL) with specificity for allo-antigens can be induced in this way [4]. In synergy with anti-CD3 mAb, antibodies to the T-cell differentiation antigen CD28 enhance T lymphocyte activation by augmenting lymphokine secretion [9, 10, 22, 23, 25]. The purpose of the present study was to analyse whether mAb to CD3 and CD28 mAb could be used in the initial expansion phase of TIL from tumour tissue. Our data show that combinations of anti-CD3 and anti-CD28 mAb very efficiently propagate and activate TIL from various tumours. Moreover, in all tumour samples tested the outgrowth of CTL could be demonstrated.

Materials and methods

Materials. All cultures were performed in Iscove's modified Dulbecco's medium supplemented with 5% human pool serum, antibiotics and 50 μM 2-mercaptoethanol. The murine mAbs CLB-T3/3 against the CD3

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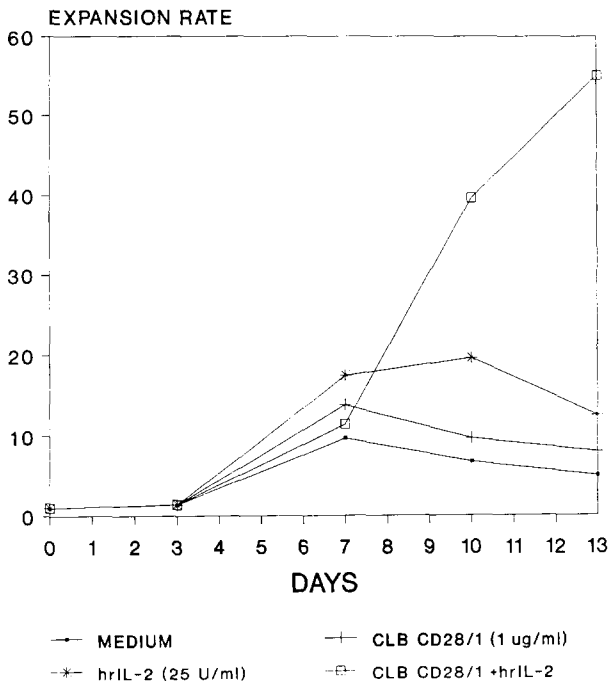

Fig. 1. Expansion rate in distinct stimulation protocols. Lymphocytes were stimulated as described in Materials and methods with immobilized CLB-T3/3 (5 μg/ml) (•) or with CLB-T3/3 to which CLB-CD28/1 (+), interleukin-2 (IL-2, *) or a combination of CLB-CD28/1 and IL-2 (□) was added from day 0 till day 7. On day 7 cells were harvested, viable cells were counted and restimulated with immobilized anti-CD3. At the indicated times cells were harvested and viable cells were enumerated. Expansion rates are represented as the number of cells at day *x* related to the number of cells at day 0. In the absence of immobilized CLB-T3/3, stimulation of cells with either CLB-CD28/1, IL-2 or their combination gave no T cell proliferation (data not shown)

molecular complex and CLB-CD28/1 against the T cell differentiation antigen CD28 have been previously described [23, 24]. Fluorescein-isothiocyanate(FITC)-labelled anti-CD3, CD4 and CD8 mAb were all prepared in our institute. Recombinant (r) IL-2 was a kind gift from Sandoz (Vienna, Austria).

Cell preparations. Peripheral blood mononuclear cells were obtained by Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density (1.078 g/cm^3) centrifugation of buffy coats from healthy blood-bank donors. Peripheral blood lymphocytes (PBL) were isolated from the mononuclear cells by counterflow centrifugation elutriation [5]. TIL were isolated from two patients with skin metastases of malignant melanoma and from one patient with a primary renal cell carcinoma. The surgically obtained specimens were mechanically homogenized and washed twice with culture medium. Subsequently, Ficoll/Isopaque density (1.078 g/cm^3) centrifugation was performed to enrich for TIL. Lymphocytes from two patients suffering from malignant melanoma disseminated into the peritoneal cavity were isolated from ascitic fluid by means of density centrifugation as described above. Cell preparations were cryopreserved and thawed according to standard procedures [3]. Cells were enumerated after brilliant crysal blue staining and brought to the desired concentration.

Lymphocyte stimulation. Anti-CD3-coated culture wells were prepared as described [24]. Briefly, 1 ml CLB-T3/3 (5 μg/ml) diluted in phosphate-buffered saline (PBS) (pH 7.8) was incubated at 4° C in Nunclon 24-well plates. After overnight incubation the supernatant was removed and the wells were washed twice with phosphate-buffered saline. PBL or TIL (1×10^6/well) were cultured on the CLB-T3/3-coated wells for 3 days in a final volume of 1 ml. Where indicated 1 μg/ml CLB-CD28/1, 25 IU/ml rIL-2 or a combination of both was added. After 3 days cells were harvested, counted, seeded at 2.5×10^5 lymphocytes/well and new culture medium with or without anti-CD28 mAb and IL-2 was added. Alternatively, TIL were stimulated with 1000 IU/ml IL-2 on day 0. On day 7, cells were harvested and viable cells were used for further experiments. Activated lymphocytes from peripheral blood of healthy donors were restimulated with immobilized anti-CD3 antibodies and cultured for another 6 days as described above. Expansion rates are given as the number of cells at day *x* divided by the initial cell number at day 0.

Immunofluorescence. For membrane antigen analysis, 2×10^5 cells were incubated for 30 min with a saturating amount of FITC-labeled mAb. Thereafter, cells were washed and 5000 cells were analysed on a FACSCAN (Becton and Dickinson, Mountain View, Calif.).

Cytotoxicity assays. After 7 days, stimulated TIL or PBL (effector cells) were harvested, viable cells were counted and cytolytic activity was tested at three effector : target (E : T) ratios, i.e. 20 : 1, 5 : 1 and 1.25 : 1. CTL activity was tested in an anti-CD3-mediated cytotoxicity assay as described [4]. In this re-directed kill assay CTL activity is detected regardless of the antigen specificity of the CTL: the ability of effector cells to lyse the Fc-receptor-bearing target cell p815 in the presence of anti-CD3 mAb (CLB-T3/4.1, ascites dilution $1 : 10^3$) is measured. LAK cell activity was tested on Daudi target cells, NK cell activity on K562 target cells. Target cells were labelled for 1 h at 37° C with $Na_2{}^{51}CrO_4$ (Radiochemical Centre, Amersham, UK) followed by three washes with assay medium (Isoves modified Dulbecco's medium supplemented with 10% fetal calf serum, antibiotics and 2-mercaptoethanol). Triplicate cultures of effector cells were incubated with 5×10^3 target cells in a final volume of 200 μl assay medium in round-bottom microtiter plated (Costar, Cambridge, USA). After 4 h incubation at 37° C, supernatants were collected with the Titertek harvesting system (Flow Laboratories, Rockville, Maryland) and counted in a gamma counter. The percentage cytotoxicity was calculated as: 100×[(experimental) – (spontaneous release)] / [(maximum release) – (spontaneous release)]. Spontaneous release is the ^{51}Cr release (cpm) in the absence of effector cells and maximum release is determined by adding saponin at a final concentration of 1%. The standard error of the mean percentage lysis did not exceed 10%.

Results

Expansion and activation of CTL from the peripheral blood by combinations of anti-CD3 and anti-CD28 mAb

To investigate whether cytolytic T lymphocytes can be efficiently grown in large numbers from peripheral blood using antibodies to the T cell membrane antigens CD3 and CD28, PBL were stimulated with immobilized CLB-T3/3 (5 μg/ml) in the absence or presence of anti-CD28 mAb (1 μg/ml) and rIL-2 (25 U/ml). After 7 days of culture, cells were restimulated with immobilized anti-CD3 antibodies. In agreement with previous proliferation experiments where [^{3}H]thymidine incorporation was used to measure T cell stimulation [24], immobilized CLB-T3/3 induced a clear expansion of T cells (Fig. 1). Whereas the addition of either IL-2 or anti-CD28 had only modest effects on cell recovery, the combination of both stimuli provided by far the best expansion in time.

The phenotype of the outgrowing cells was analysed during costimulation with rIL-2 and anti-CD28. Interestingly, although only approximately 50% of the peripheral blood CD8$^+$ cells express CD28, it was found that the percentage of CD8$^+$ cells rose from 30% to 60%, whereas the percentage CD4$^+$ lymphocytes dropped from 40 to 30%.

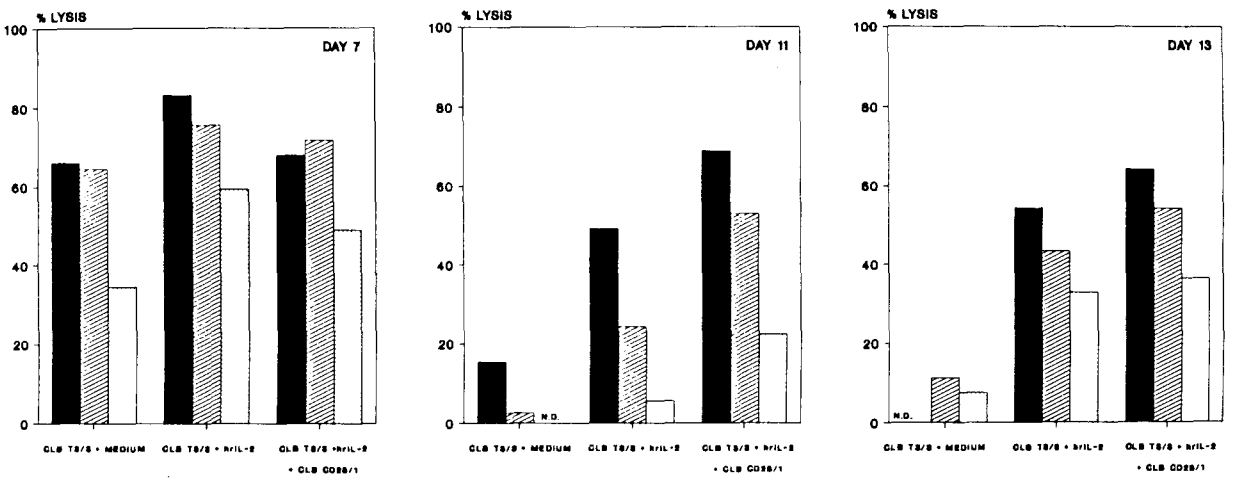

Fig. 2. Development of CTL activity as a function of time. Cells were stimulated as described in the legend of Fig. 1. At four consecutive time points cytotoxic T lymphocyte activity was measured in an anti-CD3-mediated cytotoxic assay against P815. Lysis was measured at three E : T ratios (20 : 1, *closed bars*; 5 : 1, *hatched bars*; 1.25 : 1, *dotted bars*). Results are shown as the percentage lysis above medium control

Table 1. Expansion of TIL from tumour samples

Patient	Tumour	ER	CD4/CD8	Cytolytic activity (%)		
				p815	K562	Daudi
1	Melanoma ascites	4.7	1.2	58.9	44.7	17.5
2	Melanoma metastasis	9.6	ND	22.0	ND	3.3
3	Renal cell carcinoma	4.3	1.4	58.6	18.8	16.5
4	Melanoma metastasis	10.4	4.1	56.3	7.0	0.9

Tumour-infiltrating lymphocytes were isolated as described in Materials and methods. The outgrowing cell population on day 7 was analysed for expansion rate (ER): lymphocytes harvested on day 7 divided by the starting cell number. The CD4/CD8 ratio was determined by fluorescence-activated cell sorting analysis, and the cytolytic activity was expressed as specific lysis in ^{51}Cr-release assays towards distinct target cells. ND, not determined

In parallel experiments, at various times after the initiation of activation, CTL activity of the stimulated PBL was measured in a redirected kill assay. The condition that gave the optimal expansion rate, i.e. cells activated via immobilized anti-CD3 and co-stimulated with CLB-CD28/1 in combination with low-dose rIL-2, also generated the highest levels of CTL activity in time (Fig. 2).

Expansion of TIL from tumour samples

Next it was tested whether TIL could be grown from tumour cell suspensions using the T cell activation systems described above. In marked contrast to PBL, TIL could not be expanded by immobilized anti-CD3 mAb alone (data not shown). Moreover, the addition of either CLB-CD28/1 or 25 U/ml rIL-2 induced only a low level of expansion (data not shown). The combination of immobilized anti-CD3 mAb with CLB-CD28/1 and low-dose IL-2, however, induced an efficient outgrowth of TIL, with expansion rates in four patients tested ranging from 4.3 to 10.4 on day 7. Interestingly, the number of tumour cells rapidly declined after anti-CD3/anti-CD28/IL2 co-stimulation leaving virtually no tumour cells in culture on day 5 (data not shown). Since the T cell numbers in the day-0 cultures were very low, we were unable accurately to determine the CD4/CD8 ratio. Nevertheless both CD4+ and CD8+ T lymphocytes were stimulated although the CD4/CD8 ratio varied between distinct samples (Table 1).

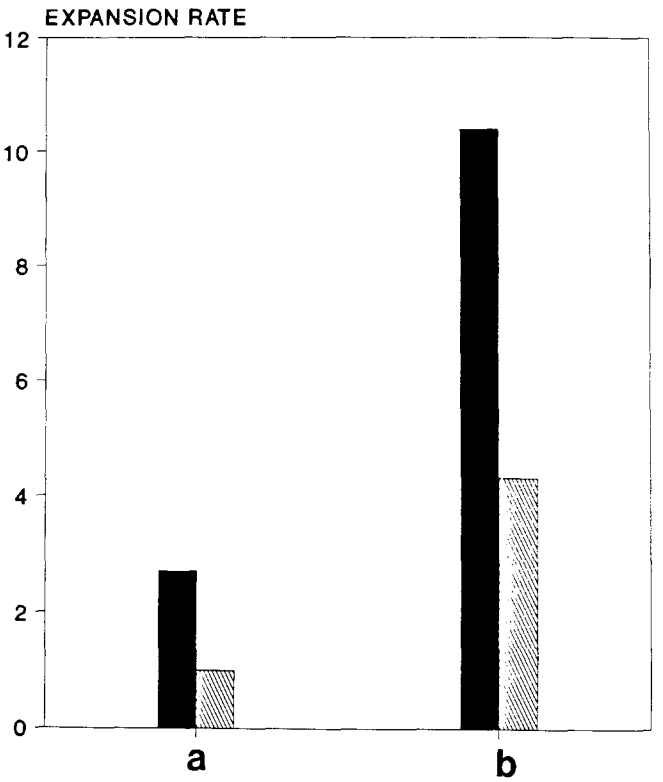

Fig. 3. Expansion rate of tumour-infiltrating lymphocytes. Cells were stimulated as described in the legend of Fig. 1. At day 7 the expansion rate and cytotoxicity were measured. Expansion rate is represented as the number of cells at day 7 related to the number of cells at day 0. *Closed bars:* patient 5 (skin metastasis from a melanoma). *Hatched bars:* Patient 3 (primary site of a renal cell carcinoma). *a* 1000 U/mlhrIl-2; *b* CLBT3/3+ hrIL-2+ CLBCD28/1

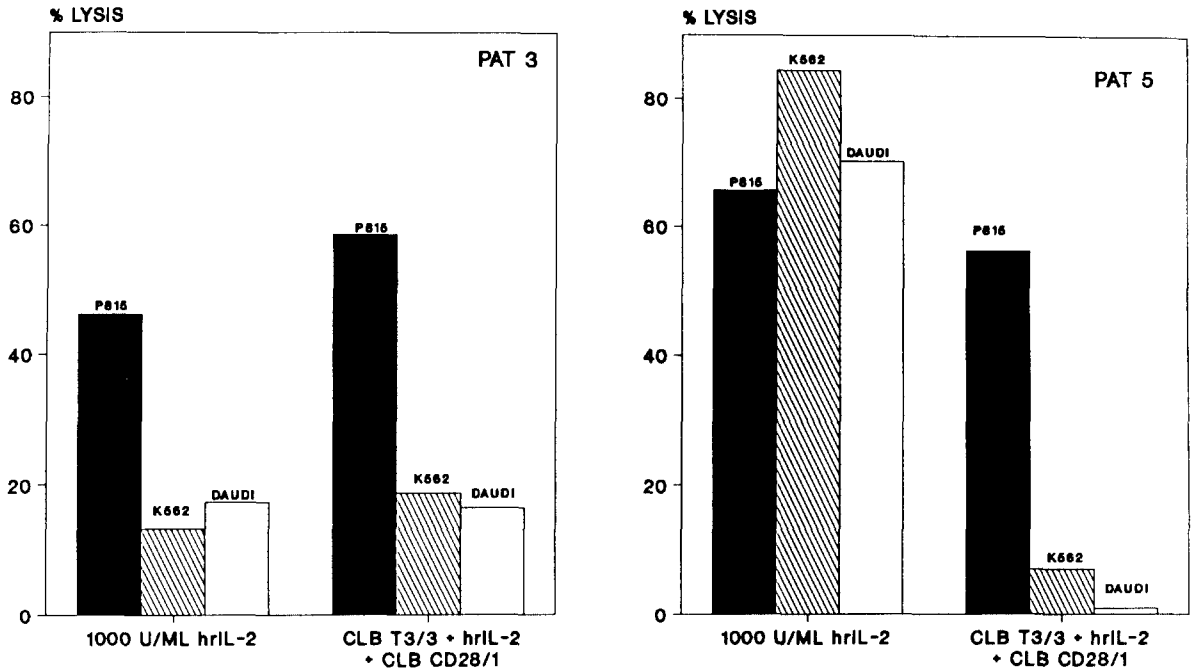

Fig. 4. Cytolytic capacity of the expanded tumour-infiltrating lymphocytes. Cytotoxicity was tested at three E:T ratios on three indicated targets. E:T ratio 20:1 is displayed only for reasons of clarity. Lysis at E:T ratios 5:1 and 1.25:1 was proportionally lower (data not shown). Cells from the same patients as described in Fig. 3 were analysed

Outgrowth of CTL by combinations of anti-CD3 and anti-CD28 antibodies

CTL activity was measured as anti-CD3-mediated cytolysis of the Fc-receptor-bearing p815 target cells after 7 days of stimulation. This assay permits detection of CTL activity regardless of the antigen specificity of the T cell receptor. No activity of non-stimulated TIL could be found in this assay (data not shown). As can be seen from Table 1, TIL populations propagated from tumour tissue by anti-CD3/anti-CD28/IL-2 co-stimulation all displayed moderate to strong cytolytic activity against p815. In most samples low levels of NK and LAK activity, measured respectively by K562 and Daudi lysis, were generated. Taken together, we conclude that stimulation with immobilized anti-CD3 mAb in the presence of anti-CD28 mAb and low-dose IL-2 can induce an efficient outgrowth of CTL from tumour tissue.

Comparison of anti-CD3/anti-CD28/IL2 activation with high-dose IL-2 stimulation

In protocols employed to culture TIL from tumour tissue most frequently high-dose IL-2 is used. Having established that co-stimulation with anti-CD3/anti-CD28/IL2 can be used to expand TIL from tumour tissue, we next compared the efficiency of this protocol with that using high-dose (10^3 IU/ml) IL-2 stimulation. Figure 3 shows that in two patients' samples analysed, CD3/CD28/IL-2 co-stimulation yielded four times more activated TIL on day 7 than did activation with high-dose IL-2. Furthermore, specific outgrowth of T cells could be demonstrated since the percentage $CD3^+$ cells in TIL culture on day 7 was on average 95%, compared to 72% in high-dose IL-2-activated tumour samples (data not shown). The experiments shown in Fig. 4 illustrate the functional heterogeneity in TIL populations. Whereas in patient 3 stimulation with high-dose IL-2 only results in the induction of CTL activity, the same protocol in patient 5 induces high levels of CTL, NK and LAK activity. Markedly, in both patients the CD3/CD28/IL2 protocol only initiates CTL activity.

Discussion

In this study we show that TIL can be efficiently expanded from tumour tissue by combinations of anti-CD3 and anti-CD28 antibodies and low-dose IL-2. In five different tumour samples tested outgrowth of CTL could be demonstrated, whereas relatively low levels of LAK and NK activity were induced. Compared to stimulation with high-dose IL-2, the described protocol was found to generate four times more CTL on day 7 of culture.

Little is known about the factors responsible for the differences found in the expansion rates of TIL. Itoh et al. [7] reported that delayed growth of TIL corresponded with rapid proliferation of autologous tumour cells in unstimulated cultures. Moreover, it is suggested that the frequency of proliferative T lymphocyte precursors is reduced in TIL [12]. Also, the production by tumours of certain factors, such as transforming growth factor β (TGFβ) that inhibit T cell activation in vitro [18], may add to the variations in TIL expansion rates.

A novel approach to the expansion of TIL is the use of anti-CD3 mAb. It has been shown in the murine model that a 10^4-10^5-fold expansion can be reached of anti-CD3-antibody-induced activated killer cells, and that these cells

have anti-tumour activity [27]. In humans, anti-CD3 mAb enhance the proliferative response of IL-2(50 U/ml)-activated TIL as well as the cytotoxicity against fresh autologous tumour targets [16]. Here we describe a stimulation protocol that also includes anti-CD28 antibodies in the initiation phase of TIL culture. It has been shown that co-stimulation with anti-CD28 antibody strongly enhances IL-2 production by T cells stimulated with anti-CD3 or anti-CD2 mAb [22, 25]. Moreover, the secretion of other cytokines such as interferon γ and tumour necrosis factor α (TNFα) are augmented by anti-CD28 antibodies [11]. When used for the expansion of lymphocytes from tumour tissue, high levels of IL-2 production will contribute to T cell proliferation directly, whereas cytokines such as TNFα may antagonize the inhibitory effects of tumour-cell-derived TGFβ [18]. Hence the advantage of anti-CD28 mAb addition in the initiation phase of TIL culture might be twofold.

The involvement of the T cell receptor/CD3 complex [7, 20], the major histocompatibility complex (MHC) restriction of the recognition [7, 8, 13, 15, 26], the existence of certain tumour antigens [13] and the inability to lyse any other tested targets [13, 26] favour the presence of MHC-restricted anti-tumour CTL in TIL propulations. The outgrowing T lymphocytes from the TIL bulk cultures, stimulated with combinations of anti-CD3/anti-CD28/IL2, all showed a high cytolytic response in the anti-CD3-mediated lysis of p815. We recently reported that immobilized anti-CD3 mAb induce the outgrowth of alloantigen-specific CTL from bulk T cell cultures [4]. Therefore, provided that the precursor frequency of HLA-restricted tumour-specific CTL is high, the CTL activity as measured by the anti-CD3-mediated lysis of p815 may well be a reflection of the specific cytotoxic capacity, including tumour-cell-specific CTL, in the day-7 cultures.

It is a crucial question whether TIL expanded in vitro are potentially able to induce tumour regression in vivo. The correlation between the in vitro specificity of TIL and their in vivo effect on tumour regression is surprisingly low. Nevertheless, since it can be envisaged that the anti-CD3/anti-CD28/IL2 activation system may induce outgrowth of other effector cells than high-dose IL-2, for these populations antigen specificity, using freshly isolated tumour cells and tumour cell lines, is also being evaluated at this moment. Our results indicate that, given the powerful preferential outgrowth of CTL from tumour tissue in the first week of culture, the time needed to obtain sufficiently high numbers of TIL for re-infusion could be substantially shortened. Since the majority of the outgrowing TIL are activated T lymphocytes, at this stage for further expansion use of moderate amounts of IL-2 seems logical and appropriate.

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