Assessment of the number of local cytotoxic T lymphocytes required for degradation of micrometer-size tumor spheroids

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Abstract

Adoptive immunotherapy with human cytotoxic T lymphocytes (CTL) is a promising cancer treatment. Previously we showed that human CTLs against various types of tumors can be efficiently produced by coculturing peripheral blood cells with target cells. The aims of this study were to simulate the interaction of CTLs and micrometer-size tumor tissues *in vitro* and to assess the required number of CTLs at local tumor sites for degradation of a tumor. Allogeneic CTLs against a human transitional cell carcinoma cell line and autologous CTLs against a renal cell carcinoma cell derived from a surgical specimen were generated. The cytotoxic activities of CTLs against tumor cells in monolayer culture and tumor spheroids formed in U-bottom 96-well culture plates were assessed. Both allogeneic and autologous CTLs showed greater destructive activity than lymphokine activated killer (LAK) cells against target tumor spheroids. CTLs inoculated at E/T ratios of 0.1 to 1 coexisted with the tumor spheroid for 5 to 6 days and then increased in number with apparently lethal activity against the tumor spheroid. In contrast to CTLs, the increase in LAK cell numbers was scarcely observed, and the proliferated LAK cells did not show cytotoxicity against the tumor spheroid. These observations suggest that, when a small number of CTLs reach a local tumor site, they can destroy micrometer-size tumors after considerable local proliferation.

Abbreviations: CTL, cytotoxic T lymphocyte; LAK cell, lymphokine activated killer cell; TIL, tumor-infiltrating lymphocyte; E/T ratio, effector/target ratio; PBMC, peripheral blood mononuclear cell; CV assay, crystal violet assay.

Introduction

Human cytotoxic T lymphocytes (CTL) can be efficiently generated *in vitro* by coculturing peripheral blood mononuclear cells (PBMC) with cultured target tumor cells or with formalin-fixed and paraffin-embedded tumor sections using a medium containing interleukin (IL)-1, 2, 4 and 6 (Liu et al., 1995, 1996, 1998). The CTLs killed over 80% of monolayer-cultured target tumor cells at a low effector/target (E/T) ratio within 24 hr (Liu et al., 1995, 1996, 1998). These results suggest that adoptive immunotherapy

with *in vitro* generated CTLs is promising for cancer patients. Indeed, preliminary clinical studies using *in vitro* activated T cells showed therapeutic benefit against several cancers such as renal cell carcinoma, glioma and malignant melanoma (Plautz et al., 1998; Chang et al., 1997). However, these reports also suggest that the present adoptive immunotherapy with CTLs is not powerful enough to completely degrade tumor mass. Several reasons for the limited efficacy were proposed: heterogeneity of the population of infused effector cells, poor localization of the transferred effector cells to tumor sites, and sub-optimal activity

of the effector cells at the local tumor sites (Yee et al., 1997).

Many investigators consider that the CTL activity of tumor-infiltrating lymphocytes (TIL), which are the most widely used effector cells in adoptive immunotherapy, plays a major role in tumor regression (Rosenberg et al., 1988; Topalian et al., 1989; Bukowski et al., 1991). However, the median percent ^{111}In injectate per gram of tumor was reported to be only 0.0021 in the study of ^{111}In -labeled TIL infusion for melanoma patients (Pockaj et al., 1994). These observations suggest that the efficacy of adoptive immunotherapy *in vivo* depends primarily on the activity of a small fraction of the effector cells. Thus, more precise knowledge of effector cell activities at local tumor sites and a more efficient method of delivery of effector cells are necessary. Two points need to be determined. First, whether the high level of cytotoxic activity of CTLs observed in monolayer culture truly carries over to tumor tissue composed of packed multilayers tumor cells. Second, how many CTLs must be delivered to the local tumor site to exert sufficient anti-tumor activity?

In order to address these questions, we conducted experiments with multi-cellular tumor spheroids as a model of micrometer-size tumor tissues. Although lacking appropriate stroma and blood vessels, the multicellular tumor spheroid model is thought to provide a useful experimental model to analyze the interaction between lymphokine activated killer (LAK) cells and solid tumor cells at the local tumor sites (Jaaskelainen et al., 1989; Iwasaki et al., 1990). In the present study, we used U-bottom 96-well culture plates (Koide et al., 1990) to evaluate the interaction between human allogeneic and autologous CTLs and solid uniform-size tumor spheroids. The results suggest that CTLs can efficiently degrade the spheroid after their numbers increased at the local site.

Materials and methods

Target tumor cells and formation of uniform spheroids

Human bladder transitional carcinoma cell line T24 obtained from RIKEN Cell Bank was maintained in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS). Renal cell carcinoma TUHR10TKB cells were derived from the surgically resected specimen of a 69-year-old man and were maintained in Dulbecco's modified Eagle's medium

(DMEM) containing 10% FBS. For spheroid formation and maintenance, the medium RHAM α containing 5% heat-inactivated FBS was used. RHAM α is a mixture of RPMI 1640, Ham's F12, and MEM α at the ratio of 3:1:1 by volume that has been developed for serum-free culture of LAK cells (Kawai et al., 1992). Spheroids were formed in U-bottom 96-well culture plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The tumor cells, 7.5×10^3 in $100 \mu\text{l}$ of medium, were seeded in each well of the plates. After one hour incubation at 37°C in a humidified CO_2 incubator, the plate was centrifuged at 1500 rpm ($140 \times g$) for 10 min and the culture was continued. A uniform-size spheroid was formed in each well within 24 hr and was maintained with a half medium change every other day.

Generation of CTLs and LAK cells

Generation of CTLs was carried out as described previously (Liu et al., 1995, 1998). Briefly, PBMCs from heparinized peripheral blood were separated by conventional Ficoll-Paque gradient centrifugation, and suspended at a concentration of 1×10^6 cell ml^{-1} in RHAM α medium supplemented with autologous plasma (5%) or heat-inactivated FBS (5%), IL-1 (Genzyme Co., 167 U ml^{-1}), IL-2 (Sionogi Co., Ltd., 67 U ml^{-1}), IL-4 (Genzyme Co., 67 U ml^{-1}), and IL-6 (Genzyme Co., 134 U ml^{-1}). The PBMCs were cocultured in 6-well culture plates which contained a confluent monolayer of the target cancer cells previously irradiated with 30 Gy. Half of the culture medium was changed every other day. After complete lysis of irradiated target cells (usually 2 weeks), the lymphocyte preparations were restimulated weekly with live target cells at an E/T ratio of 10.

LAK cells were prepared by culturing PBMCs (1×10^6 cell ml^{-1}) in RHAM α medium supplemented with autologous plasma (5%) or heat-inactivated FBS (5%) and IL-2 (500 U ml^{-1}) for 2–4 weeks. The mouse anti-human CD3 monoclonal antibody (OKT3, Nichirei Co.) at a final concentration of $0.1 \mu\text{g ml}^{-1}$ was added weekly for the first 4 weeks. Allogeneic CTL and LAK cells against T24 (T24-CTL and T24-LAK, respectively) were induced from PBMCs of a healthy volunteer, and autologous CTL and LAK cells (TU-CTL and TU-LAK, respectively) against the target TUHR10TKB cells were induced from PBMCs of the renal cell carcinoma patient TUHR10.

Figure 1. Phase-contrast photomicrographs and histology of spheroids. (a) A typical T-24 spheroid 1 hr after inoculation of T24-CTL at E/T ratio of 0.1; (b) and (c) T24-spheroids after 7 days coculture with LAK cells (b) or with CTL (c) at the initial E/T ratio of 0.1. Lymphocytes proliferated around the spheroids. Note that the surface has become indistinct and lost integrity in the spheroid treated with CTL (c).

Figure 1d-e. (d) and (e) Hematoxylin-eosin stained cross-sections ($\times 200$, the length of one grid is $500\ \mu\text{m}$) of control T24-spheroid (d) and TU-spheroid (e) at 7 days after the initiation of spheroidal culture. Note that viable cancer cells arranged in multilayers lack central necrosis.

Cytotoxicity of effector cells against monolayer target cells

The cytotoxic activity of CTLs against target cells in monolayer culture was assayed by crystal violet (CV) staining as described previously (Liu et al., 1995). This low-cost CV assay is as sensitive for assessment of the killing activity of lymphocytes against adherent target cells as the standard ^{51}Cr -release cytotoxicity assay (Liu et al., 1995) in the range of E/T ratios of 10 or below. Above this E/T ratio, underestimation, though slight, of the killing activity of effector cells was observed because of the firm attachment of a small number of the effector cells to the adherent target cells.

Briefly, the target cells, 1×10^4 cells/well in $200\ \mu\text{l}$ culture medium, were seeded in each well of 96-well plates and were precultured overnight. After the culture plate was washed with PBS, the cultured lymphocytes suspended in $200\ \mu\text{l}$ of LAK cell or CTL culture medium were added as effector cells to each well at the indicated E/T ratio. The cells were cocultured for 12 hr, and then were washed once gently with PBS. Adherent target cells were fixed for 1 hr with 10% (v/v) formalin ($200\ \mu\text{l}$ /well), then stained with crystal violet solution (0.4% in water, $100\ \mu\text{l}$ /well) for 30 min at room temperature. The plate was washed with tap water and dried at room temperature. To each well, $200\ \mu\text{l}$ of 80% methanol was added and the OD at 570 nm of each well was determined.

Figure 2. Number of cells per spheroid and percentage of viable cells in the spheroidal culture. Each data point represents the mean of triplicated determinations with SD bars.

The mean of triplicated samples was determined, and the percentage of surviving target cells was expressed as follows:

$$\text{Surviving target cells (\%)} = (B-C)/A \times 100$$

where A is the absorbance of control target cells pre-cultured in a separate plate just before the addition of the effector cells, B is that of remaining target cells to which effector cell were added, and C is that of effector cell only. Each value in the figures represents the mean of triplicates accompanied by a SD error bar. Note that some results show more than 100% survival because of rapid growth of target cells during the 12 hr incubation. Experiments for the determination of the cytotoxicity were repeated twice or more.

Determination of spheroid volume and cell numbers in a spheroid

In the spheroid culture, all tests were performed after confirming the formation of a single spheroid in each U-bottomed well of the 96-well culture plate. After incubation with or without effector cells, the tumor spheroid was gently aspirated with micropipette tip and transferred to the well of another 96-well plate and fixed with 50 μ l of 10% formalin. The spheroid size

was measured under a microscope. The volume (V) of the spheroid was calculated from the 2 orthological diameters (a and b) using $V = 3.14 \text{ aXb}^2/6$ where b is the longer axis. Morphological analysis was carried out after hematoxylin-eosin (H-E) -stained sections were cut from formalin-fixed paraffin-embedded spheroids.

To count cells, the spheroids were dissociated with 0.2% trypsin, and the total number of viable cells was counted on a hemocytometer by the conventional trypan-blue staining method. Each experiment was repeated at least twice.

Flow cytometry

For cytometric analyses, 2×10^5 effector cells were stained with FITC-conjugated monoclonal antibody at 4 °C for 30 min. Flow cytometry was performed with a FACScan (Becton-Dickinson). FITC-conjugated monoclonal antibodies used were anti-CD3, anti-CD4 and anti-CD8 (Nichirei Co., Tokyo, Japan, OKT-3, OKT-4 and OKT-8, respectively) and anti-CD45 (Dako Japan Inc.).

Figure 3. Cytotoxicity of CTL and LAK cells against monolayer-cultured target tumor cells. (a) and (b) Cytotoxicity of CTL and LAK cells against monolayer-cultured T24 cells (a) and TUHR10TKB cells (b) determined by the 12-hr killing assay. (c) Specific cytotoxicity of allogeneic T24-CTL determined by the 12-hr killing assay. Each data point represents the mean of triplicated determinations with SD bars.

Results

Formation and viability of the micrometer-size tumor cell spheroids

Both T24 and TUHR10TKB cells formed a spheroid in each well without satellite aggregate formation or fragmentation (Figures 1a, d and e). Diameters of T24-spheroids and TU-spheroids were $382 \pm 35 \mu\text{m}$ (mean $\pm$ SD, $n = 9$) and $589 \pm 95 \mu\text{m}$, respectively, just after the spheroid formation. The spheroids retained their morphology for at least 2 weeks in the culture. T24 cells continued proliferation in the spheroidal culture for two weeks and, consequently, the cell number in the spheroid doubled. In contrast,

TUHR10TKB cells did not exhibit obvious proliferation in the spheroid (Figure 2). Trypan blue vital staining showed that over 90% of cells forming the spheroids were viable (Figure 2, dotted lines). H-E staining of the spheroids after 7 days of culture revealed no central necrosis in the spheroids (Figures 1d and e).

Cytotoxic activities of CTLs against monolayer target tumor cells

Both allogeneic T24-CTL and autologous TU-CTL were generated against monolayer cultured target T24 cells and TUHR10TKB cells, respectively. They consisted of over 95% CD3⁺, over 95% CD8⁺ and 1–5%

Figure 4. Cytotoxicity of allogeneic CTL and LAK cells against the tumor spheroids at the E/T ratio of 4. (a) Cytotoxicity of CTL and LAK cells against T24-spheroids determined by sphere volume. (b) Total number of cells and CD45-negative cells per well in which T24-spheroids were treated with LAK cells or CTL for 24 hrs. Each point represents the mean of triplicated determinations with SD bars.

CD4⁺ cells. T24-CTL showed higher cytotoxicity than T24-LAK cells derived from the PBMCs of the same subject. Higher cytotoxicity was also observed in TU-CTL than in TU-LAK cells generated from the autologous PBMCs of the patient TUHR10. These two CTLs killed over 90% of the corresponding target tumor cells within 12 hr at E/T ratios of 4 and 10, respectively (Figures 3a and b). The cytotoxicity of T24-CTL was specific to T24 cells so far examined. T24-CTL did not kill two uterine cancer cell lines, OMC-1 and MIN-2, both of which have been established in our laboratory (Figure 3c). TU-CTL were also specific to

the autologous renal carcinoma, TUHR10TKB cells, and did not kill renal carcinoma cell lines Hpt.10, Hpt.15, TUHR3TKB or TUHR4TKB, which were established in our laboratory as previously reported (Liu et al., 1998).

Cytotoxicity of CTLs against tumor cell spheroids

T24-CTL inoculated at an E/T ratio of 4 (3×10^4 cells/well) significantly reduced the volume of T24-spheroids within 24 hr, and the T24-spheroids had totally disintegrated by 48 hr (Figure 4a). In contrast, T24-spheroids treated with T24-LAK cells showed

Figure 5. Cytotoxicity of CTL and LAK cells against the tumor spheroids at the E/T ratio of 0.1 and 1. (a) Effector cell proliferation in the coculture. Allogeneic CTL or LAK cells were inoculated at 0.75×10^3 cells/well into each well containing a T24 spheroid (E/T ratio = 0.1 at day 0). Autologous CTL or LAK cells were inoculated at 7.5×10^3 cell /well to each well containing a TU-spheroid (E/T ratio = 1 at day 0). (b) and (c) Cytotoxicity of CTL and LAK cells against T24-spheroid (b) and TU-spheroid (c) determined by the sphere volume.

a transient decrease in volume at 24 hr, but their volume increased again with further incubation up to 48 hr (Figure 4a). When cocultured with T24-CTL, the surface of the T24-spheroids became indistinct and lacked integrity under microscopic examination. These morphological changes were hardly observed in the T24-spheroids treated with T24-LAK cells.

To determine the number of tumor cells in each well, spheroids with or without effector cells were dissociated by trypsinization to form single-cell suspensions after 24 hr coculture, and the number of whole cells in the well was counted with a hemocytometer. The number of the tumor cells was calculated from the ratio of CD45-negative cells in the cell suspension after staining with anti-CD45 monoclonal antibody and the flow cytometry. As shown in Figure 4b, the number of tumor cells was significantly

decreased when T24-spheroids were cocultured with T24-CTL. In contrast, there was no significant decrease in the number of tumor cells in the wells treated with T24-LAK cells.

When T24-CTL and TU-CTL were inoculated into spheroid cultures at the E/T ratios of 0.1 and 1, respectively, they did not instantly reduce the volume of the spheroids. At these E/T ratios, both of the T24- and TU-spheroids were morphologically stable for at least 5 days. During this period, the number of T24-CTLs and TU-CTLs increased constantly in the coculture (Figure 5a). However, both T24-LAK cells and TU-LAK cells maintained very slow growth for 8 and 12 days in coculture, respectively.

In contrast to the CTL growth, the increase in the volume of the CTL-treated T24-spheroids was suppressed for 5 days and began to decrease rapidly

after 5 day in coculture. Complete degeneration of the T24-spheroids was observed after 7 days in coculture, although an apparent volume increase was observed in the control T24-spheroids and the LAK cell-treated T24-spheroids (Figures 1b and c, Figure 5b). In the TU-spheroids, as well, rapid destruction was observed after 5 days of coculture. T24-LAK cells and TU-LAK cells did not show destructive activity against corresponding tumor spheroids (Figures 5b and c).

Phenotypic analyses of the increased number of CTLs cocultured with tumor spheroids revealed that T24-CTL consisted of over 99% CD3⁺, 1.4% CD4⁺ and 95% CD8⁺, and TU-CTL consisted of over 99% CD3⁺, 21% CD4⁺ and 86% CD8⁺.

Discussion

In the present study, we cultured tumor-cell spheroids as an experimental model of micrometer-size tumor tissues in order to evaluate activities at the local tumor sites. First, in the spheroid model, the CTL showed higher destructive activity than LAK cells against the allogeneic and autologous tumor spheroids, as has been observed against the monolayer culture of the target tumor cells. Second, CTLs inoculated at E/T ratios of 0.1 and 1 initially coexisted with the tumor spheroids for 5–6 days, and then rapid reduction of the volume of the tumor spheroids was observed (Figure 5). Third, the proliferated lymphocyte population was CD8⁺ dominant, similar to the population at the start of the coculture. In contrast to CTLs, proliferation was not observed in the allogeneic T24-LAK cells (Figure 5a). Autologous TU-LAK cells showed moderate proliferation, but the proliferated LAK cells did not exhibit apparent destructive activity against the tumor spheroids (Figure 5c). At the E/T ratios of 0.1 and 1, the number of CTLs may have not been sufficient to degrade the tumor spheroid in the initial 5 days of coculture (Figures 5b and c). After 5 days of coculture with the tumor spheroid, T24-CTLs numbered 3×10^4 per well. Thus, the estimated local E/T ratio at day 5 was above 5. Similarly, the estimated local E/T ratio in the coculture of TU-CTL and the TU-spheroid exceeded 6 at day 5. These observations suggest that some minimum E/T ratio in which the effectors cells are definitively superior to the target cells is essential at the local tumor site to degrade the tumor tissues.

While CTLs showed promising activity against solid-tumor spheroids in the present *in vitro* study, it may be difficult to directly deliver sufficient numbers

of effector cells to tumor tissue to achieve high enough E/T ratio *in vivo* because of non-specific entrapping of infused lymphocytes (Lotze et al., 1980). Although there has been some controversy about tracking patterns of activated lymphocytes, recent results of *in vivo* gene marking studies did not support tumor-homing ability of bulk TILs (Economou et al., 1996; Merrouche et al., 1995). To overcome this limitation, two methods to achieve definitively superior E/T ratio *in vivo* are possible: One is to increase the homing capacity of effector cells to localize preferentially into the tumor tissues, and another is to support the proliferation of effector cells at the local tumor site. Several animal studies suggested that the *in situ*-activated CTLs but not bulk TILs may have significant capacity to localize to tumor tissues (Matsumura et al., 1994; Park et al., 1995). In addition, recent analysis of the cell-surface phenotypes of TILs revealed that the TIL which expresses high levels of several adhesion molecules including cutaneous lymphocyte antigen, showed enhanced integrin-mediated adhesion to dermal micro-vascular endothelium *in vitro* (Adams et al., 1997). These observations suggest that modification of the cell-surface adhesion molecules may increase a phenotype with selective homing capacity *in vivo*. The present multicellular tumor spheroid is thought to be a useful experimental model for investigating the adhesion molecules that mediate interaction between CTL and solid tumor cells. The expansion of CTLs *in situ* would be the other possible strategy to achieve the high local E/T ratio. The present study demonstrated that the CTL is a promising effector cell having superior ability to multiply at the local tumor site while retaining cytotoxicity compared to LAK cells. Although the results were obtained under the optimal environment for CTL growth, the present model might be useful to investigate the optimal microenvironment that supports CTL proliferation and activity at local tumor sites. These findings will be quite helpful for developing combination treatment adjunctive to CTL adoptive immunotherapy.

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References

- Adams DH, Yannelli JR, Newman W, Lawley T, Ades E, Rosenberg SA and Shaw S (1997) Adhesion of tumour-infiltrating lymphocytes to endothelium: a phenotypic and functional analysis. *Br J Cancer* 75: 1421–1431.
- Bukowski RM, Scharfman W, Murphy S, Rayman P, Tubbs R, Alexander J, Budd GT, Sergi J, Bauer L, Gibson V, Stanley J, Boyett J, Pontes E and Finke J (1991) Clinical results and characterizations of tumor infiltrating-lymphocytes with or without recombinant interleukin-2 in human metastatic renal cell carcinoma. *Cancer Res* 51: 4199–4205.
- Chang AE, Aruga A, Cameron MJ, Sondak VK, Normolle DP, Fox BA and Shu S (1997) Adoptive immunotherapy with vaccine-primed lymph node cells secondarily activated with anti-CD3 and interleukin-2. *J Clin Oncol* 15: 796–807.
- Economou JS, Beldegrun AS, Glaspy J, Toloza EM, Figlin R, Hobbs J, Meldon N, Kaboo R, Tso C, Miller A, Lau R, McBride W and Moen RC (1996) *In vivo* trafficking of adoptively transferred interleukin-2 expanded tumor-infiltrating lymphocytes and peripheral blood lymphocytes. *J Clin Invest* 97: 515–521.
- Iwasaki K, Kikuchi H, Miyatake S, Aoki T, Yamasaki T and Oda Y (1990) Infiltrative and cytolytic activities of lymphokine-activated killer cells against a human glioma spheroid model. *Cancer Res* 50: 2429–2436.
- Jaaskelainen J, Kalliomaki P, Paetau A and Timonen T (1989) Effect of LAK cells against three-dimensional tumor tissue: *In vitro* study using multi-cellular human glioma spheroids as targets. *J Immunol* 142: 1036–1045.
- Kawai K, Sasaki T, Saijou K, Akaza H, Koiso K and Ohno T (1992) Additive effects of antitumor drugs and lymphokine-activated killer cell cytotoxic activity in tumor cell killing determined by lactate-dehydrogenase-release assay. *Cancer Immunol Immunother* 35: 225–229.
- Koide N, Sakaguchi K, Koide Y, Asano K, Kawaguchi M, Matsushima H, Takenami T, Shinji T, Mori M and Tsuji T (1990) Formation of multicellular spheroid composed of adult rat hepatocytes in dishes with positively charged surfaces and under other nonadherent environments. *Exp Cell Res* 189: 87–92.
- Liu SQ, Saijo K, Todoroki K and Ohno T (1995) Induction of human autologous cytotoxic T lymphocytes on formalin-fixed and paraffin-embedded tumor sections. *Nature Med* 1: 267–271.
- Liu SQ, Shiraiwa H, Kawai K, Hayashi H, Akaza H, Kim BS, Oki A, Nishida M, Kubo T, Hashizaki K, Saijo K and Ohno T (1996) Tumor-specific autologous cytotoxic T lymphocytes from tissue sections. *Nature Med* 2: 1283.
- Liu SQ, Kawai K, Shiraiwa H, Hayashi H, Akaza H, Hashizaki K, Shiba R, Saijou K and Ohno T (1998) High rate of induction of human autologous cytotoxic T lymphocytes against renal cell carcinoma cells cultured with an interleukin cocktail. *Jpn J Cancer Res* 89: 1195–1201.
- Lotze MT, Line BR, Mathisen DJ and Rosenberg SA (1980) The *in vivo* distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): Implications for adoptive immunotherapy for tumors. *J Immunol* 125: 1487–1493.
- Matsumura T, Sussmann JJ, Krinock RA, Chang AE and Shu S (1994) Characteristics and *in vivo* homing of long-term T-cell lines and clones derived from tumor-draining lymph nodes. *Cancer Res* 54: 2744–2750.
- Merrouche Y, Negrier S, Bain C, Combaret V, Mercatello A, Coronel B, Moskovtchenko J, Tolstoshev P, Moen R, Philip T and Favrot MC (1995) Clinical application of retroviral gene transfer in oncology: Results of a French study with tumor infiltrating lymphocytes transduced with the gene of resistance to neomycin. *J Clin Oncol* 13: 410–418.
- Plautz GE, Barnett GH, Miller DW, Cohen BH, Prayson RA, Krauss JC, Luciano M, Kangisser DB and Shu S (1998) Systemic T cell adoptive immunotherapy of malignant gliomas. *J Neurosurg* 89: 42–51.
- Park JA, Brown RA, Kurt RA and Akporiaye T (1995) Studies of *in vivo* recruitment and activation of cytotoxic lymphocytes using a gelatin-sponge model of concomitant tumor immunity. *Int J Cancer* 62: 421–427.
- Pockaj BA, Sherry RM, Wei JP, Yannelli JR, Carter CS, Leitman SF, Carasquillo JA, Steinberg SM, Rosenberg SA and Yang JC (1994) Localization of ¹¹¹Indium-labeled tumor infiltrating lymphocytes to tumor in patients receiving adoptive immunotherapy. *Cancer* 73: 1731–1737.
- Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon D, Lotze MT, Yang JC, Seipp GA, Simpson C, Carter C, Bock S, Schwartzentruber D, Wei JP and White DE (1988) Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *N Engl J Med* 319: 1676–1680.
- Topalian AL, Solomon D, Rosenberg SA (1989) Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. *J Immunol* 142: 3714–3725.
- Yee C, Riddell SR and Greenberg PD (1997) Prospective for adoptive T cell therapy. *Curr Opin Immunol* 9: 702–708.