Adoptive Immunotherapy of Cancer

A New Approach For Cancer Treatment

Ying Q. Chin
ADOPTIVE IMMUNOTHERAPY OF CANCER

A New Approach For Cancer Treatment

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To: Jingwu, Linda and my mother
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### List of abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytolytic T lymphocytes</td>
</tr>
<tr>
<td>Cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>$^{51}$Cr</td>
<td>$^{51}$Chromium</td>
</tr>
<tr>
<td>DCC</td>
<td>Dextran-coated charcoal</td>
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<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescein Activated Cell Sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-ethane sulfonic acid</td>
</tr>
<tr>
<td>HLA</td>
<td>The human major histocompatibility complex</td>
</tr>
<tr>
<td>$^{3}$H-TdR</td>
<td>Tritiated Thymidine</td>
</tr>
<tr>
<td>rIL-2</td>
<td>recombinant Interleukin-2</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
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<tr>
<td>PR</td>
<td>Progesterone receptor</td>
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<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocytes</td>
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Chapter 1

General Introduction
1.1 Tumor Immunology

Over the past 20 years concepts in tumor immunology have undergone fundamental changes. However, the advances are still in short to provide effective treatment for cancer. It has been said that cancer has done more for the science of immunology than immunology has ever done for cancer. Recent developments in immunology promise to change this perception. New insights regarding antigen presentation to T cells, T cell recognition, activation, growth, traffic and interaction with endothelial cells suggest how tumor antigens might be recognized by the immune system and what requirements must be met to apply these principles to develop future immunotherapeutic strategy.

Role of CD4+ and CD8+ T cell subsets in tumor eradication

T cells bearing the T cell receptor αβ heterodimer can be divided into two major subpopulations based on surface phenotypes: the CD4+CD8- subset and the CD4-CD8+ subset. With a few exceptions, the phenotypes are most often associated with the T cell functions. The majority of T cells with helper functions are derived from the CD4+ subset, while cytotoxic/suppressor T cells are tightly associated with CD8 phenotype. Functionally, both CD4 and CD8 act as accessory molecules that interact with MHC class I and MHC class II in T cell activations. CD4+ T cells recognize antigens in the context of MHC class II molecules and CD8+ T cells are class I-restricted (1, 2).

It is evident that both CD4+ and CD8+ T cells are the major effectors in attacking tumors. Tumor rejection by noncytolytic CD4+ T cells in the absence of cytolytic CD8+ T cells has been confirmed in tumor models (3). CD4+ T cells have been shown to mediate rejection of a wide variety of tumors, including leukemia, plasmacytoma, sarcoma, and hepatoma (4-7). There are many effector mechanisms by which CD4+ T cells can mediate and/or promote tumor eradication. The most direct evidence is the lysis of tumor cells bearing MHC class II molecule by CD4+ T cells with cytolytic activity, and the release of short-range soluble factors mediating the lysis (8, 9). The release of these factors
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results in the lysis of bystander targets, as well as the cells being directly recognized. In addition to the in vivo antitumor activity of cytolytic CD4+ T cells, other effector functions of this subset may also contribute to the tumor eradication, such as the secretion of lymphokines that interact and recruit other effectors to mediate tumor eradication.

Unlike CD4+ T cells, cytotoxic CD8+ T cells directly lyse tumor targets in the absence of bystander killing. This specificity of target killing results from the release of cytotoxic granules into synapse-like regions formed between the cytolytic CD8+ T cells and the target cells, with the consequent formation of pores in the target membrane and disruption of the cell (10-14). The antitumor activity of cytolytic CD8+ T cells has been demonstrated in many tumor models against a very wide range of tumors, including leukemia, lymphoma, hepatoma, plasmacytoma, sarcoma, glioma, mastocytoma, melanoma, and carcinoma (15-32). In many of these models, the therapeutic activity of CD8+ T cells is dependent on the presence of CD4+ T helper during the generation and/or expression of the antitumor response, consistent with the availability of endogenous IL-2.

CD4+ and CD8+ effector T cells, in addition to recognizing potentially distinct tumor antigens and being restricted by different MHC molecules, differ in lymphokine production, cytokine responsiveness, and requirements for antigen presenting cells (APC) and accessory cells. Thus, the capacity of the cells and microenvironment at or near a tumor site to induce and support antitumor responses by each T cell subset influences whether CD4+ or CD8+ effector T cells will be effective at eliminating tumor.

Role of B lymphocytes in tumor elimination

B cells and their antibody products unquestionably have an important role in the field of tumor immunology. The antibodies may occur in the patient's serum or they may be raised in the experimental animals by immunization with the tumor. Such antibodies may be directly lytic for tumor cells through distinct mechanisms or may recruit cells carrying Fc receptors (e.g. NK cells and
macrophages) or may act in conjunction with complement to lyse tumor cells (33). Antibody-dependent cellular cytotoxicity (ADCC) appears to be the major effector pathway mediated by B cells (34). Lysis by ADCC proceeds via binding of the Ig variable region to the tumor target and of the Fc portion to an Fc receptor (FcR)-bearing effector cell, with subsequent signal transduction through the FcR and activation of the cytolytic mechanism of the effector cell. FcR+ macrophages and NK cells are the major ADCC effector cells. T cell responses to tumor promote ADCC both by the secretion of lymphokines that provide help for B cell antibody responses and by secretion of lymphokines that activate ADCC effector cells (35-39).

**Tumor-associated macrophages in tumor elimination**

Studies in experimental tumor models have suggested that tumoricidal macrophages contribute substantially to tumor eradication. Following antigen recognition, T cells secrete a number of lymphokines that have coordinating effects on macrophages, including the induction of tumoricidal activity by two distinct pathways. One of these pathways requires only a single signal, and can be induced by IL-4, GM-CSF, TNF and possibly by IL-2 (40-42). In general, macrophage killing induced by this one signal pathway proceeds via a mechanism requiring macrophage production of TNF which can directly mediate lysis following binding to specific receptor on tumor cells (40, 42-44). The second pathway of macrophage activation requires two signals for induction of cytotoxicity, and such activated macrophages lyse a broader range of targets than TNF-secreting macrophages, and rarely permit the outgrowth of tumor cells resistant to lysis (45). The first priming signal, γ-IFN (also described as macrophage-activating factor in the literature), renders the macrophages capable of selectively binding to transformed cells and makes them responsive to a second signal, a triggering signal, for cytolysis (46-48).
NK cell functions

NK cells are characterized as large granular lymphocytes that express no CD3 molecules or T cell receptor chains. They express CD16 and NK1.1/2.1 (or Leu19 in humans), and mediate cytolysis of targets in the absence of MHC expression (49). They require two signals for optimal activation. The first signal can be delivered via interaction with the surface of cultured cell lines such as K562 or lymphoblastoid cell lines and the second signal is provided by T cell growth factors. NK activity in solid human tumors is low or absent (50, 51). NK cells may kill de novo arising lymphoproliferative tumor cells induced virally. The viruses induce interferon production, which in turn stimulates the induction of NK cells.

1.2 Tumor-Associated Antigens

Tumor-associated antigens (TAA) are tumor cell constituents responsible for the interaction with the host immune system. They are found in spontaneous human tumors in man and in animals, but also in virally or chemically induced tumors of experimental animals. Their expression is associated with the processes that turn normal cells into tumor cells.

Oncogenes are small DNA fragments that are present in tumor cells and they are able to transform a normal cell into a tumor cell (52). The protein products of oncogenes resemble normal cellular proteins that govern cell growth and cell functioning, such as growth factors, their receptors, and protein kinases (53). Many chemical substances can induce tumors in experimental animals, but their antigenicity is actually not detected in the tumor-bearing host. It is suggested that carcinogen-induced tumor cells have common TAA, but many of these antigens are present in low amounts and are shaded by a few strongly immunogenic TAA (54). In virally transformed tumor cells, viral gene(s) can act as potential TAA (55). A majority of TAA can be classified as differentiation antigens that are characteristic for normal cells. They have a common fetal origin, or are in a particular phase of their differentiation, or belong to the same
tissue, for instance, the cell surface molecules found on T-lymphocytes and their precursors (56), and fetal antigens: carcinoembryonic antigen (CEA) and alpha-fetal protein (α-FP) (57).

There is a discrepancy between the detection of TAA in vivo and the detection and analysis of TAA in vitro using monoclonal antibodies. Not all 'antigens' detected in vitro will necessarily provoke an in vivo immune response against cells that carry them. There is evidence suggesting that TAA are usually normal molecules and their expression is disturbed by a failure in the control of cellular gene transcription.

1.3 Immunosurveillance and Suppression of Antitumor Immunity

Tumor cells do not arise frequently (58), but when a tumor cell arises immune surveillance and natural resistance mechanisms may resist the development of a tumor. Tumor cells may be killed by NK cells, cytotoxic T cells or cytotoxic macrophages. However, the ability of a tumor to escape from immunological control may depend on a balance between the effectiveness of the immune system and a variety of factors promoting an escape. Tumor products, genetic factors, tumor kinetics, antigenic modulation, antigen masking, antigen shedding are the factors that may contribute to an immunological escape. Local functional and systemic tolerance to certain antigens common to the viruses can also lead to tumor outgrowth (59). Suppressor T cells can inhibit T and B cell responses to tumor antigens resulting in accelerated tumor growth (60). Loss or alterations of MHC expression on tumor cells can also influence immune recognition of tumors (61).

1.4 Adoptive Immunotherapy for Cancer

Following a series of novel and imaginative investigations conducted at the National Cancer Institute (NCI), the use of adoptive immunotherapy for
malignant tumors has, almost overnight, become a realistic therapeutic option (62). Initially, lymphokine-activated killer (LAK) cell activity was described in animals and humans. Variable results were observed in practical applications ranging from lacking of response to remarkable shrinking of the tumor mass (63, 64). Soon after that, another alternate form of adoptive immunotherapy: tumor infiltrating lymphocytes (TIL) therapy, was reported. TIL have quickly moved from the laboratory bench to the hospital bedside (65, 66). Meanwhile, other forms of adoptive immunotherapy were also being investigated, monoclonal antibodies against tumor antigens were injected alone or linked to toxins, drugs or isotope to the patients (67).

Interleukin 2 and lymphokine-activated killer cell therapy

Mononuclear cells from various body compartments are transformed into potent, though nonspecific, cytotoxic cells. They are able to kill fresh autologous or allogeneic tumor cells after in vitro stimulation with interleukin 2 (IL-2) (68, 69). By infusing a large numbers of in vitro expanded LAK cells and high doses of recombinant IL-2 or high doses of IL-2 administration alone therapeutic effects have been obtained in animal models as well as in the treatment of several metastatic human tumors, especially melanoma, renal cell carcinoma and lymphoma (63-65, 67). Unfortunately, the number of patients who benefit from this therapy is rather low, and the toxicity for many of the patients receiving the treatments is unacceptable (70). Immunotherapy based on the administration of LAK cells plus IL-2 or in some cases of IL-2 alone can help about 20 percent of patients with certain advanced cancers, but accompanied with side effects (71). The administration of high doses of interleukin-2 leads to the leakage of fluid from blood into tissues, usually resulting in weight gain, and occasionally impairing lung function. Studies with LAK cells and IL-2 have led cancer workers search for immune cells with improved specificity and therapeutic effectiveness.
Monoclonal antibody targeted treatment

Monoclonal antibodies reacting with malignant cells offer new approaches for targeting anti-cancer agents (72). Therapeutic efficacy may be improved through localization of conjugates in tumors (especially metastatic deposits). The use of conjugates may also minimize toxicity of drugs for normal tissues, which is a major limitation in systemic cancer chemotherapy. Antibodies have been conjugated to plant toxins (73) or chemotherapeutic agents (72, 74). But a major obstacle to antibody targeting encountered is the development by patients of antibodies against mouse monoclonal antibody (75). Methods for reducing antibody responses have been exploited including treatment with immunosuppressive drugs and genetic engineering to construct humanized antibodies, which combine the binding sites from murine antibodies with human antibody elements (76). However, antibody targeting of therapeutic agents requires that the chosen antibody localizes effectively at tumor site in relation to its non-specific distribution in body fluids and normal tissues. Essentially, none of the monoclonal antibodies currently in clinical trials react with cancer-specific antigens (77). The search for monoclonal antibodies with tumor-restricted specificity is important for the improvement of antibody-targeted cancer treatment.

Tumor infiltrating lymphocytes therapy

Studies performed in animal models with chemically induced syngeneic transplantable tumors have shown that the host immune system could specifically recognize and reject malignant tumor cells, suggesting a crucial role of the immune system in regulating the outgrowth of tumor cells. Frequently peripheral blood lymphocytes (PBL) do not reflect the tumor-host relationship and cell mediated immunity in the PBL does not often correlate with prognosis. However, the observation that an abundant lymphocytic infiltration is associated with a less aggressive tumor behavior has supported the notion that the infiltrating cells, particularly T cells, may be a contributing mechanisms to the host's immune defense (78). Lymphocytic infiltration are demonstrated in
many experimentally induced neoplasm (78-81). Advanced enzyme immunohistochemical techniques reveal that the infiltrates are heterogeneous and frequently comprise functional T lymphocytes of different subtypes, B cells, NK subsets and macrophages (79, 82-85). The tumor infiltrating lymphocytes (TIL) are class of lymphocytes with unique antitumor activity, interacting most closely with the tumor cells, and are likely to be more accurately reflecting tumor-host interactions (86, 87). TIL are cells that infiltrate into growing tumors and can be grown by culturing single-cell suspensions obtained from tumors in the presence of IL-2. Extensive in vivo studies performed in animal models demonstrated that TIL were 50 to 100 times more effective in treating established lung and liver metastases than were LAK cells (88). That is, if 100 million LAK cells were needed to produce a 50 percent regression in animal cancer, only from one million to two million TIL would be required to achieve the same degree of success. First report of human trial using adoptively transferred TIL involving 20 melanoma patients showed that 55 percent of the patients responded well to the therapy (65). That is more than two times more effective than LAK therapy. In addition, TIL requires much less IL2 to remain alive and active in the body, the side effects caused by high dose of interleukin 2 can thus be reduced. Further studies with in vitro expanded TIL derived from different histological tumor types have shown that TIL has the ability to lyse specifically the autologous tumor cells but not normal cells from the same patients (88-90). A variety of experiments provide a rational for the use of TIL in the treatment of humans with advanced cancer (65, 66, 90-93).

1.5 Aim of This Thesis

Although adoptive immunotherapy has already established itself as a potential treatment of cancer, it is far to early to hypothesize what its real value in the therapy of human cancer. Much remains to be done before the biology of TIL is fully understood. One of the major problems with currently used TIL
immunotherapy is the heterogeneity of the IL-2 expanded cells being infused. In most studies TIL are grown in high doses of IL-2 (around 1000 U/ml), resulting in heterogeneous populations of lymphocytes, composed of several subsets varying in number and phenotype within one culture over time and from one patient to another (65, 66). The search for tumor-specific effectors may greatly enhance the efficacy of the therapy, and it will also facilitate the identification of tumor-specific antigens. The aim of this thesis was to study tumor infiltrating lymphocytes isolated from human solid tumors with respect to their functional properties and potential use as a therapy. The study was performed in three parts: study of the phenotypic profiles of tumor infiltrating lymphocytes by flow cytometry; in vivo distribution of infused TIL using imaging techniques; and the fine specificity analysis of TIL at clonal and molecular level.
References


Chapter 2

Large Scale Expansion of Human Tumor Infiltrating Lymphocytes with Surface-modified Stimulator Cells for Adoptive Immunotherapy
Abstract

Expansion of tumor infiltrating lymphocytes (TIL) in vitro is hampered by several factors, including a limited amount of lymphocytes obtained from different tumors, unknown target antigens and limited supply of antigen-presenting cells (APC) which are generally believed to be essential in the classical way of T cell stimulation and expansion. In approaching these difficulties, we have recently used surface-oxidized allogeneic PBL to stimulate the TIL periodically in the presence of a low dose of rIL-2 (200 IU/ml). TIL derived from 22 (out of 23) tumor specimens could be expanded with $20 \cdot 10^7$ fold increases over 6 - 16 weeks, to a sufficient amount of $10^9 - 10^{11}$ cells for adoptive immunotherapy. In contrast, only 2 - 100 fold increases were observed in six tumor specimens (out of 23) when 200 IU/ml rIL-2 was used only. The phenotypes, autologous tumor reactivity and cytolytic capability of TIL propagated with surface-oxidized stimulators were similar to those expanded in the presence of rIL-2 alone. These data suggest that expanding TIL with surface-modified stimulator cells could be a useful alternative method to obtain large amount of tumor specific cytolytic T cells for clinical immunotherapeutic use, irrespective of tumor-antigen stimulation and MHC restriction.
Introduction

Tumor-infiltrating lymphocytes (TIL) are immune cells harbored at tumor sites which are believed to represent a marker of host response to neoplastic growth (1). They can be isolated from human solid tumors and selectively grown in vitro in the presence of recombinant interleukin 2 (rIL-2) (2, 3). Animal studies showed that the TIL expanded in rIL-2 were 50-100 times more potent than LAK cells to induce tumor regression (4). Therefore, TIL have quickly moved from laboratory site to clinical trials (5, 6). Both animal studies and preliminary clinical trials have shown promise of adoptive TIL therapy as a potential approach of treatment for human cancers.

Extrapolation from murine model, on a weight basis, suggests that $10^9$-$10^{11}$ TIL may be required, in conjunction with IL-2, to mediate tumor regression in patients with advanced cancer (7). In our experience, the tissues obtainable from early cancer stage were usually small, the average recovery of TIL from different kinds of solid tumors ranged from $10^4$-$10^7$. Therefore, an expansion of $10^3$ to $10^7$-fold is required.

However, with the standard TIL culturing methods (rIL-2 alone), it is difficult to achieve such an amount of TIL for in vivo therapeutic use. The failure of long-term culturing and expanding of these TIL could be due to lacking of tumor-specific antigen stimulation and limited supply of autologous antigen presenting cells, as long-term growth of T cells requires stimulation of specific antigen and MHC encoded molecules besides interleukin-2 (8, 9). Several alternative methods have been described to promote the outgrowth of cytolytic TIL such as cytokines (10, 11, 12) and monoclonal antibodies (13). We have tried to stimulate TIL with mitogenic lectins such as phytohemagglutinin (PHA) or concanavalin A (ConA) and monoclonal antibodies to CD2 and CD3 in the presence of Fc receptor-bearing accessory cells, but none of them showed significant improvement on TIL growth, possibly due to a weak response of TIL to mitogens and a low proliferative frequency as compared with peripheral T cells (14).

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Recently, non-specific propagation of human antigen-dependent T lymphocyte clones stimulated by surface-modified stimulators, in addition to growth factors, has shown to be an effective alternative approach to maintain long-term growth of T lymphocytes in vitro without losing specificity (15). Introduction of aldehydes into galactose residues on stimulator cells (16) leads to the stimulation of T cells by modified stimulator cells in the absence of a mitogen and accessory cells. In the present investigation, we have studied the use of surface-oxidized stimulators for non-specific propagation of TIL in the presence of rIL-2. We now report that TIL generated from tumor sites can be maintained as tumor reactive and specific when propagated with oxidized PBL and rIL-2, but in the absence of tumor antigen and antigen presenting cells. TIL can be successfully expanded in vitro to sufficient amounts as required for adoptive immunotherapy.

Materials and Methods

Tumor infiltrating lymphocytes

Tumors excised from patients were immediately transported steriley from hospitals to our laboratory. They were then minced into 1-2 mm pieces and further dissociated with an enzyme solution containing hyaluronidase type V 0.01%, collagenase type IV 0.1% (Sigma, Val, Belgium), DNase type I 0.002%, gentamicin 50 µg/ml and fungizone 250 ng/ml dissolved in RPMI 1640 medium (Gibco, Life technologies, Belgium). The mixture was incubated for 2-4 hours at 37°C or overnight at room temperature. It was then filtered through a sterile coarse wire grid, washed four times with RPMI 1640 medium, and resuspended in culture medium which was RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 µg/ml gentamicin, 250 ng/ml fungizone (Gibco, Life Technologies), and 10 mM Hepes buffer (Flow Laboratories, Belgium). rIL-2 was added at a final concentration of 200 U/ml (Eurocetus). TIL were cultured in 24-well plate (Costar, ElsColab) in 2 ml aliquots the first 4 weeks and then
divided into two portions for the comparison study of standard TIL culture and culture with oxidized PBL stimulators in 24-well or 12-well plate.

**Oxidation of PBL stimulators and propagation of TIL**

Oxidation of PBL was performed according to Novogrodsky and Fleischer. Briefly, irradiated allogeneic PBL (4.6 x 10^7 cells/ml) from normal subjects were incubated with galactose oxidase 0.05 U/ml (Sigma) and neurominidase 0.02 U/ml (Boehringer Mannheim, Germany) in RPMI 1640 medium for 90 min at 37°C and shaked at 15 min intervals to prevent formation of clump. The cells were washed three times with RPMI 1640 containing 0.01 M galactose (Sigma) to remove residual galactose oxidase. Oxidized PBL were added to above TIL cultures at a ratio of 5-10 Stimulators to 1 TIL. Cells were restimulated with oxidized PBL in culture medium containing fresh rIL-2 on a weekly basis, and viable cell concentrations were returned to 0.5 x 10^6 cells/ml at each passage.

**Proliferative assay**

10^4 cells of each TIL were cultured in triplicate in the presence of 10^5 PBL stimulators in a 96-well plate (Costar) for 72 hours at 37°C in 5% CO2. To examine the specificity of the TIL, autologous or allogeneic tumor cells were added. To evaluate the response of TIL to different stimulators, PHA or monoclonal antibodies were added. Microcultures were then pulsed with 1 μCi of [3H]Tdr (Radiochemical Center, Amersham, England) per well 4 hours prior to harvesting and thymidine uptake was measured by scintillation techniques.

**51 Chromium release cytotoxicity assay**

TIL were examined for specific cytotoxicity with autologous and allogeneic tumor cells, NK-sensitive K562 cell line, and NK-resistant Daudi cell line. Target cells were labeled with 200 μCi 51Cr(Na_2Cr_3O_7, Amersham, England) for 60 min at 37°C and washed four times with medium. Target cells were reincubated for another 30 min and washed twice before use. 5 x 10^3
labeled target cells were incubated with TIL in 96-well plate in triplicate at a various effector:target ratios in a total of 200 μl volume. Supernatants were harvested with Skatron-Titertec system after 4 hour incubation at 37°C and counted in a gamma counter. The maximum release and spontaneous release of chromium were measured in wells containing target cells in the presence of detergent or medium alone.

The specific release was calculated as

\[
\% \text{ specific lysis} = \frac{\text{exp.release - spon.release}}{\text{max.release - spon.release}}
\]

Phenotype analysis

TIL (5 x 10^5) were washed and resuspended in 100 μl of cold phosphate-buffered saline (PBS) containing 5% fetal calf serum and 0.1% sodium azide. The cells were incubated then with fluorescein- or phycoerythrin-conjugated monoclonal antibodies to lymphocyte surface antigens (Becton-Dickinson, Belgium) on ice for 30 min. The antibodies used were: anti-CD3 (T-cells), anti-CD16 + 56 (NK and some activated cytotoxic cells), anti-CD4 (T helper/inducer cells), anti-CD8 (T cytotoxic/suppressor cells), anti-HLA-DR (activated T cells) and anti-CD25 (activated T cells). Cells were washed twice after staining and analyzed immediately on FACS 420 with consort 30 computer (Becton-Dickinson).

Large scale expansion of TIL for in vivo immunotherapy

Single cell suspensions derived from enzymatically digested tumor tissues were cultured in the 24-well plates for the first 3-4 weeks. When tumor cells were gradually disappearing from culture, oxidized allogeneic PBL were added and medium were refreshed with rIL-2. The cultures were split and transferred to 6-well plates or flasks (Costar) and restimulated with oxidized
PBL and rIL-2 on a weekly basis. The medium were changed twice a week or whenever it was necessary. Different assays were carried out during expansion period. When the cultures reached $10^9$-$10^{11}$ TIL, they were harvested and ready for use in clinical trials.

Figure 1. Growth of TIL derived from a breast tumor stimulated by oxidized PBL. TIL ($0.5 \times 10^6$/well) were stimulated weekly with $3 \times 10^6$ oxidized PBL in medium containing rIL-2 and were split and restimulated in weekly intervals.
Table 1. Proliferation of TIL in response to several stimulators.

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>cpm $^3$H-TdR incorporated (day 3)</th>
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<tbody>
<tr>
<td>No stimulator</td>
<td>626</td>
</tr>
<tr>
<td>PBL</td>
<td>9268</td>
</tr>
<tr>
<td>PBL, oxidized</td>
<td>100896</td>
</tr>
<tr>
<td>PBL + PHA 10 μg/ml</td>
<td>68429</td>
</tr>
<tr>
<td>PBL + CD3 MAb 0.1 μg/ml</td>
<td>63324</td>
</tr>
<tr>
<td>PBL + TcR-1 MAb 0.1 μg/ml</td>
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</tr>
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</table>

Results and Discussion

The recovery of TIL obtained from tumor-derived single cell preparations of 17 breast, 2 stomach, 3 melanomas and 1 ovarian tumors was about 1.6% to 56% (mean 12%) with a higher recovery in breast adenocarcinoma in situ or with minimum invasiveness (76%), as estimated with trypan blue exclusion staining. The outgrowth of TIL were much more marked in cultures stimulated with oxidized PBL than those cultured with rIL-2 alone. 22 of the TIL derived from 23 tumor samples could be successfully expanded upon stimulation with oxidized PBL and rIL-2, and a total amount of $10^{10} - 10^{11}$ TIL could be recovered for therapeutic uses between week 6 to week 16. One of the TIL failed to expand because of infection. However, only six of the TIL were able to grow to a limited extent (4 - 12 weeks) in the presence of rIL-2 alone, and with very low increasing fold of 2-100 (mean 10). Hot thymidine uptake showed that proliferation of TIL stimulated with oxidized PBL were significantly higher than those stimulated with anti-CD3 antibody, lectin or TIL alone (Table 1). The cell
numbers were remarkably increased after stimulation of oxidized PBL. These TIL could be expanded for more than 4 months (Fig. 1) and sustain their specific proliferative response (Fig. 2) or specific cytotoxicity (Fig. 3).

The phenotypes of expanded TIL were analyzed and some were found to express predominantly CD4+ T cells while the others were CD8+ T cells, and the CD 45RA expression was comparable before and after in vitro expansion. No significant phenotypic shifting of CD3, CD4, CD8 and CD16 + CD56 could be detected with the exceptions of higher expression of CD25-marker which is associated with the activation of T cells (Table 2).

Table 2. Phenotypic comparison of oxidized-PBL-stimulated TIL growth with standard culture conditions.

<table>
<thead>
<tr>
<th>No.</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD16+56</th>
<th>HLA-DR</th>
<th>CD25</th>
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<tbody>
<tr>
<td></td>
<td>SCa</td>
<td>MCb</td>
<td>SC</td>
<td>MC</td>
<td>SC</td>
<td>MC</td>
</tr>
<tr>
<td>51</td>
<td>73c</td>
<td>89</td>
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<td>74</td>
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<td>35</td>
<td>33</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.5</td>
<td>p &gt; 0.5</td>
<td>p &gt; 0.5</td>
<td>p &gt; 0.5</td>
<td>p &gt; 0.5</td>
<td>p &lt; 0.5</td>
</tr>
</tbody>
</table>

aSC = Standard culture
bMC = Modified TIL culture stimulated with oxidized PBL
cPercentage of TIL surface marker expression
dND = Not detected
The expanded TIL were tested periodically for cytolytic activity against
various target cells including autologous and allogeneic tumor cells, oxidized-
allogeneic PBL, the NK-sensitive K562 and NK-resistant Daudi cell lines.
About 83% of the TIL cultures containing CD8+ T cells exhibited cytolytic
activity against autologous tumor cells, and none of CD4+ dominated TIL
culture showed cytolysis towards autologous tumor targets. A representative
experiment is illustrated in Fig. 3 indicating the potent cytolytic activity of TIL
from a breast adenocarcinoma. No cytolysis towards oxidized-PBL was
observed. Lysis of autologous tumor cells ranged from 15.2% to 47.8% at an
effector:target ratio of 50:1.

Figure 2. Autologous tumor (auto-tu) cell specificity after *in vitro* stimulation with
oxidized-PBL. No proliferative response was exhibited when allogeneic tumor (allo-
tu) cells were added to TIL sample A and TIL sample B.
Figure 3. Cytotoxic activity of TIL expanded with the stimulation of oxidized-PBL. Autologous tumor targets were lysed by TIL. TIL failed to lyse the allogeneic tumors with the same or different histology and oxidized-PBL, although they lysed K562 and Daudi tumor cells.

The difficulties of expanding TIL to large amounts in vitro using IL-2 alone, caused by lacking of specific stimulation of tumor antigen and autologous antigen presenting cells which were needed for the classical T cell activation and proliferation, have slowed down the development and further evaluation of the immunotherapy in a clinical stage. The low response to polyclonal T cell
activators like mitogenic lectins or antibodies could hardly solve the problems either. However, surface-oxidation by galactose oxidase to modify the stimulator cells has provided an alternative way to stimulate T cells by giving the short triggering signal to the responder T cells via the short reaction of the surface aldehydes (15). TIL stimulated in this way could be expanded to a sufficient amounts without losing auto-tumor reactivity.

In summary, we have demonstrated that TIL can be propagated in vitro with the stimulation of oxidized PBL and rIL-2 and expanded to a sufficient amount required for clinical therapy. The use of surface-oxidized stimulator cells provides a useful tool to facilitate large scale in vitro expansion of TIL with obvious advantages. It precludes the need for a specific tumor antigen which is largely undefined and the use of HLA-matched antigen presenting cells. It increases the efficiency of large scale expansion of TIL for the therapeutic use. Importantly, TIL expanded by this method can sustain their specificity and functional properties.
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Chapter 3

Phenotypic Analysis of Tumor-infiltrating Lymphocytes from Human Breast Cancer
Abstract

Suspensions of fresh tumor-infiltrating lymphocytes (TIL) were prepared from 31 human breast ductal adenocarcinomas. To evaluate the phenotypic pattern of the isolated TIL, lymphocyte surface markers including CD19, CD3, CD4, CD8, CD16 and HLA-DR were examined by flow cytometry. Lymphocyte recovery ranged from 1.1% to 44% was independent of tumor size. TIL most often scored high for CD3+ with varying number of CD4+ and CD8+ cells. 3 out of 31 samples expressed up to 44% of CD19+ B cells while CD3-CD16+ NK cells were scarce. CD4 and CD8 expression was significantly different between the lymph node metastases patient group and the lymph node negative group (p < 0.01). 67% of the TIL with a CD4/CD8 ratio greater than 1 showed lymph node metastases. Furthermore, the CD4 expression of TIL and CD4/CD8 ratio correlated with tumor size (p < 0.01), but not with tumor differentiation and hormone receptor expressions. Although there was considerable diversity of TIL among breast tumors, our data suggest that a high expression of CD4+ T cells may imply progression of the tumor, and an increased CD4/CD8 ratio of the TIL isolated from human breast adenocarcinoma may indicate development of metastases.
Introduction

Lymphocytes infiltrating tumors are believed to represent a marker of the host immune response to neoplastic growth (1, 2). Although the exact role of the infiltrating lymphocytes is not fully understood, an abundant lymphocytic infiltration is often associated with a less aggressive tumor behavior (3). Studies in experimental animals have shown that the adoptive transfer of TIL was 50-100 times more potent than LAK cells in mediating tumor regression (4). Preliminary studies on humans suggested that 50% of the patients had a major response (5-8). Thus, tumor-infiltrating lymphocytes are potentially promising for adoptive immunotherapy. To understand the role played by TIL in the host anti-tumor immunological response, several attempts have been made to analyze and characterize these tumor-infiltrating lymphocytes (9-15). An important observation was that the composition of the infiltrated lymphocytes was rather heterogeneous including helper, suppressor and cytotoxic T cells, B cells, natural killer cells and macrophages as well. Most of the infiltrating cells were CD3+ T cells with variable number of CD4+ and CD8+ cells (16-24). In most of the tumors, no B cells were found and natural killer cells constituted only a small minority of TIL.

However, each histological type of human cancer has its own set of biological properties and natural history and the immune response towards tumor could be variable among different patients. In the present study attempts were made to determine the phenotypic patterns of TIL in human breast adenocarcinoma and to evaluate whether these patterns were correlated with clinical data.

Materials and Methods

Patients and Tumor tissues

Fresh tumor tissues were obtained from 31 patients with breast ductal adenocarcinoma, aged 23-79, who were undergoing therapeutic surgery. 15 of
the patients were found to have a metastases in the lymph nodes. The tumor tissues used for this study were examined by the pathologists and immediately placed in RPMI 1640 medium containing 50 μg/ml of Gentamicin and 250 ng/ml of Fungizon (Gibco, Life Technologies, Belgium). Hormone receptor analysis was performed with the dextran-coated charcoal (DCC) method.

**Preparation of single cell suspensions**

Tumorous tissues about 5 mm³ were minced into small fragments and further disassociated with an enzymatic solution containing hyaluronidase type V 0.01% (Sigma), collagenase type IV 0.1% (Sigma), DNase type I 0.002% (Gibco), gentamicin 50 μg/ml and fungizone 250 ng/ml dissolved in RPMI 1640 medium (Gibco). The mixtures were incubated for 2-4 hours at 37°C or incubated overnight at room temperature. They were subsequently filtered through a sterile coarse wire grid and washed two times with RPMI 1640 medium. The recovered cells were checked for viability with trypan blue and counted.

**Monoclonal antibodies**

Monoclonal antibodies used for flow cytometric analysis were purchased from Becton Dickinson (Belgium). These included monoclonal antibodies against CD45, CD3, CD4, CD8, CD19, CD16 and HLA-DR as shown in Table 1. An irrelevant antibody control (goat anti-mouse IgG1-FITC and goat anti-mouse IgG2a-PE) was utilized to control for nonspecific binding.

**Flow cytometric analysis**

Monoclonal antibody to each surface marker conjugated with phycoerythrin were incubated with cells at a concentration of 2 x 10⁵ cells/ml and monoclonal antibody to CD45 conjugated with fluorescein for 30 min at 4°C and then washed two times in phosphate-buffered saline containing 2% fetal calf serum and 0.1% sodium azide. Two-color cytometry was performed on a FACScan (Becton Dickinson, Belgium) utilizing an argon ion laser (Cyonics) with
15 mW of 488 nm excitation. Triggering was set on the forward scatter channel and the threshold adjusted to exclude debris. $10^4$ events acquired for lymphocytes and tumor cells were measured for each suspension. Leucogate which stains lymphocytes, mononuclearcytes and neutrophils differentially was used to measure the proportion of lymphocytes in the sample being studied without any scatter gates. Then, the gate was set around the lymphocytes (CD45$^+$CD14$^-$) to exclude tumor cells from further analysis of the tumor sample and results were reported as a percentage of antibody positive cells of total lymphocytes in suspension corrected for nonspecific binding by antibody controls.

Table 1. Monoclonal antibodies used in the study

<table>
<thead>
<tr>
<th>Simultest</th>
<th>Main cellular reactivity</th>
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<tbody>
<tr>
<td>IgG1-FITC + IgG2a-PE</td>
<td>Control</td>
</tr>
<tr>
<td>Leucogate (anti-CD45-FITC + anti-CD14-PE)</td>
<td>Lymphocytes, mononuclearcytes and neutrophils</td>
</tr>
<tr>
<td>Anti-CD45-FITC + anti-CD3-PE</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>Anti-CD45-FITC + anti-CD19-PE</td>
<td>B lymphocytes</td>
</tr>
<tr>
<td>Anti-CD45-FITC + anti-CD16-PE</td>
<td>NK cells</td>
</tr>
<tr>
<td>Anti-CD45-FITC + anti-CD4-PE</td>
<td>T helper/inducer cells</td>
</tr>
<tr>
<td>Anti-CD45-FITC + anti-CD8-PE</td>
<td>T cytotoxic/suppressor cells</td>
</tr>
<tr>
<td>Anti-CD45-FITC + anti-HLA-DR-PE</td>
<td>Activated T cells and B cells</td>
</tr>
</tbody>
</table>

Statistical analysis

The patients were divided into two groups according to their lymph node metastatic status. The statistical analysis was carried out by data analysis software Minitab 7.2 (Minitab, Inc.). Two-tailed student’s t-test, Mann-Whitney U test and ANOVA were applied in the data analysis.
Table 2. Clinical and Pathological Findings of the Patients

<table>
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<th>L.N. C</th>
<th>Age</th>
<th>Tumor size (cm)</th>
<th>WHO&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ER/PgR&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>54</td>
<td>I</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
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<td></td>
<td>16</td>
<td>66</td>
<td>II</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>WHO classification  
<sup>b</sup>Estrogen receptor and Progesterone receptor  
<sup>c</sup>Lymph node spread  
<sup>d</sup>Not detected
Results

Tumor-infiltrating lymphocytes were isolated from 31 breast cancer patients and evaluated for their TIL composition using monoclonal antibodies with two-color cytometry. Patients were divided into two groups according to their lymph node metastatic status. Clinical and pathological findings listed in Table 2 showed no significant differences in age, tumor size, WHO classification and hormone receptor concentrations between the two groups.

Flow cytometry of TIL suspensions showed a low recovery of lymphocytes from breast cancer with an average of 8.2% ranging from 1.1% to 44% as estimated by CD45+CD14+ expression. The TIL isolated were composed of CD3+ T cells (mean 41%) with varying amounts of CD4+ and CD8+ cells. B lymphocytes (CD19+) were found in 27% of the breast cancers evaluated and mostly in early breast cancer, while CD3+CD16+ natural killer cell marker were expressed in less than 5% of the tumors. HLA-DR+ cells were usually elevated (mean 23%) in most of the tumors examined.

Statistical analysis of pathological findings and flow cytometry results revealed differences between the lymph node metastases group and the lymph node negative group with respect to CD4 and CD8 expression on T cells (p < 0.05) as showed in Table 3. The CD4/CD8 ratio ranged from 0.2 to 2.1 with an average of 1.2 and it was significantly higher in lymph node metastases group than in lymph node negative group (p < 0.01). No difference was observed between these two groups regarding to CD3, CD19, CD16 and HLA-DR expression (p > 0.05) as shown in Table 3. Both the CD4 expression on T cells and the CD4/CD8 ratio were correlated with the size of the tumor. In large tumors most often a higher percentage of CD4+ TIL and increased ratios of CD4/CD8 were noticed as compared to smaller tumors (Fig. 1, 2).
Figure 1. Comparison of CD4+ T cells of tumor-infiltrating lymphocytes versus diameter of the tumors ($r = 0.54$, $p < 0.01$).

Figure 2. Comparison of CD4/CD8 ratio of T cells from tumor-infiltrating lymphocytes versus diameter of the tumors ($r = 0.75$, $p < 0.01$).
Table 3. Flow cytometry analysis of TIL surface marker expression (%).

<table>
<thead>
<tr>
<th>CD</th>
<th>L.N. (+)(^a)</th>
<th>L.N. (-)(^b)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>41 ± 21</td>
<td>32 ± 26</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>CD4</td>
<td>29 ± 14</td>
<td>15 ± 14</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>CD8</td>
<td>25 ± 13</td>
<td>15 ± 14</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>CD19</td>
<td>15 ± 12</td>
<td>9 ± 14</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>CD16</td>
<td>5 ± 4</td>
<td>3 ± 2</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>25 ± 12</td>
<td>22 ± 20</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

\(^a\) Lymph node metastatic
\(^b\) Lymph node negative

Discussion

The phenotypes of tumor-infiltrating lymphocytes from human breast carcinomas have been characterized by flow cytometric analysis. In good agreement with the findings of others (18, 19, 25), the considerable proportion are T cells and more of these are of the CD3\(^+\)CD4\(^+\) subset than CD3\(^+\)CD8\(^+\) subset. Only few CD3\(^-\)CD16\(^+\) NK cells are found. Unlike the TIL patterns from other origins (10-18, 20, 21), CD19\(^+\) B cell population is remarkably elevated up to 44% in breast tumors. 27% of the TIL samples taken at different stages contained more than 10% CD19\(^+\) B cells. The function of these infiltrated B cells is not known. It is unclear if these B cells contribute to the antibody response and by this token to the rejection of tumor.

TIL are supposed to mediate tumor rejection and their cytocytic activity is probably mostly due to CD8\(^+\) as well as some CD4\(^+\) T cells. The CD4/CD8 ratios of tumor-infiltrating lymphocytes are quite variable and this variation could be due to several factors such as differences in tumor stage, location, and
some other factors, but it is also not unlikely that CD4/CD8 ratio could reveal anti-tumor reactivity in situ. The CD4/CD8 ratios of TIL from head and neck cancer appear to be related to cervical metastatic status (12). In the latter study 75% patients with a CD4/CD8 ratio of greater than 1 developed cervical metastases and 83% exhibited extracapsular spread. In our study, an increased CD4/CD8 ratio was significantly associated with lymph node metastases and correlated with the size of the tumors. The flow cytometry patterns of TIL from breast cancer were in good agreement with pathological findings. An increased CD4/CD8 ratio with decreased CD8$^+$ T cells might reflect the deficiency of a local immune reactivity. It is possible that a local deficiency of CD8$^+$ cytotoxic T cells may lead to an impaired local immune response and tumor progression.

Metastatic lymph nodes are usually present in late stages of breast cancer patients and it is a sign of poor prognosis. Our present study shows a high expression of CD4$^+$ lymphocytes and elevated CD4/CD8 ratios in breast cancer with lymph node metastases. Since the CD4 expression and CD4/CD8 ratios of tumor-infiltrated lymphocytes can be detected in early stages, they could imply the risk for regional metastases earlier before lymph node metastases can be detected. Thus, they could be useful prognostic indicators and be helpful in considering the therapeutic strategies.
References


Chapter 4

Characterization of Tumor Infiltrating Lymphocytes in Human Breast Cancer
Abstract

Tumor infiltrating lymphocytes (TIL) were isolated from 89 human breast cancer tissues and were analyzed for their surface markers. Monoclonal antibodies against CD19, CD3, CD4, CD8, CD16, HLA-DR were used to evaluate the expression of lymphocyte surface markers by flow cytometry. Lymphocyte recovery from tumor tissues ranged from 0.6% to 52.6% (average 10.7%) and did not correlate with the size of the tumor. A heterogeneous pattern of TIL population was observed among 69 TIL suspensions examined, containing different amount of CD3+ T cells with a varying number of CD4+, CD8+ subsets. An increased CD4 expression with decreased CD8+ cells was noticed when tumor size increases (p < 0.05). Most of the suspensions contained CD19+ B cells. CD16 NK cell marker was expressed in well-differentiated tumors and reciprocally correlated with tumor size (r = 0.2, p = 0.01). Estrogen receptor positive samples usually contained more NK cells and less CD4+ cells than the receptor negative samples (p < 0.05). The amount of CD8+ T cells was higher in progesterone receptor positive samples than that in progesterone receptor negative samples (p < 0.05). Our finding suggests that antigen-specific CD8+ T cells and natural killer cells might play an important role in anti-tumor immune response in early breast cancer and progression of tumor might be related to the immune deficiency of antigen-specific T cells and non-specific NK cells.
Introduction

The presence of lymphocyte infiltrates within many malignant tumors has been argued as indicative of an in vivo anti-tumor response (1). Cytolysis of autologous tumor cells by in vitro cultured tumor infiltrating lymphocytes in interleukin 2 (IL-2) has been described (2-5). The ability to proliferate lymphocytes in vitro with IL-2 has given rise to studies in cellular immunotherapy of cancer. Infusion of tumor infiltrating lymphocytes has shown powerful effect in killing tumor targets in animal models (2). In human melanomas, TIL were associated with a response of 55% (3-6). Thus, studies of phenotypic patterns and functional properties of TIL have attracted the interest of many cancer researchers. Many efforts have been made to characterize these tumor-infiltrated lymphocytes and to better understand their role in anti-tumor activity (7-12).

Till now, breast cancer is most often treated by surgery, radiotherapy, hormone therapy and chemotherapy. The results of systemic treatments for metastatic disease are still far from encouraging. Therefore, great efforts are made in the search for new effective therapeutic approaches. Immunotherapy is one of the possibilities. However, little has been known about the characteristics of TIL in breast cancer. To better understand the properties and the possible role of infiltrating lymphocytes in breast cancer and thus to use them efficiently in immunotherapy, attempts were made to analyze these infiltrating lymphocytes and to evaluate their possible relationship with pathological grade and clinical findings.

Materials and Methods

Patients and clinical pathological findings

Tumor samples were obtained from 89 breast cancer patients aged from 19 to 82. Of the 69 samples suitable for analysis, 5 were ductular adenocarcinomas in situ, 5 minimum invasive adenocarcinomas, 4 distant
metastases, 51 small and large invasive ductular adenocarcinomas and 4 other primary carcinomas, including 3 lobular and one mucinous carcinomas. 22 out of the 69 patients had lymph node metastases. Fresh tumor tissues were transported in RPMI medium to our laboratory immediately after operation. Estrogen and progesterone receptors were determined with a DCC biochemical assay.

Preparation of single-cell suspension

Single-cell suspensions were prepared using the enzymatic digestion method as described previously (13). Briefly, dissected tumor fragments were incubated overnight at room temperature with Collagenase IV (0.1%), Hyaluronidase (Sigma) (0.01%), DNase I (0.002%), Gentamicin (50 μg/ml) and Fungizone (Gibco, Life Technologies, Belgium) (250 ng/ml) dissolved in RPMI 1640 medium. The suspension was subsequently filtered through a coarse wire grid, washed three times, stained with trypan blue to evaluate the viable cells.

Antibody staining

Fresh TIL were suspended in 100 μl of phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.1% sodium azide. Mouse monoclonal antibodies to CD3, CD4, CD16, CD19 and HLA-DR conjugated with phycoerythrin (Becton-Dickinson, Erembodegem, Belgium) were incubated for 30 min at 4°C with cell suspension together with mouse monoclonal antibody to CD45 conjugated with FITC which was used to distinguish leucocytes from tumor cells. Antibody-stained cells were washed twice and ready for flow cytometric analysis.

Flow cytometric analysis

Flow cytometry was performed on FAScan (Becton-Dickinson) using an argon ion laser with 15 mW of 488 nm excitation. Triggering was set on the forward scatter channel and the threshold adjusted to exclude debris. 10⁴ events of lymphocytes and tumor cells were measured for each suspension.
Leucogate which stains lymphocytes, mononuclearcytes and neutrophils differentially was used to measure the proportion of lymphocytes in the sample without any scatter gate. Then, the gate was set around the lymphocytes to exclude tumor cells. The results were expressed as percentage of antibody positive-stained cells of the gated lymphocytes. Isotypic controls (goat anti-mouse IgG1-FITC and goat anti-mouse IgG2a-PE) were used to control for nonspecific binding.

**Statistical analysis**

The statistical analysis performed throughout this study was carried out by data analysis software Graph and Statwork. Student's t test and ANOVA were used in the data analysis.

**Results**

Single-cell suspensions were prepared from 89 breast tumor tissues. 20 samples were discarded due to fewer lymphocytes. The recovery of TIL from 69 suspensions ranged from 0.6% to 52.6% (mean of 10.7%) as estimated by CD45+CD14- molecule expression. Flow cytometric analysis was performed using mouse monoclonal antibodies to lymphocyte antigen (CD45), T cell antigen (CD3), helper/inducer T cell marker (CD4), cytotoxic/suppressor T cell marker (CD8), NK cell antigen (CD16), B cell antigen (CD19) and activated T cell and B cell marker (HLA-DR). CD3+ T cells are the major population of the TIL samples examined with varying amount of CD4+ and CD8+ T cells. Some of the cells were activated in vivo as evidenced by elevated HLA-DR expression. CD19+ B cell marker were also expressed in most of the samples but in a relatively low degree. CD16+ NK cells were usually found in small and well-differentiated tumors.

Statistical analysis of the expression of TIL surface markers and pathological findings revealed that CD4 expression of TIL increased with the size of the tumors \( r = 0.3, p < 0.05 \). A tendency of increased CD3+ T cells with
a decreased CD8 expression was noticed when tumor size increases (Fig. 1). The number of CD16+ NK cells were relatively high in small tumor samples and it declined when the tumor size increased ($r = 0.4$, $p = 0.01$). The expression of CD16 NK marker was significantly higher in well-differentiated tumors than in poor-differentiated tumors ($p < 0.01$) as showed in Fig. 2.

Figure 1. Correlation between TIL surface marker and the size of the tumors.
Figure 2. Phenotypic expression of cell surface antigens in different stage of WHO classification ($n = 16$ in I; $n = 22$ in II and $n = 15$ in III).

A high expression of CD16 NK cell marker with a low amount CD4+ T cells were observed in estrogen receptor positive tumor samples, while less NK cells and more CD4+ T cells were noticed in estrogen receptor negative samples (Fig. 3). The amount of CD8+ T cells was significantly higher in progesterone positive tumor samples than that in progesterone receptor negative samples ($p < 0.05$). No difference was seen between phenotypes of TIL population and lymph node metastatic status (Fig. 4).
Figure 3. Comparison of TIL populations with different estrogen receptor (ER) / Progesterone receptor (PR) status (n = 11 in ER+/PR+ group; n = 5 in ER-/PR- group and n = 5 in ER-/PR+ group).

Discussion

Phenotypic analysis of TIL in human breast cancer specimens shows a great variation with regard to both the type and the absolute and relative amounts of lymphocytes present. The tumor-infiltrating lymphocytes from breast cancers have shown to be heterogeneous composed of different kinds of immune cells. Like in melanoma, renal cell carcinoma, colon cancer and ovarian carcinoma, CD3+ T cells were a major proportion in breast cancer (7, 9, 12, 14-
CD4+ T cells predominated in most especially in large tumors and they usually increased with the size of tumors. This is in agreement with our preliminary study and others (7, 17), although Whitford et al has reported predominant CD8+ T cells presented in breast TIL (18). An increased tendency of CD3+ T cells in large tumor samples is probably contributed by elevated CD4+ subpopulation with a declined CD8+ cells (Fig. 1). As tumor rejection is mostly mediated by CD8+ cytolytic T cells, an increased CD4+ T cells with a decreased CD8+ T cells might reflect the deficiency of local immune reactivity and thus lead to tumor progression.

Figure 4. CD3, CD4, CD8 and CD19 expression of TIL from patients with and without lymph node metastases (n = 15 in pN-; n = 22 in pN+).

The presence of natural killer cells in small and well-differentiated breast cancer is pronounced in our study. Natural killer cells (NK) are
lymphocytes that possess unique and characteristic antigenic and functional properties (19). NK cells mediate non-MHC restricted cytolytic activity against virus-infected cells and a variety of cultured and fresh neoplastic cells (20). They comprise an important immune effector population that has been implicated in surveillance against tumor metastases and virally infected host cells, suppression of the humoral immune response, and regulation of hematopoiesis (21). The NK cell has distinct morphologic, phenotypic and ultrastructural properties that distinguish it from T and B lymphocytes (22). IL-2 activated NK cells (LAK) are highly effective in eliminating tumor cells both in vitro and in vivo in animal models of tumor metastases. In the phase I clinical trial LAK therapy also showed response in patients with metastatic melanoma and renal cell carcinoma. The NK cells participate either directly or indirectly in multiple developmental, regulatory networks of the immune system and thus is import in human health and disease (20-22). Decreased NK cell amounts in large and poor-differentiated tumors may suggest a deficiency of natural, spontaneous cytolytic activity in the progression of tumor. However, the exact role of NK cells played in anti-tumor response in early breast cancer is not clear, and the relationship between NK cell amount and progression of tumor is awaited to be defined.

Steroid hormones are thought to regulate the metabolism of hormonally responsive cells through the mediation of receptor proteins (23, 24). The expression of estrogen and progesterone receptors of human primary breast tumor are of great clinical value because they yield prognostic information and also because they may help to predict the response to endocrine therapy (25). Patients whose primary breast cancers are estrogen receptor positive have a longer disease-free interval and longer survival than those patients whose carcinomas lack this receptor (23). Since the synthesis of progesterone receptors appears to be augmented by estrogen, progesterone receptor values are used as additional evidence to indicate the existence or absence of an "operative" estrogen receptor system in mammary cancers (24). Our findings of elevated CD4+ T cells with declined CD8 expression in estrogen receptor negative tumors
and decreased NK cell amount in progesterone receptor negative samples may in some way be related to poor differentiation of tumors. The expression of CD4, CD8 and CD16 molecules in tumor infiltrating lymphocytes may be of clinical implication in assisting of prognosis of the disease and selection of endocrine therapy for breast cancer patients.

In our preliminary study of 31 breast cancer patients, we found that CD19+ B cell population in TIL was remarkably elevated (17). Phenotypic analysis of TIL obtained from 76 breast cancer has confirmed this observation. The recovery of CD19+ B cells from breast tumor was indeed higher than TIL from other origins (9, 12). This is similar to the findings of Balch et al (7). Accumulation of B cells in breast cancer may reflect an local anti-tumor antibody response. Antibodies may have important applications for host resistance and such antibodies may be directly lytic for tumor cells or recruit cells carrying Fc Receptor. It maybe interesting to evaluate the function of these B cells and thus to understand their role in local anti-tumor reactivity.

We observed also in our preliminary study difference between patients lymph node metastases and those without lymph node metastases regarding to CD4 antigen expression. Higher amount of CD4+ cells were usually found in lymph node metastases patients than those nodular negative ones (17). Statistical analysis in present large sample scale study showed no significant difference between lymph node status of the patients and particular lymphocyte surface antigen expression.

In summary, tumor infiltrating lymphocytes in human breast cancer are composed of heterogeneous populations. A decreased CD8+ T cells and NK cells in large tumors may reflect an deficiency of local cytolytic reactivity against tumor cells, and increased CD4+ T cells in poor-differentiated large tumors might be related to the progression of tumor. Elevated CD4 T cell antigen expression in estrogen receptor-negative tumors and declined NK cell amount in progesterone receptor-negative tumors may be of clinical implication in predicting endocrine therapy response and prognosis of the patients.
References


Chapter 5

In Vivo Distribution of Radio-labeled Tumor Infiltrating Lymphocytes in Cancer Patients
Abstract

Tumor-infiltrating lymphocytes appear to be effective in murine tumor models and preliminary clinical trials. To evaluate the efficacy of these lymphocytes in therapy, attempts were made to study the in vivo migration and distribution of these lymphocytes. Tumor-infiltrating lymphocytes isolated from five patients with malignant metastatic breast cancer or melanoma, cultured and expanded in vitro with low-dose of recombinant interleukin-2 were labeled with $^{111}$Indium-oxine and infused to the patients. A large view gamma camera was used to evaluate the distribution and localization of the infused TIL. Localization of $^{111}$In-labeled TIL in the lungs was seen within two hours after infusion and high levels of radioactivity were observed at 24 hours in lungs, liver and spleen. The activity in the lungs diminished after 72 hours. No specific localization of $^{111}$In-labeled TIL was observed in the metastatic sites.
Introduction

Tumor-infiltrating lymphocytes are immune cells which have proven to be effective in the treatment of metastatic cancer after transfer to the tumor-bearing host (1, 2). Animal models and preliminary clinical trials showed that adoptive immunotherapy with TIL was more effective than with lymphokine-activated killer (LAK) cells (3). Moreover, TIL often exhibited cytolytic activity towards autologous tumor cells in vitro. It is not clear if these cells can migrate to tumor-deposits in vivo and exert their anti-tumor efficacy. Attempts were made to study the in vivo distribution of TIL. Successful isolation of TIL from solid tumor tissues and expanding them to large amounts have facilitated the study (4). $^{111}$Indium-oxine was used as labeling isotope to monitor the in vivo traffic of TIL because its advantages as compared to other radio isotopes (5-11). Five patients were selected as they met the criteria set for the study.

Materials and Methods

Patients

Patients with at least two metastatic lesions were eligible for the study. One metastatic lesion was used to harvest TIL, and the other(s) were used to evaluate the possible radioactive uptake in vivo. The protocol used was approved by the Ethical Committee and written informed consents were obtained from patient before they entered the study.

Preparation of TIL

Tumors excised from patients were transported in sterile conditions from the hospitals to the laboratory. They were minced into 1-2 mm$^3$ fragments and further disassociated with a solution containing hyaluronidase type V 0.01%, collagenase type IV 0.1% (Sigma), DNase type I 0.002%, gentamicin 50 µg/ml and fungizone 250 ng/ml dissolved in RPMI 1640 medium (Gibco, Life
technologies). The mixture was incubated overnight at room temperature. The suspensions were filtered through a sterile coarse wire grid, washed three times with RPMI 1640 medium, and resuspended in culture medium. TIL were cultured in 24-well plate (Costar) at 2 ml/well of RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μg/ml gentamicin, 250 ng/ml fungizone (Gibco, Life Technologies), and 10 mM Hepes buffer (Flow Laboratories, Belgium). Recombinant IL-2 was added at a final concentration of 200 IU/ml. The medium was refreshed every 4 days for the first three weeks. When tumor cells were gradually disappearing, the cultures were stimulated with oxidized-allogeneic PBL (4) on a weekly basis. The expanded cells were fed with fresh medium containing rIL-2 and split or transferred to 12-well, 6-well plates or culture flasks (Costar).

**Phenotype Analysis**

TIL (5 x 10⁵) were washed and resuspended in 100 μl of cold phosphate-buffered saline (PBS) containing 5% fetal calf serum and 0.1% sodium azide. The cells were incubated on ice for 30 min with fluorescein- or phycoerythrin-conjugated monoclonal antibodies to lymphocyte surface antigens (Becton-Dickinson, Belgium). The antibodies used were: anti-CD45 (leucocytes) and CD45RA (virgin cells), anti-CD3 (T-cells), anti-CD16 + 56 (NK and some activated cytotoxic cells), anti-CD4 (T helper/inducer cells), anti-CD8 (T cytotoxic/suppressor cells), anti-HLA-DR (activated T cells) and anti-CD25 (activated T cells). Cells were washed twice after staining and analyzed immediately on FACScan (Becton-Dickinson).

**Radiolabeling of TIL with ¹¹¹Indium-oxine**

Labeling of TIL with ¹¹¹Indium oxine was performed according to Müller et al (11). Approximately 1-10 x 10⁸ cultured TIL were used for labeling. Cells collected from in vitro cultures were washed five times with phosphate-buffered saline (PBS) and resuspended in 20 ml of PBS. 100 - 200 μCi of ¹¹¹Indium-oxine (Amersham, England) was added to the cell suspension and
incubated for 15 minutes at room temperature without disturbing. The labeled cell suspensions were withdrawn into a syringe and transferred to another tube, and the residue containing any clumps of cells which have settled during incubation was discarded. The labeled cells were washed five times with normal saline containing 10% autologous plasma, and resuspended in 50 ml of normal saline. 1 x 10^6 IU of rIL-2 was added to the cell suspension before infusion. An aliquot of 1 ml labeled cell suspension was kept to evaluate the viability of labeled cell by trypan blue exclusion. All manipulations were carried out at room temperature in sterile conditions.

**Infusion of ¹¹¹Indium-labeled TIL**

All patients received a single intravenous (IV) infusion of Cyclophosphamide (CPM) (25 mg/kg) 24-36 hours before receiving TIL infusion. Starting from 4 hours before infusion of TIL, rIL-2 at 60,000IU/kg were administered IV every 8 hours for 48 hours. Labeled cells were infused over 10-20 minutes via peripheral IV.

**Gamma Scintigraphy**

We used a large field of view camera CLS750 (Siemens, Germany) equipped with a medium energy collimator. Gamma camera scintigraphy was usually carried out at 2-4 hours after infusion and then at day 1, day 2, day 3 and day 7. Scans were obtained from whole-body and regions of the anterior and posterior chest, abdomen, pelvis, and spots of selected sites which were known to be involved with tumor.

**Results**

**Characterization of the patients**

Three men and two women aged from 19 to 52 who had advanced breast cancer or melanoma with metastatic lesions were recruited for the
present study. The characteristics of the patients are listed in Table 1. All of the patients had undergone prior surgery and received chemotherapy and/or radiotherapy before receiving TIL infusion.

Table 1. Summary of the patients treated

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Sex</th>
<th>Tumor</th>
<th>Site of TIL Harvested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>F</td>
<td>Breast</td>
<td>Breast</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>F</td>
<td>Melanoma</td>
<td>Skin</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>M</td>
<td>Melanoma</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>M</td>
<td>Melanoma</td>
<td>Skin</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>M</td>
<td>Melanoma</td>
<td>Arm</td>
</tr>
</tbody>
</table>

In vitro TIL culture and phenotypic analysis

Infiltrating lymphocytes were isolated from enzyme-digested tumor tissues suspensions. The lymphocytes recovered were cultured in the presence of oxidized PBL stimulators and rIL-2. The phenotypes of TIL before and after culture were examined using monoclonal antibodies to cell surface antigens. Fresh TIL were usually heterogeneous, containing varying amount of CD3+ T cells, CD19+ B cells and CD16+ NK cells. After culture CD3+ T cells became the predominant population. Of the six TIL cultures, two of them contained a majority of CD3+CD8+ T cells while the other four were dominated by CD3+CD4+ cells. All cultured cells were activated as evidenced by the high expression of the HLA-DR surface molecule. NK cells were seldom encountered after culture (Table 2). When the number of cultured cells reached $10^8$ to $10^{11}$ after four to eight weeks, they were harvested and labeled with $^{111}$Indium-
oxine. The amount of cells and the dose of radioactivity used for labeling are indicated in Table 3.

Viability test of $^{111}$Indium-labeled TIL

A primary study was performed to examine the viability of TIL after $^{111}$Indium labeling. Figure 1 shows that more than 40% radio-labeled TIL could survive for 48 hours in the absence of rIL-2 as evaluated with Trypan Blue staining.

![Graph showing cell viability over time](Image)

Figure 1. The cell viability of TIL were examined with Trypan Blue after labeling with $^{111}$Indium-oxine.

Gamma camera scintigraphy

The first scintigraphic image, taken 2 hours after infusion, showed an initial uptake of radioactivity by the lungs. Most radioactivity was concentrated
in lungs, liver and spleen 24 hours post-infusion. By 48 hours post-infusion, the radioactivity decreased in lungs and liver, increased slightly in spleen. No specific localization of $^{111}$In-labeled TIL was observed at metastatic sites at no time after infusion.

Table 2. Phenotypic analysis of tumor-infiltrating lymphocytes before and after culture.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CD3 (%)</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>CD16 (%)</th>
<th>HLA-DR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60/99</td>
<td>28/98</td>
<td>38/3</td>
<td>1$^a$/0$^b$</td>
<td>52/95</td>
</tr>
<tr>
<td>2$^c$</td>
<td>15/88</td>
<td>32/72</td>
<td>11/19</td>
<td>0/0</td>
<td>39/67</td>
</tr>
<tr>
<td>2$^d$</td>
<td>ND/99</td>
<td>ND/29</td>
<td>ND/63</td>
<td>ND/2</td>
<td>ND/74</td>
</tr>
<tr>
<td>3</td>
<td>13/98</td>
<td>16/72</td>
<td>20/20</td>
<td>2/3</td>
<td>23/81</td>
</tr>
<tr>
<td>4</td>
<td>53/95</td>
<td>26/1</td>
<td>0/96</td>
<td>12/0</td>
<td>30/ND</td>
</tr>
<tr>
<td>5</td>
<td>ND/99</td>
<td>ND/20</td>
<td>ND/80</td>
<td>ND/0</td>
<td>ND/88</td>
</tr>
</tbody>
</table>

$^a$Surface marker expressed on fresh TIL
$^b$Surface marker expressed after in vitro expansion
$^c$First trial
$^d$Second trial
$^e$Not detected

Discussion

Six radioactive scans were performed on five patients with metastatic melanoma or breast carcinoma. $^{111}$Indium-oxine was used as labeling material because it can be efficiently incorporated by cells and bind tightly to cytoplasmic
proteins with little spontaneous release (6-13). Moreover, its gamma emissions are abundant and suitable for external detection and the half-life of 2.8 days is long enough for in vivo distribution, but short enough to minimize the radiation exposure to the patients (5, 14). A 100μCi - 200μCi of $^{111}$In labeled was used to label TIL in order to achieve a good image by the gamma camera but with least radiation-induced cellular damage (7, 15).

The distribution pattern of TIL in tumor-bearing patients is somewhat similar to that observed from normal lymphocytes in normal human subjects (6, 7, 13, 16, 17). $^{111}$In-labeled normal lymphocytes demonstrated a significant uptake by lungs, liver and spleen two hours after injection. Liver and spleen uptake remained constant up to 96 hours while radioactivity in the lungs decreased at about 24 hours. Lymph nodes showed a progressive uptake after 24 to 48 hours. $^{111}$In-labeled TIL showed a similar pattern of distribution in lungs, liver and spleen, but the uptake of TIL by the lungs was prolonged to 24-48 hours. However no uptake by lymph nodes was observed throughout the imaging. There was no visible accumulation of radioactivity at lesions with known metastases.

Table 3. **Labeling parameters of TIL**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Total amount of TIL</th>
<th>Total amount $^{111}$In-TIL</th>
<th>Activity (μCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$2.4 \times 10^9$</td>
<td>$2.4 \times 10^9$</td>
<td>200</td>
</tr>
<tr>
<td>2a</td>
<td>$1.0 \times 10^9$</td>
<td>$1.0 \times 10^9$</td>
<td>200</td>
</tr>
<tr>
<td>2b</td>
<td>$1.5 \times 10^{10}$</td>
<td>$1.0 \times 10^8$</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>$5.0 \times 10^8$</td>
<td>$2.5 \times 10^8$</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>$2.0 \times 10^8$</td>
<td>$1.0 \times 10^8$</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>$1.0 \times 10^8$</td>
<td>$1.0 \times 10^8$</td>
<td>100</td>
</tr>
</tbody>
</table>

*a*First trial  
*b*Second trial.
Several factors might have impaired the tumor localization of $^{111}$In-labeled TIL in our study such as the effect of prior treatment, the size and the site of the tumor, ongoing tumor regression, quantity of rIL-2 given and tumor-specificity of the TIL infused. Findings obtained from other groups (16, 18) suggest that TIL localization to tumor sites does not simply depend on altered vascularity of tumor sites or on nonspecific homing of lymphoid cells to the tumor. Homing and migration appear to be complex processes dependent on many factors such as vascularity, cellular activation status, cell type and subtype and pretreatment of the host with CPM (19-23). In addition, in vitro expanded TIL usually contain a considerable amount of large activated lymphocytes. These large activated lymphocytes might lose their ability to traffic to the tumor sites because it is possible that they are promptly removed from the circulation by the reticuloendothelial system and therefore might be retained by lungs, liver and spleen as demonstrated by Lotze et al (24) and Mazumder et al (25). Cell damaging during labeling and infusion might be responsible for the preferential migration to liver and spleen as well(7, 13). Furthermore it is not unlikely that the specific localization of TIL in lung tissue was concealed by the nonspecific traffic of TIL since three of the five patients have metastases in the lungs.

The infused cell type and subtype infused may also play an important role in the selective migration of TIL to tumor. TIL are composed of heterogeneous cells including T cells, B cells, NK cells and other mononuclearcytes (26-28). After in vitro culture in the presence of a low dose of rIL-2, CD3+ T cells become a major population with varying amount of CD4+ and CD8+ subpopulations, but the frequency of autologous tumor-specific T cell precursors among these CD3+ T cells is rather low (29-33). The "dilution effect" of the non-specific TIL populations on tumor-specific cells could result in a nonspecific traffic of TIL to lungs, liver and spleen rather than to tumor sites. If this is the case, cloning and expanding of the tumor-specific T cells might be an alternative way to increase the specific localization and anti-tumor efficacy of TIL thus to increase the therapeutic value of TIL in immunotherapy.
References


Chapter 6

Clonal Analysis of Tumor Infiltrating T Lymphocytes from Human Breast Cancer
Abstract

51 T cell lines/clones were established from tumor-infiltrating lymphocytes of nine breast tumors by limiting dilution. All the lines/clones were exclusively CD8+ and expressed either CD4 (57%) or CD8 (26%) phenotype. In addition, 17% of the lines/clones displayed a dual expression of CD4+CD8+ antigens. No CD3-CD16+ NK clones were obtained. A vast majority of the T cell lines and clones (84%) exhibited cytolytic activity in a lectin-dependent assay which allows the detection of cytolytic T cells of any antigen specificity. 17% of the lines/clones lysed two allogeneic breast tumor cell lines, MCF-7 and HBL-100. 11% of the cells showed NK-like cytolysis by lysing an NK-sensitive cell line K562. Of the 17 lines tested against autologous tumor cells, only two exhibited cytolytic activity via T cell receptor and CD3 molecule in an MHC-restricted manner. Southern blot analysis of T cell receptor of 35 lines/clones revealed a limited heterogeneity of TCR-β chain gene rearrangements, which suggests oligoclonal expansion of T cells infiltrating into the tumor.
Introduction

Tumor infiltrating lymphocytes (TIL) may represent an attempt of the tumor-bearing host to develop an immune attack against a particular tumor. These TIL consisted of heterogeneous populations of diverse effector and immunoregulatory lymphocytes and mononuclear cells (1-3). Recent evidence obtained in animal studies has shown that the adoptive transfer of TIL into the tumor-bearing host is able to mediate significant anti-tumor effects or even to induce total tumor regression (4). In humans, however, clinical investigations revealed that administration of TIL induces anti-tumor responses in some patients, but the number of patients who benefit from this immunotherapy is rather low (5-7). The failure to achieve a complete response has been suggested to be related to the relatively low proportions of specific effector cells in the TIL preparation, to possible suppressive factors produced by regulatory cells present in TIL, or to tumor cell-surface modulation which enables tumor cells to escape from the immune attack (8, 9). To improve the efficacy of this immunotherapy attempts have been made to search for effective tumor-killers among the TIL (10-15). Limiting dilution and microculture system have been applied to establish T cell clones and lines from TIL and the frequency of tumor-specific T cell precursors has been analyzed (16-18). A number of studies have revealed the existence of specific anti-tumor cytolytic effector cells among human melanoma and ovarian carcinoma ascites (19-22). Tumor antigens recognized by autologous cytolytic T cell clones were defined by immunoselection and biotechnological techniques (23-25). However, little is known as to whether such autologous tumor-specific cytolytic cells are present in breast cancer. The analysis of the subtypes of TIL populations and their function may help to understand the cellular and molecular mechanisms involved in the lymphocytes-breast tumor interactions and may help to improve the cellular immunotherapy of breast cancer.
Materials and Methods

TIL preparation and cloning

Tumor tissues excised from breast cancer patients were mechanically and enzymatically dissociated. Twenty to two hundred TIL lymphocytes recovered from the suspensions were seeded in 96-well microtiter plates (Costar) with $10^5$ irradiated oxidized stimulator cells as described elsewhere (26) and 2 x $10^3$ autologous tumor cells in 200 μl culture medium containing 100 IU/ml rIL2 (Eurocetus). The cells were restimulated every 10 days, with autologous tumor cells and rIL2. After three to four cycles, microcultures positive for cell growth were expanded in RPMI1640 medium containing 10% fetal calf serum, 100 IU/ml rIL2. The medium was refreshed on a weekly basis or whenever it was necessary. TIL concentration was maintained in 2 x $10^6$/ml. All lines were expanded to at least $10^7$ cells for further studies.

Phenotypic analysis of cell surface markers

5 x $10^5$ cells were suspended in 100 μl of ice-cold phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.1% NaN₃. Mouse monoclonal antibodies against T cell surface markers conjugated with FITC or PE were added to the cell suspension, and incubated for 30 min at 4°C. The surface markers included CD3, CD4, CD8, CD16, CD56, HLA-DR, TcR-αβ and TcR-γδ (Becton Dickinson, Erembodegem, Belgium). Cells were subsequently washed twice and measured on a FACSscan (Becton Dickinson, Belgium).

Cytotoxicity assay

Cytotoxic activity was evaluated in a conventional 4-hr $^{51}$Cr-release assay. Target cells were labeled with 200 μCi $^{51}$Cr (Amersham) for 60 min at 37°C and then washed 4 times with medium. For autologous target cells, another 30 min incubation at 37°C and two more times washing were processed. Cytolytic activity was tested against following target cells: the NK-sensitive cell line K562; the murine cell line P815 in the presence of PHA at 1
μg/ml (in a lectin-dependent cytolytic assay); the breast cancer cell lines MCF7 and HBL100, and cryopreserved autologous tumor cells of two patients. Effector cells were incubated with $^{51}$Cr-labeled target cells ($4 \times 10^3$/well) in 200 μl microwells for 4 hours at 37°C at several effector to target ratios. The maximum release and spontaneous release of chromium were measured in wells containing target cells and either detergent or medium only. The specific release was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{exp. release} - \text{spon. release}}{\text{max. release} - \text{spon. release}} \times 100$$

**Inhibition of cytotoxic activity**

Monoclonal antibodies specific for human T cell surface markers CD3, T-cell receptor (WT31, Becton-Dickinson) and MHC class I antigens (W6/32) were incubated first with effector cells or target cells (W6/32) for at least 1 hour at 37°C. The cytolysis activity was then tested as described above. The specific inhibition was calculated as:

$$\% \text{ Inhibition} = 1 - \frac{\text{specific cytolysis in the presence of the antibodies}}{\text{specific cytolysis in the absence of the antibodies}} \times 100$$

**DNA preparation and Southern blot analysis**

The DNA from $2-3 \times 10^7$ cells was extracted after cell lysis and deproteination overnight at 37°C in lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM EDTA; pH 8.2) containing 0.6% sodium dodecyl sulfate (SDS) and 0.5 mg/ml proteinase K. Saturated NaCl was used to remove protein impurities.
before purification of DNA with chloroform. DNA was precipitated in absolute ethanol, washed with 70% ethanol and digested to completion with the restriction endonucleases EcoRI (to demonstrate rearrangements to Cβ1) and HindIII (to demonstrate rearrangements to Cβ2) at 37°C. The digested DNA was electrophoresed through a 0.8% agarose gel and then transferred to nylon membranes (Bio-Rad, Belgium) using 5 inches of Hg for 90 min in 10 x SSC (1.5 mol/L NaCl, 0.15 mol/L sodium citrate) with a Vacuum Blotter (Bio-Rad). Membranes were neutralized in 2 x SSC, fixed and dried with a vacuum Gel Dryer (Bio-Rad) at 80°C for 30 min. The blots were prehybridized for 10 min at 65°C in hybridization buffer (0.25 M Na₂HPO₄ pH 7.2, 7% SDS) containing

![Graph showing percentages of antibody positive cells for CD3+, CD4+, CD8+, HLA-DR+, CD25+ and CD16+ cells.](image)

**Cell surface markers**

*Figure 1. Phenotypic pattern of freshly isolated TIL from breast tumor.*
denatured herring sperm DNA 100 \mu g/ml. A monocytic cell line U937 was used as the germ line. Hybridization of the blots with an [\alpha-^{32}P]-labeled TcR-\beta probe was carried out in hybridization buffer overnight at 65°C. Autoradiography was performed by exposing the blots to Hyperfilm (Amersham) at -70° in Cusing intensifying screens. The TcR-\beta probe used was the HPB 32 probe (27).

Figure 2. Cytolytic activities of the lines/clones in a lectin-dependent cytotoxicity assay (LDCC) on P815 and on NK-sensitive cell line-K562.
Figure 3. Specific cytotoxic activity of TIL line YJSA2 against autologous tumor (auto-tu), NK-sensitive cell line-K562 and allogeneic (allo-tu) target cells at different effector to target ratios.

Results

Phenotypic analysis of fresh TIL

Freshly isolated TIL were stained with FITC- or PE-labeled monoclonal antibodies specific for leukocyte surface antigens and analyzed by flow cytometry. Figure 1 shows that most of the TIL preparations contained CD3+ T cells with elevated CD4+ and declined CD8+ subsets. Some of the TIL expressed HLA-DR antigen and CD25 antigen. CD3-CD16+ natural killer cells were ranged from 1 to 17% with an average of 7% of TIL obtained from breast tumors.
Cloning and characterization of tumor-infiltrating lymphocytes from breast cancer patients

51 T cell lines were generated from suspensions of nine breast tumor tissues using limiting dilution and microculture system. The phenotypic analysis of T cell lines obtained showed that all lines expressed CD3+ and TcR \( \alpha/\beta \) molecule reflecting the T cell lineage. 57% of the lines obtained were found to be CD4+ and 26% were CD8+. 17% of the lines contained CD4+CD8+ double positive cells. None of the lines were positively stained with an antibody to CD16, a NK cell surface marker. 35 lines were examined for cytolytic activity in a PHA-dependent assay which allows the detection of cytolytic T cells of any specificity and NK-like activity on a NK-sensitive cell line K562. 26 out of 35 lines exhibited cytolytic activity in the lectin-dependent assay and 8 lines were cytotoxic against K562 (Fig. 2). Most cell lines with NK-like activity expressed CD8+ phenotype and exerted cytolytic activity in LDCC assay. Among the lines which exhibited cytolytic activity in LDCC, some were CD8+ cells and some were CD4+ cells. All the LDCC negative lines were CD4+.

Detection of specific lysis and involvement of cell surface molecules

Autologous tumor cells and allogeneic breast cancer cell lines such as MCF7 and HBL100 were used to detect the specificity of the cytolytic lines obtained. As shown in Table 1, all of the CD4+ T cell lines failed to lysed the autologous and allogeneic target cells although they displayed cytotoxicity in lectin-dependent assay. 9 lines (17%) showed cytolytic activity to allogeneic breast tumor cells. Two CD8+ T cell lines exhibited specific lysis towards autologous tumor targets but not to allogeneic tumor cells (Fig. 3). Cytotoxicity against autologous tumor targets was inhibited by preincubating these two lines with monoclonal antibodies specific for T cell receptor and CD3 molecules or by preincubating the tumor cells with anti-MHC class I antigen monoclonal antibody (Figure 4).
Figure 4. Inhibition of lytic activity of CTL lines YJSA2 and YEBA3 by monoclonal antibodies: anti-CD3, anti-TcR and anti-MHC-I. Cytotoxic assays were performed at an effector to target ratio of 15:1.

Analysis of TCR β gene rearrangements of T cell lines/clones derived from TIL of breast tumors

The results obtained from 39 lines/clones derived from nine breast cancer patients revealed rearranged fragments with the TcR-β probe. 16 lines/clones (41%) showed rearrangements to TcR Cβ1 reflected in nongermline bands in the EcoRI digests, while 4 lines (10%) showed nongermline bands in HindIII digests indicating rearrangements to Cβ2. 12 lines/clones (31%) had rearrangements to both TcR Cβ1 and Cβ2. Five lines/clones lost 11-kb germline bands but no new rearranged bands in EcoRI, however, two of them had
rearrangements to Cβ2. One line showed germline pattern in both EcoRI (11-kb and 4-kb) and HindIII (8-kb, 6.5-kb and 3.5-kb) digests. 61% of the TIL lines established from different patients showed a band of 8.5-kb due to a partial digestion of EcoRI restriction site. Of the twenty-eight lines/clones with rearrangements to Cβ1, 9 lines (32%) had 6.5-kb dominant rearranged nongermline band in EcoRI digests (Figure 5). While among the 5 lines which showed 9.5-kb rearranged nongermline band in EcoRI, 3 were derived from the same individual (Figure 6). Of the 16 lines established from different patient tumor tissues with rearrangements to Cβ2, nongermline bands 11-kb and 5.5-kb bands were observed in 50% and 37.5% of the analyzed lines/clones respectively (Figure 7).

![EcoRI-digested bands (kb)](image)

**Figure 5.** EcoRI digests demonstrate rearrangements to Cβ1: Dominant nongermline rearranged 6.5- and 9.5-kb bands in addition to retained 4-kb germline band. A partial digestion band of 8.5 kb from EcoRI digests is observed in 61% of the T cell lines/clones.
Figure 6. T cell receptor β-chain gene rearrangements of TIL lines/clones derived from breast tumor tissues. Endonucleases EcoRI (A) and HindIII (B) were used to digest the DNA. GL shows germline 4-kb and 11-kb bands in EcoRI digests, and 3.5-kb, 6.5-kb and 8-kb bands in HindIII digests.
Figure 7. HindIII digests demonstrate that most of the T cell lines/clones retained germline bands of 3.5-kb, 6.5-kb and 8-kb. 50% and 37.5% of the 16 T cell lines/clones with rearrangements to Cβ2 showed nongermline bands 11-kb and 5.5-kb, respectively.

Discussion

By establishing T cell lines and clones, we were able to analyze tumor infiltrating lymphocytes at the clonal level. Phenotypic analysis of cell surface markers showed that 57% of the lines/clones derived from breast TIL were CD3+CD4+ and 26% of the lines/clones were CD3+CD8+. The CD4:CD8 ratio was higher than 1. It is not clear if this is caused by a selective enrichment in CD3+CD4+ cells during the TIL isolation procedure or a selective outgrowth of CD3+CD4+ clones in the microculture system (28). Nevertheless, the CD4:CD8 ratio is in accordance with the ratio found in fresh breast TIL (3). Surprisingly,
we found that 17% of the lines/clones displayed a dual expression of CD4+CD8+ antigen, and five lines/clones (10%) contained up to 46% of the CD4+CD8+ double-positive cells accompanied by a single-positive population either CD4+ (one of the five lines) or CD8+ (four of the five lines). The coexpression of both CD4 and CD8 surface antigens appears usually on immature cells such as thymocytes and accounts for approximately 70% of the thymocytes, or on incompletely differentiated cells found in lymphoblastic lymphomas (29). The majority of normal peripheral blood T lymphocytes express either CD4 or CD8, but not both. It is not clear whether these cells are generated from different precursors or derived from a single clone but in a transitory state. Blue et al demonstrated that prolonged culture of CD4+CD8+ cells in the absence of lectin resulted in the loss of the CD4CD8 phenotype (30). It is also not clear whether this unusually high proportion of CD4+CD8+ double-positive cells appeared in tumor-infiltrating lymphocytes is related to the immune deficiency at the tumor sites.

Cytotoxicity assays revealed the existence of high percentage of T infiltrating lymphocytes with cytolytic potential: 84% of the lines/clones derived from TIL preparation displayed cytolytic activity as assessed by a lectin-dependent cytotoxicity assay. The results are in good agreement with the findings of others who demonstrated an increased frequency of cytolytic precursors in several solid tumors (22, 28). It seems that a selective accumulation of cytolytic precursors occurs in solid tumors. Interestingly, most of the cytolytic lines/clones generated from TIL had CD3+CD4+ phenotype. They exhibited significant cytolytic activities and lysed P815 cells as efficiently as CD3+CD8+ T cells in the LDCC assay (Fig. 2). Although previous reports have showed a dissociation between phenotype and function in peripheral blood, the amount of CD3+CD4+ CTL is remarkably increased in breast tumors as compared to normal PBL which has only a small population of CD3+CD4+ CTL (2, 28). However, a frequency analysis is essential to confirm this. The exact
Table 1. Phenotype of T Lymphocyte Lines and Their Cytolytic Activity against Different Target Cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>LDCC</th>
<th>K562</th>
<th>MCF7</th>
<th>HBL100</th>
<th>Auto-tu</th>
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<tr>
<td>LY A1</td>
<td>5</td>
<td>1</td>
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<td>4</td>
<td>0</td>
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</tr>
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<td>6</td>
<td>4</td>
<td>39</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup>Effector: Target = 50:1

<sup>b</sup>Percent of positively stained cells
function of these CD3+CD4+ CTL gathered at tumor sites with heterogeneous antigen specificity is still not clear at the moment.

In contrast to the finding by others who reported depressed NK activity in human breast tumors (31), we observed that 20% of CTL had exhibited NK activity, although their cytolytic activity was mild (ranged from 9% to 66% with an average of 27%) as compared to the CTL positive for LDCC. Moreover, 17% of the CTL showed an MHC-unrestricted cytotoxicity by lysing allogeneic breast tumor cell lines like MCF-7 and HBL-100. This type of cells had the same properties as the CD3+CD4+ or CD3+CD8+ T cells in peripheral blood which can develop LAK activity as described by Geller et al (32). Of the 17 lines obtained from three patients, only two CD8+ T cell lines showed specific cytotoxicity against autologous tumor cells. It is not clear whether this low frequency of auto-tumor specific T cells reflect the immune response at the tumor site in vivo or whether it is caused by cloning and microculture system. However, their cytolytic activity was inhibited by monoclonal antibodies against the T cell receptor, the CD3 molecule and the MHC-I class molecule (33). This would suggest that these T cell lines recognize tumor antigens via the T cell receptor and CD3 molecule in an MHC-restricted manner.

The results of the southern blot analysis of the T cell lines/clones derived from breast cancer patients are characterized by the presence of dominant TcR-β gene rearrangements. The "dominance" we observed might not reflect the in vivo situation, but rather a result of the in vitro selection since these cells were cultured in the presence of rIL-2, and IL-2 may induce oligoclonal expansion of T cells. However, this oligoclonal selection may be related to the presence of T cells within the TIL preparation that have been exposed to antigen for which they are specific, "immune-primed" T cells, and that these cells make up a part of the tumor immune response (34). On the other hand, it is not unlikely that the identical pattern of TcR-β gene rearrangements within an individual may be the result of clonal expansion of T cells in breast tumor tissue. Furthermore, the predominant TcR-β gene rearrangements in different tumor samples may imply that these T cells are
sensitized by relatively common antigens in breast tumor tissues (35, 36). However, it is impossible to determine at this moment if they share an identical antigen reactivity, and we will be able to prove whether these clones share the TcR only with sequencing of the expressed gene.

In summary, the present study of tumor infiltrating lymphocytes at clonal level shows that lymphocytes infiltrated into human breast tumors are mostly CD3+ T cells with varying amount of CD4+ and CD8+ subsets. A vast majority of the cells possess cytolytic activity towards heterogeneous antigens. The molecular data indicate that an oligoclonal expansion of “immune-primed” T cells might exist at the tumor site.
References


Chapter 7

T Cell Receptor Vβ Usage of Tumor Infiltrating Lymphocytes of Human Breast Tumor and Melanoma
Abstract

A total of twenty-eight tumor infiltrating T cell lines (TIL) were established from four breast tumor and three melanoma tissues with limiting dilution and microculture system in the presence of interleukin-2. Their T cell receptor (TcR) β-chain variable region gene usage was analyzed using polymerase chain reaction (PCR) technique and oligonucleotide primers specific for 20 Vβ families. The amplified PCR products were further detected by Southern blot hybridization with a Cβ2 DNA probe. T cell lines generated from peripheral blood lymphocytes (PBL) under similar condition were examined in parallel. Our data showed a limited heterogeneous usage of TcR Vβ genes with preferential expression of Vβ8 and Vβ17 in breast tumor-derived TIL lines, and Vβ5 and Vβ17 in melanoma-derived TIL lines. A random TcR Vβ usage profile was observed among 11 control T cell lines derived from PBL of breast cancer and melanoma patients. This study provide new evidence that suggests an oligoclonal expansion of tumor infiltrating lymphocytes in breast tumor and melanoma, as a result of the recognition to related antigens in tumors.
Introduction

Tumor-infiltrating lymphocytes as a manifestation of the host immune response have been proven to be effective in mediating anti-tumor response in animal models and preliminary clinical trials (1-3). Titration of TIL in mice with established lung and liver metastases indicated that these cells were from 50 to 100 times more potent than lymphokine-activated killer (LAK) cells (4-5). Clinical trials showed that TIL were associated with a response rate of 50% in melanoma and other cancer when administered to patients after in vitro culture with Interleukin-2 (6-8).

T cells are believed to be a predominant population of TIL and thought to play a crucial role in host anti-tumor cellular immune response (9-12). The specific recognition of antigens of T cells is generally mediated through their T cell receptor (TcR) consisting of either α-β or γ-δ heterodimer in association with major histocompatibility gene complex proteins. The highly diversified T cell receptor repertoire is generated during T cell ontogeny in the thymus by rearrangement of TcR genes, which consist of variable (V), diversity (D), junctional (J) and constant (C) gene regions in β-chain of the TcR and V, J and C gene regions in α-chain of the TcR (13). Analysis of T cell receptor Vα and Vβ chain expression may provide an indirect assessment of antigen specificity. It has been reported that preferential use of specific Vβ products occurs in the T cells involved in the pathogenesis autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (14, 15) as well as in animal models of human disease such as experimental autoimmune encephalitis (16, 17). TcR Vα7 was also found to be predominantly expressed by TIL in seven of eight uveal melanomas, suggesting that a specific antigen in these melanomas provokes the clonal expansion of TIL upon antigen recognition (18).

To better understand possible mechanisms of the anti-tumor activity of in vitro expanded TIL and the molecular nature of TcR involved in antigen recognition, we have established a panel of T cell lines from tumor-infiltrating lymphocytes of breast cancer and melanoma patients, examined their
phenotypic profile and cytolytic activity towards different targets, and analyzed their TcR Vβ gene expression in comparison with T cell lines derived from peripheral blood lymphocytes of the patients.

Materials and Methods

Establishment of T-cell lines from TIL and PBL

Tumor tissues were obtained from four breast cancer patients and three melanoma patients. The specimens were extensively washed to minimize the possible contamination of blood and cut into small fragments. The fragments were enzymatically digested with enzyme solution containing hyaluronidase type V 0.01%, collagenase type IV 0.1% (Sigma, Vel, Belgium), DNase type I 0.002%, gentamicin 50 µg/ml and fungizone 250 ng/ml dissolved in RPMI 1640 medium (GIBCO, Life technologies, Belgium) by incubation overnight at room temperature and then filtered through a sterile coarse wire grid, washed four times with RPMI 1640 medium and resuspended in culture medium which was RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 µg/ml gentamicin, 250 ng/ml fungizone (GIBCO), and 10 mM Hepes buffer (Flow Laboratories, Belgium). Lymphocytes recovered from the suspension were seeded in 96-well microtiter plates (Costar) at concentrations of 50, 10 and 2 cells/well with 10^5 irradiated and oxidized stimulator cells as reported elsewhere (19) and 2 x 10^3 autologous tumor cells in 200 µl culture medium containing 100 IU/ml rIL2 (Eurocetus). The cells were restimulated every 10 days, with autologous tumor cells, feeder cells and rIL2. After three to four cycles, microcultures with cell growing were split and expanded in culture medium and 200 IU/ml of rIL2. The medium was refreshed on a week basis or whenever it was necessary. TIL concentration was maintained in 2 x 10^6/ml. All T cell lines were expanded to at least 10^7 cells for the study. PBL lines from the same patients were established under the same conditions.
Phenotypic analysis of established TIL lines and PBL lines

Phenotypic analysis was performed using the following monoclonal antibodies: anti-Leu 4 (CD3), anti-Leu 2 (CD8), anti-Leu 3 (CD4), anti-Leu 11c+19 (CD16+56) and anti-HLA-DR directly conjugated with FITC or PE (Becton Dickinson, Erembodegem, Belgium). The samples were analyzed on a FACscan (Becton Dickinson).

Assays of cytolytic activity

Cytolytic activity was measured in a conventional 4-hr assay against $^{51}$Cr-labeled tumor cell targets. Target cells labeled with $^{51}$Cr were aliquoted at $5 \times 10^3$/well into wells of 96-well U-bottomed microtiter plates (Costar), to which the effector cells had been aliquoted at different concentrations. In the LDCC assay, PHA was added to the $^{51}$Cr-labeled P815 cells immediately before placing them in microwells to avoid the formation of cell clumps. Microtiter plates were incubated at 37°C for 4 hr. The supernatants were then harvested from each well and radioactivity was counted in a Cobra II auto-gamma counter (Parkard Canberra, Meriden, CT). The maximum release was determined by incubating target cells with 2N $\text{H}_2\text{SO}_4$, and spontaneous release was determined by incubating these target cells in medium alone. Specific lysis was calculated according to the formula:

$$\begin{align*}
\text{% specific lysis} & = \frac{\text{Exp. release} - \text{spont. release}}{\text{Max. release} - \text{spont. release}} \times 100
\end{align*}$$

Isolation of total RNA and synthesis of first strand cDNA

RNA were isolated from TIL lines and PBL lines using RNAzol B (Biotecx Laboratories, Texas). Single-stranded cDNA was prepared from 2 μg of total RNA using 50 ng of random hexanucleotides and Superscript Reverse Transcriptase (GIBCO BRL, Life Technologies) under reaction conditions
specified by the supplier in a reaction volume of 20 μl. Reaction products were purified by phenol-chloroform extraction.

Table 1. Sequence of T cell receptor primers. Nucleotide sequence of primers used for PCR (20-22).

<table>
<thead>
<tr>
<th>Primer</th>
<th>5' → 3' sequence</th>
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<tbody>
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<td>Vβ1</td>
<td>AAGAGAGAGCAAAAGGAACATTTCTTGAAC</td>
</tr>
<tr>
<td>Vβ2</td>
<td>GCTCCAGGCCCCACATACGAGCAAGCGTCG</td>
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<tr>
<td>Vβ3</td>
<td>AAAATGAAGAAAAGGAGATATTCTTGAG</td>
</tr>
<tr>
<td>Vβ4</td>
<td>CTGAGGCCCACATATGAGAGTGGAATTTGTC</td>
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<tr>
<td>Vβ5</td>
<td>CAGAGAAACAAGGAAAACTTCCCTGCTGGA</td>
</tr>
<tr>
<td>Vβ6</td>
<td>GGGTGCGCCAGATGACTCAAGGCGCTGCCAA</td>
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<td>Vβ7</td>
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<td>Vβ8</td>
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<td>Vβ9</td>
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<td>Vβ10</td>
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<tr>
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<tr>
<td>Vβ13.2</td>
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<td>Vβ15</td>
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<tr>
<td>5'Cβ</td>
<td>COGAGGTCGCTGTTTGAGCCAT</td>
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</table>
PCR amplification

Polymerase chain reaction (PCR) amplification of 1 μl of the cDNA preparation was done in 100 μl reaction volume containing PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.4, 4 mmol/L MgCl₂ and 0.01% gelatin) 0.5 mmol/L of each dNTP, 15 pmol/L TcR Vβ primers or 5’Cβ, 15 pmol/L 3’Cβ and 0.5 units AmpliTaq DNA polymerase (Perkin Elmer Cetus). PCR primers covered all published Vβ families (Vβ1 to Vβ20) as shown in Table 1. The TcR-β chain constant region primer, 3’Cβ (5’-CTCTTGACCATGGCCATC-3’) was used for amplification of TcR β chain cDNA to generate PCR products of 400-550 bp. The amplification was done for 35 cycles (90°C, 1 min; 55°C, 1 min; and 72°C, 2 min) using a thermal cycler (Techne Incorporated, Princeton, New Jersey).

Southern blot analysis

15 μl of PCR amplified cDNA were electrophoresed in 1% TAE agarose gels. After electrophoresis, the gels were soaked in 0.5 N NaOH for 30 min. The DNA was transferred onto positively charged Nylon membranes (Boehringer Mannheim, Belgium) using 5 inches of Hg for 90 min in 10 x SSC (1.5 mol/L NaCl, 0.15 mol/L sodium citrate) with a Vacuum Blotter (Bio-Rad, Belgium). Membranes were neutralized in 2 x SSC, fixed and dried with vacuum Gel Dryer (Bio-Rad) at 80°C for 30 min. Blots were prehybridized for 3 h at 42°C in hybridization buffer (50% Formamide, 5 x SSC, 2% Blocking reagent, 0.1% N-lauroylsarcosine and 0.02% SDS) and herring sperm DNA (100μg/ml) and hybridized overnight at 42°C with DIG-labeled Cβ2 DNA probe (400bp). The filters were washed two times in 2 x SSC and 0.1% SDS for 5 min, two times in 0.1 x SSC and 0.1% SDS for 15 min at 68°C and once in washing buffer (Buffer 1: 0.1 mol/L maleic acid, 0.15 mol/L NaCl, pH 7.5 + 0.3% Tween-20) for 5 min. The filters were then subsequently incubated with Buffer 2 (10% Blocking reagent diluted in Buffer 1) for 30 min, anti-digoxigenin-AP Fab fragments 75 mU/ml diluted in Buffer 2 for 30 min, washed two times 15 min with washing buffer, equilibrated in Buffer 3 (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 50 mmol/L MgCl₂, pH 9.5) for 5 min and incubated in substrate solution (100μg/ml
AMPPD, Boehringer Mannheim) for 10 min. The membranes were exposed for 1 h at room temperature to Hyperfilm-MP (Amersham, Belgium) following 10 min preincubation at 37°C.

Figure 1. Detection of PCR-amplified cDNA from pooled peripheral blood lymphocytes stimulated with PHA using dot blot hybridization with a Cβ2 probe.

Results

Twenty-eight T cell lines were established from TIL of four breast tumor and three melanoma tissues. As shown in Table 2, most of the T cell lines expressed CD4 T cell surface molecule and only 28% of the lines exhibited CD8+ phenotype. Two of the T cell lines had a proportional CD4CD8 dual positive population. Functional analysis of the TIL lines showed that 71% of the lines displayed potent cytolytic activity towards heterogenous antigens as evaluated by LDCC assay while three lines exhibited LAK-like activity by killing K562 and MCF7 target cells. Ten lines established from PBL of three patients expressed comparable T cell surface molecules.

Primers specific for 20 different human TcR Vβ families were used for PCR amplification of cDNA reversely transcribed from mRNA of PHA-stimulated
### Table 2. Phenotype of T lymphocyte lines and their cytolysic activity against different target cells

<table>
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<tr>
<th>Line</th>
<th>Phenotype (%)</th>
<th>Percent of lysis (T/E = 50/1)</th>
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pooled peripheral blood lymphocytes as a positive control. The amplified PCR products were further identified by hybridization with a Cβ2 DNA probe. The result of dot blot analysis of PCR amplified products shows that all the TcR Vβ gene families were efficiently amplified in the control (Fig.1).

The results of TcR Vβ gene analysis of the T cell lines derived from TIL are summarized in Table 3. Of the twenty-eight T cell lines tested, a heterogeneous usage of most Vβ families were observed in both breast tumor and melanoma samples. However, Vβ8 (5/16) and Vβ17 (5/16) in breast TIL lines, and Vβ5 (3/12) and Vβ17 (3/12) in melanoma TIL lines were overexpressed (Fig. 2). The TcR Vβ gene usage profile of the T cell lines derived from PBL of one breast cancer patients and two melanoma patients showed heterogeneity with no marked preferential expression of certain Vβ families (Table 4).

![Figure 2. Comparison of the numbers of TcR Vβ RNA transcripts rearranged in TIL lines derived from breast cancer and melanoma patients.](image-url)
Discussion

Tumor-infiltrating lymphocytes are powerful mechanisms that restrain tumor outgrowth through T cell recognition to tumor antigens and that can be potentially adapted to a specific form of immunotherapy (23). Like conventional antigen-specific T cells, TIL may represent a T cell population that acquires the capability of infiltrating to tumor site by specific interactions and clonally expanded in situ. Our strategy to examine this notion is to analyze the TcR Vβ gene usage of TIL, an indicative feature of clonally expanded T cells. In this regard, activation of TIL would selectively expand IL-2 receptor positive (IL-2R+) T cells which represent an active population of T cells undergone an activation in situ. These IL-2R+ T cells are presumably activated by interacting with tumor antigens and clonally expanded and are thus more representative of the anti-tumor activity. In support of this theory, freshly isolated tumor infiltrating T cells from human solid tumors are often unable to mediate antitumor cytotoxicity (24-26). Activation of TIL with rIL-2 enhanced their antitumor cytotoxicity (26 -28). Thus, analysis of TcR gene expression of TIL after IL-2 activation may provide an indirect assessment of antigen specific expansion.

Upon analysis of seven specimens obtained from breast cancer and melanoma patients, we demonstrated a limited heterogeneous usage of TcR Vβ genes with a preferential expression of Vβ8 in breast TIL lines, Vβ5 in melanoma lines, and Vβ17 in both types of TIL lines. By contrast, peripheral T cells prepared concurrently under similar conditions showed a heterogenous expression of different Vβ genes, as expected. Thus, our data suggest that TIL derived from a tumor site may have undergone a clonal activation and expansion, most likely by encountering tumor antigen(s). Consistent with this observation are similar reports in organ-specific autoimmune diseases where autoreactive T cells are clonally expanded and infiltrate into a target organ by reacting to a specific autoantigen (29). It has been suggested by other studies that T cells use a limited Vβ gene to recognize a particular antigen and to
develop specialized functional properties in encountering an antigen (14-16, 22). The preferential expression of Vβ8 in breast TIL lines, and Vβ5 in melanoma TIL lines may be the result of clonal expansion of T cells reactive to breast tumor antigen, and melanoma antigen. Similarly, preferential use of Vβ17 in both breast tumor and melanoma TIL lines may suggest a common pathway by which some infiltrating T cells undergo clonal expansion. This activation pathway is more likely associated with the capability required by T cells to infiltrate into the tumor, and alternatively a recognition of a common tumor antigen.

Thus, it is of considerable interest to define whether these T cells share a related antigen specificity. Further experiments to determine an unique DNA sequences of the VαJα and VβDβJβ of the T cell receptor would provide evidence to clarify this issue (30-32).
References


Chapter 8

A Murine Monoclonal Antibody to Human Breast Cancer Cells Associated with the DNA Ploidy Status
Abstract

A murine monoclonal antibody (5D10) raised against the human breast cancer cell line MCF7 was found to react preferably with mammary carcinomas and weakly with normal epithelial cells. The antigens recognized by the antibody had molecular weights of approximately 28 kD and 90 kD. The reactivity of the antibody to human breast carcinomas correlated with the DNA ploidy status of the tumor cells. Upon analysis of 54 breast carcinoma specimens, we found that the percentage of antibody (5D10) positive cells was significantly higher in tumors with an aneuploid stemline than in those with a diploid DNA content (P < 0.001). This antibody therefore could be a useful tool to evaluate the prognosis of breast carcinomas.
Introduction

A large number of monoclonal antibodies to normal and cancerous breast cells have been developed with different immunogens such as human milk fat globule membranes, breast cancer cell lines, membrane-enriched fractions of metastatic carcinoma lesions, hormone receptors or even by fusing human lymphocytes, obtained from axillary lymph nodes of patients, with a murine myeloma cell line (1-8). Each of these antibodies reacts with mammary tumors but also with nonmammary tumors or normal tissues (9). Nevertheless some of these monoclonal antibodies have provided additional diagnostic help or have been useful to classify human breast carcinomas.

The major goal for making monoclonal antibodies to breast cancer cells has been the development of specific probes to make a differentiation among the breast tumors possible, to identify markers that correlate well with the progress of the disease and to help resolving important therapeutic dilemmas (2, 21). There is evidence suggesting that the DNA ploidy status is one of the useful prognostic factors yet identified in breast cancer (10-21).

Previously we reported a panel of murine monoclonal antibodies raised against human breast cancer cell line MCF7 (22, 23). The antibodies showed a high degree of specificity for epithelial cells. Breast tumor cells were often stained cytoplasmatically while in normal and benign breast tissues the staining was concentrated at the luminal sites and in the secretory products. One of the antibodies 5D10 was shown to be very effective to evaluate the invasiveness in vitro of MCF7 cells (26).

The antibodies were examined further on post-operative specimens from breast cancer patients to evaluate their clinical value for diagnosis and prognosis. The analysis of the nuclear DNA content of these specimens was also performed in this study. In the study, we found that antibody 5D10 was correlated with the DNA ploidy status of the breast carcinomas. The antibody therefore could be a useful tool for both the diagnosis and prognosis of mammary tumors.
Materials and Methods

Production and Characterization of the Monoclonal Antibody

The method used to develop the monoclonal antibody (5D10) has been previously described (22, 23). Briefly, male mice were immunized with 1-10 x 10^6 exponentially growing MCF7 cells. Splenocytes of the immunized mice were fused with SP2/0 myeloma cells and the resulting hybridomas reactive with MCF7 cells and unresponsive to lymphocytes were selected and cloned twice by limiting dilution. A panel of cell lines and cells were tested to determine the specificity of the antibody.

Immunoblotting

MCF7 cells were extracted by Triton X-100 (0.5%), acetone or NorMidet P40 (1%) and eletetrophoresed in a vertical 10% polyacrylamide slab gel. After electrophoresis, the separated constituents were transferred electro-photetically to nitrocellulose membranes in a trans-blot cell (Pharmacia, Belgium) for 2 hours at 180 mA at room temperature. The membranes were then incubated with monoclonal antibody, biotinylated-rabbit anti-mouse Ig and Alkaline Phosphatase conjugated Avidin-Biotin Complex. The alkaline phosphatase reaction was developed using naphthol AS-MX phosphatate and Fast Red as substrate.

Indirect Immunocytochemical Assay on Frozen Sections

Indirect peroxidase staining was carried out on formalin or acetone-fixed frozen sections. After washing with PBS, the slides were incubated consecutively with normal rabbit serum (Dakopatts, Prosan, Belgium) for 20 min, MAb 5D10 (2μg/ml) for 30 min, rabbit anti-mouse Ig (Dakopatts, Prosan, Belgium) as a bridging antibody for 30 min and finally a peroxidase conjugated mouse anti-peroxidase complex (Dakopatts, Prosan, Belgium) for 30 min. All incubation steps were carried out at room temperature. Each step was followed by a brief wash with PBS. The enzymatic reaction was carried out with a mixture of 0.5
mg/ml diaminobenzidine (Janssen Chimica, Beerse, Belgium) and 0.03% hydrogen peroxidase for 5 min. The slides were washed with running tap water for 5 min and counterstained with Methyl Green (Aldrich, Belgium). Mouse immunoglobulin was substituted for primary antibody as a control for non-specific binding. The immune stainings were evaluated with a quantitative image analysis system (CAS 200) (Becton Dickinson, Belgium). The antibody reactivity was expressed as a percentage of positive stained cells in totally measured cells.

**DNA Ploidy Analysis**

The air-dried cytological preparations of the breast tumor tissues were primarily stained with May-Grünwald-Giemsa (MGG) and examined microscopically to confirm the diagnosis. The MGG stain was then removed in absolute methanol for 60 min, refixed with 10% neutral buffered formalin (Sigma, Filter Service, Belgium) for 30 min and hydrolyzed for 60 min in 5N HCl (24). DNA Feulgen staining was performed with the CAS Quantitative DNA Staining Kit (CAS Inc. Lombard, Illinois, USA) according to the instructions of the manufacturer. One calibrator slide was included for each batch of stainings to calibrate the normal DNA content and to control the staining quality as well. The nuclear DNA staining results were analyzed on CAS 200 with assistance of the CAS Quantitative DNA Program. The program was initialized by counting 20-150 cells on the calibrator slide. A minimum number of 20 normal cells within the specimen were used as internal control. The slides in which no normal diploid cells were present were discarded. The optical density of the Feulgen stained nuclear DNA was measured for at least 100 single tumor cells/slide and the results are presented in histograms and DNA indices.

**Histogram Analysis of DNA Content**

Histogram analysis was performed without knowing the results of the immunochemical assay with monoclonal antibody 5D10. The histograms were grouped as diploid (DNA index 0.9 - 1.1), 'tetraploid' (near-tetraploid with DNA
index 1.8-1.9, and tetraploid with DNA index 2.0), and 'other DNA-an euploid' (DNA indices 1.2-1.7, and $= 2.1$) as proposed by Baildam et al (25).

Results

Characterization of MAb 5D10

The reactivity of 5D10 with several normal and neoplastic tissues was reported previously (22, 23). Generally, positive staining was mainly confined to epithelial cells and their related adenocarcinomas. The pattern of the antibody reactivity was heterogeneous which means that not all cells were stained and that the staining intensity itself was heterogeneous in the positively stained cells. The antigens recognized by the antibody had molecular weights of approximately 28,000 Dalton and 90,000 Dalton as determined by immunoblotting (Figure 1).

DNA Content Analysis

DNA histograms were determined for the imprints of 54 breast tumor specimens. Of these tumors 19 had a diploid DNA content, 16 were 'tetraploid' and 19 were 'other DNA-an euploid' tumors. Figure 2 shows these three types of histograms as determined by the CAS Quantitative DNA Analysis program after Feulgen nuclear DNA staining. The incidence of 'tetraploid DNA' and 'other DNA an euploid' tumors showed no significant differences with the data obtained by others (25).

Immunocytochemical Reactions of Antibody 5D10

Most of the breast carcinomas with non-diploid DNA content were stained by 5D10 to a variable degree and most of the staining was confined to the cytoplasm and a few to the perimembrane. Of the 19 DNA diploid specimens, 15 specimens were either unstained or stained weakly. However, the percentages of the stained cells in these weakly stained specimens were less
than 10%. Among 35 non-diploid tumors, 25 were stained by the antibody. When tumors with 'tetraploid' DNA content were not considered, all of the 'other DNA-aneuploid' tumors were positively stained and the percentage of stained cells in each specimen was more than 10%. In tumors with 'tetraploid' DNA content, some specimens were positively stained by the antibody and some were not. The statistical analysis revealed significant differences among the three groups tested (P < 0.01) (Fig. 3). The percentage of the stained cells was significantly higher in tumors with an aneuploid stemline than in those with a diploid stemline (P < 0.001).

Figure 1. Immunoblotting of antigens extracted from MCF7 cells with Triton X-100 (track 1); Acetone (track 2); Norindet P40 (track 3). Molecular weight markers (X 10^3) are shown to the left.
Figure 2. Histograms determined with CAS 200. 2A shows a histogram of diploid cells with a DNA Index (DI) of 1.0. Only few proliferating cells are seen in S and G2/M phases. 2B shows a histogram of aneuploid cells with the main cell population (G0/G1) at DNA index of 1.53 and more proliferating cells in S and G2/M phases. The first peak of the cells in the histogram with a DNA index of 1.0 represents normal cells in the specimen. 2C demonstrates a histogram of tetraploid DNA cells with most cells at DNA index 2.0, and a lot of cells in the proliferation stage.
Figure 3. The relationship of MAb 5D10 staining of breast specimens and their DNA ploidy status. (*) represents an individual specimen. (-) represents the mean staining percentage of each group.

Discussion

A monoclonal antibody against human breast tumor-associated antigens was evaluated with respect to DNA ploidy status of the tumor cells. Breast carcinomas containing tumor cells with a high degree of aneuploidy showed a high reactivity towards MAb 5D10. On the other hand, Mammary carcinomas with a diploid DNA content showed low or no reactivity to the antibody. Most of the tumor specimens tested revealed a heterogeneous pattern of staining. These results are highly suggestive for a good correlation between
the nuclear DNA content and one or more expressed antigen(s) recognized by 5D10 in primary breast carcinomas. The significance of this correlation could be of great importance. Since alterations of the normal nuclear DNA content may reflect chromosomal changes (27), it is reasonable to postulate that an alteration of the genetic material could give rise to an increased expression of a specific antigen, which is also present on normal cells but at a relatively low density.

The development of analytical techniques for the evaluation of the nuclear DNA content, such as Flow Cytometry (FCM) and Image Cytometry (CAS), has added to the importance of the DNA ploidy status of tumor cells as a diagnostic and prognostic parameter for many solid tumors (10-21,28). Mammary adenocarcinomas with a diploid DNA content progress slowly and consequently have a better prognosis as compared with non-diploid tumors. In contrast, tumors with an aneuploid DNA content progress rapidly and have a poor prognosis (10-12). The preferential reactivity of antibody 5D10 towards aneuploid mammary carcinomas could be of considerable help to assist in refining the diagnosis and prognosis of breast carcinomas.

Since both the G2/M phase of the diploid cells and aneuploid cells can give tetraploid patterns, it is often difficult to distinguish these two populations by cytometries. Antibody reactive with aneuploid cells like 5D10 could thus be used to distinguish a real aneuploid stemline with tetraploid pattern from a G2/M phase of diploid cells. Tetraploid mammary tumors recently have attracted much interest because some of these tumors are responsive to endocrine therapy while tumors with diploid or true aneuploid DNA content showed no response at all (19,25,29,30).

In conclusion, antibody 5D10 could be of considerable diagnostic and prognostic value for breast tumors. This antibody can be used, in combination with hormone receptor analysis, to select the endocrine therapy-sensitive breast tumors with tetraploid DNA content. The evaluation of the properties of the antigen recognized by antibody 5D10 and its expression mechanism in mammary carcinoma cells are in progress.
References


Chapter 9

General Discussion
Adoptive immunotherapy differs conceptually from surgery, radiation therapy, or chemotherapy as it acts not by directly attacking the tumor, but by stimulating natural host defense mechanisms, the immune system, to mediate tumor regression (1,2). The therapy has emerged as a mode of treatment capable of mediating the regression of cancer in some patients. Tumor-infiltrating lymphocytes, as a manifestation of the host immune response, are potentially the most powerful form of immunotherapy, when a substantial number of TIL are infused (3,4). However, expansion of TIL in vitro to sufficient amount required for immunotherapy is often hampered by the small size of the tissue samples that can be obtained at early stage of cancer and the limited amount of TIL recovered from the tumor tissues. Lack of known tumor-specific antigen stimulation and limited supply of antigen presenting cells, which are required for classical T cell activation, further hamper the effort to obtain specific TIL lines. Different alternative approaches have been proposed, but few has been effective so far. Stimulation with surface-oxidized allogeneic PBL appears to be an alternative way to obtain sufficient amounts of TIL for therapeutic use without losing their specificity. This approach precludes tumor antigen stimulation and MHC-matched antigen presenting cells, otherwise required for expansion of antigen-specific TIL (chapter 2).

The elucidation of the phenotypic profiles of TIL and the relationship with their functional properties might help us to understand the immune defense mechanisms of the host against neoplasm. The phenotypic analysis of freshly isolated TIL from human breast cancer as described in chapter 3 and chapter 4 demonstrates that TIL from breast tumors consist of heterogeneous populations of mononuclear cells. They are mainly CD4+ and CD8+ T cells, as has been observed in TIL derived from tumors of other histological origin. Other immunocompetent cells, such as B cells and NK cells are also present in TIL (5-8). A disproportionately increased B cell infiltration was found in breast cancers, although it does not clearly correlate with any of the clinical or pathological findings. At this time, it is not clear whether these infiltrated B cells play a role in tumor rejection by producing a specific antibody response. As CD8+ cytotoxic
T cells and NK cells are the major effector populations, a reciprocal correlation of their numbers with the tumor size would support the notion that the presence of functionally active CD8+ cytotoxic T cells and NK cells provide effective host immune responses against neoplasm as described in chapter 1, and a decrease in CD8+ T cells and NK cells observed in large tumors may be the result of local immune deficiency against established tumors.

In vitro culture of TIL in the presence of low doses of IL-2 (200 U/ml) induces predominant expansion of CD3+ T cells. However, these cells fail to home to the tumor sites after infusion back to the patients (9). Several factors may account for this failure as discussed in chapter 5. Homing of TIL to the tumor sites does not simply depend on altered vascularity of the tumor sites or on nonspecific trafficking of lymphoid cells to the tumor (10,11). TIL may acquire certain functional and structural features to home specifically to the tumor in a in vivo scenario. For instance, it could be influenced by the expression of T cell activation markers and antigen specificity of the infused TIL, which may be lost during in vitro stimulation and expansion. Indeed, fine specificity studies of TIL at the clonal level showed a heterogeneous antigen specificity and the frequency of autologous tumor target-specific T cell precursors was found to be rather low as described in chapter 6 and as has been reported by others (12-18). Although it is not clear as to whether this low frequency is the result of local immune deficiency at tumor site, it could be a reason of nonspecific localization when bulk cultured TIL was infused back to the patients. The ‘dilution effect’ of the overwhelming non-specific T cells over tumor-specific cells might attribute to a non-specific trafficking of TIL to other organs. In this regard, cloning and selective expansion of tumor-specific T cells as described in chapter 6 could provide an effective way to improve the efficacy of immunotherapy.

Like T cell recognition to other antigens, TIL recognize tumor antigens in the context of TcR and MHC interactions, as evidenced in chapter 7. Lysis of tumor targets by specific TIL clones can be blocked by monoclonal antibodies to CD3, TcR and MHC-I antigen which further suggests the mediation of T cell
receptor and CD8 molecule in antigen recognition in the context of MHC antigen. Within the tricomplexes of TcR-MHC-antigen, the α/β heterodimer of the TcR is the crucial element in antigen recognition. Furthermore, the TcR used by a T cell must rearrange to an unique sequence upon antigen stimulation (19,20). Thus, analysis of TcR can be used to distinguish different T cell clones. The identification of dominant TcR gene rearrangements may then be interpreted as indicative of oligoclonal expansion of T cells that are sensitized by relatively common antigens (21,22). Analysis of TcR Vα and Vβ chain expression provides an indirect assessment of TIL specificity. So far, the Vα and Vβ chain of the TcR can be classified in about 18 and 20 families respectively. Predominance of one particular family in a group of lymphocytes in TIL may provide a clue as to their state of activation/clonal expansion and antigen specificity. It has been suggested that T cells use a limited Vβ genes to recognize a particular antigen and to develop specialized functional properties in encountering an antigen (23-25). Data obtained in chapter 7 may imply that TIL derived from tumor sites have undergone a clonal activation and expansion, most likely by responding to tumor antigen(s).

Adoptive immunotherapy using monoclonal antibodies has also attracted the interest of cancer researchers. However, the use of monoclonal antibodies in cancer treatment has been largely unsuccessful in producing long-term therapeutic effects so far. Several forms of antibody therapy, including heteroconjugates, drug-targeted antibodies and humanized antibodies have been tried out (26-28). However, most of the antibodies currently in clinical trials do not react with cancer-specific antigens. Rather, they react with antigens preferentially or inappropriately expressed upon malignant cells when compared with normal tissues. The tumor-restricted specificity is very important to prevent normal tissue damage. Monoclonal antibody 5D10 described in chapter 8 could be a candidate as it reacts preferably with breast tumor cells displaying aneuploid DNA content, a characteristic of malignant cells. Further investigations of the antibody are required before its therapeutic application is considered.
In conclusion, the clinical applications of adoptive immunotherapy of cancer will have significant therapeutic impact on the treatment of cancer. However, despite the enormous potential and clinical benefits that the therapy could provide, it is clear that many obstacles still exist before this approach is fully applicable. With the currently available methodologies, tumor-specific T cells can only be generated in a fraction of cancer patients, and complete tumor eradication is only achieved in a limited number of patients treated with such cells. Attempts to alter the immune reactivity in the cancer-bearing host have recently turned to genetic manipulation of immune cells and tumor cells in which a functional gene is inserted into the cells of the patient to add a new functional property to the cell (29). The development of techniques for inserting and expressing foreign genes in eukaryotic cells and the increased understanding of the regulation of gene expression have opened new possibilities for gene therapy of cancer. Transfection of TNF-α and IFN-γ may regulate the MHC expression on tumor cells which directly affect the ability of T cells to recognize and to reject the malignant cell (30,31). Induction of a tumor specific immune response by cytokine-secreting tumor cells has also been demonstrated for interleukin-2, interleukin-4, interleukin-6, and granulocyte colony-stimulating factor (32-34). It remains prospective that these early approaches can be developed into effective and practical therapies for the treatment of cancer patients.
References


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List of publications


Curriculum vitae

The author was born in Shanghai, the People's Republic of China, on November 1, 1960. From 1978 till 1984, she studied Medicine and obtained her M.D. degree in Shanghai Second Medical University. After success in the annual state qualification exam in 1984, she registered in the Post-graduate school of Shanghai Second Medical University and completed the post-graduate programme in Hematology in 1985 (Promotor: Prof. Dr. Zhengyi Wang). From 1985 to 1987, she worked at the Department of Internal Medicine of Reijin Academic Hospital as a resident. In April of 1987, she started research work at Dr. L. Willems Insituut, Diepenbeek, Belgium. (Director: Prof. Dr. Jef Raus).