THE ROLE OF CELL-MEDIATED CYTOLYSIS IN ANTITUMOR RESPONSES

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THE ROLE OF CELL-MEDIATED CYTOLYSIS

IN ANTITUMOR RESPONSES

De rol van cytotoxische lymfocyten
in antitumor reakties

Proefschrift

Ter verkrijging van de graad van Doctor
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Aan Jan en Mark

Ter nagedachtenis
aan mijn ouders

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CHAPTER 1

GENERAL INTRODUCTION

IMMUNOSURVEILLANCE OF CANCER

1.1 Introduction

The demonstration of specific immunity to foreign cell surface antigens provided the experimental basis for the theory of immunosurveillance of tumor cells. First proposed by Thomas (1) and expanded by Burnet (2), this theory states that the immune system maintains "surveillance" over the body by rejecting small clones of malignant cells that are antigenically sufficiently distinct from normal cells.

Tumors can be induced by irradiation, chemical substances, viruses, or arise spontaneously *in vivo*. Depending on the origin of tumor cells, various types of "foreign" antigens can be expressed which are generally termed tumor-associated antigens (TAA) (for review see 3). Tumor-specific transplantation antigens (TSTA) and viral capsid proteins located at the cell surface of chemically or virally induced tumors, respectively, are recognized by the immune system (3). However, not all TAA are recognized, as for instance, oncofetal gene-products or T-cell antigens that are "hidden" in the nuclei of tumor cells. Spontaneous tumors have very little *in vivo* immunogenicity (3).

The cellular immune response comprises two major components: adaptive immunity and natural immunity. Adaptive immunity is governed by T cells which are antigen-specific (4,5), i.e. foreign antigens expressed on tumor cells are recognized in the context of self-major histocompatibility complex (MHC) determinants by the T-cell receptor (TCR) of T lymphocytes. Experimental results showed that tumor cells can induce activation and proliferation of cytotoxic T lymphocytes (CTL), which in turn lyse these tumor cells (3,5). Natural or MHC-unrestricted immunity is expressed by natural killer (NK) cells, monocytes/macrophages and granulocytes (4,5).

Non-immunogenic tumor cells, may be recognized and destroyed by NK cells (5). These NK cells can lyse tumor cells *in vitro* without prior

activation. Biological response modifiers (BRM) such as interleukin-2 (IL-2) and interferons (IFN) enhance NK cell lysis.

This chapter deals with the different types of cytotoxic lymphocytes and their role in immunosurveillance. The membrane structures of these cells involved in activation and recognition in relation to their CTX against tumor cells are discussed. Finally, some possibilities how to modulate lytic functions of T and NK cells with the ultimate aim of immunotherapy of cancer patients, are suggested.

1.2 Classification of cytotoxic lymphocytes

Cytotoxic lymphocytes can be classified in different types based on their lytic mechanisms and the expression of cell surface cluster differentiation (CD) antigens (for review, see 6). The most extensively studied classes of cytotoxic lymphocytes are: (1) MHC class I-restricted CTL expressing TCR$\alpha\beta$/CD3 and CD8 antigens; (2) MHC class II-restricted CTL expressing CD3 or CD4 antigens; (3) MHC-unrestricted T cells expressing TCR$\gamma\delta^+$/CD3 but not CD4 or CD8 antigens; and (4) MHC-unrestricted NK cells which lack the expression of TCR/CD3, CD4 and mostly CD8 antigens (Table 1.1). The MHC-restricted CTL recognize target cells via their TCR$\alpha\beta$ (see section 1.1). MHC-unrestricted T cells recognize target cells via other receptors than the "classical" TCR (as detailed in section 1.3). MHC-restriction has not been shown yet for TCR$\gamma\delta^+$/CD3$^+$4$^-$8$^-$ T cells. The MHC-unrestricted NK cells recognize target cells via an as yet undefined receptor.

1.3 How do T cells recognize and bind foreign structures?

How can a great variety of foreign structures on cell surfaces be recognized by the T-cell? The answer to this question has emerged during the last decade and appeared to involve 2 groups of molecules: MHC determinants on the target cell and the TCR on the effector cell (for review see 7). T lymphocytes recognize foreign antigen, e.g. degraded virus particles, only in the context of self-MHC determinants. These CTL are "antigen-specific".

TABLE 1.1

GENERAL CLASSIFICATION OF CYTOTOXIC LYMPHOCYTES

phenotype	cell type			
	CTL	CTL	CTL	NK
CD2	+	+	+	+
CD3	+	+	+	-
CD4	+	-	-	-
CD8	-	+	-	-/+
CD16	-	-	+/-	+
TCR	αβ	αβ	γδ	-
lytic mechanism				
MHC class I restricted cytolysis	-	+	unknown	-
MHC class II restricted cytolysis	+	-	unknown	-
MHC unrestricted cytolysis	-	+	+	+
ADCC	-	-	+/-	+
LDCC	+	+	+	unknown

However, in allogeneic combinations, e.g. in transplantation immunity or in a mixed lymphocyte culture (MLC), the target cell MHC determinants are recognized as foreign by CTL which are called "alloreactive" T lymphocytes.

It has been proposed that MHC proteins act as a receptor for a foreign peptide, thereby holding it in a position that favors detection by antigen-specific CTL (8,9). It appeared that MHC proteins select similar peptide sequences from diverse antigens for presentation to these CTL (10). With respect to allospecific T cells, it was suggested that a foreign MHC molecule looks like a "self" molecule carrying an antigenic peptide (8). Small differences from the "self" sequence are enough to trigger an allo-immune reaction, i.e. recognition by the TCR and subsequent activation of the CTL.

The TCR is composed of two polypeptide chains, TCRα and TCRβ, which together determine the specificity of the T-cell (11-13). The β chain gene is composed of variable (V), diversity (D), joining (J), and constant (C) region segments in the germ line configuration (14-16). The α chain gene contains V, J and C region segments, but it is not clear whether it contains D segments. Rearrangement of these gene segments is a prerequisite for the functional expression of the TCR. These regions join through rearrangement during T-cell ontogeny and different combinations of the V, (D), J and C DNA segments define their antigen specificity. The structural diversity of TCR is determined by (a) the combination of V, (D), J and C DNA segments, and (b) the different sites at which the gene segments are joined. Both chains are required for recognition of foreign structures. The TCR is non-covalently associated with the CD3 complex which is involved in the transmission of activation signals (12,16,17). The CD3 complex is composed of at least 4 invariable non-covalently linked polypeptide chains γ, δ, ε and ζ (18,19). It has been suggested that the CDδ chain transduces activation signals (19,20).

Recently, a CD3 complex was found on $CD4^-8^-$ T lymphocytes that did not express the TCR$\alpha\beta$ heterodimer (21-24). These cells appeared to express CD3 in association with:

(a) a disulphide heterodimer consisting of a 40K γ chain and a 40K δ chain;
(b) a non-disulphide heterodimer consisting of a 40K γ chain and a 40 K δ chain, or
(c) a non-disulphide heterodimer consisting of a 55K γ chain and a 40 K δ chain.

The TCRγ polypeptide component of the disulphide form is encoded by a gene that utilizes the Cγ1 gene segment, while the non-disulphide form is encoded by a gene that utilizes the Cγ2 gene segment (25). The differences in molecular weight between the 45 and 40K γ-proteins is primarily due to

differences in glycosylation (26).

The TCR γ and δ genes are essentially similar in structure to those encoded by the TCR α and β genes, although with less variability. The antigen receptors expressed on different types of cytotoxic lymphocytes are schematically depicted in Figure 1.1.

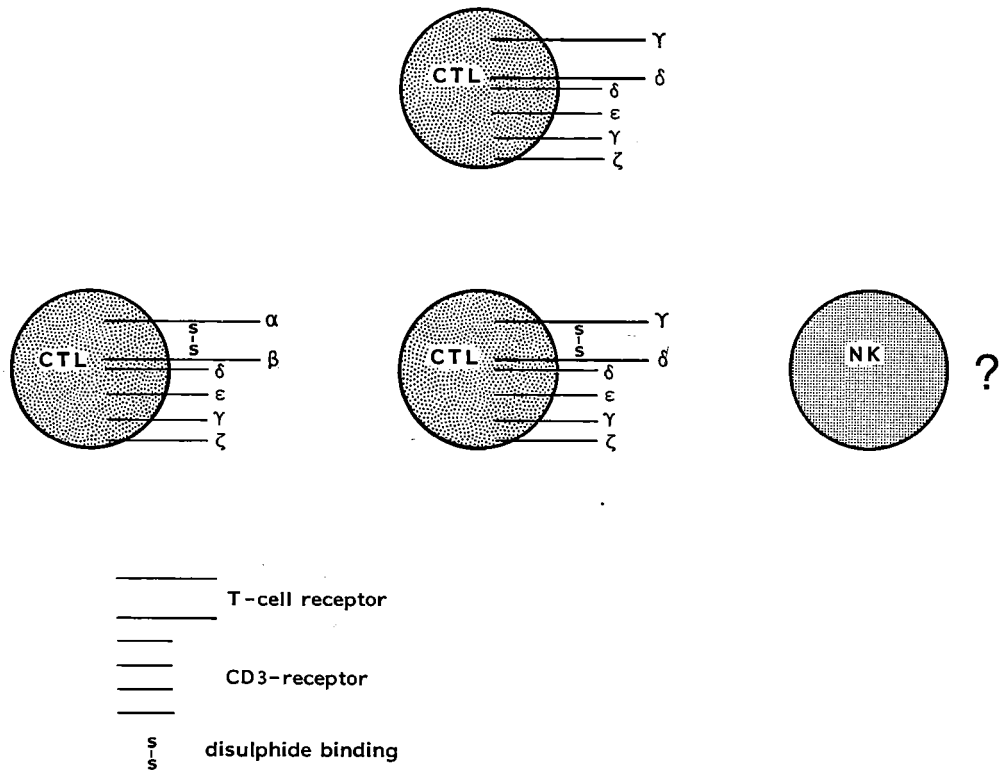


Figure 1.1 Antigen receptors on different types of cytotoxic lymphocytes. The T-cell receptors (TCR) are shown in association with a CD3 receptor. For all T cells, the CD3 receptor consists of a δ, ε, γ and ζ chain. The TCR are different for the various T cells. CTL express disulphide linked α-β chains, disulphide linked γ-δ chains or non-disulphide-linked γ-δ chains. For NK cells an antigen receptor has not been identified yet.

1.4 How do NK cells recognize and bind foreign structures?

The cytotoxic activity of NK cells is not restricted by MHC determinants on their target cells. It has been shown that in NK cells the genes encoding α, β, γ and δ are in the germline configuration. NK cells express no TCRα δ or γ mRNA, and express an aberrant TCRβ mRNA, the product of which is therefore not functional. Furthermore, no CD3-like structures have been found on NK cells. At present, it is not known which receptor structures on these $TCR^-/CD3^-$ NK cells and their target cells are involved in recognition and killing. Recently it has been shown that IgG-Fc receptors (FcR or CD16), T200 molecules (CD45) and the receptor for the sheep red blood cells (SRBC or CD2) are involved in the regulation of cellular cytotoxicity (CTX) of $TCR^-/CD3^-$ NK cells (27-30). We demonstrated that anti-CD16 monoclonal antibodies (mAb) can induce lytic activity of target cells (27). In contrast, mAb directed against structures associated with the CD45 molecule can block NK-cell lysis (31), while anti-CD2 mAb block or induce cytolysis, depending on effector-target cell combinations used (29,31 for review, see 32).

1.5 Mechanisms of cytolysis

The various types of cytotoxic cells described in section 1.2 are endowed with different lytic mechanisms (for review, see 6). CTX can be induced in various ways, depending on the activation process and the stimulator cell type (32). $TCR\alpha\beta^+/CD3^+,4^+,8^-$ and $CD3^+,4^-,8^+$ CTL require allostimulation before they are able to exert MHC-restricted CTX. Some $TCR\alpha\beta^+/CD3^+,4^-,8^+$ CTL exert MHC-restricted as well as unrestricted CTX after allostimulation (33). In contrast to $TCR\alpha\beta^+/CD3^+$ CTL, $TCR^-/CD3^-$ NK cells exert MHC-unrestricted CTX without any prior ex vivo stimulation. $TCR^-/CD3^-$ NK cells also mediate antibody-dependent cellular cytoxicity (ADCC), i.e. they lyse NK-resistant target cells when these are coated with IgG antibodies. Only $TCR^-/CD3^-$ NK cells (34) and some $TCR\gamma\delta^-/CD3^-4^-,8^-$ CTL (27,35) express CD16. Like NK cells, these $TCR\gamma\delta^+/CD3^+,4^-,8^-$ CTL can exert MHC-unrestricted lysis of target cells but need to be activated, e.g. by IL-2 (21).

TCR$\alpha\beta$ and TCR$\gamma\delta$ CTL lyse target cells which have been precoated with phytohemagglutinin (PHA), i.e. so called lectin-dependent cellular cytotoxicity (LDCC). Only those effector cells which have intrinsic lytic capacity (activated but not specifically recognizing the target cell) can lyse PHA-coated target cells. The lytic mechanisms employed by different cytotoxic lymphocytes in vitro are listed in Table 1.1.

The mechanism of in vivo killing, either by TCR$\alpha\beta^+$/CD3$^+$ CTL or by TCR$^-$/CD3$^-$ NK cells, is not well understood. As stated, TCR$\alpha\beta^+$/CD3$^+$ CTL recognize foreign antigens in vitro in the context of MHC determinants (for review, see 7). Before recognition of specific antigen and MHC, nonspecific conjugate formation between TCR$\alpha\beta^+$/CD3$^+$ CTL and target cell takes place (36,37). The nonspecific adhesion stage is Mg-dependent (38). Recent data suggest that in this stage LFA-2 (CD2) on the effector cell interacts with LFA-3 as its ligand on the target cell (39), or LFA-1 on the effector cell interacts with the Inter Cellular Adhesion Molecule-1 (ICAM-1) on the target cell (40). After binding a target cell, the TCR$\alpha\beta^+$/CD3$^+$ CTL becomes activated and rapid Ca-mobilization takes place. After the nonspecific adhesion, a specific TCR/MHC-antigen interaction takes place followed by programming for lysis and the lethal hit. The "programming for lysis" stage is Ca-dependent. Once the lethal hit has been delivered the target-cell-lysis is CTL-independent (41). Activation of TCR$\alpha\beta^+$/CD3$^+$ CTL by the tumor cells also induce the expression of interleukin-2 receptors (IL-2R) which are required for proliferation.

Two different models for T-cell lysis are presented in Figures 1.2.1 and 1.2.2 (42). The currently most common view is that upon specific recognition TCR$\alpha\beta^+$/CD3$^+$ CTL release poreforming molecules in the intercellular space between CTL and the target cell. These molecules subsequently attach to the target cell and induce cytolysis. However, 2 groups independently showed that target cell lysis by TCR$\alpha\beta^+$/CD3$^+$ CTL does not require exocytosis of cytolytic granules containing perforins, esterases or cytolysin (43,44). For instance, antibodies that inhibit perforin-mediated cytolysis do not inhibit T-cell mediated cytolysis (42).

An alternative view is that TCR$\alpha\beta^+$/CD3$^+$ CTL induce the target cells to commit suicide. When target cells are presented to TCR$\alpha\beta^+$/CD3$^+$ CTL, it is their nuclear and not the cytoplasmic membrane that is damaged (45) followed by rapid DNA fragmentation (46). Recently, it has been shown that glucocorticoids can activate an endogeneous suicide process in target cells by degradation of chromosomal DNA (47).

The interaction site on TCR$^-$/CD3$^-$ NK cells for their target cell structures is unknown but, like TCR$\alpha\beta^+$/CD3$^+$ CTL, target cell recognition is Mg-dependent and effector cell activation is Ca-dependent (48,49). After binding of poreforming molecules on the target cell, lysis subsequently takes place, even after the effector cells are detached (Figure 1.3) (49).

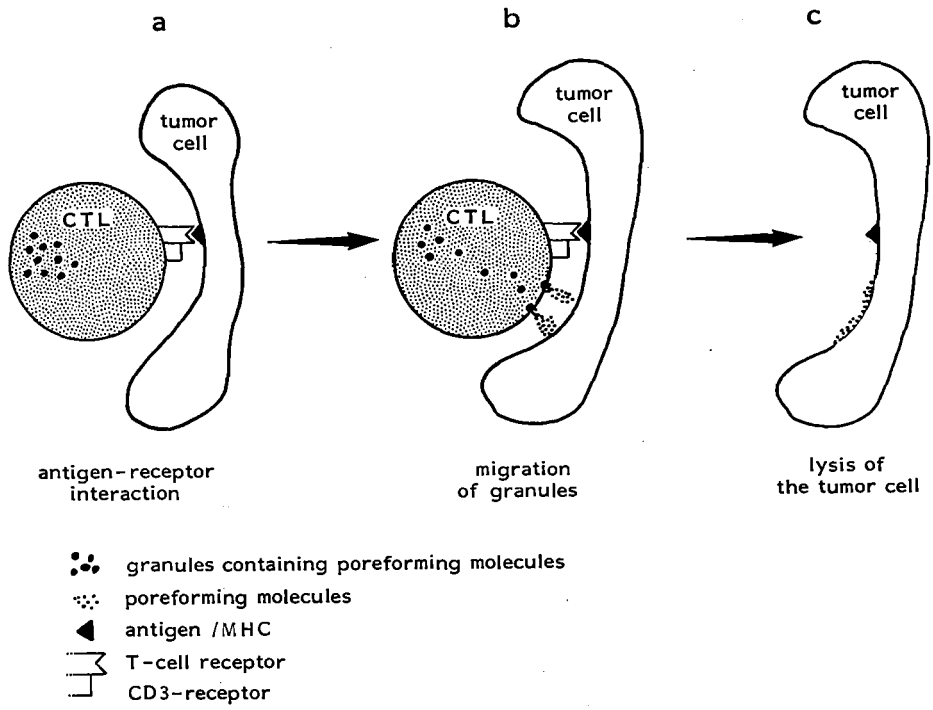


Figure 1.2.1 Poreforming molecules from CTL can induce lysis of tumor cells. After antigen-receptor interactions (a) signals are transduced via the CD3 receptor, inducing migration of lysosomes to the membrane surface of the CTL (b). Binding of poreforming molecules (cytolysins) lead to lysis of the tumor cell (c). For this final process of cell lysis the presence of CTL is not required.

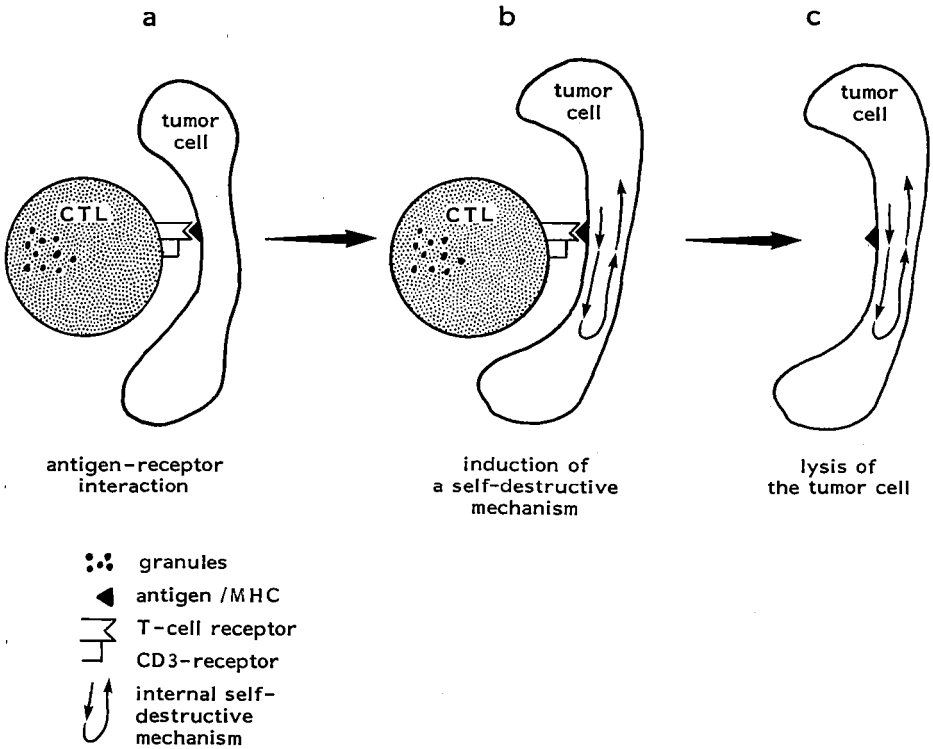


Figure 1.2.2 CTL trigger tumor cells to commit suicide. After antigen-receptor interaction (a) an internal selfdestructive mechanism is triggered in the tumor cell (b), which causes cytolysis (c). The latter step can occur without the presence of CTL.

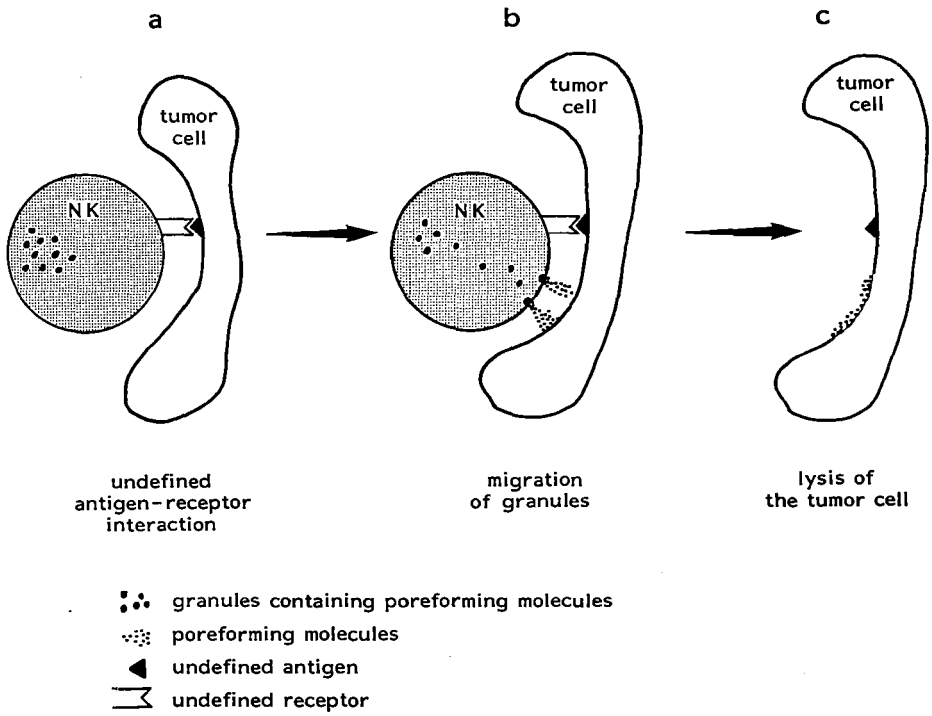


Figure 1.3 Poreforming molecules from NK cells induce lysis of tumor cells. It is not known which antigen-receptor interactions are involved in recognition. Like for CTL, after binding of NK-cells to tumor cells (a) internal signals induce migration of lysosomes containing poreforming molecules (cytolysins) (b). Binding of the poreforming molecules to the cell surface of the tumor cell (c) induces cytolysis of the tumor cell. Again, the latter step can occur without the presence of NK cells.

1.6 Growth factors

IL-2, previously termed T-cell growth factor (TCGF), provides the necessary signal for the transition of activated T cells from the G1 to the S phase of the cell cycle (50). Activation of resting $TCR\alpha\beta^+/CD3^+$ CTL by antigens or mitogens leads to the release of several lymphokines, including IL-2, and to the induction of IL-2R (51). The interaction between IL-2 and the IL-2R is an absolute requirement for proliferation of T and NK cells. Recent studies have indicated that $TCR^-/CD3^-$ NK cells, in contrast to $TCR\alpha\beta^+/CD3^+$ CTL, can proliferate for about 2 weeks in response to IL-2 alone, i.e. without the need for a separate activation signal (52-54).

Three types of IL-2R have been identified i.e., a P55 α-chain with a

low affinity for IL-2, a P70 β-chain with an intermediate affinity for IL-2, and a P55-P70 αβ heterodimer with a high affinity for IL-2 (55). On resting NK cells, only the IL-2R β-chain is expressed. Upon interaction of IL-2 with the β-chain the IL-2 α-chain receptor is induced and functional high affinity IL-2R are formed (56). The growth signal is delivered by IL-2 bound to the high affinity and not to the low or intermediate affinity receptor (57-59). The fate and function of the low-affinity type is unknown. Anti-CD25 mAb, called anti-TAC mAb, specific for the IL-2 α-chain receptor have been produced (60,61). Molecular cloning of the TAC antigen has formally proven that this receptor (CD25) binds IL-2 (62,63). In addition to its key role in proliferation of T lymphocytes, IL-2 also exerts other immune-regulatory functions. (see section 1.8.).

1.7 Oncogenes

Proto-oncogenes are conserved cellular genes homologous to the transforming (onc) genes of acute, oncogenic retroviruses (for review, see 64). It is now widely accepted that upon transcriptional or mutational activation proto-oncogenes can function like viral oncogenes. In oncogene research, activation refers to the conversion of a non-oncogenic proto-oncogene, into a carcinogenic variant. Indeed, mutations in proto-oncogenes or alterations in their level of transcription have been found in certain tumors. However, mutated proto-oncogenes are structurally different from viral oncogenes. Moreover, mutated proto-oncogenes are less efficient in transforming rodent cells than viral oncogenes (65). The proto-oncogenes are regarded as a subset of a larger group of cellular genes thought to be involved in differentiation events and in growth control of cells (66,67).

The physiological role of some (proto-)oncogenes have been established. For example, v-erbB is known to be derived from the normal cellular gene that encodes the epidermal growth factor (EGF) receptor (68,69), and the v-sis oncogene encodes a growth factor that is structurally and functionally homologous to platelet-derived growth factor (PDGF) (70,71). Similarly, the v-fms oncogene is probably derived from the gene for the macrophage colony-stimulating factor receptor (MCSF) (72). Many oncogene products identified as growth factors, influence the expression of other oncogenes. For example, the product of v-sis (PDGF) stimulates accumulation of c-myc and c-fos transcripts in growth arrested cells (73-76). Other work indicates that transforming growth factor β (TGF-β) increases the number of cell surface EGF receptors (77). Myc-like genes encode nuclear DNA binding proteins that may in turn regulate the expression of other oncogenes involved in cell

proliferation (78,79). Stern et al (80) suggested a model for the interplay of c-myc and c-ras oncogenes in which the ras-like genes induce growth factor production, while myc-like genes increase the responsiveness of cells to these factors.

The mechanism(s) by which oncogenes act in the growth-regulating processes are not very well understood and remain to be investigated. Recently, it has been found that products of ras genes are located at the inner surface of the plasma membrane of fibroblast cells and may be involved in transduction of extracellular signals (81-84). It has been suggested that the c-ras proto-oncogene encodes for a G-protein (81,85), also present in the plasma membrane of T cells, and involves the regulation of external signals induced by PHA or allostimulation (86).

The induction of many oncogene transcripts in T cells has been studied extensively in vitro. Kelly et al. (87) reported that in the mouse system c-myc mRNA levels in PBL can be augmented by mitogenic lectins. We and others found similar results for human PBL of healthy individuals (88,89). Possible routes of the induction of c-myc expression in normal and malignant $TCR\alpha\beta^+/CD3^+$ and $TCR^-/CD3^-$ lymphocytes have been described in chapter 5 of this thesis. Reed et al (89) showed that in human PHA-stimulated PBL the genes for c-fos, c-myc, IL-2 and IL-2R are expressed early, whereas c-myb, N-ras and transferrin-receptor (TRF) are expressed later in PHA stimulated PBL. IL-2 augmented the expression of the genes for c-myb, P53, N-ras and TRF in PHA stimulated cultures of PBL, as well as the early genes for c-myc and the IL-2R but not c-fos (89,90).

1.8 Modification of the cytolytic response

Cytokines involved in proliferation of T and NK cells may also affect their CTX and are therefore called BRM. The effects of BRM such as IL-2, IFN and OK-432 on the cytolytic response have been extensively studied in vivo and in vitro both in man and mice (91-96).

OK-432 (a streptococcal preparation) can reduce carcinomateus effusions in the pleural or peritoneal cavities (97,98). It can affect the immune system by activation, increase or restoration of the activities of various immune effector cells, or by inhibition or abrogation of its functions (for review see 99). Most studies were performed with fresh PBL or with NK-cell enriched fractions. Because of the heterogeneity of the responder cell populations it is not clear which particular cell types respond to OK-432. $TCR^-/CD3^-$ NK cells are considered most responsive to OK-432, i.e. augmentation of NK-cell activity against tumor cells (92,100), but others suggested that OK-432

eliminates the suppression of NK-cell activity by monocytes (101).

With the introduction of lymphocyte cloning techniques, it became possible to investigate whether TCR$\alpha\beta^+$/CD3$^+$ T-cell subsets as well as TCR$^-$/CD3$^-$ NK cells can respond to OK-432 (see chapter 6 of this thesis).

IFN have been classified into 3 categories i.e. α (leucocyte), β (fibroblast) and γ (immune). They posses numerous biological activities, including modulation of the immune response as well as antiproliferative capacities (94). IFNβ strongly augments NK-cell activity against tumor cells (96,102). IFNγ also enhances the expression of MHC and Ia antigens on various cell types (103). Increased MHC expression leads to an increased susceptibility to antiviral and antitumor cytotoxic lymphocytes (104-106). IL-2 can potentiate IFN-γ production by mitogen stimulated T cells.

IL-2 has been shown to augment the cytotoxic activity of TCR$^-$/CD3$^-$ NK cells against numerous tumor cell lines (105,107). It augments the CTX of NK cells from healthy subjects (108,109) as well as from patients with acquired immune deficiency syndrome (110).

IL-2 can also enhance the lytic activity of fresh PBL against some fresh autologous tumor cells (111-114). Such responder cells are called "lymphokine-activated killer" (LAK) cells. LAK cells, first described by Grimm et al. (114) and Rosenberg et al. (115) were reported to express CD3, CD8 and CD16 markers. However, the majority of human cells with activated killer (AK) activity, as reviewed by Hersey and Bolhuis (32), have now been proven to be derived from the TCR$^-$/CD3$^-$ 16$^+$ NK cells. These authors suggest that activated lymphocyte killing (ALK) defines an activity rather than a distinct effector cell population (32).

The adoptive transfer of LAK cells into mice with established pulmonary sarcoma metastases was highly effective in reducing the number (and size) of these tumor nodules when combined with separate injections of IL-2 (116). These findings provide a rationale for clinical application, i.e. the infusion into patients of IL-2 activated lymphocytes together with the systematic infusion of IL-2.

1.9 Cytotoxic lymphocytes and their antitumor reactivity

We described that in vitro the interaction between TCR$\alpha\beta^+$/CD3$^+$ CTL and tumor target cells may activate CTL which lead to IL-2R expression, proliferation of these CTL and lysis of tumor target cells. Such mechanisms were proposed to be operative in vivo to prevent outgrowth of tumor cells and are called immunosurveillance (see section 1.1). A concept of a generalized immunosurveillance by CTL predicts that

(a) tumors that arise in spite of immunosurveillance in normal hosts are poorly immunogenic and do not provoke an immune response;
(b) any clinical or experimental condition associated with depressed T-cell immunity will be associated with an increased tumor incidence; and
(c) many tumors arising in such immunodeficient hosts are immunogenic.

Thus far, some results obtained in experimental studies support the immunosurveillance theory and others argue against it. A phenomenon that has been used as an argument against the immunosurveillance theory is the fact that a small number of immunogenic tumor cells injected into a syngeneic recipient can often escape from immune lysis, whereas a larger number of tumor cells is rejected (117,118). A phenomenon which supports the immunosurveillance theory is the poor immunogenicity of the majority of spontaneous tumors arising in immunocompetent hosts. Another phenomenon which supports the immunosurveillance theory is the increased incidence of tumors in immunosuppressed patients. In hereditary immunodeficient patients and in individuals receiving chronic immunosuppressive therapy after transplantation, the incidence of tumors was found to be strongly increased in comparison to the general population (119,120). An example of a naturally occuring immunodeficiency state associated with an increased incidence of malignancies is Wiskott-Aldrich syndrome (128-fold increase). In renal transplant recipients, receiving immunosuppressive drugs, the incidence of tumors was also found to be increased (119,121). Dialysis-patients showed a 26-fold increase for non-Hodgkin lymphoma, while for patients with autoimmune diseases the increased incidence of tumors appeared to be variable (122). An excess of tumors has also been found in patients with an acquired immunodeficiency syndrome (AIDS), such as Kaposi's sarcoma, Burkitt lymphoma, reticulum sarcoma, cancer of mouth, head and neck, cloaogenic cancer and sometimes hepatoma (123-125). Another facet of the immune response is the tumor-induced activation of suppressor T cells leading to the inhibition of a CTL response. This phenomenon has been demonstrated for melanoma in vitro by Mukherji et al. (126,127).

Besides $TCR\alpha\beta^+/CD3^+$, also $TCR^-/CD3^-$ NK cells were considered to participate in the immune response against tumor cells (see section 1.1). Many studies have shown that $TCR^-/CD3^-$ NK cells may inhibit the growth of tumor cells in vivo (128). NK activity is strongest in blood and spleen, but relatively poor or undetectable in lymph nodes, thymus and bone marrow (129). These observations lead to the suggestion that metastases present in blood or spleen are the most likely targets for $TCR^-/CD3^-$ NK cells. The observed increased invasiveness and frequency of metastases in patients with diminished natural immunity mediated by $TCR^-/CD3^-$ NK cells or natural antibodies or both

(128) support this suggestion. Like CTL-responses, NK-responses can be inhibited by suppressor cells or by soluble factors such as prostaglandin E (PGE), produced by tumor-activated suppressor cells (130,131).

What could be the role of $TCR\gamma\delta^+/CD3^+$ CTL in the immune response against spontaneous or virus-induced tumors? $TCR\gamma\delta^+/CD3^+$ CTL appear early in the ontogeny and may provide protective immunity against viral or bacterial infections (132). There is evidence that $TCR\gamma\delta^+/CD3^+$ CTL mediate MHC-unrestricted killing of target cells in vitro (133). Unlike NK cells, these $TCR\gamma\delta^+/CD3^+$ CTL do require prior activation to display MHC-unrestricted CTX (134-136). Although not proven yet, the structure of the TCR$\gamma\delta$ receptors predicts antigen specificity and MHC restriction (for a review see 10).

1.10 Introduction to the experimental work

An important objective in tumor immunology is to develop effective immunotherapy for cancer patients by means of BRM, either naturally present or artificically administered. In order to achieve this goal, fundamental and practical questions need to be answered. Some strategic questions are why do transformed cells develop into tumors when there is an adaptive and natural immune response to destroy them? Is the immune system deficient? If not, are tumor cells non-immunogenic or do they lack expression of antigenic determinants? Do tumor cells activate mechanisms of immunosuppression? Answers to these questions will allow to modulate and mobilize the host immune system in a controlled way so that efficient reduction or even elimination of the tumor is achieved.

The purpose of the studies described in this thesis was to increase the insight in the potential usefulness of cytotoxic lymphocytes as antitumor agents. First, we studied the involvement of $TCR\alpha\beta^+/CD3^+$ and $TCR\gamma\delta^+/CD3^+$ CTL and $TCR^-/CD3^-$ NK cells in tumor cell lysis and the regulation of their lytic functions by other T-cell subsets (137,138, chapter 2 and 3 of this thesis). Next, we investigated which strategy was most efficient to reduce or eliminate tumor cells by using cytotoxic lymphocytes such as $TCR^-/CD3^-$ NK cells or $TCR\alpha\beta^+$/CD3 CTL.

Although in the last few years much has been learned about TCR recognition of foreign antigens in association with MHC determinants on cultured tumor cell lines (see section 1.3), little is known about recognition of fresh tumor cells in vivo by CTL and NK cells. To use T and NK cells for immunotherapeutic aims, detailed studies into the mechanisms by which fresh tumor cells can be recognized and killed are necessary. Therefore, the availability of large numbers of functionally stable T and NK cells is a

prerequisite. Unfortunately, the growth capacity of T and NK cells in tissue culture systems is limited and requires the presence of feeder cells, lectins, IL-2 and other unknown growth factors. The establishment of immortalized T and NK cells is therefore of paramount importance. Immortalization by somatic cell hybridization seemed to be a promising method to obtain permanent cytotoxic cell lines. This technique has proven to be successful in immortalizing mouse B lymphocytes for the production of mAb (139). Although less successful, hybridoma technology has also been used for immortalization of human B cells and human T cells which produce growth factors (140-143). However, immortalization of the lytic function of lymphocytes by hybridoma technology has been proven to be extremely difficult. This could be due to nonspecific lysis of the tumor fusion partner by the cytotoxic lymphocytes during or after the fusion procedure (144). Moreover, the production of human-human hybridomas in general is difficult, due to (1) the slow establishment of initial growth of fusion products (145, 146) and (2) chromosomal instability (147). Thus far, hybridoma cell lines with cytolytic activity have been described only in the mouse-mouse and mouse-human system (148-150).

In view of these problems we first developed a new technique for the efficient production of human-human T-T cell hybridomas (151,152). This protocol, described in chapter 4, is based on the electric field-induced cell fusion used by Zimmermann (153) for plant cell fusions. Using this approach it was possible to efficiently produce human-human T-cell hybridomas. However, the few hybridomas that were cytolytic lost this activity within a short period of time. On the basis of the large number of hybridomas tested (more than 500) we concluded that immortalization of cytotoxic lymphocytes by somatic cell hybridization is not feasible (chapter 4 of this thesis). DNA-mediated gene transfer is an alternative approach. In this respect it is important to identify the genes involved in proliferation. Chapter 5 describes the expression of oncogenes and growth factors in normal and malignant $TCR\alpha\beta^+/CD3^+$ and $TCR^-/CD3^-$ lymphocytes. The expression of the c-myc and c-ras oncogene as well as IL-2R levels has been studied on the mRNA and protein level, respectively. To screen large numbers of T and NK-cell clones on the expression of the different oncogenes, we (88) modified the "quick blot" technique developed by Bresser et al. (154). This technique only requires low cell numbers and experiments can be performed in a short time. IL-2R levels have been measured using anti-CD25 mAb.

In the future, immortalized cytotoxic lymphocytes may be used for immunotherapeutic aims. The other approach to eliminate or reduce tumor cells is to modulate the lytic function of cytotoxic lymphocytes by application of BRM. Chapter 6 describes the enhancement of the CTX of $TCR^-/CD3^-$ NK cells and $TCR\alpha\beta^+/CD3^+$ CTL by the BRM IL-2 and OK-432, using cloned lymphocytes. We

identified which lymphocyte cell types are suitable to use for therapeutic aims when treated with BRM (155).

CHAPTER 2

LYTIC ACTIVITY OF TCR$\gamma\delta^+$/CD3$^+$ CYTOTOXIC T-CELL CLONES AGAINST FRESH AND CULTURED TUMOR CELLS

2.1 Introduction

Tumors induced in experimental animals by carcinogens or by viruses often express tumor-associated antigens (TAA) which can activate the immune system in the host (156,157). In contrast, most "spontaneous" tumors are non-immunogenic, i.e. do not provoke an immune response (3,157). TCR$\alpha\beta^+$/CD3$^+$ cytotoxic T lymphocytes (CTL) recognize and lyse immunogenic tumor cells expressing TAA in the context of major histocompatibility complex (MHC) determinants (adaptive immunity), whereas TCR$^-$/CD3$^-$ natural killer (NK) cells recognize and lyse also non-immunogenic tumor cells (natural immunity) (158).

Recently, a third subset of cytotoxic lymphocytes, termed TCR$\gamma\delta^+$/CD3$^+$ CTL, has been cloned from peripheral blood lymphocytes (PBL) (134) and from immunodeficiency patients (159). TCR$\gamma\delta^+$/CD3$^+$ CTL share several features with TCR$^-$/CD3$^-$ NK cells as well as with "classic" TCR$\alpha\beta^+$/CD3$^+$ CTL. Like TCR$^-$/CD3$^-$ NK cells, most TCR$\gamma\delta^+$/CD3$^+$ CTL are CD4$^-$ and CD8$^-$ and are CD16$^+$, although this latter receptor is readily lost during *in vitro* culture (134). TCR$\gamma\delta^+$/CD3$^+$ CTL, upon activation, also exert MHC-unrestricted cellular cytotoxicity (CTX) (21,22,134-136). The TCR$\gamma\delta$ protein chains are distinct in composition from the TCR$\alpha\beta$ but similarly noncovalently linked to the CD3 complex (23,24,160). The TCR$\gamma\delta$ structure may be involved in specific interactions (136,160).

Recently, results from others and ourselves have shown that TCR$\gamma\delta^+$/CD3$^+$ CTL kill cultured tumor cells (23,134,162). In this study we compared the lytic capacity of cloned TCR$\gamma\delta^+$/CD3$^+$ CTL with that of cloned TCR$\alpha\beta^+$/CD3$^+$ CTL and TCR$^-$/CD3$^-$ NK cells using a variety of fresh and cultured tumor target cells.

2.2 Materials and methods

2.2.1 Cells and cell culture

Cloned lymphocytes studied are listed in Table 2.1. They were cultured as described previously (163). Briefly, responder cells ($2x10^3$ cells/200 µl) were expanded in RPMI medium supplemented with 10% human serum (not heat-inactived), 4 mM glutamin, 1 µg/ml indomethacin, 25 U/ml recombinant interleukin-2 (IL-2) and antibiotics. Gamma-irradiated (20 Gy) B-LCL ($5x10^4$ cells/200 µl) and γ-irradiated (20 Gy) allogeneic lymphocytes (10^5 cells/200 µl) were added as feeder cells.

TABLE 2.1

CHARACTERISTICS OF HUMAN CLONED CYTOTOXIC LYMPHOCYTES

clones	CD3	CD4	CD8	CD16	TCR	derived from	ref.
CTL4	+	-	-	+	γδ	PBL healthy donor	134
CTL119	+	-	-	+	γδ	pleura exudate	134
CTL1005	+	-	-	+	γδ	$CD4^-CD8^-$ enriched fraction	134
CTL1011	+	-	-	+	γδ	$CD4^-CD8^-$ enriched fraction	134
CTL1012	+	-	-	+	γδ	$CD4^-CD8^-$ enriched fraction	134
CTL1015	+	-	-	+	γδ	CD4-CD8- enriched fraction	134
CTLWi-K	+	-	-	-	γδ	liquor	134
NK76	-	-	-	+	-	PBL Tγ lymphocytosis	163
NK77	-	-	-	+	-	PBL Tγ lymphocytosis	163
NK472	-	-	-	+	-	PBL SCID	163
CTL11	+	-	+	-	αβ	PBL healthy donor	166
CTL20	+	+	-	-	αβ	PBL healthy donor	167

SCID = severe combined immunodeficiency disease
TCR = T-cel receptor

2.2.2 Target cells

Fresh tumor cells. Tumor cells were isolated from pleura or ascites of cancer patients with ovarian, mammary, lung, endometrium and one mesothelioma carcinoma (Table 2.2). To separate tumor cells from exudate lymphocytes in pleura or ascites, cells were centrifuged on a Ficoll-gradient with 2 densities (d=1.077 g/cm and d=0.8078 g/cm). The low density cell fraction contains the tumor cells and the high density cell fraction the lymphocytes.

The latter were not used in these experiments. Tumor cells isolated from pleura or ascites, were identified by microscopic analysis of smears stained with May Grünwald-Giemsa. Contamination of the low density fraction with lymphocytes, detected by using anti-CD3 monoclonal antibodies (mAb), was less than 2%. In most of the preparations contamination of the tumor cell fraction with low percentages of macrophages was observed. Tumor cells were frozen and stored in liquid nitrogen. After thawing, dead cells were removed by Ficoll-Isopaque (d=0.8078 g/cm) and viability of the cells was measured by trypan blue staining.

TABLE 2.2

CHARACTERISTICS OF FRESH TUMOR CELLS

type of tumor	isolated from	malignancy
lung1685	pleura	pap V
endometrium1689	ascites	pap V
endometrium1784*	ascites	pap V
ovarian1690	ascites	pap V
ovarian1762	ascites	pap V
ovarian1781	ascites	pap V
mammary1721	ascites	pap V
mammary1806	pleura	pap V
mesothelioma1791	acites	pap V

* including sarcoma components
pap V = malignant cells were found in pleura or ascites

Cultured tumor cells. Tumor cell lines used as targets in the CTX tests are listed in Table 2.3. Tumor cell lines were cultured in RPMI-medium supplemented with 10% fetal calf serum (FCS; heat-inactivated), 2 mM glutamin and antibiotics.

TABLE 2.3

CHARACTERISTICS OF CULTURED TUMOR CELLS

tumor cell line	Human origin	source
K562	myeloid cells	Cannon, Litton Bionetics Kensington,USA.
MOLT	T-lymphoma	Hagemeyer, Erasmus Univ. The Netherlands.
HSB	T-lymphoma	Foung, Stanford Univ. Stanford, U.S.A.
JURKAT	T-lymphoma	Foung, Stanford Univ. Stanford, U.S.A.
DAUDI	EBV-transf B cells	de Vries, NKI, Amsterdam The Netherlands.
APD	EBV-transf B cells	Giphart, Leiden, The Netherlands.
BSM	EBV-transf B cells	Giphart, Leiden, The Netherlands.
LICRON-HMY-2	B-cells	Croce, Wistar Institute, U.S.A.
GLC1	small lung carcinoma	de Ley, RUG, Groningen, The Netherlands.
GLC2	small lung carcinoma	de Ley, RUG, Groningen, The Netherlands.
GLC3	small lung carcinoma	de Ley, RUG, Groningen, The Netherlands.
IGR137*	melanoma cells	de Vries, Amsterdam, The Netherlands.
IGR139*	melanoma cells	de Vries, NKI Amsterdam The Netherlands.
SKMEL*	melanoma cells	de Ley, RUG, Groningen, The Netherlands.
OMEL*	melanoma cells	de Vries, NKI, Amsterdam The Netherlands.
T24	bladder carcinoma cells	Perlman, Wenner-Gren Institute,Stockholm
U937	monocytic cells	Roozemond, AMC Amsterdam, the Netherlands

* melanoma cells were cultured for a few passages

2.2.4 Determination of cytotoxic activity

CTX was determined in a ^{51}Cr-release assay in round-bottom microtiter plates, as described previously (164). Briefly, varying cell numbers in 150 µl RPMI-medium, supplemented with 10% FCS (heat-inactivated), 2 mM glutamin and antibiotics, were seeded in the wells of the microtiter plate before the

addition of $2x10^3$ ^{51}Cr-labeled target cells (100 µl). Cells were incubated for 3 h at 37°C and centrifuged. Supernatant (100 µl) was collected and radioactivity determined. Specific ^{51}Cr-release was calculated as:

$$\frac{\text{experimental cpm - spontaneous cpm}}{\text{maximum cpm - spontaneous cpm}} \times 100\%$$

The maximum ^{51}Cr-release was obtained by incubation of the target cells with 10% (v/v) Triton X-100.

2.3 Results

Lysis of fresh tumor cells

We have tested in 3 separate experiments the CTX of 6 $TCR\gamma\delta^+/CD3^+$ CTL clones, 2 $TCR^-/CD3^-$ NK and 2 $TCR\alpha\beta^-/CD3^-$ CTL clones against fresh tumor cells from different histological type. All $TCR\gamma\delta^+/CD3^+$ CTL, $TCR^-/CD3^-$NK and $TCR\alpha\beta^+/CD3^+$ CTL clones expressed CTX against at least some of the fresh tumor cells used. The results of a representative experiment are provided in Table 2.4. In the 3 experiments the level of CTX by $TCR\gamma\delta^+/CD3^+$ was virtually as high as that of $TCR^-/CD3^-$ NK cells, and in some cases higher (endometrium 1784 and ovarian 1690). Clear differences were observed in lysis of different tumors. For instance, mammary carcinoma 1721 was not susceptible to lysis but mammary carcinoma 1806 was lysed by both cloned $TCR\gamma\delta^+/CD3^+$ CTL and $TCR^-/CD3^-$ NK cells. Ovarian carcinomas 1762 and 1781 were both lysed by $TCR\gamma\delta^+/CD3^+$ CTL and $TCR^-/CD3^-$ NK clones, and the latter also by $TCR^-/CD3^-$ NK clones. Moreover, both tumor targets were also lysed by the $TCR\alpha\beta^+/CD3^+$ CTL clones CTL11 and CTL20, whereas ovarian carcinoma 1690 was only lysed by $TCR\gamma\delta^+/CD3^+$ CTL clones. Endometrium carcinoma 1689 was efficiently lysed by clones of all lineages, but endometrium carcinoma 1784 was only susceptible to lysis by $TCR\gamma\delta^+/CD3^+$ CTL clones. Mesothelioma 1791 was lysed by some of the $TCR\gamma\delta^+/CD3^+$ T-cell and $TCR^-/CD3^-$ NK clones but not by $TCR\alpha\beta^+/CD3^+$ CTL clones. Lung tumor cells were not lysed by any of the clones.

TABLE 2.4

LYSIS OF FRESH TUMOR CELLS BY DIFFERENT EFFECTOR CELLS

tumor target cells	effector cell clones									
	$TCR\gamma\delta^+/CD3^+$						$TCR^-/CD3^-$		$TRC\alpha\beta^+/CD3^+$	
	CTL4	CTL1005	CTL1011	CTL1012	CTL1015	AKWi-K	NK76	NK77	CTL11	CTL20
	percent lysis of target cells*									
lung1685	3	nt	0	nt	5	7	nt	nt	6	1
endom1689	20	50	26	66	31	40	42	80	33	15
endom1784	14	20	12	nt	20	20	5	9	9	10
ovarian1690	5	10	10	nt	10	22	7	7	7	2
ovarian1762	0	nt	6	nt	29	40	nt	nt	16	61
ovarian1781	10	24	17	15	32	29	14	22	10	11
mammary1721	1	6	1	2	2	7	1	6	3	0
mammary1806	10	26	6	14	17	22	9	27	4	5
mesothe1791	1	12	2	1	3	15	8	12	6	3

* Results expressed as percent ^{51}Cr-release; effector:target ratio 27:1

Lysis of cultured tumor cells

The clones described in the previous section, and 2 other clones (CTL119 and NK472), were tested for their CTX against different tumor cell lines. All $TCR\gamma\delta^+/CD3^+$, $TCR\alpha\beta^+/CD3^+$ and $TCR^-/CD3^-$ clones showed CTX against at least some of the cultured tumor target cells used (Table 2.5).

As found for "fresh" tumor cells, the levels of CTX of $TCR\gamma\delta^+/CD3^+$ CTL and $TCR^-/CD3^-$ NK clones against cultured tumor cells were comparable. K562 tumor cells (MHC^-) were lysed by cloned $TCR\gamma\delta^+/CD3^+$ CTL and $TCR^-/CD3^-$ NK cells, and by the $TCR\alpha\beta^+/CD3^+8^+$ clone CTL11, but not by the MHC class II specific $TCR\alpha\beta^+/CD3^+,4^+$ clone CTL20 (Table 2.5). T-lymphoma cells were lysed by $TCR\gamma\delta^+/CD3^+$ CTL and $TCR^-/CD3^-$ NK clones and (one tumor cell line (HSB) marginally), by the $TCR\alpha\beta^+/CD3^+$ clone CTL11 (Table 2.5).

B-lymphoma cells, which were efficiently lysed by cloned $TRC^-/CD3^-$ NK cells, appeared resistent to $TCR\gamma\delta^+/CD3^+$ CTL clones. Two exceptions were observed: clones CTL4 and CTL1005 efficiently lysed Daudi B-lymphoma cells (Table 2.5).

TABLE 2.5

LYSIS OF CULTURED TUMOR CELLS BY DIFFERENT EFFECTOR CELLS

percent lysis of tumor cells*

effector cell clones	erythroid	T cells			B cells			
	K562	MOLT	HSB	JUR	DAU	APD	BSM	LICR
$TCR\gamma\delta^+/CD3^+$								
CTL4	46	51	33	18	59	13	9	0
CTL119	56	53	39	33	0	5	0	0
CTL1005	82	57	nt	nt	63	3	3	16
CTL1011	72	32	nt	nt	3	0	0	5
CTL1012	71	41	nt	nt	0	3	3	0
CTL1015	36	13	nt	nt	0	0	0	2
AKWi-k	nt	nt	nt	nt	0	0	0	nt
$TCR^-/CD3^-$								
NK76	57	50	nt	74	68	46	80	7
NK77	66	65	nt	84	44	25	74	5
NK472	58	35	nt	57	53	57	22	13
$TCR\alpha\beta^+/CD3^+$								
CTL11	65	0	18	0	0	0	62	77
CTL20	1	0	nt	nt	0	58	0	0

* Results expressed as percent ^{51}Cr-release; effector:target ratio.

The (Drw6) MHC class II-specific $TCR\alpha\beta^+/CD3^+$ clone CTL20 only lysed the relevant APD target cells ($Drw6^+$). Lung tumor cells (GLC1-3) were lysed by both $TCR^-/CD3^-$ NK and $TCR\gamma\delta^+/CD3^+$ CTL clones, but not or only slightly by the $TCR\alpha\beta^+/CD3^+$ clone CTL11 (Table 2.6).

TABLE 2.6

LYSIS OF CULTURD TUMOR CELLS BY DIFFERENT EFFECTOR CELLS

percent lysis of tumor cells*

effector cell clones	lung			melanoma				bladder	mono
	GLC1	GLC2	GLC3	IGR37	IGR39	SKMEL	OMEL	T24	U937
$TCR\gamma\delta^+/CD3^+$									
CTL4	56	15	16	31	9	24	8	23	29
CTL119	41	22	18	21	8	9	46	39	1
CTL1005	57	20	nt	56	nt	49	15	38	26
CTL1011	24	3	nt	13	nt	21	11	15	13
CTL1012	52	51	nt	66	nt	11	27	6	11
CTL1014	42	47	nt	11	nt	68	47	93	20
CTL1015	4	1	nt	3	nt	14	5	12	3
CTLWi-K	32	35	nt	4	nt	nt	24	nt	nt
$TCR^-/CD3^-$									
NK77	62	40	40	8	0	16	67	30	82
$TCR\alpha\beta^+/CD3^+$									
CTL11	12	0	0	8	0	30	70	0	0
CTL20	nt	nt	nt	4	1	11	7	0	2

* Results expressed as percent ^{51}Cr-release; effector:target ratio 27:1

Most of the melanoma cells (IGR37, SKMEL and OMEL) were lysed by $TCR\gamma\delta^+/CD3^+$ CTL clones. However, SKMEL and OMEL, but not IGR37 and IGR39, were also lysed by the $TCR^-/CD3^-$ clone NK77 and $TCR\alpha\beta^+/CD3^+$ clone CTL11. The bladder carcinoma T24 and the monocytic cell line U937 were efficiently lysed by $TCR\gamma\delta^+/CD3^+$ CTL and $TCR^-/CD3^-$ NK clones but not by the $TCR\alpha\beta^+/CD3^+$ clone CTL11. Except for APD, none of the tumor cell lines used were lysed by the MHC class II-restricted $TCR\alpha\beta^+/CD3^+$ clone CTL20.

2.4 Discussion

Thus far, little is known about the physiological function of $TCR\gamma\delta^+/CD3^+$ CTL in the overall immune response. Although $TCR\gamma\delta^+/CD3^+$ CTL have been implicated in the cytolysis of virus-infected target cells (132), we found no increased susceptibility of virus infected cells in vitro (unpublished data). Janeway et al. (161) have proposed that $TCR\gamma\delta^+/CD3^+$ CTL may demonstrate specificity for class I MHC antigens and mediate immunological surveillance of epithelia. Since activated $TCR\gamma\delta^+/CD3^+$ lymphocytes have also been isolated from the joint fluid of patients with juvenile rheumathoid arthritis (165), they may be involved in the pathogenic mechanisms of this disease.

Experimental results (23,134) suggest that $TCR\gamma\delta^+/CD3^+$ CTL may also be involved in antitumor responses. In the present study we have compared $TCR\gamma\delta^+/CD3^+$ CTL clones with $TCR^-/CD3^-$ NK and $TCR\alpha\beta^+/CD3^+$ CTL clones with respect to their CTX against different freshly biopsied as well as cultured tumor cells. Comparison of cloned $TCR\gamma\delta^+/CD3^+$ CTL with cloned $TCR^-/CD3^-$ NK and $TCR\alpha\beta^+/CD3^+$ CTL showed that the CTX of activated cloned $TCR\gamma\delta^+/CD3^+$ CTL against fresh tumor cells was comparable to that of $TCR^-/CD3^-$ NK clones. Our data indicate that $TCR\alpha\beta^+/CD3^+$ antitumor CTX is generally lower than that of $TCR\gamma\delta^+/CD3^+$ clones. With respect to cultured tumor cells, the target cell spectrum of $TCR\gamma\delta^+/CD3^+$ clones was virtually as wide as that of $TCR^-/CD3^-$ NK cells. However, an exception was observed for B-tumor cells, i.e. EBV-transformed B cells (APD, BSM and Daudi) and a leukemia plasma cell line (Licr), which in general were efficiently lysed by cloned $TCR^-/CD3^-$ NK cells, were not lysed by cloned $TCR\gamma\delta^+/CD3^+$ CTL. Why this $TCR\gamma\delta^+/CD3^+$ clones do not kill B tumor cells remains to be elucidated.

Different fresh and cultured tumor target cells show differential susceptibility to lysis by an individual effector clone (Tables 2.4, 2.5 and 2.6). Also different cultured tumor cells of the same histological type showed differential lysability by $TCR\gamma\delta^+/CD3^+$ clones and $TCR^-/CD3^-$ NK cell clones. Vice versa, individual effector clones showed particular target cell spectra, i.e. lysed different tumor cells with different efficacies. How can these differences be explained? A large body of data show that CTX can be inhibited by mAb directed against effector cell surface molecules which were not directly involved in antigen recognition, i.e. non-TCR structures. Furthermore the heterogeneity of recognition by the cloned effector cells may be explained by differences in the (level) of expression and/or nature of (a) MHC determinants, (b) TAA and (c) tumor cell ligands for effector cell accessory

molecules, such as ICAM-1 and LFA-3.

An additional level of complexicity results from synergistic activations between distinct activation sites such as CD2 and CD16, CD2 and CD3, suggesting that (intracellularly) the activation sites converge (27). Therefore, the target cell selectivity of a particular (cloned) lymphocyte may critically be determined by the relative expressions of these structures on effector as well as on tumor target cells and their interplay.

CHAPTER 3

IN VITRO AND IN VIVO ACTIVATION OF $CD4^+$ LYMPHOCYTES BY AUTOLOGOUS TUMOR CELLS

3.1 Introduction

Tumor cells can escape destruction by the host immune defense system in various ways. First, tumor cells can be non-immunogenic when they lack expression of major histocompatibility complex (MHC) or cognate antigens, and are thus incapable of provoking an immune response (157). Second, the host can be immunodeficient (168). Third, the tumor cells may induce immunosuppression mechanisms lowering or abrogating the activity of tumor directed cytotoxic T lymphocytes (CTL) (169). Evidence for this latter possibility was provided by Damle et al. (170,171) who showed that alloantigen-primed $CD4^+$ (inducer) lymphocytes activate autologous $CD8^+$ cells to differentiate into suppressor cells that specifically inhibit the response of fresh T cells to the original allogeneic stimulator cells. Also Mukherji et al. (126,127) reported such a down regulatory mechanism generated by autologous tumor cells in man. These investigators used $CD4^+$ (inducer) lymph node lymphocytes, derived from a patient with melanoma, which prevented the generation of a CTL-response against the melanoma cells, when added to a mixture of fresh peripheral blood lymphocytes (PBL) (containing pre-CTL) and autologous tumor cells.

Thus far, most of the autotumor-directed $TCR\alpha\beta^+/CD3^+$ CTL reponses have been observed against melanoma cells (126,127,172-176), but a few have been reported against larynx and bladder carcinoma (177). Expression of antigens characteristic for a tumor cell type, termed tumor-associated antigens (TAA), depends on the origin of the tumor, i.e. chemically or virally induced tumors are more immunogenic than spontaneous tumors (3,178,179). The existence of specific antigens on spontaneous tumors remains uncertain. Reports published sofar, demonstrate the difficulty to develop specific CTL responses against tumor cells arisen spontaneously *in vivo*. It is not clear whether the number of tumor-directed $TCR\alpha\beta^+/CD3^+$ CTL and/or the strength of their response to autologous tumor cells is insufficient. Therefore, it is of importance to test PBL of cancer patients for the presence of activated T cells and subsequently

assay them against autologous tumor cells.

Here, we have immunophenotyped PBL of 22 cancer patients and 21 healthy individuals. Lymphocytes derived from 9 of the patients had an increased percentage of $CD25^+$ lymphocytes. The CD25, i.e. interleukin-2 receptor (IL-2R) is an activation marker for T cells (51). This raised the possibility that the tumor cells had activated these lymphocytes in vivo, resulting in the induction of IL-2R. Indeed, fresh PBL from these cancer patients could be stimulated in vitro by autologous tumor cells, which led to a further increase of $IL\text{-}2R^+$ lymphocytes. We present evidence that $CD4^+$ lymphocytes are preferentially activated by autologous tumor cells in vitro and in vivo.

3.2 Materials and methods

3.2.1 Lymphocytes

PBL were obtained from blood of healthy donors and patients by centrifugation on Ficoll-Isopaque (d=1.077g/cm). All cells were frozen and stored in liquid nitrogen before their use as responder in mixed-lymphocyte-tumor-culture (MLTC) assays.

3.2.2 Fresh tumor cells

Tumor cells were isolated from pleura or ascites of cancer patients with ovarian, mammary, lung, gall-bladder, mesothelioma, and endometrium carcinoma (Table 3.1). To separate tumor cells from pleura and/or from peritoneal exudate lymphocytes, cells were centrifuged on a Ficoll-gradient with 2 densities (d=1.077g/cm and d=0.8078g/cm). The low density cell fraction represents the tumor cells and the high density cell fraction the lymphocytes. The latter were not used here. Presence of the tumor cells, isolated from pleura or ascites was confirmed by microscopic analysis of May Grünwald-Giemsa stained cytospins. Lymphocyte contamination of the low density tumor fraction was assessed with fluorescein isothiocyanate (FITC)-labeled anti-CD3 monoclonal antibodies (mAb) and was always less than 2%. Tumor cells were frozen and stored in liquid nitrogen. After thawing, dead cells were removed by Ficoll-Isopaque centrifugation (d=0.8078g/cm) and viability of the cells was measured by trypan blue staining. Tumor cells were used for stimulation of autologous PBL in a MLTC and as target cells in cytotoxicity assays.

TABLE 3.1

CHARACTERISTICS OF CANCER PATIENTS

patient	carcinoma	malign.	isolated from	chemo treatment	percent CD25
1685	lung	pap V	pleura	no	nd
1689	endometrium	pap V	ascites	yes	17
1784	endometrium*	pap V	ascites	no	12
1690	ovarian	pap V	ascites	no	nd
1747	ovarian	pap V	ascites	no	29
1754	ovarian	pap V	ascites	yes	0
1757	ovarian	pap V	pleura	yes	10
1762	ovarian	pap V	ascites	yes	nd
1782	ovarian	pap V	ascites	yes	7
1790	ovarian	pap V	ascites	yes	3
1743	ovarian	pap V	ascites	yes	22
1798	ovarian	pap V	ascites	yes	6
1702	mammary	pap V	ascites	yes	nd
1718	mammary	pap III	ascites	yes	8
1721	mammary	pap V	ascites	yes	2
1758	mammary	pap V	pleura	no	14
1783	mammary	pap V	pleura	yes	0
1789	mammary	pap III	pleura	yes	22
1806	mammary	pap V	pleura	no	0
1746	mesothelioma	pap V	ascites	no	24
1791	mesothelioma	pap V	ascites	no	6
1775	gall-bladder	pap III	ascites	no	7
1715	none	pap II	ascites	yes	0
1716	none	pap I	pleura	no	6
1759	none	pap II	ascites	yes	11
1767	none	pap II	ascites	yes	11

pap 1 and 2 = nonmalignant cells were found in pleura or ascites
pap 3 = observed cells were suspected of malignancy
pap 4 and 5 = malignant cells were found in pleura or ascites
*including sarcoma components

3.2.3 Cultured tumor cell line

K562, a human erythroid cell line (provided by Dr. Grace Cannon, Litton Bionetics, USA) was used as natural killer (NK)-sensitive target cell line in cytotoxicity assays. Cells were cultured in RPMI supplemented with 10% fetal calf serum (FCS; heat-inactivated), 2 mM glutamin, and antibiotics.

3.2.4 MLTC/^{14}C-Thymidin incorporation/expansion

The presence of MHC determinants on fresh tumor cells was tested by a allogeneic mixed-lymphocyte-tumor-culture (MLTC). Stimulation of PBL by

autologous tumor cells was examined in an autologous MLTC. MLTC assays were performed in 96-well round-bottom microtiter plates (Greiner). Responder cells (10^5) in 0.2 ml RPMI, containing 10% normal human serum (not heat-inactivated), 4 mM glutamin and antibiotics, were mixed with $2x10^4$ γ-irradiated (25 Gy) allogeneic or autologous tumor cells and cultured for 1 week. To measure stimulation of PBL by allogeneic or autologous tumor cells, lymphocytes in triplicate wells were pulsed with ^{14}C-Thymidin and 18 h later harvested on glass fiber filters. To select for autotumor-reactive T cells, MLTC-derived cells (from autologous combinations) were cultured with 25 U recombinant IL-2/ml (Biogen, Geneva, Switzerland) for 1 week and expanded in a T-cell culture system described earlier (33). Briefly, responder cells ($2x10^3$ cells/well) were cultured in 200 µl RPMI medium supplemented with 10% human serum (not heat-inactivated), 4 mM glutamin, 1 µg indomethacin/ml, 1 µg leucoagglutinin/ml, 25 U recombinant IL-2/ml and antibiotics. Gamma-irradiated (20 Gy) Epstein-Barr virus-transformed human lymphoblastoid B-cell lines (B-LCL) ($5x10^4$ cells/200 µl) and allogeneic lymphocytes (10^5 cell/200 µl) were added as feeder cells. Feeder cells and medium weekly were refreshed. In each well 10^3 MLTC-derived lymphocytes were seeded. These MLTC-derived lymphocytes were expanded for 4 weeks, after which they were immunophenotyped and used as effector cells in cytotoxicity assays.

3.2.5 Determination of cytotoxic activity

CTX was determined in a ^{51}Cr-release assay in round-bottom microtiter plates (Greiner) as described earlier (164). Briefly, various numbers of cells in 150 µl RPMI medium supplemented with 10% FCS (heat-inactivated), 2 mM glutamin and antibiotics, were seeded in the wells of the microtiter plate before addition of 2500-5000 ^{51}Cr-labelled target cells (in 100 µl). Cells were incubated for 3 h at 37°C and centrifuged. Supernatant (100µl) was collected and radioactivity was determined. Specific ^{51}Cr-release was calculated as follows:

$$\frac{\text{experiment cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100\%$$

The maximum ^{51}Cr-release was obtained by incubation of the target cells with 10% (v/v) Triton X-100.

3.2.6 Phenotypic analysis and separation of lymphocyte subsets

MAb used for phenotyping the lymphocytes and tumor cells are listed in Table 3.2.

TABLE 3.2

MONOCLONAL ANTIBODIES

mAb	antigen recognized	dilution/amount used	obtained from
OKT3	CD3	1/4 culture sup.	Ortho Pharmaceuticals, N.J. Raritan.
OKT4	CD4	1/10 culture sup.	Ortho Pharmaceuticals, N.J. Raritan.
OKT8	CD8	1/4 culture sup.	Ortho Pharmaceuticals N.J. Raritan.
VD2	CD16	1/500 ascites	Central Laboratory Red Cross The Netherlands.
TAC	CD25	1/1000 ascites	T. Uchiyama, Kyoto, Japan.
MHC Class I	HLA-A,B,C	1/100 culture sup.	Seralab, Uden, The Netherlands.
MHC Class II	HLA-Dr	1/1000 culture sup.	M. Jonker, TNO, The Netherlands.

Cells ($2x10^5$) were incubated for 30 min with the mAb, washed with phosphate-buffered saline supplemented with bovine-serum albumin, and incubated for 30 min with 40-fold diluted fluorescence isothiocyanaat conjugated (FITC) goat-anti mouse IgG (Nordic Tilburg, The Netherlands). Cells were washed again and analysed using a fluorescent activated cell sorter (FACS IV, Becton & Dickinson). Tumor cells were analysed by light microscopy. To separate $CD4^+$ from $CD8^+$ lymphocytes, fresh PBL were labeled with anti-CD4 and anti-CD8 mAb and separated on the FACS. To eliminate possible influences of anti-CD4 and anti-CD8 mAb on the proliferation of the lymphocytes, the $CD4^-$ fraction was used for experiments to analyse $CD8^+$ cells and vice versa. The $IL\text{-}2R^+$ lymphocytes were separated from the $IL\text{-}2R^-$

lymphocytes by FACS using FITC anti-TAC mAb. In this case it was not possible to use the negative selection procedure and thus IL-2R$^+$ fractions were used.

3.3 Results

3.3.1 ^{14}C-Thymidine incorporation

Stimulation of PBL with autologous tumor cells (1690, 1702, 1718, 1721, 1743, 1754, 1762 and 1784) in MLTC did not result in increased ^{14}C-Thymidine incorporations (Table 3.3).

TABLE 3.3

^{14}C-THYMIDINE INCORPORATION IN PBL AFTER AUTOLOGOUS AND ALLOGENEIC MLTC

		autologous tumor cells	
		-	+
PBL	carcinoma	(cpm)	(cpm)
1690	ovarian	410	320
1702	mammary	415	325
1718	mammary	422	616
1721	mammary	200	185
1743	ovarian	353	685
1754	ovarian	215	655
1762	ovarian	290	390
1784	endometrium*	445	395
		allogeneic tumor cells	
		-	+
		(cpm)	(cpm)
HD1701	ovarian1689	1320	1640
HD1701	ovarian1690	925	5760
HD1701	mammary1702	605	4705
HD1707	ovarian1689	740	1765
HD1707	ovarian1690	1060	5120
HD1707	mammary1702	995	1170
HD1708	ovarian1689	815	1965
HD1708	ovarian1690	815	5770
HD1708	mammary1702	1150	1740

HD = healthy donor
*including sarcoma components

Significant enhancement of ^{14}C-Thymidin incorporation was found in PBL of healthy donors when stimulated with fresh allogeneic tumor cells.

3.3.2 Levels of IL-2R$^+$ cells

Fresh PBL of 22 patients and 21 healthy individuals were phenotyped using mAb against CD3,4,8,16 and 25. With respect to the IL-2 R$^+$ (CD25) cells, it appeared that in 9 (1689, 1743, 1746, 1747, 1758, 1759, 1767, 1784 and 1789) out of 22 patients their percentages were augmented varying between 11 and 29% (Table 3.4).

TABLE 3.4

PHENOTYPE OF FRESH PBL OF CANCER PATIENTS

		percentage of positive cells				
patient	carcinoma	CD3	CD4	CD8	CD16	CD25
1689	endometrium	70	43	25	18	17
1784	endometrium*	nt	36	18	11	12
1743	ovarian	61	35	41	22	22
1746	ovarian	64	54	30	28	24
1747	ovarian	62	49	24	14	29
1754	ovarian	56	47	15	6	0
1757	ovarian	77	62	9	3	10
1782	ovarian	70	53	27	4	7
1790	ovarian	70	42	23	9	3
1798	ovarian	85	65	18	0	6
1718	mammary	51	43	11	13	8
1721	mammary	11	5	0	15	2
1758	mammary	57	37	14	10	14
1783	mammary	68	46	24	11	0
1789	mammary	74	53	20	9	22
1806	mammary	57	33	17	8	0
1775	gall-bladder	46	26	39	27	7
1791	mesothelioma	80	39	35	4	6
1759	none	60	53	10	3	11
1767	none	64	56	15	5	11
1715	none	72	65	7	0	0
1716	none	60	22	34	6	6
healthy individuals (n=21)						0-10

*including sarcoma components

From patient 1689 the IL-2R$^+$ and IL-2R$^-$ cells were fractionated and immunophenotyped again. The IL-2R$^+$ fraction predominantly contained CD4$^+$ lymphocytes and CD3$^-$ NK cells, while the IL-2R$^-$ fraction comprised CD8$^+$ lymphocytes (Table 3.5).

TABLE 3.5

CELLULAR COMPOSITION OF IL-2 RECEPTOR POSITIVE AND NEGATIVE FRACTION OF PATIENT 1689

days of culture	IL-2R fraction	percent of positive cells				
		CD3	CD4	CD8	CD16	CD25
0	unfractionated	70	43	25	18	17
14	-	68	11	69	8	nt
14	+	nt	22	23	27	nt

3.3.3 Cytotoxic activity

From 10 patients (1685, 1689, 1690, 1702, 1721, 1743, 1754, 1762, 1784 and 1806) PBL were stimulated with autologous tumor cells in MLTC, cultured with recombinant IL-2 and expanded during two weeks. Cellular CTX of these MLTC-derived cells was tested against autologous and allogeneic fresh tumor cells as well as against K562 tumor cells (Table 3.6). In 5 out of 10 cases (1685, 1689, 1690, 1762 and 1784) fresh tumor cells were killed by MLTC-derived autologous lymphocytes; 4 out of these 5 (1689, 1690, 1762 and 1784) also lysed K562. In 1 case, only K562 tumor cells were killed. In 2 out of 5 cases autologous tumor cells but also allogeneic fresh tumor cells were lysed (PBL1690 and PBL1762) by MLTC-activated lymphocytes.

TABLE 3.6

CTX OF MLTC-DERIVED PBL FOR FRESH AUTOLOGOUS, FRESH ALLOGENEIC AND CULTURED K562 TUMOR CELLS

percent lysis of target cells*

PBL** Patient	cultured K562	fresh tumor cells 1685	1689	1690	1702	1721	1743	1754	1762	1784	1806
1685	9	20	4								
1689	16		13	5							
1690	35		23	17							
1702					7						
1721	9					0	0	1			
1743	9					0	0				
1754	4					1	0	0			
1762	19								11	12	
1784	65								9	15	
1806	35										8

* Results are expressed as percent ^{51}Cr-release; effector:target ratio 27:1; >10% of lysis is considered as relevant;
** PBL were derived from autologous MLTC

To test whether the observed autologous tumor cell lysis was tumor-selective PBL of patient 1689 were expanded without prior MLTC; the lysis of autologous tumor cells and K562 cells was 22% and 21%, respectively. However, MLTC-derived lymphocytes (autologous combination) showed 13% and 16% lysis of autologous and K562 tumor cells, respectively. Moreover, when PBL of patient 1689 were expanded without PHA in the culture system, the level of autologous tumor cell lysis was only 2% (Table 3.7). In this experiment we also tested the CTX of a $TCR\alpha\beta^+/CD3^+$ and a $TCR^-/CD3^-$ NK-cell clone against tumor 1689. Both types of clones showed high CTX, i.e. 44 and 43% lysis of fresh tumor cells, respectively, and 65 and 54 % lysis of K562 tumor cells, respectively (Table 3.7). Furthermore, PBL of a healthy individual incubated either with or without tumor cells of patient 1689 and expanded in the presence of PHA showed 23 and 27% lysis of this tumor.

TABLE 3.7

CTX OF DIFFERENT EFFECTOR CELLS AGAINST FRESH AUTOLOGOUS AND K562 TUMOR CELLS

		percentage lysis of target cells*	
effectors	tumor 1689	tumor 1689	K562 tumor cells
HD	-	23	36
HD	+	27	43
NK76	-	44	65
CTL11	-	43	54
1689/PBL-lect	-	2	3
1689/PBL+lect**	+	13	16
1689/PBL+lect	-	22	21

* Results expressed as percent ^{51}Cr-release; effector:target ratio 27:1
** Results were obtained from another experiment
HD = healthy donor

3.3.4 Influence of tumor cells on the cellular composition of autologous PBL

The in vitro influence of tumor cells on the cellular composition of autologous PBL of 4 patients was tested by phenotypic analysis with anti-CD3,4,8,16 and 25 mAb. The PBL were stimulated in a MLTC, cultured with recombinant IL-2 and expanded during 4 weeks.

Patient 1718

After 2 weeks of expansion of MLTC/IL-2 derived lymphocytes a decrease in the number of $CD16^+$ lymphocytes and a slight increase in the number of $CD3^+$ and $CD4^+$ lymphocytes was observed (Table 3.8).

TABLE 3.8

THE IN VITRO ACTIVATION OF $CD4^+$ LYMPHOCYTES BY AUTOLOGOUS TUMOR CELLS

(PBL were used as responder cells in the MLTC)

patient	tumor cells	fresh PBL percent of positive cells				
		CD3	CD4	CD8	CD16	CD25
1718	-	51	43	11	13	8
1721	-	11	5	0	15	nd
1743	-	61	35	41	22	22
1806	-	57	33	17	8	0
		MLTC/IL-2 derived cells after 2 weeks of expansion absolute number of positive cells* x 10^3				
		CD3	CD4	CD8	CD16	CD25
1718	-	126.7	106.4	35.5	141.9	167.2
1718	+	200.1	190.0	2.5	5.1	195.0
1721	-	336.0	336.0	33.6	0.0	268.8
1721	+	442.0	442.0	0.0	0.0	243.7
1743	-	215.8	137.8	96.2	0.0	215.8
1743	+	340.0	300.0	124.0	0.0	316.0
1806	-	617.2	379.9	251.8	113.7	592.8
1806	+	893.1	571.2	332.3	62.3	820.4

* absolute numbers of positive cells were calculated by: initial number of cells per well x percent positive cells x multiplification factor after one week of culture.

After 4 weeks, a strong increase of $CD3^+$, $CD4^+$ and $CD25^+$ cells but no change in the number of $CD8^+$ cells was found (Table 3.9).

Patient 1721.

For this patient only a slight stimulatory influence of the tumor cells on the cellular composition of autologous PBL was found. An increase in the number of $CD3^+$, $CD4^+$ and $CD25^+$ cells after stimulation with the tumor cells, could only be observed after 2 weeks of expansion of MLTC/IL-2 derived

lymphocytes (Table 3.8). Low numbers of $CD8^+$ cells were present in fresh PBL but tumor cells could not induce activation. The $CD16^+$ lymphocytes were no longer detected after MLTC and 4 weeks of expansion (Table 3.9).

TABLE 3.9

THE IN VITRO ACTIVATION OF $CD4^+$ LYMPHOCYTES BY AUTOLOGOUS TUMOR CELLS

(PBL were used as responder cells in the MLTC)

patient	tumor cells	fresh PBL percent of positive cells				
		CD3	CD4	CD8	CD16	CD25
1718	-	51	43	11	13	8
1721	-	11	5	0	15	nd
1743	-	61	35	41	22	22
1806	-	57	33	17	8	0
		MLTC/IL-2 derived cells after 4 weeks of expansion absolute number of positive cells* x 10^3				
		CD3	CD4	CD8	CD16	CD25
1718	-	123.5	39.0	136.5	152.8	39.0
1718	+	560.6	414.4	268.1	146.3	349.4
1721	-	739.5	714.0	51.0	0.0	493.0
1721	+	494.5	440.9	53.6	0.0	268.1
1743	-	610.0	291.0	465.5	0.0	318.9
1743	+	401.4	233.4	275.3	9.3	242.7
1806	-	36.7	18.6	14.7	0.0	21.6
1806	+	79.0	18.0	33.0	4.0	30.0

* absolute numbers of positive cells were calculated by: initial number of cells per well x percent of positive cells x multiplification factor after one week of culture.

Patient 1743

For this patient, an increase in the number of $CD3^+$, $CD4^+$ and $CD25^+$ cells was observed after 2 weeks of expansion of the MLTC/IL-2 derived lymphocytes (Table 3.8), while $CD8^+$ cells responded weakly to autologous tumor cells. After 4 weeks the number of $CD4^+$ and $CD8^+$ as well as $CD25^+$ cells were decreased (Table 3.9).

Patient 1806

After 2 weeks of expansion of the MLTC/IL-2 derived lymphocytes, a strong increase was observed in the number of $CD3^+$ and $CD4^+$ as well as $CD25^+$ cells and a much smaller increase in the number of $CD8^+$ cells (Table 3.8). After 4 weeks of expansion, no further influence of the tumor cells on autologous lymphocytes could be detected i.e. differences in the number of $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$ or $CD25^+$ were not observed when cultured either with or without tumor cells (Table 3.9).

3.3.5 Influence of tumor cells on the proliferation of $CD4^-$ and $CD8^-$ fractions

$CD8^-$ lymphocyte fraction of patient 1806

After 2 weeks of expansion of the MLTC/IL-2 derived $CD8^-$ ($CD4^+$-enriched) lymphocyte fraction, a strong increase in the number of $CD3^+$, $CD4^+$ and $CD25^+$ cells, but also in the number of $CD16^+$ cells was found (Table 3.10). Expectedly, no $CD8^+$ lymphocytes were found. After 4 weeks of expansion, differences in the number of $CD3^+$, $CD4^+$, $CD16^+$ and $CD25^+$ lymphocytes could no longer be detected.

TABLE 3.10

THE IN VITRO ACTIVAITON OF $CD4^+$ LYMPHOCYTES BY AUTOLOGOUS TUMOR CELLS

($CD4^+$-enriched lymphocytes were used as responder cells in the MLTC)

day of culture after MLTC/IL-2	tumor cells	$CD4^+$ enriched lymphocytes percent of positive cells				
		CD3	CD4	CD8	CD16	CD25
0**	-	5	33	17	8	0
		MLTC/IL-2 derived cells absolute number of positive cells*x 10^3				
14	-	181.8	1755.0	23.4	140.4	1755.0
14	+	266.4	2607.8	34.3	377.4	2642.1
28	-	57.2	49.6	0.8	9.6	34.4
28	+	94.9	88.4	0.0	6.5	61.1

* absolute numbers of positive cells were calculated by: initial number of cells per well x percent positive cells x multiplification after one week of culture

** unfractionated

$CD4^-$ lymphocyte fraction of patient 1806

No proliferation of MLTC-derived $CD4^-$-fractionated lymphocytes (i.e. $CD8^+$ lymphocytes) was obtained.

3.4 Discussion

In the first part of this study we have shown that uncultured, i.e. fresh tumor cells derived from lung, ovarian, mammary and endometrium carcinomas could not induce tumor-reactive CTX in autologous PBL. Low levels of CTL by MLTC-derived PBL were found against autologous as well as allogeneic fresh tumor cells (Table 3.6). Limiting dilution of autotumor stimulated PBL did not yield clones with autotumor-directed cytotoxicity (data not shown).

Phenotypic analysis of tumor cells revealed that MHC expression varied from 17-83% for class I and from 26-82% for class II antigens (tumor 1689,

1721, 1743 and 1806). Tumors 1689, 1690 and 1702 induced high ^{14}C-thymidin incorporation in allogeneic PBL when used for stimulation in a MLTC (Table 3.3). This phenomenon functionally demonstrated the expression of MHC determinants on these tumor cells. Phenotyping of fresh PBL from cancer patients suggest that their "spontaneously" in vivo arisen tumor cells express aberrant antigens. Evidence for this are the augmented levels of IL-2R$^+$ lymphocytes (11-29%) found for fresh PBL from 9 out of 22 cancer patients, while fresh PBL from healthy individuals comprised 0-10% IL-2R$^+$ cells. Induction of IL-2R on PBL in vivo by chemotherapeutic reagents can be excluded, since no correlation exists between the presence of enhanced levels of IL-2R$^+$ cells and chemotherapeutic treatment of the cancer patients (Table 3.1). Moreover, PBL from most of the treated patients did not comprise enhanced levels of IL-2R$^+$ cells. PBL of 2 patients showed 11% of IL-2R$^+$ cells while malignant cells could not be detected in acites smears by microscopic analysis. However, in these cases the presence of malignant tumor cells in the body cannot be ruled out. In this respect, the use of primary tumors and/or metastases for experiments is more reliable, but other problems such as less viable tumor cells after enzymatic or mechanic treatment and possible subsequent destruction of foreign antigens favours the use of tumor cells from pleura and ascites.

The question arises why enhanced numbers of IL-2R$^+$ lymphocytes were found in PBL of only 9 out of 22 cancer patients. Unsuitable stimulation circumstances in vivo, i.e. optimal antigen/receptor interactions, insufficient IL-2 concentration as well as other factors, which can be optimized in vitro, may be an explanation for this failure.

Separation and phenotypic analysis of the IL-2R$^+$ and IL-2R$^-$ fresh PBL showed that CD4$^+$ cells and CD16$^+$ cells were mainly present in the IL-2R$^+$ and CD8$^+$ cells in the IL-2R$^-$ fraction. In vitro stimulation of PBL with autologous tumor cells in a MLTC showed a similar pattern. In all 4 cases CD4$^+$ lymphocytes were stimulated by the tumor cells, as shown by enhanced proliferation as well as by an augmented level (absolute number) of IL-2R$^+$ cells, although this occured with different strengths (patients 1718, 1721, 1743 and 1806) (Tables 3.8 and 3.9). By contrast, CD8$^+$ lymphocytes were not stimulated at all, or at a much lower level than CD4$^+$ lymphocytes. Similar results were observed for CD4$^+$ and CD8$^+$ enriched cell fractions (Table 3.10).

These results suggest that CD8$^+$ (cytotoxic/suppressor) lymphocytes may become inactivated in vitro or in vivo by autologous tumor cells. In the mouse system, it has been shown that soluble suppressor factors (Ts 1, 2 and 3) produced by each suppressor T cell subset may inhibit the CTL response against

autologous tumor cells (180). In humans, it has been shown that tumor-stimulated $CD4^+$ inducer cells (derived from lymph nodes) may activate $CD8^+$ suppressor cells (derived from fresh PBL), which in turn prevent the CTL response against autologous melanoma tumor cells *in vitro* (126,127). Suppressor activity by T lymphocytes has also been reported to be present in metastatic lymph nodes of patients with cancer of the larynx or bladder (177). However, others reported the absence of suppressor activity by T lymphocytes among tumor infiltrating lymphocytes (TIL) (181).

On the basis of the data presented here, we conclude that $CD8^+$ lymphocytes may be inactivated *in vivo* and do not respond to stimulation signals provided by the *in vitro* activated $CD4^+$ lymphocytes. The latter phenomenon, i.e. poor *in vitro* lymphocyte functions and mitogenic responses, has also been found for TIL (181).

CHAPTER 4

ATTEMPTS TO PRODUCE HUMAN CYTOTOXIC LYMPHOCYTE HYBRIDOMAS BY ELECTROFUSION AND PEG FUSION

4.1 Introduction

The study of lymphocyte-target cell interactions and their interaction sites requires large numbers of functionally stable lymphoid cells. An important development has been the determination of culture conditions allowing the long term maintenance of T lymphocyte clones after cloning in limiting dilution (182). In a number of laboratories, including our own, culture systems for both specific and nonspecific human cytotoxic T-cell clones have been developed (183-185). However, the growth capacity of cloned lymphocytes is limited and requires the presence of feeder cells, interleukin-2 (IL-2) and other undefined growth factors. To circumvent these requirements we attempted to prepare human cytotoxic T-cell hybridomas i.e. immortalize the lytic function after somatic cell hybridization.

Thus far, hybridoma cell lines with cytolytic activity have been described occasionally in mouse-mouse and mouse-human systems (144,148-150). Failures to establish human cytotoxic T-cell hybridomas have been attributed to nonspecific lysis of the tumor cells by the cytotoxic lymphocytes during or after the fusion procedure (144). However, the production of human-human hybridomas is associated with other difficulties, including establishment of initial growth, i.e. proliferation of fused cells was observed only after one month (145,146). Moreover, human-human hybridomas frequently showed chromosomal instability (147).

We attempted to overcome these difficulties by using both a modified polyethylene glycol (PEG) method and electrofusion (151-153). To avoid the putative lysis of the fusion partners during the fusion procedure (144), $TCR\alpha\beta^+/CD3^+$ cytotoxic lymphocytes (CTL) were pretreated by heat shock to reversably inactivate the lytic machinery. Tumor cells were not lysed by lymphocytes during electrofusion (151,152) and hence pretreatment of the lymphocytes was not required. We also used allospecific $TCR\alpha\beta^+/CD3^+$ CTL clones (33,183) without and $TCR^-/CD3^-$ NK cell clones with lytic activity for the

tumor fusion partner. Finally, mouse myeloma B cells were used as (PEG) fusion partner for human TCR$\alpha\beta^+$/CD3$^+$ CTL clones.

4.2 Materials and methods

4.2.1 Cells and cell culture

Human and mouse tumor cell lines used as fusion partners (Table 4.1) were cultured in RPMI medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamin and antibiotics. Peripheral blood lymphocytes (PBL) were isolated from blood of healthy donors on a Ficoll-Isopaque layer.

TABLE 4.1

HUMAN AND MOUSE TUMOR CELL LINES USED AS FUSION PARTNERS

Human T-lymphoma cell lines	HAT^s	Oua^r	Mutagenized by
CEM-1	+	+	S.K.H. Foung, Stanford University, USA.
CEM-2	+	-	C.M. Croce, Wistar Institute, USA.
JURKAT-1	+	+	S.K.H. Foung, Stanford University, USA.
JURKAT-2	+	-	C.M. Croce, Wistar Institute, USA.
HPB-1	+	-	S.K.H. Foung, Stanford University, USA.
HPB-2	+	-	C.M. Croce, Wistar Institute, USA.
HSB-1	+	-	A. Hagemeyer, Erasmus University, The Netherlands.
Mouse B-myeloma cell lines	**HAT^s**	**Oua^r**	**Mutagenized by**
SP20	+	-	M. Shulman, Basel Institute for Immunology, Switzerland.
NS1/X63	+	-	J.F. Kearny, University of Alabama, USA.

r = resistant
s = sensitive

PBL (10^6/ml) were cultured in RPMI medium supplemented with 10% human serum, 4 mM glutamin and antibiotics, and activated by phytohaemagglutinin (PHA) with 10 µg PHA/ml, for 2 days. Bulk cultured cells and cloned cells are listed in Table 4.2. Cloning and expansion was performed in RPMI medium supplemented with γ-irradiated (40 Gy) PBL and/or B-LCL (lymphoblastoid B-cell line) as described elsewhere (33,183,186).

TABLE 4.2

HUMAN AND MOUSE TUMOR CELL LINES USED AS TARGET CELLS IN CTX ASSAYS

Human origin	Source	Obtained by
K562	erythroid cell line	G. Cannon, Litton Bionetics Kengsinton, U.S.A.
MOLT	T-lymphoma	A. Hagemeijer, Erasmus University Rotterdam, the Netherlands
APD	EBV-transformed B-cell line	M. Giphart, Dept. of Immunohematology University Hospital, Leiden, the Netherlands
Licron-My-2	B-leukemia	P.A.W. Edwards, Ludwig Institute for Cancer Research, Sutten, England
Mouse origin		
P815*		MacDonald, Swiss Institute for Experimntal Cancer Research, Lausanne, Switserland

* P815 mouse cells precoated with PHA were used for the lectin-dependent cellular cytotoxicity (LDCC)

Human and mouse tumor cell lines used in cytotoxicity assays (Table 4.3) were cultured in RPMI medium supplemented with 10% (v/v) FCS, 2mM glutamin and antibiotics.

TABLE 4.3

HUMAN CYTOTOXIC LYMPHOCYTES USED AS FUSION PARTNERS

Cloned cytotoxic lymphocytes	CD3	CD4	CD8	CD16	TCR	Derived from
CTL11	+	-	+	-	αβ	PBL healthy donor
CTL20	+	+	-	-	αβ	PBL healthy donor
NK76	-	-	-	+	-	PBL Tγ-lymphocytosis
NK77	-	-	-	+	-	PBL Tγ-lymphocytosis
CSF1-1	+	-	+	-	αβ	cerebrospinal fluid
CFS1-7	+	-	+	-	αβ	multiple sclerosis
Uncloned cytotoxic lymphocytes						
CSF-1						cerebrospinal fluid
CSF-2						multiple sclerosis
fresh PBL						PBL healthy donor
PHA-blasts						PBL healthy donor
MLC-blasts						PBL healthy donor

4.2.2 Heat treatment

To transiently reduce lytic activity, lymphocytes were pretreated by heat shock at 46°C for 10 min (144).

4.2.3 PEG fusion

The fusion procedure described by Oi et al. (187) for B-B cell fusions was used with minor modifications as described earlier (151). Briefly, lymphocytes were mixed with tumor cells at a ratio of 5:1. After PEG fusion, the cells were resuspended in RPMI medium supplemented with 20% FCS, 4 mM glutamin and cultured ($6x10^4$ cells/100 µl/well) in 96-well microtiter plates (Greiner). Gamma-irradiated (40 Gy) murine bone marrow cells ($4x10^4$ cells/100 µl/well) were added as feeder cells to initiate proliferation of the fused cells. Hybridomas were selected in 0.1 M hypoxanthine, 0.1 M aminopterin, 100 nM thymidine (HAT), or in 1 µg/ml azaserine, 0.1 M hypoxanthine (AH) medium. In some experiments, ouabain ($5x10^{-7}$M) was added directly after fusion to eliminate unfused lymphocytes. One week later, the culture medium was supplemented with HAT or AH (or ouabain) at double strength. After 14 days,

the medium was replaced by the same medium without azaserin, aminopterin (or ouabain). One week later the thymidine and/or hypoxanthine was ommitted from the culture medium. In each fusion experiment, tumor-tumor cell fusion products, unfused tumor cells and lymphocytes were cultured in HAT or AH medium as negative controls.

4.2.4 Electrofusion

4.2.4.1 Technique

The experimental set up used for electrofusion is shown in Figure 4.1. It consists of a fusion chamber, a function generator (type 7707, P.Toellner Electronics), and a pulse generator (Devices, type 521).

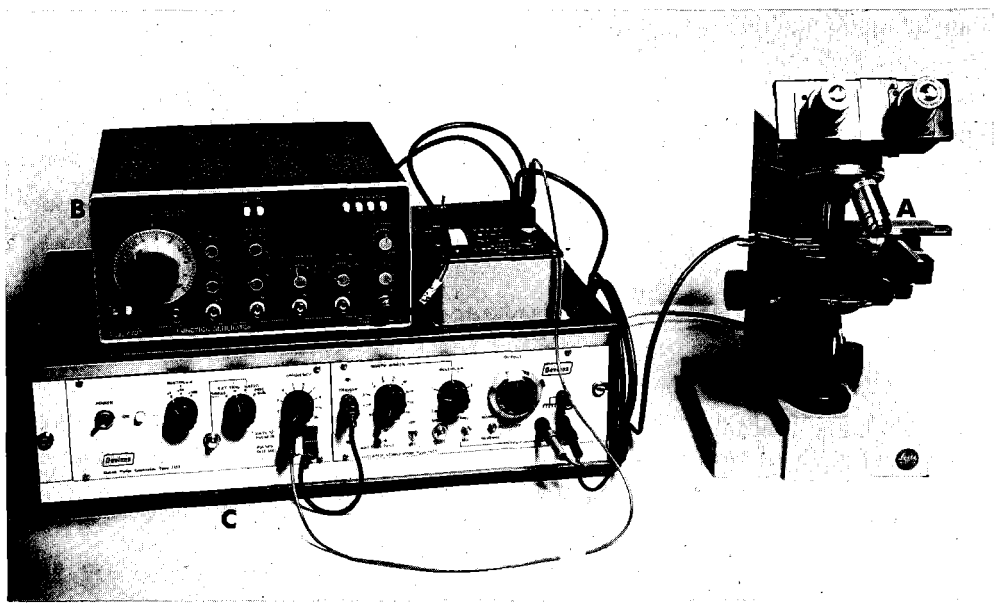


Figure 4.1 The experimental setup for electrofusion. (A) The fusion chamber, under the microscope, connected with (B) a function generator used for pearl chain formation. (C) Pulse generator, connected in parallel with the function generator, for induction of the electrical breakdown.

The fusion chamber (Fig. 4.2) consists of 2 platinum wires (diameter 0.2 mm) mounted in parallel on a perspex slide at a distance of 0.2 mm. This chamber is connected to the function generator, which is used as voltage source for an alternating electrical field.

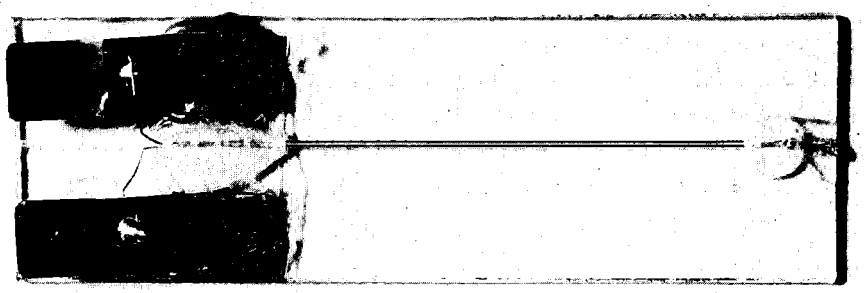


Figure 4.2 A fusion chamber. Two platinum wires (diameter 0.2 mm) are mounted in parallel on a perspex slide at a distance of 0.2 mm.

A pulse generator was connected in parallel for application of square pulses of high field intensities. The electrofusion process can be devided into two stages:

1. "Pearl chain" formation. Cells are brought into close contact with each other in a nonuniform alternating electrical field of low intensity (field strength 100-250 V/cm; frequency 1-5 MHz). In the alternating electrical field, a dipole moment is induced in the cells because of charge separation or because of orientation of dipoles in the membrane. Cells then migrate in the nonuniform electrical field because the field intensity is not equal on both sides of the cells. This phenomenon is known as dielectrophoresis. Migration of the dipole-induced cells occurs in the direction of the highest field intensity.

2. "Fusion". Fusion is induced by the application of a pulse of high field intensity (3000-5000 V/cm) and of short duration (10-50 µsec). An electrical breakdown takes place on that part of the membrane (lipid domains) where the cells have come into contact with each other. Fusion takes place within seconds to minutes, depending on the cell type and species.

4.2.4.2 Fusion procedure

The electrofusion procedure of Zimmermann et al. (153) was adapted for human T-cell fusions and described earlier (151). To reduce the conductivity of the cell suspension without substantially affecting the proper osmolarity, tumor cells and lymphocytes were washed three times in a 0.3 M mannitol solution before fusion. However, cell aggregates were formed due to cell death. After 1 hr, the viability of the cells in the mannitol solution was 50%. Therefore, the washing procedure was shortened and carried out at 4°C to lower the cell metabolism. Furthermore, other sugars such as glucose (enhances survival) and sorbitol were also tested in the washing procedures. A 0.3 M mannitol: glucose solution [8:1.25 (v/v)] appeared to be optimal.

Formation of lipid domains (particle free areas) is required for pearl chain formation and fusion. It is in these membrane areas that cell-cell contact and electrical breakdown takes place. To create lipid domains in the membrane, cells were pretreated with pronase (1 mg/ml). The proteolytic activity of pronase causes partial degradation of membrane proteins, so that mobility of the remaining proteins increases (153). This increase in the mobility may facilitate the emergence of the lipid domains in the membrane during the exposure to the electrical field. Other enzymes, such as dispase and neuraminidase, had no effect. After the washing procedure and pretreatment, the cells were transferred to the fusion chamber.

Pearl chain formation was induced by a field strength of 250 V/cm and a frequency of 5 MHz. The intensity of the field pulse applied to induce fusion appeared to be critical for subsequent proliferation of the cells. Although successful fusions were obtained by pulses of 5000 V/cm during 10 µsec, no proliferation of fused cells could be induced. Lower pulses of prolonged duration were tested. Pulses of 4000 V/cm during 25 µsec were found to be optimal. After fusion, the cells were transferred to the culture medium and hybridomas were selected as described for PEG fusion.

4.2.5 Determination of cytotoxic activity

Cytotoxic activity was assayed in a ^{51}Cr-release test in round-bottom microtiter plates as described earlier (164). Briefly, varying cell numbers in 150 µl RPMI supplemented with 10% FCS were seeded in the wells before the addition of 10^3 ^{51}Cr-labelled target cells in 100 µl. Plates were incubated for 3 h at 37°C and centrifuged. Supernatant (100 µl) was collected and radioactivity was determined. Specific ^{51}Cr release was calculated as:

$$\frac{\text{experimental cpm - spontaneous cpm}}{\text{maximum cpm - spontaneous cpm}} \times 100\%$$

The maximum 51-Cr release was obtained by incubation of the target cells with 10% (v/v) Triton X-100.

4.2.6 Chromosome analysis

Cells were arrested at the mitotic stage by treatment with 0.025 µg colchicin/ml for 20 min and treated with a hypotonic solution of KCl (0.075 M) for 10 min at 37°C. Slides were prepared immediately after fixation, air-dried, and chromosomes were R-banded with acridin-orange (0.01%) after heat denaturation at 87°C.

4.2.7 Analysis of membrane antigens

The immune phenotype of parental cell lines and hybridomas was determined by immunofluorescence analysis using monoclonal antibodies (mAb) against CD3 antigens expressed by mature T cells, but not NK cells, CD4 antigens expressed by helper/inducer T cells, CD8 antigens expressed by cytotoxic/suppressor T cells, and CD2 antigens expressed by virtually all T lymphocytes, HNK-1 antigens expressed by HSB-1 tumor cells, and MHC class I and II antigens expressed by the T-cell clones used. Anti-TAC mAb was used for

the detection of IL-2 receptors (IL-2R) (CD25), expressed by activated T cells. Cells ($2x10^5$) were incubated for 30 min with 40-fold diluted fluorescence-isothiocyanate-conjugated (FITC) goat anti-mouse IgG (Nordic, Tilburg, The Netherlands). Cells were washed again and analysed on a fluorescence-activated cell sorter (FACS II, Becton & Dickinson).

4.2.8 Invasiveness in hepatocyte cultures

Invasiveness of the hybridomas was tested in rat hepatocyte cultures as described elsewhere (188). Briefly, $6x10^6$ cells were added to a 16 mm well containing $2x10^5$ rat hepatocytes that had been cultured for 24 hr after their isolation. After 4 h the cultures were washed, fixed, dehydrated, detached from the dish, and embedded in Epon. Sections of 1 µm were examined by light microscopy. Hybrid cells that were completely surrounded by hepatocytes, or located between hepatocytes and substrate were considered as "infiltrated cells". As a measure of invasiveness we used the infiltration index (infiltrated cells/hepatocyte nuclei). At least 150 hepatocyte nuclei were counted in four sections cut at a distance of 20 µm.

4.3 Results

4.3.1 Hybridomas obtained by PEG fusion

Different human and mouse tumor cell lines were tested for their suitability as tumor partner in PEG fusion experiments with cytotoxic lymphocytes. Proliferating hybridomas were obtained at between 3-6 weeks after fusion with the following tumor cell lines used: HSB-1, CEM-1, CEM-2 and JURKAT-2 (Table 4.4). No hybridomas could be generated using JURKAT-1, HPB-1 and HPB-2 cells as tumor fusion partner. Fusions between cloned CTL20 cells (without cytotoxic activity against HSB-1) and HSB-1 tumor cells resulted in proliferating hybridomas in 29% of the wells seeded. However, fusions between NK76, NK77 or MLC-blasts (with high cytotoxic activity against HSB-1) also resulted in proliferating hybridomas (in 17, 37 and 17% of the wells, respectively); these cytotoxic lymphocytes were not pretreated by heat shock to reduce cytotoxic activity (Table 4.4). Hybridomas of "effector" cells and CEM-1 or JURKAT-1 tumor cells are ouabain resistant. "Normal" effector lymphocytes were expected to rapidly die in the presence of ouabain. However, it was found that this only occurred after 2-3 days, during which period

cytotoxic activity was not depressed (data not shown). No differences were found in the yield of hybridomas with or without ouabain (Table 4.4); in both cases 3% of the wells seeded contained hybridomas, using CEM-1 cells as tumor fusion partner, and none with JURKAT-1 tumor cells. Fusions with JURKAT-2 tumor cells yielded hybridomas in 30% of the wells seeded with all CTL used.

TABLE 4.4

YIELD OF HUMAN-HUMAN HYBRIDOMAS AFTER PEG FUSION

Lymphocyte fusion partner	Tumor fusion partner	Percent yield of hybridomas
fresh PBL	HSB-1	0 (900)
fresh PBL*	HSB-1	11 (900)
PHA-blasts	HSB-1	0 (900)
MLC-blasts	HSB-1	17 (350)
clone CTL20	HSB-1	29 (400)
clone NK76	HSB-1	17 (100)
clone NK77	HSB-1	37 (100)
fresh PBL	CEM-1**	3 (100)
clone CTL20	CEM-1**	3 (100)
MLC-blasts	CEM-2	10 (180)
uncloned CSF-1	CEM-2	10 (172)
uncloned CSF-2	CEM-2	10 (196)
clone 1, CSF-1	CEM-2	10 (180)
clone 7, CSF-1	CEM-2	10 (172)
fresh PBL	JURKAT-1**	0 (100)
clone CTL20	JURKAT-1**	0 (100)
uncloned CSF-1	JURKAT-2	30 (190)
uncloned CSF-2	JURKAT-2	30 (180)
clone 1, CSF-1	JURKAT-2	30 (96)
clone 7, CSF-1	JURKAT-2	30 (96)
MLC-blasts	JURKAT-2	30 (190)

Numbers between parentheses indicate total number of wells seeded.
* effector cells were pretreated by heat shock
** tumor cells were HAT-sensitive as well as ouabain-resistant

Finally, SP20 and NS1/X63 mouse myeloma cell lines were also tested for their suitability as fusion partners in PEG fusion experiments with human CTL clones. None of the human CTL clones lysed mouse tumor cell partners, and thus heat shock was not necessary. PEG fusion between SP20 mouse myeloma cells and the human cytotoxic T-cell clones CTL11 and CTL20 resulted in rapidly proliferating hybridomas in 19-28% of the wells at between 1-2 weeks after

fusion (Table 4.5). Using NS1/X63 mouse myeloma cells as fusion partners, proliferation was observed in 1-2% of the wells (Table 4.5) at between 2-3 weeks. These latter hybridomas proliferated very slowly and died at between 1-2 months.

TABLE 4.5

YIELD OF HUMAN-MOUSE HYBRIDOMAS AFTER PEG FUSION

Human T-lymphocyte fusion partner	Mouse tumor fusion partner	percent yield of hybridomas
clone CTL11	SP20	19 (250)
clone CTL11	SP20	28 (250)
clone CTL20	SP20	20 (250)
clone CTL11	NS1/X63	2 (250)
clone CTL20	NS1/X63	2 (250)

Numbers between parentheses indicate the total numbers of wells seeded.

4.3.2 Comparison between PEG fusion and electrofusion

A comparison was made between PEG fusion and electrofusion with respect to the frequency of hybridomas obtained for the different tumor cell lines used (Table 4.6).

TABLE 4.6

YIELD OF HUMAN-HUMAN HYBRIDOMAS BY PEG FUSION

AND BY ELECTROFUSION

fusion partners		percent yield of hybridomas	
T lymphocytes	tumor cells	PEG fusion	electrofusion
fresh PBL	HSB-1	0 (900)	99 (200)
PHA-blasts	HSB-1	0 (900)	1 (200)
clone CTL20	HSB-1	29 (400)	69 (400)
clone CTL20	CEM-1	3 (100)	3 (100)
clone CTL20	HPB-1	0 (100)	0 (100)
clone CTL20	JURKAT-1	0 (100)	0 (100)

Numbers between parentheses indicate the total numbers of wells seeded.

Rapidly proliferating hybridomas were obtained at between 3-6 weeks after fusion. Using HSB-1 as tumor fusion partner, electrofusion appeared to be more efficient than PEG fusion, as illustrated by a higher yield of hybridomas obtained under the same culture conditions. The electrofusion process, i.e. collection and fusion of cells, is shown in Figure 4.3.1 and 4.3.2. Microscopy revealed no lysis of the tumor cells, and thus heat shock of the lymphocytes was not necessary. The best results with electrofusion were obtained with uncultured PBL; 99% of the wells seeded yielded hybridomas. This corresponded with the high fusion frequency observed in microscopy. Using PEG, no hybridomas were obtained between HSB-1 tumor cells and uncultured PBL, which may be due to non-specific lysis of HSB-1 tumor cells by the PBL. The yield of hybridomas between HSB-1 and cloned CTL20 cells (without cytolytic activity against HSB-1) was also higher after electrofusion than after PEG fusion (69 and 29%, respectively). No hybridomas were obtained from PEG fusions between HSB-1 tumor cells and PHA-activated lymphocytes. During electrofusion, a low fusion frequency was observed between these fusion partners and only a few hybridomas were obtained (1% of the wells seeded). In PEG fusion and electrofusion a low yield (3% of the wells seeded) of hybridomas was observed using CEM-1 as tumor fusion partner, and no yield with JURKAT-1 and HPB-1 tumor cells (Table 4.6). Nevertheless, a high frequency of fusions was observed under the microscope with CEM-1 tumor cells during electrofusion. JURKAT-1 tumor cells collapsed during the electrofusion process and HPB-1 tumor cells did not fuse.

4.3.3 Cytotoxic activity of hybridomas

More than 500 of the human-human hybridomas obtained were tested for CTX in ^{51}Cr release assay. Only three (obtained by electrofusion of clone CTL20 and HSB-1 tumor cells) showed CTX (albeit at low levels) against APD, the original stimulator cells of clone CTL20. Hybridomas of MLC-responder blast cells with CEM-2 or JURKAT-2 tumor cells showed low levels of CTX against K562 and also against the stimulator cells after overnight incubation of the hybridomas with non-T stimulator cells.

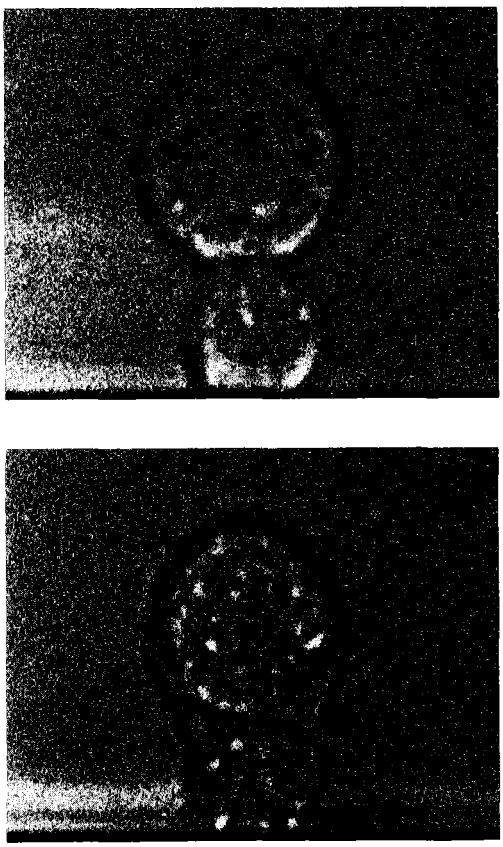


Figure 4.3.1 Pearl chain formation of a large tumor cell and a small lymphocyte in an alternating electrical field.
Figure 4.3.2 Fusion is induced between a large tumor cell and a small lymphocyte after application of an electrical breakdown pulse.

Of more than 100 human-mouse hybridomas tested for CTX only 1 (between CTL11 and SP20) showed high CTX (49%) against Licron Hmy-2 at an effector/target ratio of 27/1. One week later the CTX of the hybrid cells had decreased to 12% lysis of target cells, and after 2 weeks no activity was left.

4.3.4 Karyotypes of hybridomas

In human-human hybridomas chromosomes derived from the tumor cells could not be distinguished. All hybridomas obtained with HSB-1 as tumor cell fusion partner showed the translocations derived from the tumor cell line. However, no specific marker chromosomes, derived from the lymphocytes, were observed, such as chromosome 16 with a centromeric polymorphism (clone CTL20). Most of the hybridomas between HSB-1 tumor cells and PBL, cloned CTL11 or cloned CTL20 cells contained the same number of chromosomes as HSB-1 tumor

Figure 4.4 R-banded chromosomes from a xenogeneic hybrid cell. The hybrid cell contains human (see arrows) and mouse chromosomes.

Some hybridomas contained more chromosomes than either of the parental cell lines. In addition, some hybridomas of HSB-1 tumor cells and clone CTL20 cells revealed newly formed polymorphisms which were absent from HSB-1 tumor cells and lymphocytes (results not shown). Most of the hybridomas of CEM-1 tumor cells and cloned CTL20 cells were not identified. Hybridomas with CEM-2 and JURKAT-2 were not analyzed.

Hybridomas between SP20 mouse myeloma cells and cloned CTL11 or cloned CTL20 cells were identified by the presence of easily distinguishable human and mouse chromosomes (Figure 4.4). These hybridomas contained 2-13 human chromosomes (Table 4.7). Chromosome 16 from clone CTL20 was also clearly identified. There was no indication of a selective loss of human chromosomes. Hybridomas between NS1/X63 mouse myeloma cells and cloned CTL11 or CTL20 cells had died before karyotyping was possible.

TABLE 4.7

CHROMOSOME ANALYSIS OF XENOGENEIC HYBRIDOMAS

(HUMAN) CLONE CTL20 - (MOUSE) SP20

Cell number	Human chromosomes
1	1 6 12 15 16 17 20 X
2	7 15 17 20 X
3	6 19 20
4	6 8 9 13 15 15
5	1 6 17 17 19
6	1 6 6 19
7	1 6 7 10 10 14 15 16 17 X
8	1 6 10 10 17 20
9	1 3 3 6 6 8 9 10 10 12 16 17 19
10	1 3 4 5 6 9 12 20
11	1 3 4 4 6 6 9 10 10 12 17 19 19
12	6 17
13	3 6 7 11 20

4.3.5 Expression of membrane antigens

Membrane antigen expression was determined on both parental cells and their fusion products. All hybridomas, obtained after PEG fusion and electrofusion using HSB-1 as parental tumor cells, expressed HNK-1 antigens derived from HSB tumor cells but none of the lymphocyte markers could be

detected. Likewise, all hybridomas obtained with CEM-1, CEM-2 and JURKAT-2 cells as tumor fusion parents expressed the membrane antigens derived from the tumor cell line. CEM-1 is $CD3^-$, but CEM-2 and JURKAT-2 tumor cells are $CD3^+$. Two of the hybridomas of cloned CTL20 cells and CEM-1 tumor cells expressed low levels of CD3 antigens derived from the lymphocytes. None of the hybridomas expressed IL-2R after PHA or allogeneic stimulation, or after incubation with high concentrations of IL-2.

Some xenogeneic hybridomas between SP20 mouse myeloma cells and cloned CTL20 cells expressed low levels of MHC class II antigens derived from clone CTL20.

4.3.6 Invasiveness of hybridomas

Some of the hybridomas between the non-invasive tumor cell line HSB-1 tumor cells and invasive cloned CTL20 cells were found to be highly invasive in cultured rat hepatocytes (Table 4.8).

TABLE 4.8

INVASIVENESS OF HUMAN-HUMAN AND HUMAN-MOUSE HYBRIDOMAS

Human-human hybridomas	Fusion nr	Infiltration index
CTL20xHSB-1	72 (1)	0.41*
CTL20xHSB-1	72 (2)	0.77*
NK77xHSB-1	76 (1)	0.09
human-mouse hybridomas		
CTL20xSP20	125 (4)	0.00
CTL11xSP20	115 (A1)	0.01
tumor cell lines		
HSB-1		0.05
SP20		0.01
T lymphocytes		
PBL unstimulated		0.06
PBL stimulated (PHA)		0.74

* Cells with invasive properties.
Infiltration index = number of cells infiltrated per 100 rat hepatocytes.

The level of invasiveness (I.I = infiltration index) of fusions between HSB-1 and CTL20 is 16 times higher (I.I = 0.77) than that of HSB-1 per se (I.I = 0.05), and equals that of stimulated T lymphocytes (I.I = 0.74). Hybridomas of CEM-1 tumor cells and cloned CTL20 cells showed low invasiveness (data not shown). Fusions using CEM-2 and JURKAT-2 as tumor fusion partner were not tested. The human-mouse hybridomas tested were not invasive (Table 4.8).

4.4 Discussion

In the present study proliferating hybridomas were obtained by fusion of human $TCR\alpha\beta^+/CD3^+$ CTL or $TCR^-/CD3^-$ NK cells and human T-lymphoma cells either by PEG or electrofusion. In experiments using HSB-1 cells as a tumor partner, more proliferating hybridomas were obtained under the same culture conditions with electrofusion than with the PEG fusion. This could be due to either higher fusion frequencies or better survival of the hybridomas after electrofusion. In contrast to PEG fusion, the electrofusion process can be visualized by microscopy and thus offers information on fusion frequencies in relation to yield of hybridomas. Remarkably high fusion frequencies using CEM-1 as tumor fusion partner did not result in a high yield of proliferating hybridomas. Thus, it appears that in addition to a high fusion frequency other factors are important for the generation of proliferating human T/NK-cell hybridomas and these are cell type dependent.

The results obtained by electrofusion unequivocally demonstrated that fusion efficiency depends both on the tumor cell and T/NK lymphocyte subset. High fusion efficiencies were obtained with HSB-1 and CEM-1 tumor cells, whereas virtually no fusions were observed with JURKAT-1 and HPB-1 tumor cells. This is consistent with the data of Roos et al. (189) who demonstrated by freeze-fracture electron microscopy that not every tumor cell line can be fused by PEG. It appears that aggregation of intermembrane particles, a prerequisite for PEG-induced cell fusion, does not occur in fusion-deficient cells, at non-toxic PEG concentrations. It is not clear from our results whether pretreatment with heat shock of the CTL to transiently reduce the CTX, is indeed a prerequisite for the production of cytotoxic T-cell hybridomas as previously suggested by Kaufmann et al. (144). These authors speculated that the lymphocytes would lyse tumor cells and/or hybridomas during or after the fusion procedure. Some of the results are in contrast with this suggestion. For instance, no differences were found in the yield of hybridomas between

fusions of untreated PHA-blast cells (with high cytotoxic activity) with HSB-1 tumor cells, and fusions of heat-treated PHA-blast cells (cytotoxic activity was transiently reduced to zero) with HSB-1 tumor cells. However, no hybridomas were obtained by either procedure. Furthermore, untreated MLC-blasts cells, clone NK76 and NK77 (with high cytotoxic activity against HSB-1 tumor cells) yielded hybridomas in 17, 17 and 37% of the wells, respectively, when fused with HSB-1 tumor cells, while no hybridomas were expected if the lytic effector cells would lyse the tumor cell fusion partner. Using CEM-2 or JURKAT-2 as tumor fusion partner, hybridomas were obtained in 10 and 30% of the wells, respectively, for each type of CTL used.

On the other hand, the results obtained with fusions between PBL and HSB-1 tumor cells supported the suggestion of Kaufmann et al. (144). Here, heat-treatment of PBL resulted in an increased yield of hybridomas; heat-treated PBL and HSB-1 tumor cells yielded hybridomas in 11% of the wells, while no hybridomas were obtained with untreated PBL and HSB-1 tumor cells. These varying results suggest that there is no relation between the presence of CTX of the T lymphocytes against the tumor fusion partner and the yield of hybridomas (cytotoxic or non-cytotoxic).

Of more than 600 hybridomas tested for CTX only 3 human-human hybridomas and 1 human-mouse hybridoma showed transient CTX against specific and nonspecific target cells, respectively. Some of the human-human hybridomas tested were highly invasive in rat hepatocyte cultures and others expressed lymphocyte derived (CD3) antigens. However, we never found these lymphocyte derived properties together in a single hybridoma. According to Foung et al. (147), most of the cytotoxic T/NK-cell hybridomas we obtained showed chromosomal instability.

The hybridomas we obtained usually had the immunophenotype of the tumor cell parent and not of the lymphocyte cell parent. This can be explained by preferential loss of the lymphocyte-derived chromosomes. Because the genetic information in lymphocytes for the process of target cell binding, recognition and lysis is located on different chromosomes, lysis can only occur if the hybridomas contain all these chromosomes. We have analyzed a number of xenogeneic hybridomas in which the human and mouse lymphocyte chromosomes could easily be distinguished. The results obtained are illustrative for the problems encountered in attempts to immortalize a complex immune function like CTX (binding, recognition and lysis). Firstly, in Table 4.7 it is shown that xenogeneic hybridomas varied greatly regarding their number of human chromosomes. Secondly, a number of xenogeneic hybridomas contained 2 copies of chromosome 6 (with genes coding for MHC antigens), while

no MHC Class I antigens were detected using mAb against these antigens. This lack of MHC expression could be the result of genetic repression of genes coding for MHC Class I antigens by tumor-derived gene products. Alternatively, the expression of MHC-genes on chromosome 6 may be co-regulated by products of genes located on other human chromosomes. In this context, van Dongen et al. (190) demonstrated that expression of the TCRαβ/CD3 receptor complex, detectable by mAb, only occurs when chromosome 11 (CD3-δ, CD3-ε and CD3-γ genes), chromosome 7 (TCRβ genes) as well as chromosome 14 (TCRα-genes) were present in the hybridomas. Finally, the MHC type of the parental cells may be altered after fusion as a result of redistribution of MHC antigens in the membrane (191). If so, the use of mAb for the detection of MHC antigens is not adequate, i.e. MHC mRNA levels in the hybridomas might be normal whereas MHC antigens on the membrane are altered or lack those epitopes recognized by the mAb used. Hence, alteration of the membrane molecule distribution on the hybridomas may lead to reduction or loss of nonspecific binding and/or recognition capacity of target cells, and thus of CTX. These various factors may account for the low success rate in the production of cytotoxic T/NK-cell hybridomas. In contrast most of the T-cell hybridomas produced in human, mouse or xenogeneic systems display immortalized immune functions, such as release of lymphokines (interleukin-2, macrophage-activating factor, and B-lymphocte replication factor) (140,143,145-147). The production of lymphokine(s) may involve fewer genes, located on less different chromosomes than cytolytic funtions. For instance, IL-2 mRNA is produced by one gene located on chromosome 4 (192).

Our results demonstrate that PEG fusion, and in particular electrofusion, are efficient tools to produce human T/NK-cell hybridomas. However, somatic cell hybridization does not allow the immortalization of CTX. Other strategies, such as DNA-mediated transfer of genes endowing human cytotoxic cells with unlimited proliferative capacity, may be more successful, since (a) chromosomal instability is not involved here, and (b) transfer of proliferative capacity seems less complicated than transfer of CTX.

CHAPTER 5

ONCOGENE EXPRESSION AND INTERLEUKIN-2 RECEPTOR LEVELS IN NORMAL AND MALIGNANT TCR$\alpha\beta^+$/CD3$^+$ AND TCR$^-$/CD3$^-$ HUMAN LYMPHOCYTES

5.1 Introduction

Several lines of evidence suggest a role for the c-myc oncogene in cell proliferation (193-195). Expression of c-myc is constitutively enhanced in Burkitt lymphomas after translocation of the c-myc gene (196,197). Enhanced c-myc expression is associated with amplification of the c-myc gene locus in human colon carcinoma APUDoma Colo 320 (198) and in the human promyelytic leukemia HL60 cells (199). The expression of c-myc in HL60 is more related to cell differentiation than to proliferation (199). The c-myc gene is inducible in normal cells and regulated by growth signals which promote proliferation, e.g. in mouse fibroblasts by platelet-derived growth factor, in mouse B lymphocytes by lipopolysaccharides and in mouse T lymphocytes by lectin activation (87).

One of the functions of c-myc protein products is to promote an acquisition of competence to respond to exogenously added growth factors (87,200,201). However, induction of myc expression in normal human lymphocytes may not be sufficient for acquisition of competence to proliferate (202). Gene transfer experiments with fibroblasts suggests that the multistep process of transition from primary cells to fully transformed ones requires functional complementation between two or more transforming genes (194,202-204). In contrast to this theory, Spandidos and Wilkie (205) claimed that mutated Ha-ras-1 gene, if linked to a strong transcriptional promotor, may not require a cooperating gene to induce malignant transformation of early passage rodent cells.

Proliferation of T and B lymphocytes requires the interaction of interleukin-2 (IL-2) with its receptors (206-209). IL-2 is the growth hormone for activated T lymphocytes (210). The expression of IL-2 receptors (IL-2R) on resting T cells can be induced by phytohaemagglutinin (PHA) (210,211). PHA-induced T-cell activation occurs via the TCR$\alpha\beta$/CD3 complex (212,213). We have shown that cross-linked anti-CD3 monoclonal antibody (mAb) and PHA are able to induce cytolytic activity in TCR$\alpha\beta^+$/CD3$^+$ but not in TCR$^-$/CD3$^-$ clones

(17), and concluded that the CD3 receptor serves as a site for signal transduction.

In the present study we have investigated the involvement of the CD3 receptor in c-myc and IL-2R expression. To this aim we used cloned normal human $TCR^-/CD3^-$ NK and $TCR\alpha\beta^+/CD3^+$ T cells as well as malignant $TCR^-/CD3^-$ and $TCR\alpha\beta^+/CD3^+$ T-lymphoma cells. To study a possible cooperation between the expression of ras and myc we compared mRNA levels of both genes in highly and poorly proliferating $TCR\alpha\beta^+/CD3^+$ T cell and $TCR^-/CD3^-$ NK-cell clones, respectively.

5.2 Materials and Methods

5.2.1 Human blood lymphocytes

Peripheral blood lymphocytes (PBL) were Ficoll-isolated from blood of a healthy donor. PBL ($1x10^6$ cells/ml) were cultured in RPMI medium supplemented with 10% v/v human serum, 4 mM glutamin and penicillin/streptomycin, and activated by PHA (10 µg PHA/ml) for 1,2 or 3 days, respectively.

5.2.2 Cloned $TCR\alpha\beta^+/CD3^+$ cells and $TCR^-/CD3^-$ NK cells

The following (cytotoxic or noncytotoxic) T cell and NK cell clones were used: TO+8 ($TCR\alpha\beta^+/CD2^+,CD3^+,CD4^+$) and CTL9, CTL11, P+2, TO+7 ($TCR\alpha\beta^+/CD2^+,CD3^+,CD8^+$), NK76, NK77, NK468 (all $CD2^+,CD3^-,CD4^-,CD8^-$). Clones were obtained by limiting dilution (163) and cultured in RPMI medium supplemented with 10% (v/v) human serum, 4mM glutamine, 1 µg/ml leucoagglutinin (purified PHA), 5% (v/v) IL-2 and penicillin/streptomycin. Gamma-irradiated (20Gy) Epstein-Barr virus-transformed human lymphoblastoid B cell lines (1×10^4 cells/200 µl) and PBL (10×10^4 cells/200 µl) were added as feeder cells. Cells were cultured in round-bottom 96-well microtiter plates at 37°C, 5% CO_2 and replated weekly with γ-irradiated (20Gy) fresh feeder cells and medium.

5.2.3 Tumor cell lines

Human T lymphoma cell lines, CEM ($TCR^-/CD2^-,3^-$), JURKAT ($TCR\alpha\beta^+/CD2^+,3^+$)(kindly provided by Dr. S.K.H. Foung, Stanford University Medical Center, USA) and MOLT-4 ($TCR^-/CD2^-,3^-$) (kindly provided by Dr. A. Hagemeyer, Erasmus University, Rotterdam, The Netherlands) were used. The JURKAT cell line (J3R7) used in these experiments did not produce IL-2 (147). Cells were cultured with and without PHA in RPMI medium containing 10% v/v fetal calf serum (FCS), heat-inactivated, 2 mM glutamin and penicillin/ streptomycin.

5.2.4 Assay for oncogene expression

Levels of c-myc and c-ras mRNA were determined by means of a dot blot assay (154) using the commercially available quick blot kit (Schleicher and Schuell). The human c-myc (DNA) probe isolated from the colon carcinoma APUDoma Colo 320 (198) was kindly provided by Dr. H.E. Varmus (Department of Microbiology and Immunology, University of California, CA). A fixed number of cells was spotted on a nitrocellulose filter (number of cells are specified in Tables 5.1-5.4. The hybridization procedure is described elsewhere (214). Briefly, the plasmid was labelled with ^{32}P-dCTP (Radiochemical Center, Amersham, UK) to high specific activity 1-5x10^8 cpm/µg), using a nick-translation kit (Amersham), according to the manufacturers instructions. The filters were preincubated in a hybridization solution containing 50% formamide, 3xSSC (1xSSC is 0.15M NaCl, 0.015M Na-citrate), 1% sodium dodecyl sulfate (SDS) and 10x Dennhardt's solution (1x Dennhardt's solution is 0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone). Radioactively labelled probe fragments were then heat-denaturated, incubated with the filters for at least 16 h at 42°C in a final concentration of 1-5x10^6 cpm/ml). After incubation, the filters were rinsed in 2xSSC and subsequently washed at 65°C in 3xSSC, 0.1% SDS, in 1xSSC, 0.1% SDS and twice in 3xSSC, 0.1% SDS, for 30 min. After a short wash in 2xSSC at room temperature, the filters were autoradiographed (Kodak X-OMATIC, AR2 film) at -70°C using X-OMATIC intensifying screens.

5.2.5 Determination of IL-2R

Cells ($2x10^5$) were incubated with the anti-Tac mAb (kindly provided by Dr. T. Uchiyama, Kyoto Universty Hospital, Kyoto, Japan) which identifies the IL-2R. The cells were then washed with phosphate buffered saline, supplemented with 1% bovine serum albumin, 0.1% sodium azide and incubated with anti-TAC mAb plus a second layer of fluorescein isothiocyanate (FITC) labelled goat-anti-mouse-antiserum (Nordic, Tilburg, The Netherlands). As a control for background fluorescence, cells were incubated with goat-anti-mouse-antiserum alone. Stained cells were analyzed using a fluorescence-activating cell sorter (FACS II, Becton & Dickinson). Percentages of $IL\text{-}2R^+$ and $IL\text{-}2R^-$ cells were calculated from the fluorescence histograms. Total levels of IL-2R were determined by cumulation of the fluorescence intensities (as measured with a logarithmic amplifier) of a fixed number of cells ($IL\text{-}2R^-$ and $IL\text{-}2R^+$ cells). This was done to allow direct comparison with the levels of c-myc mRNA, which were also measured as an average of a fixed number of cells. To obtain linear values, the cumulative fluorescence intensities were calculated by multiplying the number of events in each channel (CHN) of the fluorescence histograms with $2^{CHN/17}$: a difference of 17 CHNs represents a 2-fold difference of fluorescence intensity. The fluorescence intensities are given in arbitrary units.

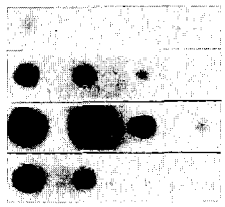
5.3 Results

5.3.1 Expression of c-myc and IL-2R in resting and PHA-activated lymphocytes

In resting PBL, expression of IL-2R and c-myc was below the detection level under the experimental conditions applied. After PHA activation, a strong increase in the expression of both c-myc and IL-2R was found over the first 2 days, but at day 3 lower levels of c-myc mRNA and IL-2R were observed as well as a drop in the percentages of $IL\text{-}2R^+$ cells (Table 5.1). In all subsequent experiments resting and PHA-activated PBL were routinely used as negative and positive control cells, respectively.

TABLE 5.1

LEVELS OF c-MYC mRNA AND IL-2R$^+$ IN PHA ACTIVATED LYMPHOCYTES

Days *** PHA	total level*	percent of IL-2R+	c-myc expression**
0	22	0	
1	32	37	
2	187	72	
3	158	62	

* total levels of IL-2R in arbitrary units of fluorescence
** each dot blot dilution series represent c-myc mRNA from 160,000, 53,000, 18,000 and 6,000 cells.
*** days of incubation with PHA

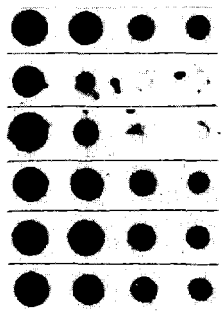
5.3.2 Expression of c-myc and IL-2R in TCR$\alpha\beta^+$/CD3$^+$ T- and TCR$^-$/CD3$^-$ NK-cell derived clones.

Table 5.2 shows the expression of c-myc and IL-2R in human TCR$\alpha\beta^+$/CD3$^+$ T-cell clones (cytotoxic and noncytotoxic) and TCR$^-$/CD3$^-$ NK cell derived clones which were cultured in the complete expansion medium, i.e. containing PHA, feeder cells and IL-2. All T-cell clones expressed both c-myc and IL-2R.

TABLE 5.2

DIFFERENT LEVELS OF c-MYC mRNA AND IL-2R IN

TCR$\alpha\beta^+$/CD2$^+$,3$^+$ AND TCR$^-$/CD2$^+$,3$^-$ CLONES

clone	phenotype	PHA	total level*	positive cells (percent)	c-myc expression**
CTL9	CD2$^+$,3$^+$	+	449	79	
CTL11	CD2$^+$,3$^+$	-	183	29	
CTL11	CD2$^+$,3$^+$	+	535	84	
P+2	CD2$^+$,3$^+$	+	602	89	
TO+7***	CD2$^+$,3$^+$	+	597	89	
TO+8***	CD2$^+$,3$^+$	+	746	91	
NK468***	CD2$^+$,3$^-$	+	n.t.	n.t.	
NK76***	CD2$^+$,3$^-$	-	260	42	
NK76***	CD2$^+$,3$^-$	+	395	53	
NK77***	CD2$^+$,3$^-$	-	182	43	
NK77***	CD2$^+$,3$^-$	+	376	58	

* total levels of IL-2R in arbitrary units of fluorescence
** each dot blot dilution series represents c-myc mRNA from 160,000, 80,000, 40,000 and 20,000 cells; for clone CTL11 the dilution series represents c-myc mRNA from 160,000, 53,000, 18,000 and 6,000 cells.
*** derived from a patient with Tγ lymphocytosis

Contamination of feeder cells in the evaluation of IL-2R and c-myc expression can be excluded because the feeder cells desintegrated in about 7 days as described in detail earlier (33). The TCR$\alpha\beta^+$/CD3$^+$ clones (CTL9, CTL11, P+2, TO+7, and TO+8) showed manifold higher levels of c-myc mRNA and a 2-fold higher level of IL-2R than the TCR$^-$/CD3$^-$ clones (NK76, NK77, and NK468). A representative example of the determination of IL-2R on a TCR$\alpha\beta^+$/CD3$^+$ and a TCR$^-$/CD3$^-$ clone is shown in Figure 5.1. The significance of the observed

levels of c-myc mRNA in $TCR^-/CD3^-$ clones was demonstrated by the absence of any c-myc specific hybridization signal with these cells after deprivation of IL-2, PHA and feeder cells (data not shown).

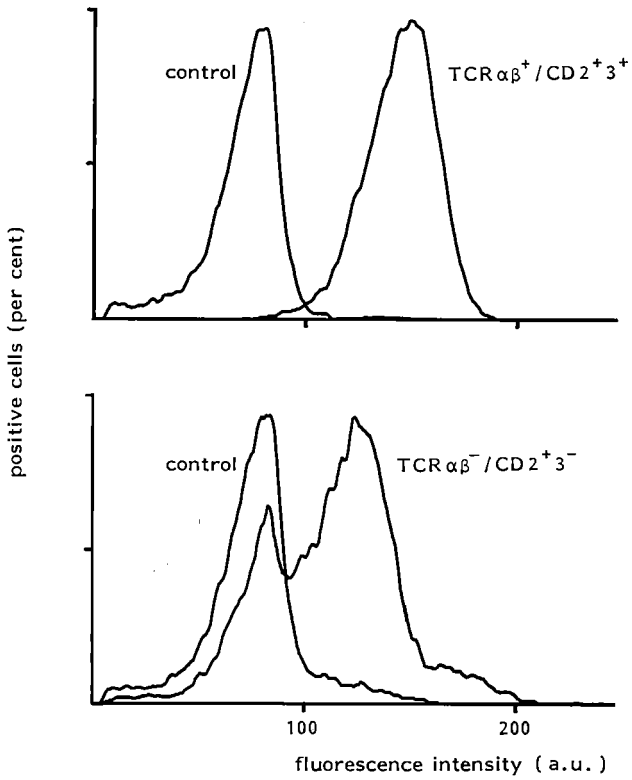


Figure 5.1 Fluorescence histograms of IL-2 receptors expressed on a $TCR\alpha\beta^+/D3^+$ T-cell clone and a $TCR^-/CD3^-$ NK-cell clone. Both clones were cultured in the presence of PHA in the expansion system.

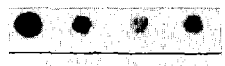
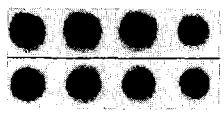
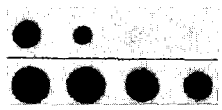
In the absence of PHA, but in the presence of feeder cells and IL-2, decreased levels of IL-2R were observed on both the $TCR\alpha\beta^+/CD3^+$ clone CTL11 and $TCR^-/CD3^-$ clones NK76 and NK77 (Table 5.2). In the same culture a decreased level of c-myc mRNA in the $TCR\alpha\beta^+/CD3^+$ clone CTL11 was found (Table 5.2). For the $TCR^-/CD3^-$ NK-cell clones such a decrease was not observed or fell below the detection level of the method.

5.3.3 Expression of c-myc and IL-2R in TCR$\alpha\beta^+$/CD3$^+$ and TCR$^-$/CD3$^-$ T-lymphoma cell lines

Table 5.3 shows the expression of c-myc and IL-2R in human T lymphoma cell lines cultured with and without PHA. All three cell lines, CEM, MOLT and JURKAT constitutively expressed c-myc. Only JURKAT (CD3$^+$) responded to PHA with a manifold increase in the c-myc mRNA level (Table 5.3). No increase was observed in the CD3$^-$ T-lymphoma cell lines. In contrast to normal lymphocytes, none of the malignant T-lymphoma cell lines expressed IL-2R.

TABLE 5.3

LEVELS OF c-MYC mRNA AND IL-2R IN MALIGNANT HUMAN TCR$\alpha\beta^+$/CD2$^+$,3$^+$ AND TCR$^-$/CD2$^-$,3$^-$ T-LYMPHOMA CELL LINES

T lymphoma cell line	phenotype	PHA	IL-2R total level*	IL-2R pos cells percent	c-myc expression**
MOLT	CD2$^-$,3$^-$	-	43	0	
MOLT	CD2$^-$,3$^-$	+	56	0	
CEM	CD2$^-$,3$^-$	-	49	0	
CEM	CD2$^-$,3$^-$	+	54	0	
JURKAT	CD2$^+$,3$^+$	-	38	0	
JURKAT	CD2$^+$,3$^+$	+	50	0	

* total levels of IL-2R in arbitrary units of fluorescence
** each dot blot dilution series represents c-myc mRNA from 160,000, 80,000, 40,000 and 20,000 cells

5.3.4 Expression of c-myc and c-ras in TCRαβ$^+$/CD3$^+$ T- and TCR$^-$/CD3$^-$ NK-cell clones

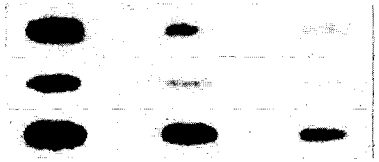
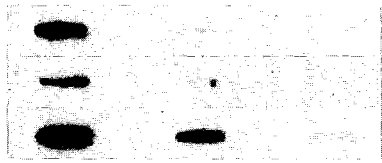
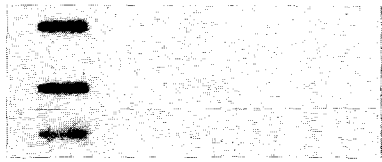
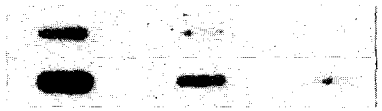
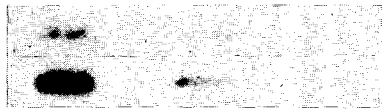
In these experiments we increased the detection level (assay sensitivity) of mRNA amounts since the expression of c-ras appeared to be much lower than that of c-myc. We therefore performed proteinase K digestion at 60°C instead of 37°C. Resting and PHA-activated lymphocytes were used as control cells. Table 5.4 shows the expression of c-myc and c-ras mRNA levels in cloned TCRαβ$^+$/CD3$^+$ T- and TCR$^-$/CD3$^-$ NK-cells. When c-myc mRNA levels were high, c-ras mRNA levels were also high and, vice versa, when the c-myc mRNA level was low, the c-ras mRNA level was low. Like c-myc expression, the c-ras mRNA level is higher in TCRαβ$^+$/CD3$^+$ T cells than in TCR$^-$/CD3$^-$ NK cells. The level of c-myc and c-ras mRNA correlates directly with the proliferative capacity of the cells, i.e. high levels of c-myc and c-ras mRNA corresponded with high cell yields.

5.4 Discussion

We studied the relative involvement of the TCRαβ$^+$/CD3 complex in the regulation of c-myc and IL-2R expression. To this aim cloned normal human TCRαβ$^+$/CD3$^+$ and TCR$^-$/CD3$^-$ NK cells as well as malignant TCRαβ$^+$/CD3$^+$ and TCR$^-$/CD3$^-$ T-lymphoma cell lines were used. PBL were used as control cells, i.e. PHA activation of resting PBL induces a sharp but transient increase in IL-2R and c-myc expression. All cloned TCR$^-$/CD3$^-$ NK and TCRαβ$^+$/CD3$^+$ T cells expressed c-myc and IL-2R when cultured in complete expansion medium. The TCRαβ$^+$/CD3$^+$ T-cell clones (CTL9 CTL11, P+2, TO+8) showed about 2-fold higher levels of c-myc mRNA than the TCR$^-$/CD3$^-$ NK cell-derived clones (NK76 and NK77 and NK468). These differences in c-myc mRNA and IL-2R levels for TCRαβ$^+$/CD3$^+$ T-cell and TCR$^-$/CD3$^-$ NK-cell clones plus the fact that PHA transduces its signal via the CD3 receptor (29,212,213), suggest that in TCRαβ$^+$/CD3$^+$ T-cell clones the lectin-induced c-myc expression occurs via the CD3 receptor. However, TCR$^-$/CD3$^-$ NK-cell clones do express IL-2R and c-myc, albeit at lower levels (Table 5.2) and hence, (an) activation site(s) other than CD3, mediating the induction of IL-2R and c-myc, must exist. Such a distinct activation site on T cells could be the CD2 50KD sheep red blood cell receptor. The CD2 receptor is present on both mature TCRαβ$^+$/CD3$^+$ T-cell clones and cloned TCR$^-$/CD3$^-$ NK cells. Under appropriate conditions CD2 serves as a regulatory site for proliferation (29). Moreover, a single anti-CD2 mAb

TABLE 5.4

c-MYC and c-RAS mRNA LEVELS IN HUMAN TCR$\alpha\beta^+$/CD3$^+$ AND TCR$^-$/CD3$^-$ CLONED LYMPHOCYTES

Clones	Phenotype	percent yield of cells	c-myc mRNA level**	c-ras mRNA level**
CTL20	CD3$^+$,4$^+$,8$^-$	4861		
CTL11	CD3$^+$,4$^-$,8$^+$	5468		
P+2	CD3$^+$,4$^-$,8$^+$	9513		
NK76*	CD3$^-$,4$^-$,8$^-$	1747		
NK77*	CD3$^-$,4$^-$,8$^-$	1500		
NK472*	CD3$^-$,4$^-$,8$^-$	1700		
PBL(-PHA)				
PBL(+PHA)				

* derived from a patient with Tγ lymphocytosis

** each slot blot dilution series represent mRNA for 160,000, 80,000 and 40,000 cells

reportedly inhibits PHA-induced c-myc expression (201). We have shown that cytolytic functions in TCR$\alpha\beta^+$/CD3$^+$ and TCR$^-$/CD3$^-$ clones can be mediated via the CD2 receptor (6,29).

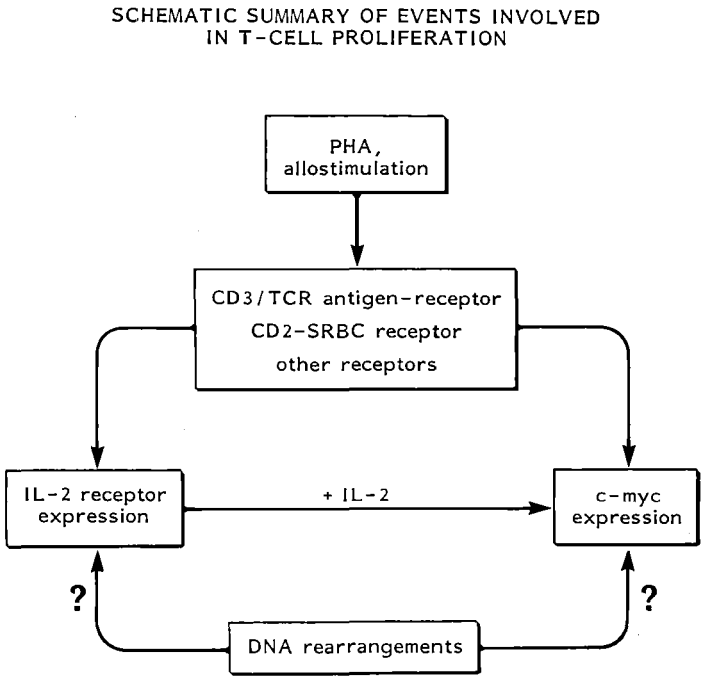


Figure 5.2 PHA or alloantigens can induce the expression of c-myc and IL-2 receptors via the TCR$\alpha\beta^+$/CD3$^+$ or CD2$^+$ receptor in TCR$\alpha\beta^+$/CD2$^+$3$^+$ T lymphocytes, via the CD2$^+$ receptor in TCR$^-$/CD2$^+$3$^-$ NK cells or via other receptors. Interaction between IL-2 with its receptor induces c-myc expression. The constitutively enhanced levels of IL-2 receptors and c-myc in malignant T cells may be a result of DNA-rearrangement(s).

The hypothesis that signal transduction via the TCR$\alpha\beta^+$/CD3 complex mediates c-myc expression was supported by the observation that the TCR$\alpha\beta^+$/CD3$^+$ T-lymphoma cell line JURKAT but not the other two TCR$^-$/CD3$^-$ T-lymphoma cell lines responded to PHA with a further increase in c-myc mRNA.

This PHA-induced c-myc expression also supports the suggestion that the c-myc gene contains two promoters from which transcription can be initiated independently (215,216). The JURKAT cell line also expresses CD2 receptors which may be involved in c-myc expression. It is noteworthy that all three lymphoma cell lines constitutively expressed c-myc at high levels in the absence of IL-2R. However, in malignant T cells the expression of c-myc is constitutively enhanced which may possibly lead to loss of IL-2R, since the presence of IL-2R has no longer a biological function. Recently, it has been observed that in human T-cell leukemias deregulation of c-myc was induced by DNA-rearrangements (217, 218). Reed et al. (90) showed that IL-2 can induce c-myc expression in cloned mouse lymphocytes. On the other hand, in human T-leukemia virus (HTLV-I)-infected cells the IL-2R as well as the c-myc-gene is permanently activated (219). Possible routes of the induction of c-myc expression in normal and malignant $TCR\alpha\beta^+/CD3^+$ and $TCR^-/CD3^-$ lymphocytes have been summarized in Figure 5.2.

The results obtained for c-ras expression in normal T and NK cells showed a similar pattern as c-myc expression, i.e. higher levels of expression of c-ras were found in cloned $TCR\alpha\beta^+/CD3^+$ T cells than in $TCR^-/CD3^-$ NK cells (Table 5.4). Recently, Stevenson and Volsky (220) showed in transfection experiments that the expression of c-myc was stabilized when cotransfected into normal human lymphocytes with the ras gene. We found for $TCR\alpha\beta^+/CD3^+$ T as well as for $TCR^-/CD3^-$ NK-cell clones a positive correlation between the expression level of c-ras and c-myc, i.e. when c-myc mRNA levels were high than the c-ras mRNA levels were high and vice versa. The differences in proliferative capacity of $TCR^-/CD3^-$ NK and $TCR\alpha\beta^+/CD3^+$ T cells was reflected by the level of IL-2R, c-myc and c-ras expression. The higher levels of IL-2R, c-myc and c-ras mRNA in $TCR\alpha\beta^+/CD3^+$ T-cell clones correlated with their higher proliferative capacity, i.e. the mean generation time and lifespan for $TCR\alpha\beta^+/CD3^+$ T-cell clones being 20-28 h and 70-100 generations, respectively, whereas for $TCR^-/CD3^-$ NK-cell clones these values are 30-40 h and 25-25 generations (163). Moreover, the level of c-myc and c-ras mRNA correlated directly with the cell yield after one week of culture.

CHAPTER 6

OK-432 AND IL-2 AUGMENTED CYTOTOXICITY OF HUMAN $TCR^-/CD3^-$ NATURAL KILLER CELLS AND $TCR\alpha\beta^+/CD3^+$ CYTOTOXIC T LYMPHOCYTES AT THE CLONAL LEVEL

6.1 Introduction

Cytotoxic T lymphocytes (CTL) directed against tumor-associated antigens (TAA) can be immune specific, i.e. antigen is recognized in the context of major histocompatibility complex (MHC) determinants and involves the α and β-chain of the T-cell receptor (TCR) (6). NK cells represent another immune defence system against tumor cell growth and, in contrast to CTL, exert MHC-unrestricted cellular cytotoxicity (CTX). It has been shown that some T lymphocytes also mediate MHC-unrestricted cellular cytotoxicity (CTX) after activation (221,222). Moreover, also lectins such as phytohaemagglutinin (PHA) or tumor cells can induce MHC-unrestricted CTX by immune specific T cells (223) and T-cell clones, (164) respectively.

The biological response modifiers (BRM) OK-432 and interleukin-2 (IL-2) are used in the clinic for treatment of cancer patients. OK-432, a bacterial product of Streptococcus pyogenes, enhances survival of patients with lung or stomach cancers (93,224). IL-2 is a growth factor for human T cells and NK cells (210,225) and it affects the lytic capacity of these cells (96). In vitro, the MHC-unrestricted CTX of peripheral blood lymphocytes (PBL) against K562 can be enhanced by OK-432 and IL-2 (155,226-228). It is assumed that this effect is due to OK-432-induced IL-2 production (229). With respect to the cell type involved in this process there is evidence that not CTL but NK cells are responsive to OK-432 (100). However, this evidence has been obtained in experiments with fresh PBL or with T-cell and NK-cell-enriched percoll fractions.

We have produced clones of various lymphocyte lineages of NK cells and T cells and tested the effects of OK-432 and IL-2 on the cytotoxic response of $TCR\alpha\beta^+/CD3^+4^+/8^+$ CTL and $TCR^-/CD3^-16^+$ NK cells at the clonal level in comparison to PBL of healthy individuals.

6.2 Materials and methods

6.2.1 Cytotoxic effector cells

PBL comprising CTL and NK cells, were obtained from blood of healthy donors by centrifugation on Ficoll-Isopaque (d=1.077 g/cm^3). The cells were resuspended in RPMI medium supplemented with 10% FCS (heat-inactivated), 4 mM glutamin and antibiotics. TCR$^-$/CD3$^-$ NK-cell clones and TCR$\alpha\beta^+$/CD3$^+$ CTL clones used are summarized in Table 6.1. NK-cell clones were obtained by limiting dilution of PBL without prior activation against stimulator cells. TCR$\alpha\beta^+$/CD3 CTL clones were obtained by limiting dilution of PBL after stimulation with Epstein-Barr virus (EBV) transformed lymphoblastoid B-cell lines (B-LCL) (167). Cloned cells were cultured as described previously (163). Briefly, responder cells ($2x10^3$ cells/200 µl) were cultured in RPMI medium supplemented with 10% human serum (not heat-inactivated), 4 mM glutamin, 1 µg/ml indomethacin, 1 µg leucoagglutinin/ml or not, 25 U recombinant IL-2/ml and antibiotics. Gamma-irradiated (20 Gy) EBV transformed B-LCL $5x10^4$ cells/200 µl) and γ-irradiated (20Gy) allogeneic lymphocytes (10^5 cells/200 µl) were added as feeder cells.

TABLE 6.1

CLONED EFFECTOR CELLS

effector cells	phenotype CD2	CD3	CD4	CD8	CD16	MHC spec	target cells CTX
TCR$^-$/CD3$^-$ NK clones							
NK76*	+	-	-	-	+	no	K562 Daudi P815-IgG***
NK77*	+	-	-	-	+	no	K562 Daudi P815-IgG
NK472**	+	-	-	-	+	no	K562 Daudi P815-IgG
NK468**	+	-	-	-	+	no	K562 Daudi P815-IgG
NK436**	+	-	-	-	+	no	K562 Daudi P815-IgG
TCR$\alpha\beta^+$/CD3$^+$ CTL clones							
CTL11	+	+	-	+	-	no	K562
CTL11	+	+	-	+	-	class I	BSM
CTL9	+	+	-	+	-	Cw3, class I	BSM
CTL20	+	+	+	-	-	Drw6, class II	APD Daudi

* NK-cell clones derived from a patient with Tγ-lymphocytosis
** NK-cell clones derived from a SCID (severe combined immunodeficiency disease) patient.
***ADCC
All CTL clones are derived from healthy individuals

6.2.2 Target cells

The target cells used are detailed in Table 6.2. These cell lines are cultured in RPMI medium supplemented with 10% FCS (heat-inactivated), 2 mM glutamin and antibiotics.

TABLE 6.2

TARGET CELLS

human tumor cell lines	expression of target MHC antigens	origin
K562	no	erythroid leukemia
Daudi	Drw6	Burkitt lymphoma
BSM	Cw3	EBV transformed B-LCL
APD	Drw6	EBV transformed B-LCL
mouse tumor cell line		
P815*	no	mastocytoma cell line

* P815 mouse cells coated with anti-P815 antiserum (IgG) were used for the antibody-dependent cellular cytotoxicity (ADCC)

6.2.3 BRM treatment of effector cells

OK-432, a lyophilized powder of penicillin-treated low virulent Streptococcus pyogenes was kindly provided by Chugai Pharmaceutical Co., Tokyo, Japan. Exposure of effector cells with OK-432 has been described in detail previously (155,227). Briefly, effector cells (10^6/ml) were resuspended in RPMI medium supplemented with 10% FCS (heat-inactivated), 4 mM glutamin, antibiotics and various concentrations of OK-432 at 37°C. Cells were incubated with OK-432 in a plastic tube (Falcon 2058) for different time periods. Thereafter, the cells were washed, resuspended in RPMI medium and tested for CTX. Human recombinant IL-2 (10^5 U/ml) was a gift from Biogen, Geneva, Switzerland. IL-2 was diluted and used at a final concentration of 25 U/ml. Effector cells were incubated with IL-2 for 20 h in microtiter plates

(Greiner) at 37°C. Then, target cells were added to the wells of the microtiter plate and CTX was determined.

6.2.4. Determination of cytotoxic activity

CTX was determined in a ^{51}Cr-release assay in round-bottom microtiter plates as described earlier (164). Briefly, varying cell numbers in 150 µl RPMI medium supplemented with 10% FCS (heat-inactivated), 2 mM glutamin and antibiotics, were seeded in the wells of the microtiter plate before the addition of 100 µl of 10^3 ^{51}Cr-labelled target cells. Cells were incubated for 3 h at 37°C and centrifuged. Supernatant (100 µl) was collected and radioactivity was determined. Specific ^{51}Cr-release was calculated as:

$$\frac{\text{experiment cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100\%$$

The maximum ^{51}Cr-release was obtained by incubation of the target cells with 10% (v/v) Triton X-100. Statistical significances of differences were calculated from the cpm values of triplicate samples by Student t-test.

6.3 Results

6.3.1 OK-432 effect on fresh PBL

Optimal concentration and incubation time of OK-432 appeared to be 0.5 Klinische Einheiten (KE) OK-432/ml and 20 h, respectively (155,227). The OK-432 effect on the level of CTX by PBL of various healthy donors against NK-susceptible K562 and NK-resistent Daudi target cells and of antibody dependent cellular cytotoxicity (ADCC) using P815-IgG target cells was determined. OK-432-treated PBL showed significantly higher CTX against K562 target cells than untreated PBL, i.e. 6 out of 9 donors responded positively to OK-432 (Table 6.3). When Daudi cells were used as targets, OK-432 augmented the CTX of PBL for all 9 donors tested (Table 6.3).

TABLE 6.3

OK-432 AUGMENTED CTX BY FRESH PBL

donor number	percent lysis of target cells*			
Fresh PBL	K562 OK-432		Daudi OK-432	
	-	+	-	+
1	27	54**	12	29**
2	36	52**	17	45**
3	14	23**	8	15**
4	32	52**	9	29**
5	27	44**	13	29**
6	29	30	9	27**
7	31	28	17	30**
8	27	44**	15	28**
9	29	30	22	42**

* Results expressed as percent ^{51}Cr-release; effector:target ratio 27:1

** Significantly higher than control cytotoxicity ($p<0.05$)

PBL of 7 out of 12 donors showed OK-432-augmented ADCC (P815 cells coated with IgG) but not of uncoated (control) P815 target cells (Table 6.4).

TABLE 6.4

OK-432 AUGMENTED ADCC BY FRESH PBL

donor number	percent lysis of target cells*			
fresh PBL	P815 OK-432		P815-IgG OK-432	
	-	+	-	+
1	7	15	52	72**
2	0	1	46	57**
3	0	1	14	25**
4	0	0	34	53**
5	3	4	47	52
6	0	4	37	37
7	0	3	28	33
8	0	1	25	30
9	0	3	48	50
10	0	0	29	47**
11	0	3	39	56**
12	3	6	38	51**

* Results expressed as percent ^{51}Cr-release; effector:target 27:1

** Significantly higher than control CTX ($p<0.05$)

6.3.2 OK-432 effect on $TCR^-/CD3^-$ cloned NK cells

Optimal concentration and incubation time of OK-432 were determined for cloned NK cells. Effector cells were incubated with 0, 0.01, 0.1, 0.5 and 1 KE OK-432/ml for 8, 16 and 20 h. Similar to fresh PBL, incubation with 0.5 KE OK-432 for 20 h appeared to the optimal for cultured NK-cell clones (Fig. 6.1).

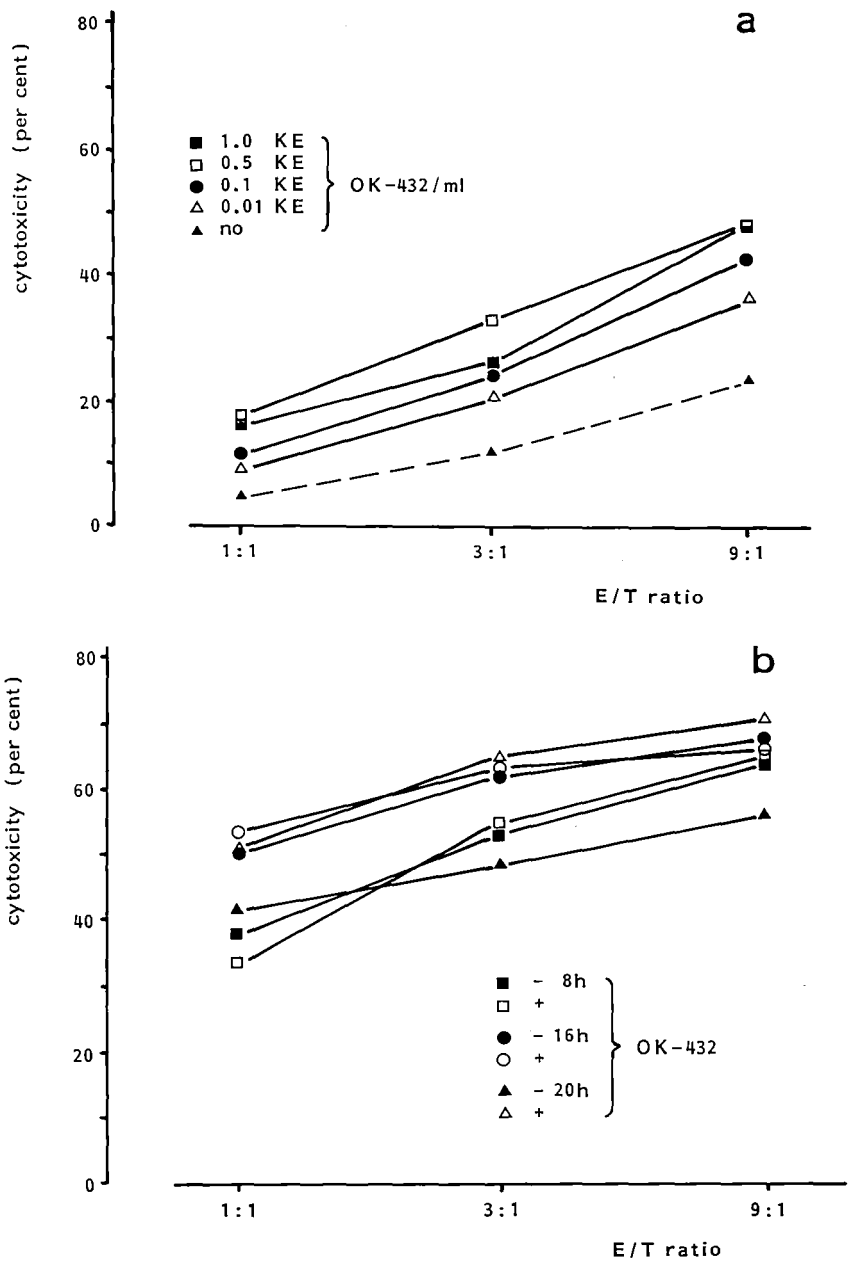


Figure 6.1: Optimal concentration of OK-432 and incubation time for cloned $TCR^-/CD3^-$ NK cells. 1A: cells were incubated for 20 h with 0, 0.01, 0.1, 0.5 and 1 KE OK-432/ml; 1B: cells were incubated for 8, 16 and 20 h with 0.5 KE OK-432/ml. Effector cell: clone NK76; target cell: Daudi.

Five NK-cell clones (NK76, NK77, NK436, NK468 and NK472) were incubated with and without OK-432. Subsequently, the CTX against K562 and Daudi target cells as well as the ADCC was determined. As shown in Table 6.5 the MHC-unrestricted CTX is increased by OK-432 using K562 and Daudi target cells, provided the effector cells were cultured without PHA (Table 6.5, Fig. 6.2).

TABLE 6.5

INFLUENCE OF PHA ON OK-432 RESPONSIVENESS OF CLONED $TCR^-/CD3^-$ NK CELLS

effector cells		percent lysis of target cells*							
NK clones***	PHA	K562		Daudi		P815		P815-IgG	
		OK-432		OK-432		OK-432		OK-432	
		-	+	-	+	-	+	-	+
NK76	-	34	45**	25	32**	0	3	33	46**
NK76	+	56	54	48	46	5	5	38	49**
NK77	-	34	56**	52	66**	nd	nd	57	69**
NK77	+	63	63	44	51	nd	nd	55	60

* Results expressed as percent ^{51}Cr-release: effector:target ratio 27:1 (NK cells against all target cells)
** Significantly higher than control cytotoxicity ($p<0.05$)
*** NK-cell clones were cultured with and without PHA
nd: not done

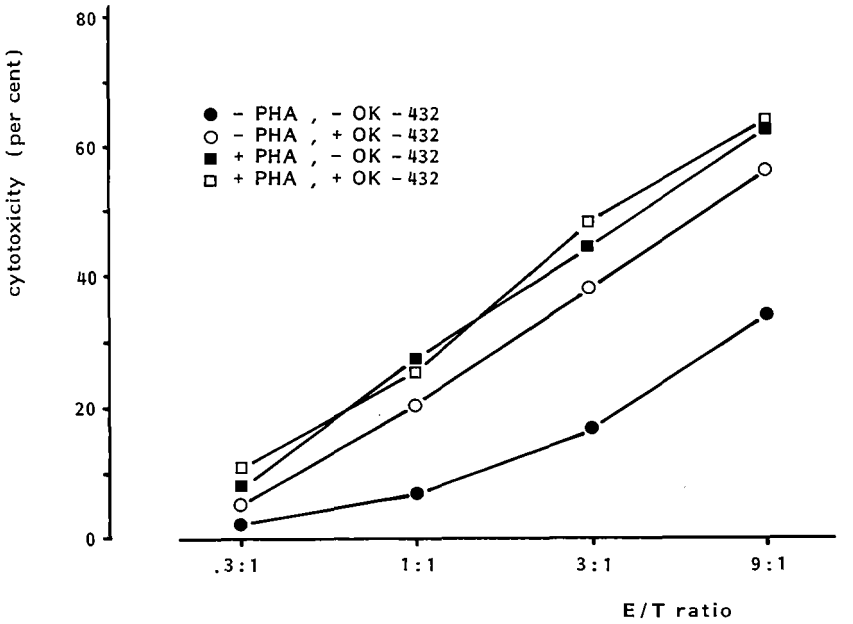


Figure 6.2: OK-432 does not affect CTX of PHA cultures of $TCR^-/CD3^-$ NK-cell clones; cloned NK cells were incubated for 20 h with 0.5 KE OK-432/ml. Effector cell: clone NK77; target cell: K562.

Moreover, in some experiments PHA inhibited the OK-432 response of cultured NK cells (Table 6.5). A complicating factor in the above mentioned relationship between OK-432 augmentation of NK-cell CTX and PHA is the influence of the proliferative capacity of these effector cells, i.e., only NK-cell clones with a cell yield higher than about 1000% after 1 week of culture responded to OK-432 with augmented CTX (Table 6.6). Indeed, the 2 NK-cell clones (NK76 and NK77) with a high proliferative capacity and cultured without PHA showed enhanced lytic activity. When the cell yield of clone NK76 was higher than about 900%, significantly augmented cytolysis of K562 and Daudi target cells was observed. With clone NK77, such a response was found when the cell yield was higher than 700%. This "growth dependency" of OK-432 responsiveness of clones NK76 and NK77 could not be demonstrated for the ADCC (Table 6.6).

TABLE 6.6

EFFECT OF OK-432 ON CTX OF $TCR^-/CD3^-$ NK CELL

CLONES IN RELATION TO PROLIFERATION

NK cell clones****	percent of cell yield	percent lysis of target cells* K562 OK-432		Daudi OK-432		P815-IgG OK-432	
		-	+	-	+	-	+
NK76							
exp.1	833	46	42	50	52	35	33
exp.2	892	31	39	23	28	42	28***
exp.3	1600	2	11**	8	25**	9	26**
exp.4	1760	36	49**	55	67**	nd	nd
exp.5	1932	8	39**	17	44**	13	27
exp.6	2300	28	41**	49	67**	30	31
NK77							
exp.1	585	53	57	54	44	37	29
exp.2	700	57	41	37	41	54	36***
exp.3	700	32	40	31	45**	29	23
exp.4	1389	12	39**	12	39**	43	59**
exp.5	1133	58	73**	46	56**	nd	nd
exp.6	1833	27	58**	26	67**	15	24**
exp.7	6000	46	61**	52	62**	26	25

* Results expressed as percent ^{51}Cr-release; effector:target 9:1
** Significantly higher than control CTX ($p<0.05$)
*** Significantly lower than control CTX ($p<0.05$)
**** NK-cell clones were cultured without PHA
nd: not done

Clones NK436, NK468 and NK472 did not respond positively to OK-432 (data not shown) when cultured in the presence of PHA. Without PHA, these NK-cell clones do not proliferate.

6.3.3 OK-432 effect on cloned $TCR\alpha\beta^+/CD3^+$ CTL

As for NK-cell clones and PBL, optimal concentration and incubation time of OK-432 appeared to be 0.5 KE/ml and 20 h, respectively (not shown). Five T-cell clones, 3 of which were cytolytic (CTL9, CTL20 and CTL11) and 2 noncytolytic (clone 317 and clone 397), were incubated with and without

OK-432. Subsequently, the CTX against K562, Daudi, APD and BSM target cells was determined. OK-432 augmented the CTX of MHC class I-specific cloned CTL9 ($CD3^+8^+$) and of clone CTL11 ($CD3^+8^+$) using BSM target cells (MHC class I^+ and II^+) (Table 6.7). Using Daudi (MHC class $I^+$and II^+) and APD (MHC class I^+ and II^+) as target cells the CTX of MHC class II specific CTL20 ($CD3^+4^+$) was not affected by OK-432 (Table 6.7).

TABLE 6.7

OK-432 AUGMENTED CTX OF $TCR\alpha\beta^+/CD3^+8^+$ BUT NOT OF $TCR\alpha\beta^+/CD3^+4^+$ T CELL CLONES

effector cells	percent lysis of target cells*					
	K562		Daudi		BSM/APD	
CTL clones****	OK-432		OK-432		OK-432	
	-	+	-	+	-	+
CTL11 ($CD3^+8^+$)	31	19***	1	1	35	54**
CTL9 ($CD3^+8^+$)	2	5	0	0	38	49**
CTL20 ($CD3^+4^+$)	12	16	55	55	36	40

* Results expressed as percent ^{51}Cr-release; effector:target ratio 81:1 (K562 and Daudi target cells) and 3:1 (BSM/APD target cells)
** Significantly higher than control CTX ($p<0.05$)
*** Significantly lower than control CTX ($p<0.05$)
****All CTL clones were cultured with PHA

Clones CTL9 and CTL20 did not lyse K562 cells, neither in the presence nor in the absence of OK-432. OK-432 inhibited the lysis of K562 target cells by clone CTL11 (Table 6.7). In contrast to the situation with NK cells, OK-432-induced CTX by none of the cloned T cells used was affected by PHA (data not shown). OK-432 was not able to induce CTX in the non-cytolytic clones 317 and 397 (data not shown).

6.3.4 Comparison of OK-432 and IL-2 effects on cloned $TCR^-/CD3^-$ NK cells and $TCR\alpha\beta^+/CD3^+$ CTL

OK-432 and IL-2 were compared for their effects on CTX and ADCC of PBL, or cloned NK cells and T cells. PBL stimulated with OK-432 or IL-2 showed augmented cytolysis of K562 target cells while the ADCC was only augmented by OK-432 and not by IL-2. In these experiments, IL-2R were not expressed on the effector cells after stimulation with OK-432 or IL-2 (Table 6.8).

TABLE 6.8

OK-432 AND IL-2 AUGMENTS THE CTX OF FRESH PBL WHILE IL-2R WERE NOT DETECTED AFTER STIMULATION

healthy donor	stimulation	percent of IL-2R	percent of lysis of target cells*		
			K562	P815	P815-IgG
1713	untreated	9	30	0	29
	OK-432	11	47**	0	47**
	IL-2	8	42**	0	35
	PHA	50	nd	nd	nd
1714	untreated	10	38	0	39
	OK-432	7	53**	3	56**
	IL-2	7	53**	0	48
	PHA	34	nd	nd	nd
1634	untreated	6	21	3	38
	OK-432	8	40**	6	51**
	IL-2	5	33**	3	44
	PHA	54	nd	nd	nd

* Results expressed as percent ^{51}Cr-release; effector:target ratio 27:1
** Significantly higher than control CTX ($p<0.05$)

With respect to NK cells, OK-432 and IL-2 augmented the CTX of NK-cell clones NK76 and NK77 against K562 and Daudi target cells. In contrast to OK-432, IL-2 never augmented the ADCC of NK-cell clones NK76 and NK77. The apparently augmented ADCC by IL-2-treated NK-cell clone NK77, as shown in Table 6.9, was due to the induction of CTX against uncoated P815 cells (control). As for OK-432, IL-2 did not further enhance the CTX of clones cultured in medium plus PHA. No correlation between IL-2 responsiveness and the proliferative capacity of NK-cell clones was observed (data not shown).

The effects of OK-432 and IL-2 were also compared for the T-cell clones (Table 6.9). OK-432 and IL-2 could not induce cytolysis of K562 target cells by the clones CTL9 and CTL20 but IL-2 augmented the CTX of clone CTL11 against K562 target cells. Using BSM as target cells, both OK-432 and IL-2 augmented the CTX of clone CTL9 and CTL11 (Table 6.9). Unlike OK-432, IL-2 augmented the CTX of clone CTL20 against Daudi and APD stimulator cells.

TABLE 6.9

EFFECTS OF OK-432 AND IL-2 ON THE CTX OF $TCR^-/CD3^-$ NK CELL CLONES AND $TCR\alpha\beta^+/CD3^+$ T CELL CLONES

effector cells	target cells	percent lysis of target cells* exp. 1 OK-432		exp.2 IL-2	
		-	+	-	+
NK clones****					
NK76	K562	27	49**	4	41**
	Daudi	23	47**	1	24**
	P815-IgG	21	53	36	38
	P815	1	2	0	1
NK77	K562	12	39**	18	32**
	Daudi	12	39**	12	49**
	P815-IgG	32	42**	19	43**
	P815	0	2	0	27**
CTL clones*****					
CTL11	K562	31	19***	8	38**
	Daudi	1	1	0	0
	BSM	34	54**	26	62**
CTL9	K562	5	2	3	6
	Daudi	0	0	1	4
	BSM	38	49**	22	34**
CTL20	K562	9	7	13	18
	Daudi	23	26	20	43**
	APD	45	42	20	43**

* Results expressed as percent ^{51}Cr-release; effector:target ratio 9:1 (NK-cell clones against all target cells), 81:1 (CTL clones against K562 and Daudi target cells) and 3:1 (CTL clones against BSM and APD target cells).
** Significantly higher than control CTX ($p<0.05$)
*** Significantly lower than control CTX ($p<0.05$)
**** NK-cell clones were cultured without PHA
*****CTL clones were cultured with PHA

6.4 Discussion

BRM are defined as cell products that modify the hosts biological response. BRM can affect the immune system by activation, increase or restoration of reactivity of various immune effector mechanisms, or by inhibition or abrogation of cellular functions resulting in reduced immune responsiveness. Among the immunopotentiating BRM, OK-432 is known as an effective bacterial product for cancer therapy (99). In vitro stimulation of fresh PBL with OK-432 augments the MHC-unrestricted CTX (155,227). In this chapter we show that with fresh PBL obtained from various healthy donors the MHC-unrestricted CTX was augmented by OK-432 but its level is donor dependent (Table 6.3). Several donors were repeatedly tested and results for individual donors were reproducable (230).

We have cloned $TCR^-/CD3^-$ NK cells and $TCR\alpha\beta^+/CD3^+$ CTL (134,163,167) to see whether both cell types respond to OK-432 and IL-2. OK-432 augmented the MHC-unrestricted cytolysis of NK-cell clones as well as fresh PBL. However, we demonstrated that several requirements have to be met to allow for augmentation of cytolysis by OK-432. First, when cloned NK cells were grown in medium plus PHA they already showed increased lytic activity, which could not be further enhanced by OK-432. This may well depend on the fact that the action of PHA involves the production of IL-2 by the effector or feeder cells which in turn augments the CTX. These results are supported by the observation that in parallel experiments PHA cultures of NK-cell clones had a higher level of CTX than those cultured without PHA (Table 6.5). Secondly, CTX of only those NK-cell clones (NK76 and NK77) with a good proliferative capacity (Table 6.6) was augmented by OK-432. The other NK-cell clones studied, NK436, NK468 and NK472 do not proliferate without PHA. It has been reported that OK-432 does not enhance the ADCC (231). Interestingly, PBL and (less frequently) cloned NK cells exerted increased ADCC after stimulation with OK-432 but not with IL-2 (Table 6.8 and 6.9), even when 100 or 1000 U recombinant IL-2/ml was used (data not shown). In contrast to the idea that OK-432-augmented CTX might occur via IL-2 production, these results suggest that OK-432 stimulation of ADCC is IL-2 independent. In addition, if OK-432 augments the CTX via IL-2 production, expression of IL-2 receptors on fresh PBL should be required for action of both BRM. Surprisingly, using anti-TAC monoclonal antibodies (mAb) expression of IL-2 receptors was not observed on OK-432 and IL-2-stimulated PBL (Table 6.8). Stimulation of fresh PBL or cloned NK cells with IL-2 and OK-432 in the presence of anti-TAC mAb did not show the expected inhibition of NK-cell activity (data not shown). Recently, NK cells has been reported with

the IL-2R β-chain (56). Anti-TAC mAb only interacts with the IL-2R α-chain (see chapter 1, section 1.6). This may explain the above mentioned results i.e. the inability to detect IL-2 receptors and the inability to inhibit the IL-2-induced NK-cell activity using anti-TAC Mab. Preliminary results indicate that OK-432 binds to the IgG-Fc receptor, among other membrane structures. Thus, augmentation of the ADCC by OK-432, which involves the IgG-Fc receptor (34) suggests that these IgG-Fc receptors function as an activation site for OK-432 stimulation. We indeed showed that human IgG-Fc receptors can transduce activation signals for induction of CTX (27).

We also found that OK-432 can occasionally enhance the CTX of CTL ($CD3^+8^+$), i.e. OK-432-augmented CTX by the MHC-restricted $CD3^+8^+$ T-cell clones CTL11 and CTL9 using BSM target cells (MHC class I^+ and II^+). It is possible that these BRM enhance the MHC-specific component of CTX by CTL since neither the lysis by either MHC negative target cells nor target cells with MHC incompatible determinants were lysed (Table 6.9). Results obtained with $Cw3^+$ lymphocytes as target cells indeed showed IL-2-induced augmentation of the MHC-specific component (Cw3) of CTL9 (data not shown). The MHC class II-restricted cloned $CD3^+,4^+$ CTL20 cells were not activated by OK-432. As yet, too few clones have been tested to prove that the observed differences in the OK-432-induced lytic response by class I and II-restricted cytotoxic T-cell clones are real.

In conclusion, cloned $TCR^-/CD3^-$ NK cells as well as cloned $TCR\alpha\beta^+/CD3^+$ CTL can respond to OK-432 and IL-2. For both types of cells it appeared that not all clones responded positively to the BRM. With respect to NK cells, the proliferative capacity of these clones may play a role in their responsiveness to OK-432 and IL-2. The OK-432 unresponsiveness of some T-cell clones remains to be elucidated. With respect to its mechanism, our results indicate that OK-432 acts independently of IL-2.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

Since 1909 when the concept of immunosurveillance of tumor cells was proposed by Ehrlich (232) for the first time, many investigators focused on the isolation and identification of lymphoid effector cells expressing antitumor activity. The aim was to use these as immunotherapeutic tools. During the sixties and early seventies it became clear that cytotoxic T lymphocytes (CTL), natural killer (NK) cells and monocytes/macrophages, alone in sequence or in concert with other cell types exerted antitumor activity (1,2,233-235). During the late seventies cloning of lymphoid effector cells with antitumor reactivity (33,183-185) enabled the study of pure effector-target cell interactions, which revealed in the eighties information as to how the immune response could be manipulated. Now, at the end of the eighties it is clear that the cellular interactions and the involvement of numerous soluble factors in the immune response against tumors are more complex than anticipated from the earlier in vitro experiments. This thesis describes approaches how to potentiate lymphoid killer activities against tumors. First, target cell susceptibility to lysis and effector cell specificity has been studied using freshly isolated and cultured tumor cells as targets. Second, T-cell regulation in cancer patients has been investigated in vitro. Third, lytic activity of T-cell receptor negative (TCR^-)/$CD3^-$ NK and $TCR\alpha\beta^+/CD3^+$ T cells has been modulated by interleukin-2 (IL-2) and OK-432. Fourth, (cytotoxic) lymphocytes have been immortalized by somatic cell hybridization to sustain their function, and finally expression of proto-oncogenes and IL-2R in resting and activated normal and malignant T cells has been studied.

7.1 Cytotoxic lymphocytes with antitumor activity: target cell susceptibility and effector cell specificity

Following the appearance of tumor cells in a host, a cascade of immune reactions is initiated involving different types of immune cells culminating in the generation of cytotoxic lymphocytes that destroy the tumor. At first, $TCR\alpha\beta^+/CD3^+$ CTL were considered as the sole effector cell type able to lyse

target cells (1,2) but later also $TCR^-/CD3^-$ NK cells were found to kill cells (233-235). Recently, a third type of cytotoxic lymphocytes has been isolated, termed $TCR\gamma\delta^+/CD3^+$ lymphocytes. The latter share features with $TCR\alpha\beta^+/CD3^+$ lymphocytes as well as with $TCR^-/CD3^-$ NK lymphocytes. While $TCR\alpha\beta^+/CD3^+$ lymphocytes and $TCR^-/CD3^-$ NK cells are known to exert antitumor activity, it is still unclear whether $TCR\gamma\delta^+/CD3^+$ lymphocytes can also be involved in antitumor responses. Several authors have suggested that $TCR\gamma\delta^+/CD3^+$ cells are involved in the immune response against viral or bacterial infections (132,161,165). In chapter 2 of this thesis evidence is provided that each of these three lymphocyte populations can lyse cultured as well as freshly isolated tumor cells. Moreover, the efficacy of lysis by $TCR\gamma\delta^+/CD3^+$ clones was generally as high as that of $TCR^-/CD3^-$ NK lymphocytes. $TCR\gamma\delta^+/CD3^+$ clones were able to lyse target cells, in a MHC-unrestricted fashion.

From the results described in chapter 2 and from literature data (37,38,41,43) the following picture emerges. When $TCR\alpha\beta^+/CD3^+$ lymphocytes encounter tumor cells in vivo, the sequence of events include: (1) conjugation to the target cell; (2) T-cell activation; (3) triggering and delivery of a lethal hit; and (4) lysis of the tumor cell. After effector-target cell conjugation, the effector cell will be triggered via the $TCR\alpha\beta^+/CD3$ complex on the cell membrane (antigen recognition and signal transduction), which initiates production of IL-2 and expression of IL-2 receptors, and subsequent proliferation and/or lysis. Upon activation, and delivery of the lethal hit the CTL detaches from the tumor cells and subsequently may recycle. Activation of $TCR\alpha\beta^+/CD3^+$ lymphocytes only occurs when the tumor target cell expresses tumor-associated antigens (TAA) in the context of MHC determinants, i.e. MHC-restricted recognition and lysis. More or less the same mechanisms could be involved in killing by $TCR\gamma\delta^+/CD3^+$ lymphocytes, although $TCR\gamma\delta^+/CD3^+$ cells are able to recognise and lyse MHC^- as well as (allogeneic) MHC^+ tumor cells (236). In both cases, $TCR\gamma\delta^+/CD3^+$ lymphocytes need to be activated, for instance, by IL-2, to trigger the lytic machinery (136).

In contrast to $TCR\alpha\beta^+/CD3^+$ and $TCR\gamma\delta^+/CD3^+$ cells, $TCR^-/CD3^-$ NK cells do not require prior activation to lyse tumor cells (for a review, see 6). Although $TCR^-/CD3^-$ NK lymphocytes lyse MHC^- and MHC^+ target cells, MHC determinants appear not to be involved in the process of recognition (for a review, see 6).

7.2 Where does the immune system fail? The role of accessory cells

In cancer patients, immune mechanisms have failed to destroy the tumor cells. The question arises is why? Are, for example, the activation and subsequent proliferation steps inhibited, directly or indirectly, by the tumor cells, or are the "end point" lytic functions blocked. With respect to $TCR\alpha\beta^+/CD3^+$ cells, the possibility should be taken into account that TAA and/or MHC determinants are not functionally expressed on tumor cells resulting in immune unresponsiveness. Results of *in vitro* experiments presented in chapter 3 of this thesis indicate that tumor cells of different histologic type can be recognized by autologous lymphocytes. This can be interpreted from the augmented levels of $IL\text{-}2R^+$ lymphocytes among "fresh" PBL derived from cancer patients. Further studies revealed that $CD4^+$ lymphocytes (helper/inducer T cells, termed accessory cells) were preferentially activated by the tumor cells, i.e. an elevated proportion of CD4 lymphocytes expressed the IL-2R in the cancer patients. The $IL\text{-}2R^-$ T cell fraction mainly comprised $CD8^+$ lymphocytes. The fact that we were able to activate this $CD4^+$ lymphocytes by *in vitro* stimulation with autologous tumor cells supports the idea that these $CD4^+$ lymphocytes were activated in vivo by the tumor cells. Apparently, the tumor cells of the patients express "non-self" antigens which can be recognized by the $CD4^+$ T cells. Failures of the immune system to destroy the tumor cells may result from lack of signal transduction pathways in the $CD8^+$ T cells.

Results described in chapter 3 indicate that $CD8^+$ suppressor T cells were not activated by autologous tumor cells, neither *in vivo* nor *in vitro*.

7.3 Potentiation of cytolytic activity of cloned lymphocytes

Knowing which populations of cytotoxic lymphocytes can be lytic against tumor cells (chapter 2) and in view of the lack of an effective antitumor immune response *in vivo* (chapter 3), one strategy is to enhance existing cellular cytotoxicity (CTX) of lymphoid effector cells. In recent years considerable progress has been made towards the *in vitro* regulation of lymphocyte function using biological response modifiers (BRM) such as IL-2, interferon (IFN) and OK-432 (94-96,99-101,110). We have studied the responsiveness of lymphocytes to OK-432 and IL-2 using cloned $TCR^-/CD3^-$ NK cells and $TCR\alpha\beta^+/CD3^+$ CTL. The results obtained (chapter 5 of this thesis) show that CTX of both $TCR^-/CD3^-$ NK-cell clones and $TCR\alpha\beta^+/CD3^+$ T-cell clones

was enhanced by OK-432. Similar results were found using IL-2. For both cell types it appeared that not all clones responded to OK-432 and IL-2. When a clone was noncytolytic towards a certain target cell, no CTX could be induced against that target by OK-432 or IL-2. Several authors suggested that OK-432-induced CTX occurs via the production of IL-2 (229). We found that antibody-dependent cellular cytotoxicity (ADCC) was enhanced by OK-432 but not by IL-2 (chapter 6), which indicates that augmentation of CTX by OK-432 can occur independently from IL-2. Preliminary results suggest that OK-432 may bind to the IgG-Fc-receptor (CD16) (involving the ADCC), in this respect Fc-receptors may function as an activation site for OK-432. Indeed, we have shown that human CD16 can transduce activation signals resulting in enhanced CTX (27). As mentioned above, not all lymphocytes (clones) responded to BRM. Maximal effectiveness, with respect to immunotherapy of cancer patients, may be obtained when the effector cells responsive to BRM are separated from nonresponsive cells by selective cloning or bulk culture procedures, expanded in vitro and reinfused into the patient.

7.4 Immortalized cytotoxic lymphocytes as potential tools for immunotherapy

In order to have access to unlimited numbers of cytotoxic lymphocyte clones for studying human effector-target cell interactions and/or for therapeutic purposes, permanently available, i.e. immortalized lymphocytes are desirable. Hybridoma technology has been applied widely and has become the method of choice to immortalize lymphocyte functions (139-142). Until now, xenogeneic (mouse-man) as well as human-human hybridomas have been produced using polyethyleneglycol (PEG) as the fusion agent (145-152). However, human-human hybridomas are difficult to expand. Chromosomal instability has frequently been observed, leading to cell death (147). Kaufmann et al. (144) reported that lysis of the tumor cell partner by the lymphocyte during the PEG fusion procedure is a major obstacle in producing cytotoxic T-cell hybridomas.

We have demonstrated that the electric field-induced cell fusion technique, adapted for human T- and NK-cell fusions (chapter 4 of this thesis), is an efficient alternative for PEG induced cell fusion. The results obtained by electrofusion demonstrated that fusion efficiency depends on the characteristics of both fusion partners, i.e. the tumor cell and lymphocyte type. Microscopic observation learned that in an electrical field the tumor fusion partners were not lysed by the cytotoxic lymphocytes. However, in

contrast to the results of Kaufmann et al. (144), our data on PEG fusion do not suggest that there is a relationship between the presence of CTX of the T lymphocytes against the tumor fusion partner and the yield of hybridomas (chapter 4).

Although we were able to produce 600 human-mouse and human-human hybridomas by PEG and electrofusion, the CTX of the obtained hybridomas appeared to be transient. In this respect, chromosomal instability in human-human as well as in human-mouse hybridomas appeared to be the causative factor (see chapter 4 of this thesis). As reviewed in section 1.5 of chapter 1 the different steps of recognition, signal transduction and induction of CTX are all required for a successful target-cell lysis. Failure of only one of these steps will not allow the generation of lytic ability. The likely loss of chromosomes containing at least one or more genes involved in these steps explain our low success rate in the production of human cytotoxic lymphocyte hybridomas. However, our results show that PEG fusion, and in particular electrofusion, are efficient tools to produce human T- and NK-cell hybridomas. Unfortunately, somatic cell hybridization did not appear to be the most suitable tool for immortalizing the lytic functions. The transfer of genes involved in cell proliferation into cytotoxic lymphocytes appears to be more promising.

7.5 Genes involved in cell proliferation

Transfection of genes involved in cell proliferation could be an alternative strategy to immortalize human cytotoxic lymphocytes. Results from such experiments suggest that the transition from primary cells to fully transformed ones requires functional complementation between several gene involved in cell proliferation (220,237,238). Primary rodent cells were efficiently immortalized by transfection with cloned viral genes, e.g. v-Ha-ras plus v-myc, v-fos, polyoma large T-antigen gene and adenovirus, or by overexpressed cellular oncogenes such as c-Ha-ras and P53 (202-204,239-242). For immortalization of human cytotoxic lymphocytes myc and ras were considered suitable candidates. In addition, the genes encoding for IL-2R seemed useful in combination with oncogenes to immortalize human cytotoxic lymphocytes.

We studied the expression of c-myc and c-ras proto-oncogenes at the mRNA level in relation to IL-2R expression, using normal human resting and activated $TCR\alpha\beta^+/CD3^+$ T cells, NK cells and human T-lymphoma cells. C-myc was

found to be permanently expressed in human T-lymphoma cell lines but only transient in normal TCR$\alpha\beta^+$/CD3$^+$ T and TCR$^-$/CD3$^-$ NK cells after PHA-stimulation (chapter 5 of this thesis). Interestingly, the c-myc and c-ras mRNA levels were 10-fold higher in TCR$\alpha\beta^+$/CD3$^+$ T cells than in TCR$^-$/CD3$^-$ NK cells. In general, a positive correlation was found between proliferative capacity of the cytotoxic lymphocytes and c-myc oncogene mRNA levels. Stevenson and Volsky showed that in transfection experiments using human lymphocytes, the expression of v-myc was stabilized when cotransfected with the v-ras gene (220). We found a positive correlation between the levels of c-ras and c-myc mRNA, i.e. when c-myc mRNA levels were high, the c-ras mRNA levels were also high and vice versa. However, very recently it has been found that transfection of both genes on one vector in normal human lymphocytes is not sufficient for the induction of unlimited cell proliferation (220). These results suggest that still other genes are required for continuous cell proliferation. We (chapter 5 of this thesis) and others (243) have found that TCR$\alpha\beta^+$/CD3$^+$ T cells expressed a two-fold higher level of IL-2R than TCR$^-$/CD3$^-$ NK cells, which correlates with their proliferative capacity. Krönke et al. (244) found that c-myc mRNA levels can be augmented by addition of IL-2. Interestingly, T lymphoma cell lines permanently expressed c-myc in the absence of IL-2R (chapter 5 of this thesis). If IL-2 is required for the induction of c-myc expression in normal cells, T lymphoma cells, which apparently express c-myc permanently, would not need IL-2R.

Another recent report by Abken et al. (245) describes the immortalization of human lymphocytes by fusion of cytoplasts from mouse L929 cells with normal human lymphocytes using dextran as the transfection agent. This is the first report ever in which the successful immortalization of human lymphocytes is described. Abken et al. concluded that the immortalizing DNA was located on extrachromosomal DNA (but not on mitochondrial DNA) which frequently occurs in the cytoplasm of tumor cells. The immortalized lymphocytes contained about 10-fold more c-Ha-ras and c-myc mRNA (245), and 6-fold more P53 mRNA than normal cells. In the future, fusion of cytoplasts from immortalized cells, for instance from T-lymphoma cells, with cytotoxic lymphocytes may be an efficient way to immortalize cytotoxic lymphocytes. However, transfection of oncogenes (c-ras, c-myc and P53) combined with IL-2R genes may also be considered.

7.6 Recapitulation and conclusions

The purpose of the work described in this thesis was (1) to study the effector cell types involved in antitumor responses; (2) to investigate whether of the immune system in cancer patient may occur at tumor-target or at lymphocyte-effector cell level; and (3) to explore new strategies for producing tools for immunotherapy of cancer, i.e. enhancement of cytotoxic activity to optimal levels by BRM, and production of immortalized cytotoxic lymphocytes with stable lytic functions.
The major conclusions that can be drawn from the results obtained are the following:

1. From the three lymphoid (sub) populations studied i.e. $TCR^-/CD3^-$ NK, $TCR\alpha\beta^+/CD3^+$ and $TCR\gamma\delta^+/CD3^+$ CTL clones, $TCR\gamma\delta^+/CD3^+$, $TCR\alpha\beta^+/CD3^+$ CTL appeared to have the widest target cell spectrum and to exert the highest level of CTX against fresh tumor cells.

2. The failure of the immune system in the cancer patients we studied seems located at the level of the $CD8^+$ lymphocyte, possibly tumor cell induced. This may be due to a defective signal transduction mechanism.

3. Cytolytic activity of $TCR^-/CD3^-$ NK and $TCR\alpha\beta^+/CD3^+$ T-cell clones against tumor cells can be successfully enhanced, i.e. lymphocyte clones that were already active could be stimulated further by OK-432 and IL-2.

4. In an attempt to immortalize cytotoxic lymphocytes by somatic cell hybridization, an efficient electrofusion system was developed for the first time, allowing to produce T- and NK-cell hybridomas routinely. In spite of the large number of hybridomas generated, only a few were transiently cytolytic. This is most likely due to chromosomal instability of the hybridomas. As suggested by our studies and those of others, DNA-mediated transfer of multiple genes, for instance c-ras, c-myc, P53 and the IL-2R gene, involved in cell differentiation and proliferation may be an alternative strategy to immortalize cytotoxic lymphocytes.

SAMENVATTING

Dit proefschrift beschrijft het mobiliseren van cytotoxische lymfocyten met als einddoel immuuntherapie van kanker. Cytotoxische lymfocyten vormen de laatste schakel in een keten van cellulaire immuun systeem interakties. De meest voorkomende typen zijn $TCR\alpha\beta^+/CD3^+4^+$ cytotoxische T-lymfocyten (CTL), $TCR\alpha\beta^+/CD3^+8^+$ CTL, $TCR\gamma\delta^+/CD3^+$ CTL, en $TCR^-/CD3^-$ "natural killer" (NK) cellen.

Tegen het einde van de jaren zestig werd duidelijk dat $TCR\alpha\beta^+/CD3^+$ CTL en $TCR^-/CD3-$ NK cellen betrokken zijn bij de immuunrespons tegen tumoren. Echter, zoals beschreven in hoofdstuk 2 van dit proefschrift zijn ook $TCR\gamma\delta^+/CD3^+$ in staat gekweekte en zelfs "vers" geisoleerde tumorcellen te lyseren in vitro. De lytische capaciteit van gekloneerde $TCR\gamma\delta^+/CD3^+$ CTL bleek soms zelfs hoger te zijn dan die van gekloneerde $TCR^-/CD3^-$ NK cellen.

Het feit dat er ondanks de aanwezigheid van cytotoxische lymfocyten toch tumoren in het menselijk lichaam onstaan kan verschillende oorzaken hebben. Ergens in de keten van cellulaire interakties, die uiteindelijk de cytotoxische cellen tot aktiviteit aanzet kan een verstoring optreden, hetzij doordat één of meerdere cellulaire funkties geremd worden, hetzij doordat de vermeerdering van een bepaald celtype geremd wordt. Ook is het mogelijk dat de tumor niet in staat is een immuunrespons op te wekken omdat tumor-geassocieerde antigenen (TAA) niet herkend kunnen worden, bijvoorbeeld omdat ze niet in voldoende mate aanwezig zijn, of door de lage frequentie of afwezigheid van zogenaamde "major histocompatibility" (MHC) determinanten. MHC determinanten zijn een voorwaarde om de CTL te aktiveren. Tenslotte is het ook nog mogelijk dat strukturen voor de niet-antigeen specifieke cel-adhesie, de zogenaamde ligand, in onvoldoende mate of in gemuteerde vorm aanwezig zijn op de tumorcel waardoor effektor-doelwitcel interakties niet tot stand kunnen komen.

Verhoogde percentages van interleukine-2 (IL-2) receptor positieve (R^+) cellen in het perifere bloed van kanker patienten (11-29%) in vergelijking met gezonde donoren (0-10%) deed vermoeden dat de tumoren van deze patienten immunogeen zijn (Hoofdstuk 3). Uit de analyse van membraanantigenen met behulp van monoklonale antilichamen (mAb) bleek dat de $IL\text{-}2R^+$ cellen voornamelijk $CD4^+$ lymfocyten waren. De in vitro stimulatie van perifere bloed lymfocyten (PBL) van deze patienten met autologe tumorcellen, gaf ook preferente stimulatie van de $CD4^+$ lymfocyten.

In tegenstelling tot $CD4^+$ lymfocyten bleken $CD8^+$ lymfocyten niet te reageren op stimulatie met autologe tumorcellen; er werd geen toename van IL-2R gevonden op "verse" $CD8^+$ lymfocyten uit het bloed van kankerpatienten. Evenmin kon in vitro de lytische aktiviteit en proliferatie van deze $CD8^+$ lymfocyten geinduceerd worden met autologe tumorcellen.

Het onderzoek beschreven in Hoofdstuk 2 geeft aldus aanleiding tot de konklusie dat tumoren in vitro en waarschijnlijk ook in vivo IL-2R kunnen induceren op autologe $CD4^+$ lymfocyten. Het onvermogen van $CD8^+$ lymfocyten om te reageren op autologe tumorcellen in vivo en in vitro zou een verklaring kunnen zijn voor de afwezigheid van lytische aktiviteit van de T-cellen tegen de autologe tumor.

Eind 1970 werd het mogelijk effektor cellen te kloneren in vitro en ze te vermeerderen in speciaal ontwikkelde kweeksystemen. Hierbij worden de lymfocyten gestimuleerd met allo-antigenen, mitogenen en/of IL-2. Gekloneerde lymfoide effektor cellen werden door ons gebruikt om effektor-doelwitcel interakties te bestuderen.

Voor biochemische alswel celbiologische studies bleken de aantallen cellen verkregen met behulp van de daartoe bestemde expansiesystemen onvoldoende. In hoofdstuk 4 wordt de immortalisatie van cytotoxische lymfocyten door middel van somatische celfusie beschreven. Cytotoxische lymfocyten werden met behulp van polyethyleen glycol (PEG) gefuseerd met tumorcellen, met als doel de onbeperkte proliferatieve kapaciteit van de tumorcel over te dragen op de cytotoxische lymfocyt met behoud van diens lytische funktie. Zeven verschillende humane T-lymfoma cellijnen en twee verschillende muize myeloom cellijnen werden gebruikt. Somatische celfusie lag het meest voor de hand omdat deze techniek al sinds 1975 met succes gebruikt wordt voor de immortalisatie van B-lymfocyten ten behoeve van de produktie van mAb. Tijdens het onderzoek werd duidelijk dat immortalisatie van humane cytotoxische lymfocyten veel moeilijker is dan van muize B-lymfocyten. Proliferatie van mens-mens fusies werd pas 3-6 weken na fusie waargenomen, en het aantal hybridomas per fusie was laag (0-30%). In eerste instantie werd verondersteld dat de cytotoxische lymfocyten in staat waren de tumor fusiepartner tijdens het fusieproces te lyseren, zoals door Kaufmann (144) gesuggereerd werd. Op basis van onze resultaten met PEG fusie kunnen wij deze mogelijkheid nu uitsluiten (zie Hoofdstuk 4).

Teneinde het aantal hybridomas per fusie sterk te verhogen werd gebruik gemaakt van elektrofusie. Deze techniek, oorspronkelijk ontwikkeld door Zimmermann (153) ten behoeve van de fusie van plantecellen, is door ons gemodificeerd voor de produktie van humane cytotoxische hybridomas. Met het

door ons ontwikkelde protocol (referentie 151, 152 en Hoofdstuk 4) werden lymfocyt en tumorcel gefuseerd in een elektrisch veld. Het voordeel van deze methode boven de PEG fusie is dat het fusieproces onder de microscoop gevolgd kan worden. Microscopisch werd waargenomen dat de tumorcel tijdens het fusieproces niet gelyseerd wordt door de lymfocyt. Met beide fusiemethoden werden prolifererende hybridomas verkregen. Wanneer de T-lymfoma cellijn HSB-1 als fusiepartner werd gebruikt lag het aantal hybridomas verkregen met elektrofusie hoger dan met PEG fusie. Van de 500 mens-mens hybridomas verkregen met elektrofusie bleken er drie een lage cytotoxische aktiviteit tegen de oorspronkelijke stimulatorcel van de lymfocyt te bezitten, terwijl één van de meer dan 100 mens-muis hybridomas een hoge lytische aktiviteit had die na drie weken kweken volledig verdwenen was. Uit karyotyperingen van de hybridomas en fusiepartners bleek dat zowel mens-mens alsmede mens-muis fusieprodukten chromosomen verloren. Gekonkludeerd kon worden dat voornamelijk chromosomen van de lymfocyt partner werden uitgestoten omdat de hybridomas in bijna alle gevallen de eigenschappen van de tumorcel bezaten en niet die van de lymfocyt. In enkele gevallen echter werden membraanantigenen (CD3) aangetoond op mens-mens, en MHC determinanten op mens-muis fusieprodukten. Deze CD3 antigenen en MHC determinanten waren afkomstig van de T-lymfocyt. Van een aantal hybridomas kon het invasieve karakter van de T-lymfocyt-fusie partner aangetoond worden.

De konklusie is dat zowel PEG als elektrofusie uitstekende technieken zijn om humane T-cellen te immortaliseren. Door het verlies van één of meer chromosomen, waarop de genen liggen die coderen voor de eigenschappen die tesamen noodzakelijk zijn voor het lytische proces, blijkt celhybridisatie ongeschikt voor immortalisatie van lytische funkties.

Het injekteren van genen met proliferatieve eigenschappen zou een alternatieve strategie kunnen zijn om cytotoxische lymfocyten te immortaliseren. Daartoe is echter inzicht nodig in de genen die betrokken zijn bij de proliferatie van cytotoxische lymfocyten. Kandidaten hiervoor zijn bijvoorbeeld IL-2R c-myc en c-ras genen. In Hoofdstuk 5 is de expressie van c-myc en c-ras genen op mRNA niveau en IL-2R op eiwitniveau in gekloneerde $TCR\alpha\beta^+/CD3^+$ T - en $TCR^-/CD3^-$ NK cellen, alsmede in T-lymfoma cellijnen, voor en na aktivatie met (PHA, allo-antigenen of met IL-2) beschreven. Uit onze resultaten bleek dat het aantal c-myc mRNA produkten en IL-2R na aktivatie van de gekloneerde lymfocyten tijdelijk verhoogd was, terwijl in T-lymfoma cellijnen alleen c-myc mRNA gevonden werden en geen IL-2R.

Produktie van c-myc mRNA door stimulatie met IL-2 in normale lymfocyten, zoals beschreven door Reed en medewerkers (89), en het permanent

produceren van c-myc mRNA in T-lymfoma cellijnen suggereert dat de afwezigheid van IL-2R op T-lymfoma cellijnen het gevolg is van het niet meer biologisch funktioneel zijn van deze receptoren. Immers, c-myc mRNA wordt al permanent geproduceerd in deze cellen, dus de interaktie tussen IL-2 en diens receptor voor de induktie van c-myc mRNA produktie is overbodig. Behalve c-myc mRNA en IL-2R is ook de produktie van c-ras mRNA bestudeerd in gekloneerde TCR$\alpha\beta^+$/CD3$^+$ T en TCR$^-$/CD3$^-$ NK cellen. De produktie van c-ras mRNA bleek parallel te lopen met de produktie van c-myc mRNA. Deze resultaten komen overeen met de resultaten van Stern en medewerkers (80), die aangetoond hebben dat in humane PBL c-myc mRNA alleen geproduceerd wordt als c-ras mRNA ook geproduceerd wordt. Ook toonden zij aan dat de aanwezigheid van c-myc en c-ras mRNA in humane PBL niet voldoende was om immortalisatie tot stand te brengen. Op grond van onze resultaten en recente literatuurgegevens lijkt de kombinatie van myc, ras en IL-2R genen op één vector zeer geschikt voor immortalisatie van cytotoxische lymfocyten. Onlangs heeft Abken (245) muize cytoplasten met sukses gefuseerd met humane lymfocyten. In deze geimmortaliseerde lymfocyten was de produktie van c-ras en c-myc alswel P53 mRNA verhoogd, wellicht als gevolg van overgedragen extra chromosomale genetische faktoren. Ook deze faktoren zouden geschikt kunnen zijn voor immortalisatie.

Manipulatie van specifieke komponenten van het immuunsysteem met "biological response modifiers" (BRM) biedt ook goede mogelijkheden voor immuuntherapie. In hoofdstuk 6 is de invloed van BRM (OK-432 en IL-2) op de lytische kapaciteit van gekloneerde TCR$\alpha\beta^+$/CD3$^+$ CTL en TCR$^-$/CD3$^-$ NK cellen beschreven. Beide typen cellen bleken in staat op OK-432 en IL-2 te reageren; verhoging van de lytische kapaciteit werd verkregen na inkubatie van de lymfocyten met OK-432 of IL-2. Eerder is beschreven dat alleen TCR$^-$/CD3$^-$ NK cellen gevoelig zijn voor OK-432. Onze resultaten tonen aan dat ook TCR$\alpha\beta^+$/CD3$^+$ CTL gestimuleerd kunnen worden met BRM. De resultaten beschreven in Hoofdstuk 6 tonen aan dat ook de antilichaam-afhankelijke cytotoxiciteit van gecloneerde NK cellen en verse PBL (bevat NK cellen) verhoogd kan worden met OK-432, maar niet met IL-2. Door verschillende auteurs is gesuggereerd dat OK-432 werkzaam is via IL-2 produktie. Onze resultaten suggereren echter, dat OK-432 onafhankelijk van IL-2 werkt.

De resultaten beschreven in dit proefschrift geven inzicht in de verschillende typen cytotoxische lymfocyten die in staat zijn tumorcellen te lyseren. Deze gegevens, alsmede de gevonden aanwijzingen dat de tumoren mogelijk immunogeen kunnen zijn in vivo, verschaffen een basis voor immuuntherapie. Geimmortaliseerde cytotoxische lymfocyten kunnen daarbij van

essentieel belang zijn. Somatische celhybridisatie blijkt echter ongeschikt als methode om immortalisatie te verkrijgen. De overdracht van genen betrokken bij de proliferatie van cytotoxische lymfocyten, zoals de genen die koderen voor IL-2R c-myc, c-ras en P53, biedt waarschijnlijk betere perspektieven. Een alternatief voor het mobiliseren van grote aantallen geimmortaliseerde cytotoxische lymfocyten met anti-tumor aktiviteit is het gebruik van geselekteerde (gekloneerde) cytotoxische lymfocyten gestimuleerd met BRM zoals IL-2 en OK-432, die in staat zijn de lytische kapaciteit van $TCR\alpha\beta^+/CD3^+$ CTL en $TCR^-/CD3^-$ NK cellen te verhogen. Deze gekloneerde $TCR\alpha\beta^+/CD3^+$ CTL en $TCR^-/CD3^-$ NK met verhoogde lytische kapaciteit tegen tumor cellen zouden gebruikt kunnen worden voor immunotherapie.

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ABBREVIATIONS

ADCC	antibody-dependent cellular cytoxicity
AH	azaserine hypoxanthine
AK	activated killer
B-LCL	lymphoblastoid B-cell line
BRM	biological response modifiers
C	constant
CD	cluster differentiation
CHN	channel
CTL	cytotoxic T lymphocytes
CTX	cellular cytotoxicity
D	diversity
EBV	Epstein-Barr virus
EGF	epidermal growth factor
FCS	fetal calf serum
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HAT	hypoxanthine aminopterin thymidine
HD	healthy donor
HLA	human leucocyte antigen
HTLV	human T-leukemia virus
IFN	interferon
II	infiltration index
IL-2	interleukin-2
J	joining
K	klinische einheiten
LAK	lymphocyte-activated-killing
LDCC	lectin-dependent cellular cytoxocicity
LFA	lymphocye function-associated antigen
mAb	monoclonal antibody
MAF	macrophage activating factor
MCSF	macrophage colony-stimulating factor
MLC	mixed lymphocyte culture
NK	natural killer
PBL	peripheral blood lymphocytes
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PHA	phytohaemagglutinin
R	receptor
SCID	severe combined immunodeficiency disease
SDS	sodium dodecyl sulfate
SRBC	sheep red blood cells
TAA	tumor-associated antigens
TCGF	T-cell growth factor
TCR	T-cell receptor
TRF	transferrin receptor
TSTA	tumor-specific transplantation antigens
V	variable

CURRICULUM VITAE

De auteur van dit proefschrift werd op 30 juni 1954 geboren te Rotterdam. In 1966 werd een aanvang gemaakt met de huishoudschool, welke opleiding werd afgerond in 1969. Na het behalen van het diploma inrichtingsassistente (INAS) in 1971, werd een aanvang gemaakt met de studie HBO medische microbiologie die in 1975 werd afgesloten met het behalen van het diploma HBO-A. Na 2 jaar te hebben gewerkt als ziekenhuishygienist en als analist op het bacteriologisch laboratorium van het Diaconessenziekenhuis te Voorburg, werd in 1977 aangevangen met de studie biologie aan de rijksuniversiteit te Leiden (op basis van een colloquium doctum). Het kandidaatsexamen werd afgelegd in 1980 en het doctoraalexamen in 1982. In september 1982 werd een tijdelijk dienstverband aangegaan met de Dr. Daniel Den Hoed Kliniek, op basis van financiering door het Koningin Wilhelmina Fonds, waar onder leiding van Dr. R.L.H. Bolhuis en Prof. Dr. D.W. van Bekkum (Radiobiologisch Instituut, TNO) het in dit proefschrift beschreven onderzoek verricht werd. In augustus 1987 werd een dienstverband aangegaan met het Koninklijk Instituut voor de Tropen te Amsterdam, als wetenschappelijk medewerkster voor onderzoek aan en diagnostiek van leptospirosen.