THE MANIPULATION OF CELLULAR IMMUNITY BY MONOCLONAL
ANTIBODIES IN CANCER PATIENTS

by

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The work on which this dissertation is based is my
own independent work except where acknowledged.

James Hollingworth
January 1994

To Eve and my parents whose constant love and support
enabled the completion of this work.

"It is a capital mistake to theorize before one has data."

The Memoirs of Sherlock Holmes. The Crooked Man.
Sir Arthur Conan Doyle 1859 - 1930

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ABBREVIATIONS

Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
BRM	Biological Response Modifier
CD	Cluster Determinant
CEA	Carcinoembryonic antigen
CMC	Cell-mediated cytotoxicity
CPM	Counts per minute
^{51}Cr	Chromium-51 sodium chromate
CSF	Colony Stimulating Factor
CTL	Cytotoxic T lymphocyte
EDTA	Ethylene diamino-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent-assay
ELAM	Endothelial lymphocyte adhesion molecule
Fab	Fraction antibody binding
Fc	Constant ('crystalizable') fragment
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate
HBSS	Hanks balanced salts solution
HLA	Human leucocyte antigen
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
LAK	Lymphokine activated killer
LFA	Leucocyte function antigen
LU	Lytic unit
mAb	Monoclonal antibody
MBq	Mega Bequerel
MHC	Major histocompatability complex
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule
$T_{c/s}$	Cytotoxic/suppressor T lymphocyte
TcR	T cell receptor complex
T_h	Helper T lymphocyte
TIL	Tumour infiltrating lymphocytes
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule

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SYNOPSIS

The role of an immune response in the development and regulation of tumour growth is outlined. The historical development of immunotherapy in the treatment of human cancer is reviewed and the mechanisms by which immunity may be manipulated *in vitro* and *in vivo* with respect to improving cancer immunotherapy are discussed. The experimental studies and laboratory methods finally used are presented and validated in chapter 3. Non-major histocompatibility-restricted cellular cytotoxicity was assessed in a standard 4-hour [51]chromium-release assay and peripheral blood cell populations were examined using fluoresceine-conjugated monoclonal antibodies and flow cytometry.

Initially *in vitro* studies of cellular cytotoxicity against cultured tumour cells were undertaken using peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers. Extremely variable levels of cellular cytotoxicity were documented in these healthy donors which were unrelated to age or sex but were positively correlated with numbers of circulating natural killer (NK) cells. The effect on cytotoxicity, of preincubating PBMC with monoclonal antibodies (mAb) and interleukin 2 (IL-2) was studied. Various mAb including those to the CD3 antigen were found to enhance cellular cytotoxicity *in vitro*. The mechanism of enhancement remained unproven. However, preliminary studies suggested that mAb redirected effector cells by interacting with Fc receptors on the surface of tumour cells. Combinations of mAb had a partially additive effect on enhancement of cytotoxicity. IL-2 also enhanced cytotoxicity in a dose dependent manner although synergy between IL-2 and anti-CD3 was not demonstrated.

PBMC were then studied from patients with advanced gastrointestinal tract cancer. Levels of cellular cytotoxicity were not significantly related to tumour extent and were similar to cytotoxicity in healthy donors. Cytotoxicity was mediated mainly by NK cells although lymphocytes coexpressing the CD3 antigen and NK surface antigens were significantly increased in cancer patients compared with healthy donors and also in patients with liver metastases compared to those without liver involvement. In cancer patients with liver metastases, lymphocytes coexpressing the CD3 antigen and NK antigens may play a more significant role in K562 cytotoxicity. Cytotoxicity was also enhanced *in vitro* by anti-CD3 mAb and Il-2.

A clinical study was undertaken to investigate the safety and the immunomodulating properties of administering between 50 μg and 0.5 μg of OKT3 or normal saline to patients with advanced cancer. Patients experienced minimal and self-limiting dose-related side effects. Only one patient developed evidence of cytokine release following OKT3 when assessed by enzyme-linked immunosorbent assays. Depletion of circulating T cells and NK cells occurred following OKT3 which was proportional to the dose administered. Lymphocyte activation assessed by interleukin 2 receptor expression was not detected. Considerable individual variation in cytotoxicity was related to variation in NK cell numbers in blood in both OKT3 and normal saline treated patients. 50 μg OKT3 was associated with a rapid decline in both circulating NK

cells and cytotoxicity at 4 hours compared with lower doses of OKT3. The lower doses of OKT3 had a variable effect on cytotoxicity at 24 hours. 20 μg and 5 μg OKT3 was associated with a significant rise in cellular cytotoxicity at 24 hours compared with untreated controls and the 10 μg dose.

These results demonstrate that anti-CD3 mAb enhance cellular cytotoxicity *in vitro*. The effect of OKT3 on lymphocyte function *in vivo* is dose-related and variable. Low doses of OKT3 may either enhance or depress cellular immunity depending upon the precise dose and time of study following administration. The relationship between the *in vitro* effects of anti-CD3 mAb on cellular cytotoxicity and their effects *in vivo* are uncertain. Although low doses of OKT3 may be safely administered to cancer patients further studies are needed to define its potential in human cancer immunotherapy.

CHAPTER ONE

THE IMMUNE RESPONSE IN THE ELIMINATION OF CANCER CELLS

1.1 INTRODUCTION.

The stages involved in the development of an effective immune response against a foreign material are similar whatever the nature of the initiating agent although, the relative components of the response may vary. The concept of an immune response against cancer cells poses several unique problems. For example, potentially malignant cells usually arise in the environment of an apparently healthy immune system. It must therefore be envisaged that although some premalignant cells may be eliminated, other cancer cells escape detection and multiply at an early stage in their development. At a more advanced stage tumour-related suppression of any subsequent immune response may then contribute to further tumour growth. Cell-mediated immunity is presently recognized to be of primary importance in the rejection of established solid tumours (North, 1984; Robins and Baldwin, 1985). Immunotherapy is an attempt to enhance immunity *in vivo* which is usually directed at enhancing the ability of immune cells to directly kill tumour cells. A broad understanding of the immune system is particularly important in the development of effective cancer immunotherapy regimens. It necessitates an understanding of how transformed and normal cells differ from one another and the types of immune cells involved in their elimination.

1.2 TUMOUR ANTIGENS.

1.2.1 Tumour Specific Transplantation Antigens (TSTA).

The concept of an immune response against cancer cells depends upon the variation in expression of antigens expressed by malignant cells compared with non-malignant cells. The existence of tumour antigens was first proposed by Ehrlich in 1909. When inbred strains of mice, immunized with a syngeneic chemically induced tumour, rejected a graft of the immunizing tumour the term tumour-specific transplantation antigen (TSTA) was introduced to describe the antigens responsible for rejection (Klein et al. 1960). These specific antigens provided effector cells of the immune system with targets through which elimination of malignant cells may occur. TSTA have been discovered in rodents, in many different chemically induced sarcomas and carcinomas, in tumours caused by oncogenic viruses, and in tumours following ultraviolet radiation (Kripke, 1989). Chemically induced TSTA are individually unique even when tumours have the same histological appearance and are induced by the same carcinogen. Tumours expressing strong TSTA have generally been induced by large doses of carcinogen, whereas low doses produce tumours with low antigenicity. Tumours induced by ultraviolet (UV) irradiation are often so immunogenic that they cannot grow when transplanted, unless the recipient has been immunosuppressed.

The nature of the individually unique TSTAs is unknown although a gene mutation probably gives rise to individually distinct antigens. TSTA identified in murine tumours induced by different chemicals, have been found to belong to a family of glycoprotein molecules (gp96) with a molecular weight of approximately 96kD which

have a human homologue in melanoma (Srivastava and Old, 1989). In contrast to chemically induced tumours most rodent tumours induced by oncogenic viruses express TSTA which are specific for the infecting virus. A causative association between certain viruses and human malignancies has also been suggested (Zur Hausen, 1987). Unfortunately with the exception of Burkitts' lymphoma (Kallin et al, 1986) and cervical carcinoma (DePalo et al, 1985) most human tumours do not have an obvious viral association nor do they express TSTA. Virally encoded TSTA do however, provide useful models for investigating the immune response to tumours *in vitro* and in murine models.

1.2.2 Tumour-Associated Differentiation Antigens (TADA).

While TSTA are specifically expressed by transformed cells, most tumours express antigens which are also found on normal cells and are termed tumour-associated differentiation antigens (TADA). These have relative rather than absolute specificity for cancer cells. They are sometimes known as oncofoetal antigens because they are often present in high levels in some foetal tissues (Goggin, 1986). By immunizing animals with human tumours Abelev et al (1963) demonstrated that human hepatomas express TADA known as alphafetoproteins (AFP) which they share with foetal liver cells. Gold and Friedman (1965) similarly discovered that colon cancers express "carcinoembryonic" antigen (CEA) which is abundant in foetal colon but almost completely absent from adult normal human tissues. The role of these molecules is uncertain. CEA is possibly thought to function as a calcium independent intercellular adhesion molecule which may be involved in tumour progression and metastasis (Benchimol et al. 1989). Because these molecules are shed into the serum they are useful as markers of disease progression and recurrence (Rieger and Wahren, 1975).

Over 40 different antigens have been defined in melanoma, one of which, p97 is related to transferrin and is an important growth factor (Brown et al, 1982). TADA may also be important in tumour elimination. Tumour infiltrating lymphocytes (TIL) have recently been reported to recognize TADA in the context of multiple HLA-A, -B, and -C molecules (Hom et al, 1991). Three different TADA which are recognized by cytotoxic T cell clones have also been detected on ovarian carcinoma cells obtained from ascites of a patient with ovarian carcinoma (Ionnides et al, 1991). The use of unmodified mAb directed at TADA for both the therapy and diagnosis of colorectal cancer has been extensively investigated and tumour responses have been occassionally seen (Lange and Martin, 1991; Mellstedt et al, 1991).

1.2.3 Proto-oncogenes.

Because the underlying defect in cancer is genetic a number of oncogenes have been identified. Proto-oncogenes generate products such as an altered c-abl gene product made by Ph1 chromosome-positive cells from chronic myeloid leukemia (Mes-Masson

and Witte, 1987) which may be important in human tumourigenesis. Other examples include GTP-binding proteins collectively referred to as p21 which are produced by the c-ras genes (Downward et al. 1992) and include a mutated p21 protein which has been observed in a variety of human tumours (Slamon et al. 1984). In certain situations oncogene products appear to be qualitatively different in cancer cells and normal cells and because they are often directly related to cell proliferation by being growth factors or growth factor receptors (Chan and Sikora, 1987; Chan and McGee, 1987) provide an attractive therapeutic target. However, their importance remains unproven. In a colon carcinoma study, CEA was reported to be present more frequently than either p21 or its mutated forms (McKenzie et al, 1987).

1.3   IMMUNOSURVEILLANCE AGAINST CANCER.

With the concept of tumour antigens Ehrlich also proposed the concept of immune surveillance. Good and Finstad (1969) and Burnet (1970) further developed the concept that an immune mechanism of the allograft type is the primary line of defence against neoplasia. The theory of immune surveillance proposes that potentially malignant cells are recognized by the immune system which destroys them. Reports of occasional spontaneous tumour regressions and attempts to correlate tumour infiltration by effector cells with a favourable prognosis (Svennevig et al, 1984) have often been explained in the context of a host immunological response. Congenital deficiency of cell-mediated immunity is associated with an increased incidence of certain malignancies. Patients with ataxia telangiectasia, the Wiskott-Aldrich syndrome, and Chediak-Higashi syndrome, all of which have a long natural history and deficiency in immunological function are predisposed to lymphoreticular neoplasia notably Non-Hodgkins Lymphoma (Roder et al, 1980; Kersey et al, 1987).

Organ transplant recipients receive immunosuppressive therapy for extended periods and overall there is a more than threefold increase in the incidence of malignancy which increases with the length of follow-up after transplantation (Starzl et al, 1985; Penn, 1988). Families with an increased incidence of cancer have been reported to have low levels of NK activity (Strayer et al, 1986). Most cancer chemotherapeutic agents have immunosuppressive side effects resulting in depression of both humoral and cell-mediated immune functions (Penn, 1982; Penn, 1988). The risk of second malignancies following chemotherapy assumes particular importance in individuals who have potentially long survival times such as children. The pattern of tumours arising in immunosuppressed patients is very different to that seen in the general population and tumours of the lymphoid system predominate. In the Aquired Immunodeficiency syndrome (AIDS) the most common malignancy is Kaposi's sarcoma, the risk of which is 100 times greater than in the general population (Report of the Centers for disease Control Task Force on Kaposi's Sarcoma and Opportunistic Infections, 1982).

Despite the large body of evidence in favour of an immunosurveillance theory it remains a controversial concept. The effectors involved in immunosurveillance also remain poorly defined. Trinchieri (1989) has emphasized the potential importance of NK cells with more general cytotoxic properties, compared with antigen-specific T-cell immune responses, in the elimination of tumour cells arising de novo. This concept is supported by evidence suggesting that cells which fail to express MHC class I antigens are more susceptible to NK cell lysis (Ljunggren and Karre, 1990; Storkus et al, 1991, Blottierre et al 1992). Alternatively, T cells providing specific immunity, may be important in the elimination of certain types of tumours which are more antigenic.

In summary there is evidence to suggest that an immune response can be generated against neoplastic cells during their development and subsequent proliferation.

1.4   MECHANISMS BY WHICH TUMOURS ESCAPE IMMUNE REJECTION.

Cancer cells may escape immune rejection by avoiding recognition or alternatively by suppressing the immune response. Although most experimental and clinical tumours are apparently immunogenic, their antigens are relatively weak and this may result in a failure by the immune system to recognise neoplastic cells. Examination of the cells within a tumour mass often reveals a heterogeneity of antigenic expression of both TSTA and TADA (Topalian et al. 1990). A form of natural selection of cells from the heterogenous population "struggling for survival" in the tumour mass probably occurs (Nowell, 1976). Variation in expression of major histocompatibility complex (MHC) antigens also affects immune responses by NK cells and cytotoxic T cells (CTL). MHC antigen expression inhibits effectors mediating non-specific cellular cytotoxicity (De Fries and Golub, 1988; Carbone et al, 1991) while selective changes in MHC expression influences CTL responses directed against melanoma *in vivo* (Goepel et al, 1991). It has been reported that 30% of gastrointestinal cancers do not express MHC class I antigens (Durrant et al, 1987) and effector cells mediating non-MHC-restricted killing may assume greater importance in eliminating these tumours.

Tumours may also possess antigens referred to as 'emergence associated tumour immunogen' (EATI) (Manson 1991) which are postulated as generating a host antibody response which impairs the ability of immune cells to recognize tumour cells. It has been postulated that the early antibody response to EATI masks tumour cell epitopes recognized by cytotoxic effector cells allowing progressive tumour growth (Manson, 1991). IgM antibodies to EATI have been shown to accumulate on experimental tumour cells in mice within the first two weeks after inoculation. Immunization of these mice with eluted antibody to induce an anti-EATI antibody response prior to tumour injection, resulted in rejection of a previously resistant tumour.

Tumour induced suppression of immunity has been noted. Patients with advanced gastrointestinal tract cancer have impaired NK cell activity as well as a decrease

in ADCC mediated by both monocytes and lymphocytes in PBMC preparations (Blottiere et al, 1990). Lymphokine activated killer (LAK) cell generation is impaired in patients with advanced gastrointestinal cancer (Monson et al. 1987). Poorly characterized substances which impair lymphocyte functions have also been derived from both tumour cell lines (Guillou et al, 1989; Ebert et al, 1990) and tumour bearing patients' serum (Bugis et al, 1990; Marubayashi et al, 1991). Circulating tumour derived antigen may also be absorbed to the surface of cytotoxic lymphocytes and inhibit their capacity to interact with the target cell or, generate suppressor cells capable of preventing the induction of an effective CTL response (Takahashi et al, 1988). Also the generation of both non-specific and tumour-specific suppressor T cells has been described (Hoon et al, 1987). Cozzolino et al, (1987) have demonstrated a population of mononuclear cells with suppressor activity in tumour infiltrated lymph nodes. Monocytes are also capable of *in vitro* suppression of effector cell activation, possibly mediated through prostaglandin release (Eisenthal, 1990).

Both escape mechanisms and host immunosuppression therefore operate *in vivo* and either may predominate depending on the stage of tumour development. A major hurdle of cancer immunotherapy is to overcome an already impaired immune system and to enhance the capacity of effector cells to recognize cancer cells.

1.5   THE MECHANISM OF CELLULAR IMMUNE RESPONSES AGAINST CANCER.

1.5.1   Introduction.

Investigation of the mechanisms of cellular immunity to a tumour requires an understanding of the types of effector cells present *in vivo*, their level of activation, and their ability to recognise the type of cancer cells being studied. It is important to consider the *in vivo* situation in humans particularly at tumour level, when interpreting results of animal and *in vitro* studies. Variation in circulating blood cells may not be indicative of events within the tumour. Cytotoxic cells have two requirements; the expression of receptors capable of triggering activation, and the ability to produce and release the effector molecules that mediate cytotoxicity following ligand binding. Lymphocyte-induced cytolysis is a complex multistep process which has been divided into a number of stages (Martz, 1975; Mentzer et al, 1987). These are;

1. Conjugation and recognition
2. Progamming for lysis ('lethal hit')
3. Effector cell-independent stage   - perforin mediated
                                        - apoptosis

Although the recognition structures used by lymphocytes may be different, their mechanisms resulting in cell death are very similar. The initial adhesion step is the rapid formation of an intercellular bond which may not involve a specific antigen interaction (Spits et al, 1986). The conjugate between the effector cell and the target cell occurs within 10 minutes at 37°C (Radcliff et al, 1991). It is nevertheless a complex reaction between adhesion molecules present on the effector and target cell (Springer et al, 1987; Springer, 1990). In the case of T cells this is followed by specific binding of the TcR-CD3 complex to the antigen on the target cell which then appears to stabilize the conjugate (Sung et al, 1986). Lymphocytes can induce target cell lysis within 3 - 10 minutes after initial contact and can then be detached or inactivated after this time without preventing the lysis of the target cells. Lymphocytes are not self annihilating and recycle after killing their targets, the rate of tumour cell destruction then depends upon the number of effector-tumour cell interactions (Berke et al, 1972).

The cytolytic activities of effector cells are most commonly assessed using cytotoxicity assays based on that described by Brunner et al. (1968) which measure radioactive (chromium-51) isotope release from tumour cells *in vitro*. This occurs some 30-90 minutes following cell death and is indicative of target cell membrane disruption.

1.5.2 Definition of Cell Phenotype.

Different populations of effector cells may be defined by their variable expression of cell surface antigens. Advances in computer and monoclonal antibody technology have enabled a precise identification of the distribution of molecules expressed on peripheral blood cell membranes. These surface molecules contain segments of amino acid sequences that have similarities with other cell surface proteins and they are probably derived by divergent evolution from common precursors. Dayhoff et al. (1983) introduced the terms "superfamily" for proteins that have 50% sequence similarity or less and "family" for those with more than 50% identity. This is an artificial classification which does not equate with function. Flow cytometry using fluorescein-conjugated monoclonal antibodies has become the basis for grouping these surface molecules into "Clusters of Differentiation" or CD antigens (Knapp et al, 1989a and 1989b). MAb that give the same patterns of labelling on different cell types are considered to label the same antigen and the naming of antigens has been formalized in workshops on human leucocytes (Knapp et al, 1989a). Definition of cell phenotype is most commonly undertaken using fluorochrome-conjugated mAbs and a flow cytometer.

The CD designation therefore defines the antibody cluster. However, it is more frequently used to describe the molecule or antigen defined by the antibody when the term 'CD molecule' or 'CD antigen' should be used. This systematic approach has been of great benefit since the CD names have allowed a common nomenclature for both human antigens and homologous antigens of other species. Using this method of analysis and classification it is now likely that most of the major surface molecules on T lymphocytes

are known (Barclay et al, 1993). This approach has also enabled the investigation of lymphocyte subpopulations which have been associated with specific immune functions. However, to avoid inconsistencies in functional studies employing mAb, the precise antibody should be stated.

Alternatively lymphocytes may be classified according to function, for example into those exhibiting MHC-restricted or non-MHC-restricted cytotoxicity (Reynolds and Ortaldo, 1987). The identification of distinct effector cells, classified by surface phenotype has been extensively used to report the behaviour of different lymphocyte populations which mediate non-MHC-restricted cytotoxicity (Lanier and Phillips, 1986; Lanier et al, 1986a and 1986b). By combining these classifications three types of cytotoxic lymphocytes have been defined (Lanier and Phillips, 1986); (1) Antigen-specific, MHC-restricted lymphocytes recognizing targets via a T cell receptor (TcR) complex. (2) Non-MHC-restricted lymphocytes which do not express a TcR but recognize targets via poorly defined receptors (NK cells), and (3) lymphocytes recognizing targets without MHC restriction but also expressing a TcR complex which is not involved directly in target cell recognition.

The four major sources of effector cells with potential antitumour activity are CTL, NK cells, activated macrophages, and activated polymorphonuclear neutrophils (PMN). Their major surface antigens and functional properties are shown in Table 1.1.

1.6   CYTOTOLYTIC PERIPHERAL BLOOD CELLS.
1.6.1   T Lymphocytes.

T lymphocytes show the classic hallmarks of immune responses, namely, antigen recognition, MHC-dependent interaction between antigen presenting cells (APC) and T cells, and a memory response. Two subsets of mature T cells can be identified based on expression of CD4 and CD8 markers which differ from one another in important functional parameters (Bierer et al, 1989). $CD4^+$ T cells are defined as expressing a 'helper' phenotype and assist with B-cell antibody secretion and responses of other T cell populations. They are usually required for the optimal induction of CTL responses, although, they may exert tumour-specific effector function by themselves. $CD8^+$ T cells are cytotoxic or are capable of suppressing the immune response of other lymphoid populations (Parnes, 1989). These subsets are referred to as T-helper (T_h) cells and T-cytotoxic/suppressor cells $(T_{c/s})$ respectively. Cytotoxic T lymphocytes (CTL) represent one of the most extensively studied and characterized cell populations in peripheral blood.

 Work on murine lymphocytes (Siegelman et al, 1989) and more recently on human lymphocyte clones (Berg et al. 1991) has led to the view that there are two major types of T_h cells producing a different spectra of cytokines. T_h^1 cells produce predominantly Interleukin 2 (IL-2), tumour necrosis factor β (TNF-β) and interferon γ (IFN-γ), while T_h^2 secrete mainly IL-4, 5, 6 and 10. Both types produce IL-3, IL-8, TNF-α, granulocyte macrophage-colony stimulating factor (GM-CSF) and other

Table 1.1 - Approximate CD antigen expression and functional characteristics of
peripheral blood cytolytic effectors

Antigen	NK Cells	T Cells	Monocytes	Neutrophils
CD2	70 - 90	> 95	< 5	< 5
CD3	0	> 95	0	0
CD4	< 5	45 - 60	< 5	< 5
CD8	30 - 40	30 - 40	< 5	< 5
CD15	< 5	< 5	> 90	> 95
CD16	80 - 90	10 - 15	> 90	> 90
CD16	80 - 90	< 5	60 - 80	> 95
CD56	> 95	< 5	10 - 15	< 5
Function				
ADCC	Yes	No	Yes	Yes
NK activity	Yes	No	No	No
Phagocytosis	No	No	Yes	Yes
Memory	No	Yes	No	No
Proliferation	Yes	Yes	No	No

From Robertson and Ritz (1990).

cytokines. Different isoforms of CD45 molecules (leucocyte common antigen) are found on all cells of haemopoeitic origin except erythrocytes. In humans two major subsets of both CD4 and CD8 lymphocytes can be detected with antibodies to the CD45RA and RO isoforms (Kasahara et al, 1990; Wallace and Beverley (1990). The activation requirements as well as the state of activation of these two subsets probably differ. Under conventional conditions of *in vitro* culture only CD45RO CD4 or CD8 cells show significant responses to recall antigens. This has led to the use of the terms naive and memory for CD45RA and RO cells respectively. It is presently unclear how T_h^1 and T_h^2 effector types relate to each other and to naive or memory populations of lymphocytes freshly isolated from animals or man.

All T cells mediating MHC-restricted cytotoxicity express an antigen recognition site known as the T cell receptor (TcR) consisting of $\alpha\beta$ or $\gamma\delta$ heterodimers in association with a nonpolymorphic group of polypeptides known as the CD3 complex (Kronenberg et al, 1986; Allison and Lanier, 1987; Brenner et al. 1988; Clevers et al, 1988). All available evidence suggests that the structures of both the $\alpha\beta$ TCR-CD3 complex and the $\gamma\delta$ TcR-CD3 complex are similar (Neerven et al, 1990). In keeping with most cell surface expressed receptors, the TcR-CD3 complex is a multichain structure composed of molecules that span the lipid bilayer. The CD3 complex comprises at least 4 invariant polypeptides non-covalently associated with each heterodimer and designated γ, δ, ϵ, and ζ (Weiss, 1989; de la Hera et al, 1991; Wunderlich and Hodes, 1991). Most anti-human CD3 antibodies available react with CD3ϵ (Transy et al, 1989; Tunnacliffe et al, 1989).

In general cell surface expression of the TcR-CD3 complex takes place only if all subunits are present. Most chains are synthesized in excess intracellularly and only a small percentage of the TcR and CD3 chains reach the cell surface (Koning et al, 1988). The δ, γ, and ϵ-chains belong to the immunoglobulin supergene family and share significant sequence homology (Gold et al, 1986). A 5th polypeptide designated η has been described as part of the CD3 complex in mice, (but not so far demonstrated in human T cells) and is found in association with the ζ chain to which it is disulphide linked (Jin et al, 1990). In contrast to the other components of the CD3 complex, the ζ subunit is a discrete polypeptide with a much larger cytoplasmic domain and probably belongs to a distinct group (Weissman et al, 1988). The structure of the $\alpha\beta$ TcR is shown in Figure 1.1.

The CD3 complex does not contribute to the antigen specificity of the T cell but appears to be responsible for the transduction of signals that result from the binding of the $\alpha\beta$ and $\gamma\delta$ heterodimer to their ligands (Clevers et al, 1988). It is notable that these chains have significant cytoplasmic domains and the γ, δ and ζ chains possess phosphorylation sites which are implicated in T cell activation (Weiss, 1989). Clevers et al, (1988) has suggested that the arginine and lysine residues on the TcR $\alpha\beta$ and TcR$\gamma\delta$

Figure 1.1 - Schematic representation of the T cell receptor complex.
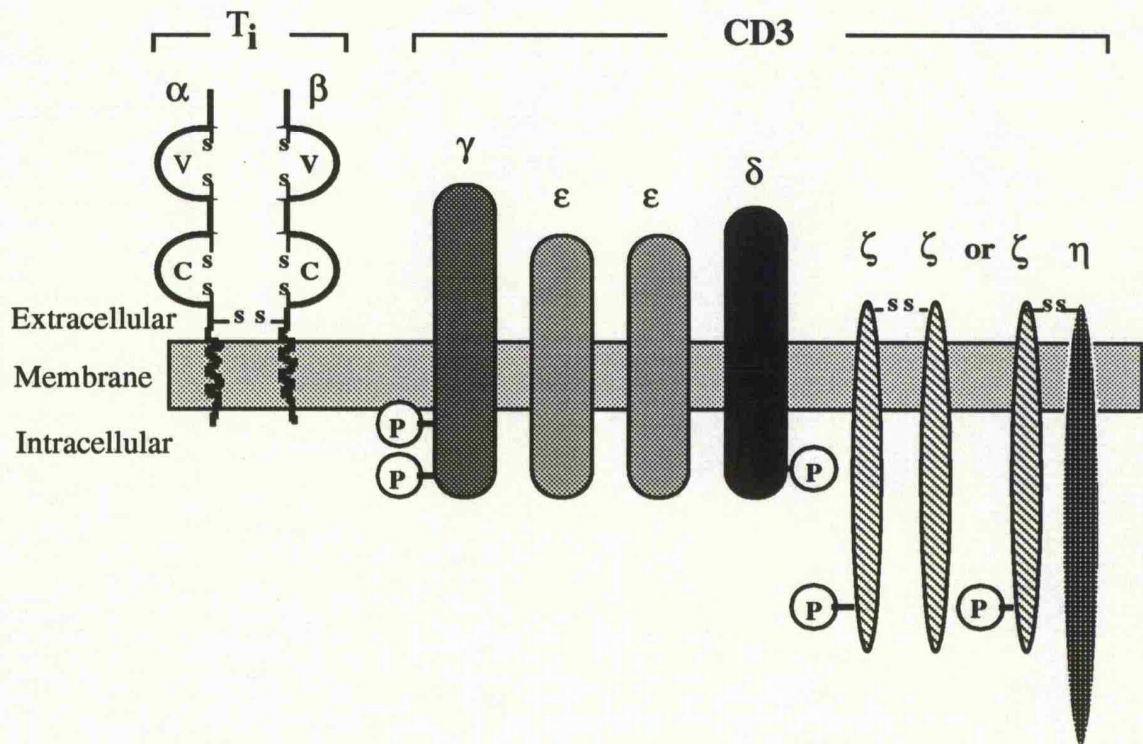
T_i = Antigen recognition site ($\alpha\beta$ shown, $\gamma\delta$ has a similar arrangement).
V = constant region
C = variable region
CD3 = CD3 complex

(P) = Phosphorylation sites

Adapted From Finkel and Kubo (1990)

interact with glutamate and aspartate residues on CD3 and through the formation of salt bridges are responsible for TcR/CD3 interactions.

Although it was originally suggested that each clonotypic, heterodimeric αβ or γδ TcR was associated with one of each of the invariant CD3γ, δ and ε chains plus one set of disulphide-linked ζζ or ζη chains (Clevers et al, 1988) it is now clear from studies in humans and mice that a number of stable subunits exist within the TcR-CD3 complex. There are CD3ε chains in close proximity in the mature TcR-CD3 complex and CD3ε-CD3γ or CD3ε-CD3δ but not CD3γ-CD3δ associations can be detected (de la Hera et al, 1991). It is proposed that every αβ or γδ TcR heterodimer associates with two chains of CD3ε, one of CD3γ, one of CD3δ and two disulphide linked ζζ (or ζ plus η chains in murine T cells) (Koning, 1991).

Knowledge of the spatial array of the subunits within the receptor complex is important in understanding function and several hypothetical models have been proposed based on the above findings (Rojo and Portoles, 1991; de la Hera et al, 1991; Koning, 1991). There are a number of consequences of a TcR with two CD3ε subunits which includes the ability of anti-CD3 mAbs to crosslink TcR-CD3 complexes in both an inter-receptor and intra-receptor mode (de la Hera, 1991). This may be the critical mitogenic property of whole anti-CD3 mAb, since antibodies monovalent for CD3 are nonstimulatory in the absence of secondary crosslinking (Roosnek and Lanzavecchia, 1989).

The TcR-CD3 complex is also associated with other cell surface antigens which are involved with both cell adhesion and provide accessory activation signals. The association with the immunoglobulin superfamily of adhesion receptors is described in Figure 1.2. Antigens including CD2, CD4, CD5, CD8 and CD28 are capable of upregulating the activation which follows TcR-CD3 stimulation (Geppert et al, 1990).

The correlation of CD4/8 status and a helper or cytotoxic function is not absolute. A stronger correlation exists between CD4/8 status and antigen-recognition specificity. CD4+ T cells recognize antigen in association with Class II MHC products (HLA-DP, DQ, and DR) and CD8+ T cells recognize Class I products (HLA-A, B, C) (Zinkernagel and Doherty, 1979; Swain, 1983). The structure of the MHC has now been extensively elucidated (Trowsdale et al, 1991). Whereas all cells express MHC class I molecules only cells specialized for antigen presentation (antigen presenting cells - APC) express class II products. Recent studies have demonstrated a recess between the two external domains of human HLA class I molecules and this is now known to present antigenic peptides to the TcR (Bjorkman et al, 1987; Davis and Bjorkman, 1988; Townsend and Bodmer 1989). Two major pathways of antigen processing and presentaton for T-cell recognition have therefore been identified. Class I MHC molecules present mostly endogenously derived cellular peptides inserted into the peptide-presenting groove of MHC class I molecules in the endoplasmic reticulum (ER). So called peptide pumps

Figure 1.2 - Immunoglobulin superfamily adhesion receptors involved in lymphocyte target cell interactions.
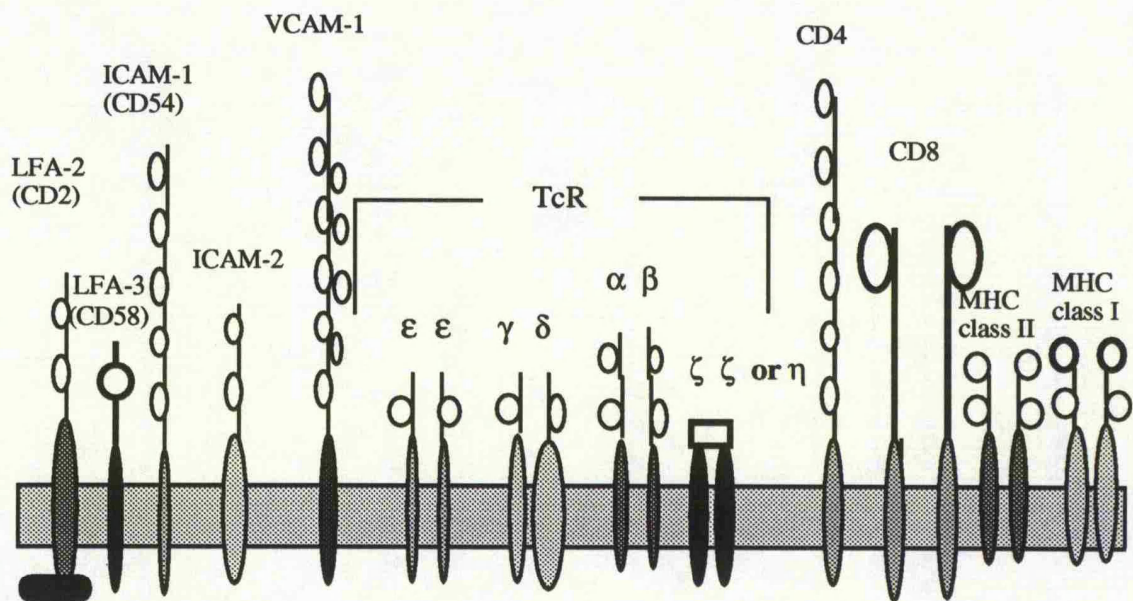
TcR = T cell receptor complex
LFA = leucocyte function associated molecule
ICAM = intercellular adhesion molecule
VCAM = vascular endothelial cell adhesion molecule
MHC = major histocompatibility complex

Adapted from Springer (1990)

responsible for active transport of peptides from the cytoplasm to the ER have recently been identified (Spies et al, 1990; Trowsdale et al, 1990). The size of peptides required for binding to class I molecules appears to be very critical and probably varies between 8 and 11 amino acids (Schumacher et al, 1991). Class II MHC molecules are specialized for the presentation of peptides derived from extracellular compartment molecules. Binding of an invariant chain (Ii) to the antigen binding groove of MHC class II molecules which subsequently becomes detached in the late endosomal compartment, prevents binding of endogenous peptides and then allows access to exogenously derived peptides (Roch and Cresswell, 1990; Teyton et al, 1990). The relationship between T cells, their interaction with MHC molecules, and subsequent events following IL-2 secretion are shown in Figure 1.3.

T cells that express $\gamma\delta$ heterodimers may mediate both MHC-restricted recognition and non-MHC-restricted recognition of targets and comprise approximately 1% of circulating T cells (Brenner et al, 1988; Scott et al, 1990). Peripheral blood $\gamma\delta$ T cells virtually all lack CD4 or CD8 antigens and although not demonstrating lysis of NK sensitive targets on fresh isolation they may do so following *in vitro* activation (Bolhuis et al, 1991). $\gamma\delta$ T cell lines isolated from peripheral lymphoid tissues display non-MHC-restricted cytotoxicity and intraepithelial $\gamma\delta$ T cells are cytolytic without stimulation. Studies in mice and humans have demonstrated a predominance of $\gamma\delta$ T cells in the epithelial tissues of the epidermis, lung, and intestine (Brenner et al, 1988; Yoshikai 1991). These cells may thus be of potential importance in response to antigenic challenge at these sites (Wunderlich and Hodes, 1991) particularly against bacterial infections (Yoshikai, 1991). $\gamma\delta$ T cells may contribute to the immunological surveillance against both infected and transformed cells (Yoshikai, 1991).

Although the molecular interactions between the TcR and other cells described above involve specific antigens, adhesion receptors account for the antigen-independent interactions that are crucial to activated lymphocytes during cytolysis (Springer, 1990) (Figure 1.2). The initial adhesion step is the rapid formation of an intercellular bond which does not involve a specific TcR-antigen interaction (Spits et al, 1986). Two major pathways have been defined (Shaw et al, 1986). In the first, leucocyte function antigen-1 (LFA-1 or CD11/18a) on the CTL surface interacts with intercellular adhesion molecule ICAM-1 (CD54) or ICAM-2 on the target cell and in the second CD2 on the CTL interacts with LFA-3 (CD58) on the target cell. CD4 and CD8 contribute to this reaction when present by interacting with MHC class II and class I molecules respectively. The CD4/CD8 glycoproteins not only increase the avidity of the TcR antigen interaction they may also play an important role as an independent signal transducing element. Their intracellular domains are physically associated with the lymphocyte specific tyrosine protein kinase (TPK) p56[lck] which plays an important role in T cell activation by phosphorylation of the ζ chain (Bolhuis et al, 1991).

Figure 1.3 - Diagramatic representation of the cellular and molecular
interactions that follow antigen presentation and the
central role of IL-2.

CTL = cytotoxic T cell
α = p55 component of IL-2Receptor
β = p75 component of IL-2Receptor
Ag = Antigen

Adapted from Borden and Sondel, 1989
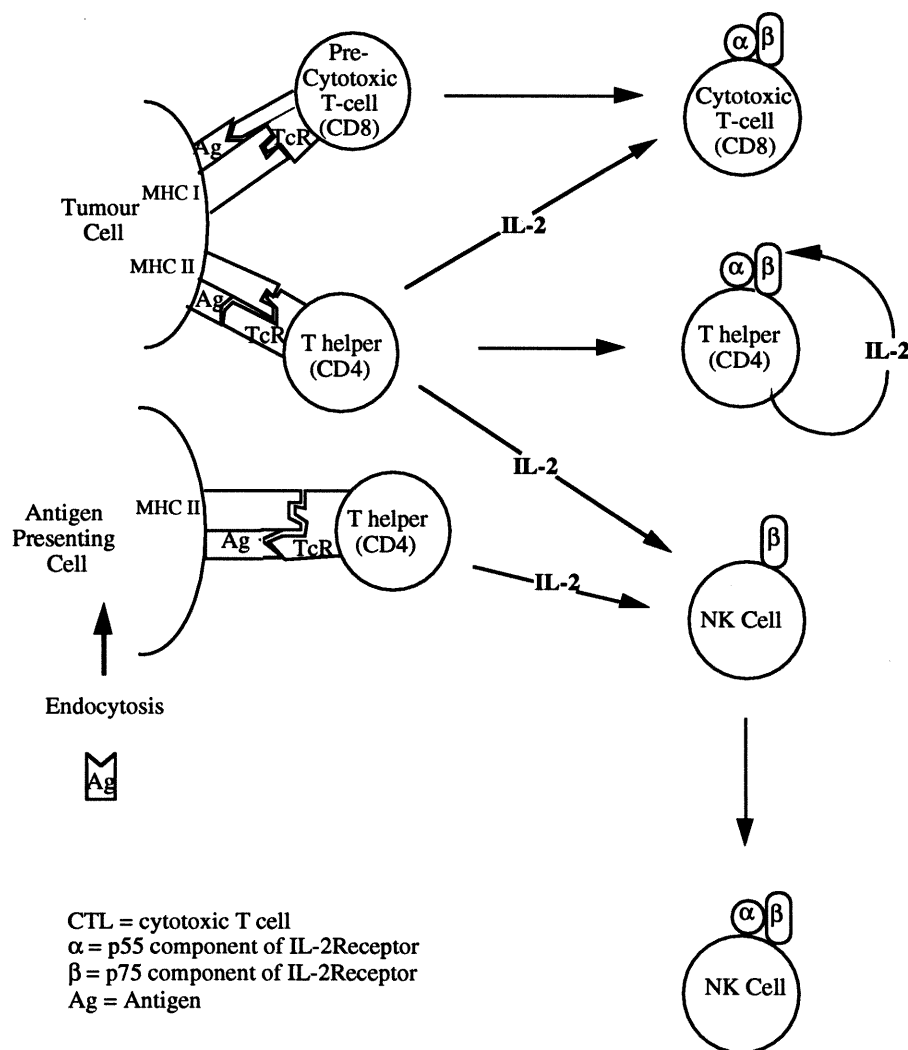
1.6.2 Natural Killer Cells.

Natural killer (NK) cells comprise between 5% and 15% of peripheral blood lymphocytes, have the morphology of large granular lymphocytes (LGL) and are capable of mediating cytolysis of tumour targets without prior sensitization or MHC-restriction (Timonen et al, 1981; Reynolds and Ortaldo, 1987; Trinchieri, 1989). They were discovered following the observation that lymphocytes isolated directly from unimmunized normal hosts were able to lyse spontaneously a variety of tumour cell targets (Herberman and Gaylord, 1973), a phenomenon termed "natural immunity". NK cells mediate the majority of non-MHC-restricted cytotoxicity in an unimmunized host (Herbermann and Ortaldo, 1981; Hersey and Bolhuis, 1987).

A minority of T cells express TcR α/β^+ receptors, mediate non-MHC-restricted killing of some tumour targets, and also demonstrate "LGL" morphology (Lanier et al, 1986a and 1986b; Schmidt et al, 1986). The term LGL strictly remains a morphological description of a cell, rather than a synonym for an NK cell. Since NK cells represent only a small fraction of PBMC and are themselves heterogenous, it has been difficult to perform meaningful experiments with NK cells in normal peripheral blood. Physical enrichment techniques such as Percoll density gradients have been useful for purifying cells with LGL morphology and have therefore been extensively used to obtain NK-enriched populations (Timonen and Saksela, 1980; Timonen et al, 1981).

No single antigen identifies all NK cells although they do not express CD3 or rearrange immunoglobulin or TcR gene products. NK cells can be identified in peripheral blood using specific fluorescent monoclonal antibodies to antigens expressed by the majority of NK cells. The CD57 (Leu-7, HNK-1) antigen was initially used to identify large granular lymphocytes most of which expressed NK activity. However, CD57 antigens are absent on approximately 50% of cells which are known to possess potent NK activity (Perussia et al, 1983). Hercend et al, (1985) described two antigens associated with the majority of NK cells termed NKH-1 and NKH-2 which are now known to be isomers of the neural cell adhesion molecule(N-CAM). NKH-1 (CD56) is identified by the monoclonal antibody Leu-19 and this has replaced anti-CD57 mAb as a pan-NK associated cell surface marker. CD56 is a 200 - 220 kD glycoprotein expressed predominantly on NK cells and a small subset of T cells mediating non-MHC-restricted cytotoxicity (Hercend et al, 1985; Schmidt et al, 1986; Lanier et al, 1986a and 1986b). Its function is unknown although it may be involved as a homotypic adhesion molecule in conjugate formation when the target cell also expresses CD56 (Nitta et al, 1989).

Another important antigen expressed by NK cells is CD16, a receptor for the Fc portion of IgG. The CD16 (Leu-11, FcγRIII) antigen is present on virtually all cells in peripheral blood that are known to possess functional NK activity (Phillips and Babcock, 1983; Lanier et al, 1986a; Trinchieri, 1989). CD16 is a low affinity receptor for the Fc region of IgG through which these cells mediate phagocytosis and antibody-dependent

cellular cytotoxicity (ADCC) (Ravetch and Kinet, 1991). Cell surface antigens expressed by NK cells are outlined in Table 1.2.

Resting NK cells are a highly heterogenous population. By measuring the density of expression of CD56 and CD16 and also CD3 antigen expression different subsets of lymphocytes with LGL morphology can be identified. Normal adult peripheral blood contains a major NK subset (>90%) with the CD16$^+$ phenotype which also coexpress a low density of CD56 (CD16$^+$CD56^{dim+} NK cells) and a minor (<10%) CD16$^-$CD56$^+$ subset which express a high density of CD56 (CD16$^-$CD56$^{bright+}$ NK cells) (Lanier et al, 1986a; Nagler et al, 1989; Inveradi et al, 1991). The majority of cells exhibiting non-MHC-restricted cytotoxicity express the CD3$^-$CD16$^+$CD56$^+$ phenotype. A small population of CD3$^+$CD16$^-$CD56$^+$ T cells are also involved (Lanier et al, 1985). However, depletion of cells bearing CD16 or CD56 abrogates 90% of NK activity as measured in ^{51}Cr-release assays (Van Tol et al, 1992).

The majority of peripheral blood CD16$^+$ NK cells also constitutively express the intermediate affinity interleukin 2 receptor (p75 or IL-2Rβ) which is the predominant IL-2 binding structure on resting NK cells (Phillips et al, 1989). CD16$^-$ NK cells constitutively express high affinity IL-2 receptors *in vivo* (IL-2Rα chain in combination with IL-2Rβ) and preferentially respond to low amounts of IL-2 (Nagler et al, 1990). This is in contrast to most CD4$^+$ T lymphocytes which express only IL-2Rα and are relatively unresponsive to IL-2 (Takeshita et al, 1989). CD16$^-$ NK cells are therefore the only population of PBL that constitutively express high affinity IL-2 receptors *in vivo*. This may account for their preferential expansion in the peripheral blood of cancer patients after *in vivo* rIL-2 therapy (Ellis and Fisher, 1989) and following activation withIL-2 *in vitro* (Warren and Skipsey, 1992).

When NK cells are further subclassified according to density of CD16 expression a CD16^{dim} subset has been identified which appears to be a distinct population from CD16^{neg}CD56^{bright} cells (Warren and Skipsey, 1992). As with CD56 expression, CD16 is expressed as a continuum from CD16$^-$ to CD16$^+$ on the CD3$^-$CD56$^+$ population. These cells are probably related with the CD16^{bright} subset representing the more mature cells (Nagler et al, 1989). Identification of these subsets does have functional significance. Density of expression of CD16 is positively correlated with K562 cytotoxicity and the proliferative capacity to respond to IL-2 is negatively correlated with CD16 expression and positively correlated with IL-2Rα (CD25) expression (Nagler et al, 1989). In these studies cytolytic activity against a colorectal cell line was also greater in the CD16^{neg} and CD16^{dim} subsets following incubation in low doses of IL-2 compared with CD16^{bright} subset. CD16 expression is unaffected by culture in IL-2 although CD56 expression is upregulated (Nagler et al, 1989).

Although NK cells are thought to be clearly distinct from the T cell lineage, more recently similarities between NK cells and T cells have been emphasised (Lanier et al, 1992). The identification of membrane bound proteins such as the ζ chain in association

Table 1.2 - Approximate % surface antigen expression by human natural killer cells.

CD	Antigen	% of NK Cells Expressing Ag	Other Cells Expressing Ag
CD2	SRBC receptor	70-90	T cells, thymocytes
CD7	p40	80-90	T cells, thymocytes
CD8	MHC I ligand	30-40	CTL, suppress. T cell
CD11a	LFA-1	> 95	All leucocytes
CD11b	C3bi receptor	80-90	Monocytes, PMN
CD11c	p150	30-60	Macrophages, PMN
CD16	Fcγ receptor (III)	80-90	Macrophages, PMN
CD18	β chain CD11a-c	> 95	All leucocytes
CD54	ICAM-1	30-50	Many activated cells
CD56	NKH-1 (NCAM)	> 95	NEC, T cells (<2%)
CD57	HNK-1	50-60	T cell subset, NEC
CD58	LFA-3	85-95	Many cells

Robertson and Ritz (1990).

SRBC = sheep red blood cell receptor.

NEC = neuroendocrine cells

LFA = leucocyte function antigen.

NCAM = neural cell adhesion molecule.

ICAM = intercellular adhesion molecule.

with CD16 on NK cells (Lanier et al, 1989; Anderson et al 1989a) supports the concept that CTL may have arisen from NK cells by acquiring a clonally distributed T cell receptor.

Unlike T cells, the receptors through which NK cells recognize their targets are poorly defined (Trinchieri, 1989). MHC expression by targets does influence their susceptibility to lysis by NK cells. MHC expression has been suggested to inhibit NK activity and NK recognition is therefore in a sense partly MHC restricted cytotoxicity (Moretta et al, 1989). This has led to the theory that one function of NK cells in immunosurveillance is to eliminate cells that fail to express MHC class I products (Ljunggren and Karre, 1990). The transmembrane form of CD16 which mediates phagocytosis and antibody-dependent cellular cytotoxicity (ADCC) (Ravetch and Kinet, 1991) also allows NK cells to selectively respond to specific antigens. Generally however, multiple receptors are involved in non-MHC-restricted cytotoxicity and these are molecules known to be important in cellular adhesion (Hersey and Bolhuis, 1987). It is the relative expression of ligands on the target cell for several cell surface molecules on the effector cell determines whether or not target cell cytolysis will occur.

Conjugate formation between NK effectors and target cells is primarily mediated by binding of the NK surface adhesion molecules LFA-1 (CD11a/CD18) and CD2 to their respective target cell ligands intracellular adhesion molecule-1 (CD54) and LFA-3 (CD58) (Springer et al, 1987; Schmidt et al, 1985; Ritz et al, 1988; Timonen et al, 1988; Sanders et al, 1988). Freshly isolated NK cells uniformly express CD11a and CD58 and the NK sensitive target K562 expresses CD54 and CD58 (Robertson et al, 1990). Anti-CD54 has been shown to inhibit partially, killing of K562 targets by impairing conjugation (Timonen et al, 1988).

Activation of NK cells is more clearly understood. Membrane expression of CD16 is dependent on the co-expression of γ or ζ chains (Lanier et al, 1989). In T cells signal transduction is mediated through the γ chain and a similar mechanism operates in NK cells whereby engagement of CD16 by IgG results in tyrosine phosphorylation of the TcRζ chain (O'Shea et al. 1991; Wirthmueller at al, 1992). CD16 therefore acts as a signalling pathway through which IL-2-dependent activation is enhanced (Harris et al, 1989). Cross-linked anti-CD16 mAb act synergistically with IL-2 to elicit the expression of the IL-2Rα chain on normal NK cells (Harris et al, 1989). They may therefore result in NK cell activation and the production of various cytokines, including interferon-γ, tumour necrosis factor (TNF), macrophage colony stimulating factor (M-CSF), and granulocyte-macrophage CSF (GM-CSF) (Trinchieri, 1989; Cassatella et al, 1989).

Alternative pathways for activation have been identified. The CD2 molecule which expresses 3 functionally important epitopes is present on NK cells and T cells and provides an alternative pathway for activation in NK cells and CTL (Meuer et al, 1984). NK cells can be triggered through the CD2 molecule using a single anti-CD2 mAb (Siliciano et al, 1985; Schmidt et al, 1987 and 1988). Only combinations of anti-CD2

mAb have been shown to induce antigen non-specific cytolytic activity by CTL (Bolhuis et al, 1986). Recently CD2 has been shown to be physically associated with CD3 following T cell activation (Moingeon et al, 1989). Not suprisingly the events by which NK target cell killing is augmented by IL-2 and mAb are also poorly understood. This reflects the inadequate understanding of the mechanisms regulating NK cytolysis. It has been suggested that NK cells are in a state of relative activation by autologous target cells even when freshly isolated (Timonen et al, 1988). They express the intermediate affinity IL-2R (β chain) in contrast to resting T cells which express very low levels of IL-2Rβ (Siegel et al, 1987; Caligiuri et al, 1990). Upregulation of CD2, CD11a/CD18, CD54, CD56, and CD58 is seen after incubation with exogenous IL-2 and this occurs through the intermediate affinity IL-2R (Robertson and Ritz, 1990). Antilymphocyte and antithymocyte immunoglobulins whilst stimulating T cells to proliferate *in vitro* appear to have no direct effect on purified NK cells (Taniguchi et al, 1990). Because NK cells do not express the CD3 antigen activation of NK cells by anti-CD3 mAb is indirect.

Alterations in natural killer (NK) cell activity can be produced by changes in the availability of NK cells in the peripheral circulation (decreased production or changes in circulating patterns between blood and immune organs) or changes in the killing capacity of NK effector cells (Whiteside and Herberman, 1989). The study of changes in NK function depends upon measurement of peripheral blood NK cell populations and assessment of NK activity using a reproducible cytotoxicity assay. Because of marked variation in NK activity between individuals measurement of NK cell numbers is not a substitute for measurement of function (Whiteside and Herberman, 1989).

One of the most extensive studies performed on NK activity was reported by Pross and Baines (1982). 539 normal healthy donors were tested from once to 213 times. NK activity was detected in all cases and was relatively constant over a seven year period for individuals repeatedly tested although for any one individual there was a broad range of activity. Individuals can be grouped into possessing consistently high or low activity. Significant NK activity was present at birth and gradually increased through adulthood. Males had higher levels of NK activity than females. The relative cytotoxic activity of NK cells was found to be a characteristic of the individual donor, and was largely independent of the target cell used for its assessment. In other words individuals with high levels of reactivity against one target also demonstrated relatively high reactivity against other targets.

NK function is affected by a multitude of diverse factors which may be relevant to monitoring hospital inpatients. Depressed patients have reduced NK activity (Caldwell et al, 1991) which appear to be gender related being restricted to male patients (Evans et al, 1992). Even minor life stresses such as academic examinations have been shown to reduce NK activity (Kiecolt-Glaser et al, 1984). Stress due both to hospitalization and participating in clinical trials may therefore be a relevant factor affecting NK activity. Healthy female subjects generally have lower NK activity than males and young healthy

individuals also have lower levels of cytotoxicity than an elderly population (Whiteside and Herberman, 1989). A positive correlation has also been reported between age and percentage of NK cells as defined by the CD16 antigen (Ligthart et al, 1986) although no significant difference in NK cell numbers was reported for elderly males and elderly females (Ligthart et al, 1986). This may account for the increase in NK activity observed with increasing age (Pross and Baines, 1982).

Whole blood transfusion causes reduced NK cell function and a decreased T helper: T suppressor lymphocyte ratio (Kaplan et al, 1984). This has been shown to remain depressed for up to 30 days following elective colorectal surgery and is associated with increased rates of postoperative infection (Jensen et al, 1992). All these factors are relevant to the study of patients in clinical trials and provide variables which must be taken into account in selecting patients and controls.

Despite their apparent lack of specificity, NK cells have been implicated in a large number of diverse immunologic functions including, cytotoxicity against tumour cells and virally transformed cells (Herberman and Ortaldo, 1981), resistance to some microbial (Nencioni et al, 1983), fungal, and parasitic agents (Hatcher and Kuhn, 1982), immune regulation through secretion of lymphokines such as interleukin 2 (IL-2) (Kasahara et al, 1983) and interferon (Timonen et al, 1980), regulation of haematopoiesis (Hermann et al, 1987), and natural resistance to allogeneic grafts (Warner and Dennert, 1982).

The significance of NK cells in human neoplasia remains unclear, despite numerous studies. Freshly isolated human tumours are generally relatively resistant to cytolysis, particularly when autologous lymphoid cells are used (Vose and Moore, 1980). Takasugi et al, (1977) noted a decline in NK activity with increasing tumour growth and Pross and Bains (1980) reported preliminary evidence for a correlation between NK activity and response to therapy in ovarian cancer. NK activity has been shown to inversely relate to disease stage (Takasugi et al, 1977; Steinhauer et al, 1982). Of the few studies that have been undertaken in which controls were matched for age, sex, and hospitalization these suggest that early tumours are associated with little or no derangement of blood NK function, whereas some impairment is noted at advanced disease stages (Hersey et al, 1980). A return to nearly normal NK cell activity has been noted following potentially curative excision of primary colorectal tumours (Tarrter et al, (1986).

Lymphocytes isolated from human solid tumours, tumour infiltrating lymphocytes (TIL), have low or absent NK cytotoxicity (Uchida and Micksche, 1981; Vose et al, 1977). In contrast lymphoid cells from neoplastic pleural effusions have a high frequency of cells with LGL morphology (Uchida and Micksche, 1981). These studies suggest that NK cells are not a prominant feature of an immune response at tumour level although variation with anatomical site may occur.

Peripheral blood NK cells appear to represent a first line of defence against the metastatic spread of blood-borne tumour cells and NK activity may be important in immunosurveillance against tumours (Whiteside and Herberman, 1989). NK cells protect against against the development of metastases in animal models (Gorelick et al, 1982; Hanna, 1982). Reduced NK activity may also be associated with the subsequent development of metastases (Schantz and Goepfert, 1987). However, results of these studies do not clearly distiguish between low NK activity as a cause or consequence of tumour development.

1.6.3 Activated Macrophages and Neutrophils.

The function of macrophages in tumour immunity is controversial. Macrophages and monocytes function as antigen presenting cells (APC), combining with antigen to initiate an immune response. This may be relevant to tumour immunity in experimental models and immunization against tumours. They are also potential effector cells capable of mediating tumour cell lysis (Mantovani et al, 1992). Although tumour-associated macrophages (TAM) represent a major component of the lymphoreticular infiltrate of tumours they play an uncertain role. Activated macrophages like NK cells and CTLs cultured with relatively high doses of IL-2, can mediate non-MHC-restricted cytotoxicity (Drysdale et al, 1988). Like NK cells monocytes can be preferentially cytotoxic for tumour cells on direct contact. Destruction of tumour cells or inhibition of their growth by activated macrophages is mediated extracellularly by the release of a variety of substances stimulated by macrophage binding to tumour cells (Nathan 1987).

Macrophages or monocytes unlike NK cells and CTL may also be phagocytic. Because monocytes express Fc receptors for IgG they are capable of mediating ADCC. The supernatants of stimulated T cells have been shown to produce a macrophage arming factor (MAF) (Dullens et al, 1989) and it is probable that this is a mixture of substances including IFNγ and GM-CSF. Effector functions of macrophages may therefore be partly dependent upon T cell immunity.

The antitumour cytotoxic activity of neutrophils has not been as widely studied as that of other effector cells but reports suggest that they are capable of mediating tumour killing by similar mechanisms to NK cells and macrophages (Becker, 1988), either through direct contact or by an antibody-dependent mechanism similar to that of macrophages and NK cells (Graziano and Fanger, 1987). IFN-γ, tumour necrosis factor-α (TNF-α), and TNF-β have been shown to be potent activators of neutrophils (Shalaby et al, 1985). The role of neutrophils in tumour cell growth is presently undefined.

1.7. MECHANISMS OF LYMPHOCYTE-MEDIATED CYTOTOXICITY AGAINST CANCER CELLS.

1.7.1 Introduction.

Following recognition by, and activation of cytolytic effectors, the events culminating in target cell death are generally similar for all cell types. These mechanisms are important both *in vivo* and *in vitro*. Two possible cytocidal mechanisms have been proposed; a receptor-triggered disintegration model in which effector target interactions trigger a programmed process of target cell death and dissolution referred to as apoptosis; and a granule exocytosis (perforin) model, in which lytic molecules are secreted by the effector cell into the intercellular space and polymerize to form lytic pores in the target cell membrane (Berke, 1991).

Whichever mechanism is proposed, lysis must account for the following experimental findings (Berke, 1991)

1. Refractoriness of the CTL to its own lytic mechanism while being susceptible to the lytic mechanism of other CTL.
2. Recycling of the effector cell to lyse additional targets while protein synthesis is blocked.
3. Target cell lysis in the absence of perforin.
4. Target DNA fragmentation prior to chromium-51 release.

1.7.2 Apoptosis.

Apoptosis refers to an autolytic cascade initiated by pre-lytic nuclear fragmentaton following a specific target cell stimulus and was first suggested by Russell in 1983. The target cell must be metabolically active (Zychlinsky et al, 1991) because DNA damage results from activation of target cell enzymes by the effector cell (Sellins and Cohen, 1991) and membrane disruption subsequently arises as an indirect event. Alteration in ion permeability of the target cell membrane, may account for the observed pre-lytic elevation of intracellular calcium prior to membrane damage (Allbritton et al, 1988).

Elevated intracellular calcium may be the mechanism for triggering internal degradation processes in the target cell. Terminal membrane permeability failure, culminating in cytolysis probably results from the inability of ATP-fuelled ionic pumps to maintain ionic gradients.

1.7.3 Perforin-mediated Cytolysis.

The theory of perforin-mediated target cell lysis initiated by the formation of pores in the target cell membrane, is based on the observation that NK cells and most CTL contain lytic molecules called perforin or cytolysin with similarity to the lytic pore-forming C9 component of the complement system (Zalman et al, 1986; Tschopp and Nabholz, 1990). These lytic molecules are stored in intracytoplasmic lysosomal granules

during CTL activation and differentiation (Berke and Rosen, 1988). In the presence of calcium, perforin has potent lytic activity capable of causing lysis of different target cells without inducing effector cell damage (Ojcius et al, 1991). It is postulated that perforin monomers secreted by effector cells bind to the target cell membrane and form pores which allow the passage of solutes into the target cell resulting in lysis (Tschopp and Nabholz, 1990).

Although perforin is an excellent candidate for a 'lytic bullet', evidence for its involvement in lymphocyte-mediated cytolysis has been largely inferential and it fails to fulfill many of the criteria previously outlined above (Berke, 1991). It is probable that both the process of apoptosis and the release of cytocidal substances from target cells are complementary in mediating target cell killing.

1.8   SUMMARY.

Lymphocytes are major participants in immune processes directed against tumour cells. The identification of lymphocyte surface molecules associated with adhesion and activation has enabled the study of these different lymphocyte populations. Although common mechanisms exist for the destruction of malignant cells, mechanisms involved in tumour cell recognition are very different. T cells are capable of recognising antigen in association with products of the MHC and may eliminate highly immunogenic experimental tumours associated with viral infections. The absence of TADA on most human tumours arising de novo probably precludes an effective T cell response in many tumours.

Cells capable of non-MHC-restricted cytotoxicity recognise a broader array of surface molecules and may play a more important role in the elimination of relatively non-immunogenic tumours. Although NK cells mediate most non-MHC-restricted killing *in vivo,* the effectors involved are extremely heterogenous and some T cells are capable of non-MHC-restricted killing in certain circumstances. The relation between cell function and phenotype has enabled an improved understanding of factors required for lymphocyte activation with important therapeutic implications. One could anticipate that expansion of lymphocyte populations capable of both MHC-and non-MHC-restricted cytotoxicity *in vivo* may optimise the prospect of tumour regression in cancer patients.

CHAPTER TWO

THE REGULATION OF THE IMMUNE RESPONSE AND ITS MANIPULATION
FOR CANCER THERAPY

CONTENTS.

2.1 INTRODUCTION.

In 1980 there were an estimated 730 000 deaths from cancer in the European community (Jenson et al, 1980). This figure is almost certainly an underestimate because the burden of cancer on society as measured by the number of new cases arising each year may be 20% higher than previously assumed. Moreover with an increasingly elderly population this figure is likely to increase over the next 20 years. Cancers of gastrointestinal tract origin form the largest single section of oncological practice. Unfortunately the prognosis of patients with tumours of epithelial origin remains poor and the rate of cancer related deaths has remained unchanged over the previous 10 years. Early systemic dissemination is a feature of many tumours and limits the potential of surgery and radiotherapy to effect a cure. Of the many drugs which have been developed, only a small number of chemotherapeutic agents with proven efficacy have been identified. This has resulted in only modest responses to chemotherapy for most solid tumours. Conventional therapies appear to have reached their limits and the development of new approaches is required.

The finding that adjuvant treatment for colon carcinoma using a combination of 5-fluorouracil and levamisole (a drug with general immunopotentiating properties) improves survival in patients with Dukes stage C disease illustrates such an approach (Moertel et al, 1990). Biological therapy of cancer may be defined as a treatment that uses biological materials which either have direct effects on tumour cell proliferation or differentiation, or modify the host biological response to the tumour (Jones and Selby, 1991). Recent advances in molecular biology have resulted in an improved understanding of immune events at the cellular and molecular level. Cancer immunotherapy is not a new concept. However, the large scale production of cytokines and monoclonal antibodies for use in clinical trials, has recently resulted in a more scientific approach with great therapeutic potential (Guillou, 1991).

2.2 DEVELOPMENT OF CANCER IMMUNOTHERAPY.

2.2.1 Non-Specific Immunotherapy.

Early physicians recognized the potential for manipulating the host response and general approaches to cancer immunotherapy have remained consistent despite recent technological advances (Oettgen and Old, 1991). Current explorations of monoclonal antibodies represent a direct extension of earlier studies with horse and rabbit antisera, while the present use of cytotoxic T lymphocytes in therapy reflect early experiments establishing that tumour-specific transplantation immunity in the mouse can be transferred with lymphocytes. Likewise, purified cancer antigen-containing vaccines are modern versions of earlier vaccines prepared from crude tumour homogenates. The present use of recombinant cytokines have their precedent in the use of bacterial vaccines since microbial products are potent inducers of cytokines. Four approaches to cancer immunotherapy may thus be defined; nonspecific enhancement of immune mechanisms,

immunotherapy with tumour vaccines, immunotherapy with antibodies, and immunotherapy with immune cells.

Immunotherapy was pioneered by an American surgeon called William Coley who observed that a recurrent inoperable sarcoma of the neck regressed following a severe attack of erysipelas, (Coley, 1891). Coley induced erysipelas in cancer patients by injecting living cultures of erysipelas streptococci and later went on to incorporate *Serratia marcescens* (Bacillus prodigiosus) into his streptococcal vaccines (Coleys' toxins). He treated substantial numbers of patients over the next 40 years with varying but documented success before this approach declined (Nauts et al, 1946). Interest was rejuvinated by a report of a small positive trial studying BCG and acute lymphoblastic leukemia (Mathe et al, 1969). Numerous studies of immunotherapy in humans followed, using bacterial adjuvants such as Bacillus Calmette-Guerin (BCG), *Corynebacterium parvum*, streptococcal "vaccines," *Pseudomonas* "vaccines" most of which yielded marginal or negative results. These studies contributed to the negative attitude of most clinicians toward immunotherapy by the end of the 1970's (Oldham and Smalley, 1983). In contrast to systemic administration reproducible effects have been observed when agents such as BCG are applied locally at the tumour site and regression of cutaneous melanoma metastases (Morton, 1970) and superficial bladder cancer (Pinsky et al, 1985) have been reported.

2.2.2 Cancer Vaccines.

Although early physicians inoculated themselves with cancer tissue in an attempt to stimulate active immunity against cancer (Oettgen and Old, 1991), cancer vaccines have since taken the form of autologous or allogeneic fresh tumour tissue or cultured tumour cells or tumour cells modified by chemicals, enzymes or virus infection (Currie, 1972). Most studies have failed to demonstrate any therapeutic benefit using this approach. However, when 40 patients with colorectal carcinoma were randomized to receive either standard resection or resection and vaccination with cryopreserved, irradiated autologous tumour cells and BCG, fewer recurrences and deaths were reported in the vaccine group compared with the control group (Hoover et al, 1985). The finding that cytotoxic immunity can be generated against intracellular as well as cell surface mutant cell products (Townsend et al, 1985) may extend the range of potential antigens for inclusion in cancer vaccines. The problem of identifying such specific mutant products remains the principle limitation to this approach.

2.2.3 Immunotherapy Using Immune Sera.

Initial attempts at treating patients with cancer using sera from immunized animals began at the turn of this century (Berkely 1914). These early attempts resulted in poorly documented and unsubstantiated claims of success . The identification of heteroantisera that reacted with cancer tissue but not normal tissues have generally proved

disappointing. Tumours with an aetiology associated with viral infections such as Burkitt's lymphoma, have been shown to produce high titres of antibody against Burkitt's lymphoma cells (Henle and Henle, 1966). Stimulated by the successful serological analysis of Burkitt's lymphoma, sera from patients with other cancers notably sarcomas (Morton et al, 1969) and melanomas (Morton et al, 1970) were claimed to contain antibodies reacting with cell surface antigens of established melanoma or sarcoma cell lines more frequently than sera from normal subjects. These studies must be interpreted with caution in view of the high levels of reactivity often seen with serum from apparently healthy individuals which probably account for the majority of positive reactions that were recorded in past immunological studies of human cancer (Rosenberg and Terry, 1977).

Consequently attention moved in the 1960's to the search for cancer-specific antibodies in the serum of cancer patients. Levine et al, (1951) first suggested that serum factors interacting with specific antigenic targets on tumour cells may inhibit tumour growth. He noted that a woman with gastric cancer, homozygous for a rare variant of the P blood group system who was given a small transfusion of incompatable blood prior to surgery achieved a prolonged survival. The subsequent finding that the tumour absorbed P_1 reactivity suggested that her survival was due to the cytotoxic action of the P_1 antibody on her tumour cells. Despite these findings serotherapy was not associated with significant tumour regressions and has been abandoned with the advent of monoclonal antibodies.

2.2.4 Immunotherapy Using Immune Cells.

Cell-mediated immunity is of major importance in the rejection of solid tumours *in vivo* (North, 1984; Robins and Baldwin, 1985). The realization that antibody-dependent cellular-cytotoxicity (ADCC), rather than complement dependent cytotoxicity is the more efficient lytic mechanism of tumour cells (Denkers et al, 1985) underlies the importance of cellular elements even with regard to antibody therapy. Cellular immunity was originally demonstrated by Landsteiner and Chase (1942) who transferred delayed hypersensitivity reactions with cells as opposed to serum from an immunized donor to a naive host. That tumours elicit immune responses was demonstrated by Gross (1943) who discovered that inbred strains of mice could be immunized against a tumour that developed in a mouse of the same inbred strain. The significance of cell-mediated immunity in cancer was subsequently confirmed in mice by the pioneering work of Mitchison (1955) and Prehn and Main (1957) who demonstrated immunity to a methylcholanthrene-induced sarcoma.

Initial studies suggested that cellular cytotoxicity against cultured cancer cells *in vitro* was specific for cancer cells of a similar histological type to that of the patient (Hellstrøm et al, 1971). Cytotoxicity to cultured allogeneic tumour cells was reported using peripheral blood lymphocytes (PBL) from more than 50% of patients with colo-

rectal cancer (Baldwin et al, 1973). Reports then appeared showing that lymphocytes from normal individuals are cytotoxic for a broad range of cultured human cancer cells and these effectors were referred to as natural killer (NK) cells (Herberman and Holden, 1978; Trinchieri, 1989). The capacity for ADCC is an important property of NK cells. Klein et al, (1980) investigated the cellular infiltrates in a variety of human cancers and identified tumour-specific T lymphocytes in high concentration.

It was the discovery of interleukin-2 (IL-2) by Morgan et al, (1976), that enabled the generation of cytotoxic T-lymphocyte (CTL) clones and investigation of their role in the generation of an immune response to specific antigen. The two mechanisms for cellular cytotoxicity based on the mechanism by which NK cells and CTL recognize their targets were then described. CTL generally recognize antigens presented in association with products of the major histocompatibility complex (MHC) (Zinkernagel and Doherty, 1979). 'Natural immunity' (principally mediated by NK cells) involves recognition of target cells without MHC restriction (non-MHC-restricted immunity) (Trinchieri et al, 1989). Whichever mechanism of target recognition is involved direct effector cell-target cell interaction was demonstrated as essential for lymphocyte-mediated cytotoxicity. Modern immunotherapy has been directed towards the expansion and activation of lymphocyte populations with anti-tumour reactivity both *in vivo* and *ex vivo* with transfer of cells back to the donor.

The term adoptive immunity is used to denote the immune state elicited by the injection of immunologically active cells from syngeneic donors by a systemic route. Initial attempts at demonstrating the anti-tumour efficacy of transferred lymphocytes in tumour bearing mice were disappointing. Only a few studies were able to demonstrate regression of established tumours and these involved the transfer of very large numbers of lymphocytes from hyperimmunized donors to animals with strongly immunogenic tumours (Smith et al, 1977). Extensive trials of adoptive immunotherapy have been undertaken in humans using IL-2 to expand and activate peripheral blood mononuclear cells (PBMC) from cancer patients, or lymphocytes obtained from their tumours (tumour infiltrating lymphocytes, TIL) (Rosenberg et al, 1986 and 1988). The benefits of this approach remain unproven. Present research involves the investigation of new biological agents, new techniques for administration, and drug synergy in an attempt to reduce toxicity and improve efficacy.

2.3   CYTOKINES.

2.3.1   Introduction.

The immune response is now known to be a precisely regulated and complex process. As previously discussed direct stimulation of the TcR complex and certain other lymphocyte receptors such as CD2 and CD16 by antigen or mAb generate primary activation signals. The events which follow are regulated by a variety of accessory signals provided by soluble mediators known as cytokines (Figure 2.1).

Figure 2.1 - Positive cytokine interactions following antigen presentation to T cells.
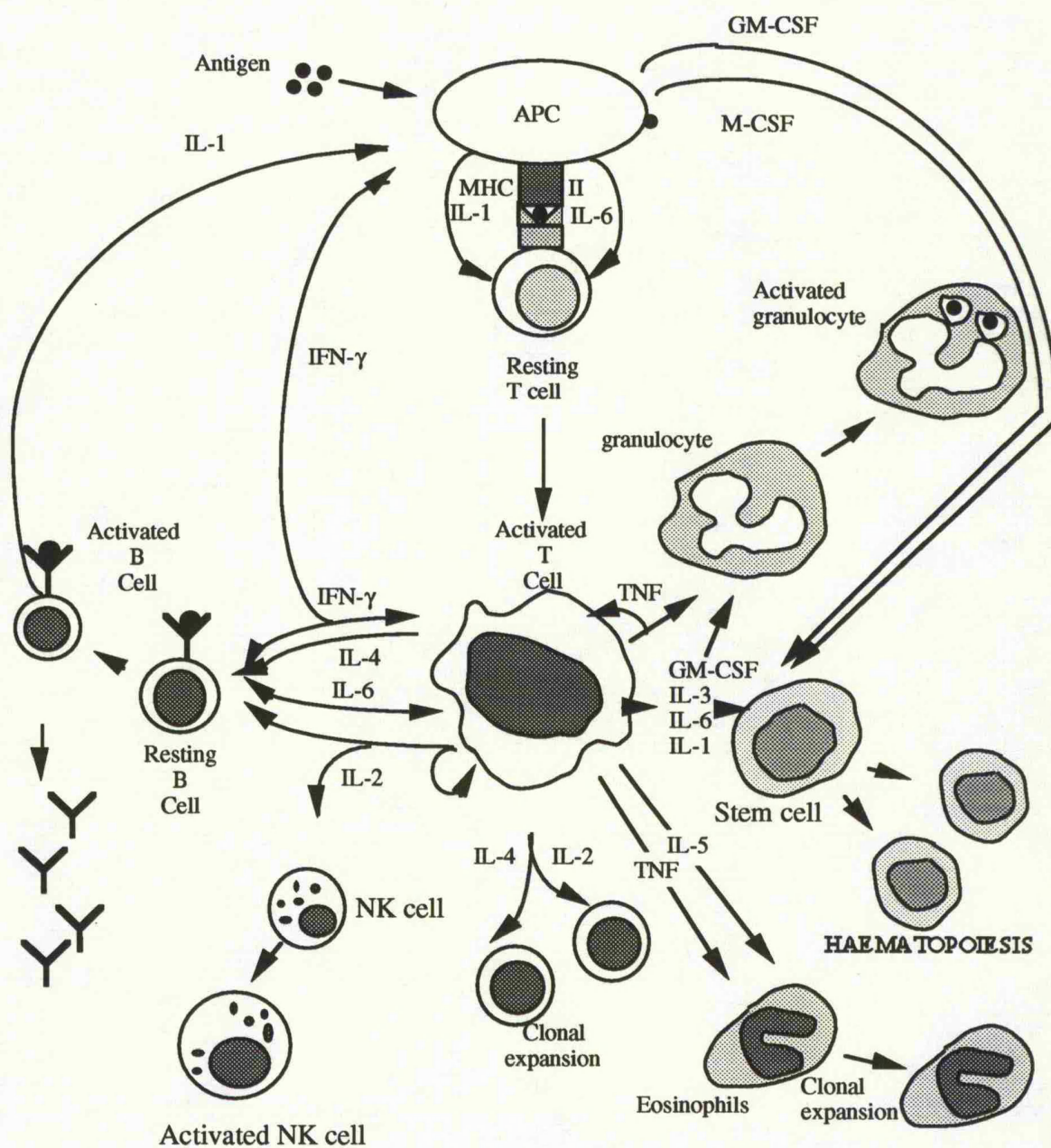
APC = antigen presenting cell
IL = interleukin
CSF = colony stimulating factor
GM-CSF = granulocyte macrophage
            colony stimulating factor
IFN = interferon
TNF = tumour necrosis factor   Adapted from Balkwill (1989a)

Cytokines are soluble low molecular weight polypeptides secreted by a wide range of cell types, which have multiple actions on cellular growth or cellular differentiation, or both (Balkwill and Burke, 1989) (Table 2.1). These are secreted by the activated cell and other blood cells and function in both an autocrine and paracrine manner. Following cell activation, expression of molecules such as the α subunit of the IL-2 receptor are induced and these play an important role in regulating responsiveness to circulating cytokines.

The *in vivo* effects of cytokines are very complex. They also have different effects on different target cells. They induce or inhibit the production of other cytokines, and antagonize or enhance the actions of other cytokines. Cytokines are important in tumour immunity because they modify host responses to malignant cells. Recent advances in recombinant DNA technology which have facilitated large-scale production of cytokines of high purity has established their importance in cancer therapy (Borden and Sondel, 1989). From a clinical perspective most progress has been achieved with IL-2, IFN, and Tumour Necrosis Factor (TNF).

2.3.2 Interferons.

Interferons (IFN) are a family of regulatory glycoproteins produced by many cell types that were originally identified as a response to viral infections (Isaacs and Lindenmann, 1957). They confer resistance upon cells against a variety of viruses. At a cellular level, IFN alter the state of differentiation, rate of proliferation and functional activity of several cell types. This is achieved through the regulation of gene expression, modulation of expression of cell surface proteins and induction of synthesis of new enzymes. These alterations result in modulation of levels of receptors for other cytokines, regulatory proteins on the surface of immune effector cells, and enzymes that modulate cellular growth (Trinchieri and Perussia, 1985). IFN are also capable of enhancing the cytotoxic activity of a variety of host immune effector cells (Borden and Sondel, 1989) including NK activity both *in vitro* and *in vivo* in patients with advanced colorectal cancer (Blottiere et al, 1990).

IFN are extremely heterogenous proteins with considerable species specificity and human IFN was originally tested on human tumours growing as xenografts in nude mice. Three major classes of IFN (α, β, and γ) have been identified on the basis of chemical, antigenic and biologic differences (Kurzrock et al, 1991). Human IFN-α and -β are structurally similar and located on chromosome 9 (Slate et al, 1982; Owerbach et al, 1981), and possess 45% homology of nucleotides and 29% homology of amino acids. IFN-γ has only minimal sequence homology with IFN-α or IFN-β and is located on chromosome 12 while the receptor for IFN-γ located on chromosome 6 has recently been cloned and sequenced (Aguet et al, 1988). Two families of more than 20 chemically related IFN-α proteins have now been identified and a high-affinity receptor for both

Table 2.1 - Human cytokines

CYTOKINE	Alternative name/abbrevation	Subtypes
Interferons	IFN	IFN-α IFN-β IFN-γ
Tumour necrosis factor Lymphotoxin	TNF (TNF-α/Cachectin) LT (TNF-β)	
Interleukin-1 Interleukin-2 Interleukin-3 to 11	IL-1 IL-2 (T Cell Growth Factor) IL-3 to IL-11	IL-1α IL-1β
Colony-stimulating factors	CSF	Multi-CSF GM (granulocyte macrophage)-CSF M (macrophage)-CSF G (granulocyte)-CSF
Transforming growth factor β	TGF	TGF-β1 TGF-β2 TGF-β3 TGF-β1.2

IFN-α and IFN-β has been identified on chromosome 21. IFN-α is mainly produced by leucocytes, IFN-β by fibroblasts and epithelial cells, and IFN-γ by T cells and NK cells. IFN-β was the first example of a specifically designed, mutant protein to be used in clinical trials when it was discovered that the substitution of cysteine for serine by site-specific mutagenesis enhanced its specific biologic activity (Mark et al, 1984).

IFN may exert their antitumour effects by direct regulation of tumour cells, by modifying the host immune response to the tumour, or alternatively by means that do not involve immune responses (Borden and Sondel, 1989). IFN augment the effectiveness of all immune effector cell types that have the potential to kill tumour targets, including, T cells, natural killer (NK) cells and monocytes (Trinchieri and Perussia, 1985). Patients given IFN demonstrate augmented NK and monocyte function 24 to 72 hours after administration of doses 30-fold to 100-fold lower than that maximally tolerated (Edwards et al, 1985; Maluish et al, 1988).

IFN-γ can profoundly alter the function of both benign and malignant cells by regulation of gene expression. This has resulted in the identification of IFN-induced proteins including modulation of HLA class I and class II molecules, and enhancement of expression of tumour-associated antigens (Greiner et al, 1984). IFN may also inhibit the expression of the oncogene *c-myc* (Jonak and Knight, 1984) and thereby modulate cell proliferation. IFN are cytostatic, and occasionally cytotoxic for a wide range of tumour cell lines and primary cell cultures *in vitro* (Borden and Sondel, 1989)). IFN can promote the differentiated state of some tumour cell lines, and an alteration in DNA content in biopsy specimens from patients with carcinoid tumours treated with IFN-α has been demonstrated (Wilander et al, 1986). Local nutritional and angiogenic factors produced by the host are essential in promoting and maintaining tumour growth. Production of factors stimulating angiogenesis has been inhibited by IFN therapy independent of the antiproliferative effect of IFN (Sidky and Borden, 1987).

Large-scale assessment of IFN as treatment for human tumours began in 1979 using a relatively crude IFN-α preparation and activity in inducing disease regression was confirmed in lymphoma (Horning et al, 1985). These results generated sufficient interest to catalyse cloning of the IFN genes and recombinant human IFN were produced by 1982. Historically, the first clinical trial with IFN in neoplasia was as an adjunct to surgery for primary osteosarcoma and final analysis of this trial showed that almost 50% of IFN-treated patients were alive and disease-free at 5 years (Strander et al, 1988).

Anticancer activity by IFN-α, (the most intensively studied IFN) has substantial single-agent efficacy against haematologic malignancies and more than 85% of patients with hairy cell leukemia have objective evidence of partial or complete haematologic response (Quaseda et al, 1988) as do 70% of patients with chronic myeloid leukemia (Talpaz et al, 1989). Results following treatment of most solid tumours with IFN are less encouraging although responses are well documented. Patients with carcinoid tumours also demonstrate a reduction in measured disease as well as improvement in biochemical

parameters (Oberg et al, 1986). Phase II studies of high-dose IFN-α in patients with advanced colorectal carcinoma have not demonstrated any therapeutic response (Silgals et al, 1984). Synergy between IFN-α and 5-fluorouracil has been demonstrated *in vitro* against human colonic cancer cell lines (Wadler et al, 1989) although clinical trials in colorectal cancer patients have reported only modest therapeutic efficacy with minimal toxicity (Wadler et al, 1991).

Two solid tumours for which effectiveness has clearly been established are melanoma and renal cell carcinoma in which treatment with systemic IFN-α results in tumour responses in the order of 20% and 12% respectively (Cregan et al, 1987). IFN-α therapy alone is not of any benefit in colorectal, lung and breast cancers. Responses to intravesical IFN-α in patients with recurrent superficial bladder cancer have been reported (Torti et al, 1988). Although IFN-γ has more potent immunoregulatory activities than either IFN-α or IFN-β its' clinical role remains uncertain (Malik and Waxman, 1992). Aulitzky et al, 1987 treated patients with once weekly, low-dose IFN-γ (0.1mg) and reported tumour regressions in 7 of 14 patients with renal cell carcinoma suggesting that IFN-γ may prove to have significant antineoplastc effects.

2.3.3 Tumour Necrosis Factor.

Two forms of tumour necrosis factor (TNF) have been identified with 30% homology in their amino acid sequence. These are TNF-α (cachectin) and TNF-β (lymphotoxin). TNF-α is the only cytokine that was initially defined on the basis of its direct antitumour activity. Originally identified as cachectin, it is a proinflammatory cytokine that derives its name from its ability to induce haemorrhagic necrosis in tumours (Balkwill, 1989b). It was initially isolated during studies aimed at defining the underlying mechanisms of cancer cachexia (Carswell et al. 1975). TNF-α can result in all the pathological changes associated with endotoxin shock and infection-induced cachexia. The major source of TNF-α is the monocyte while TNF-β is produced by activated lymphocytes. Both forms have antiproliferative, cytostatic, and cytolytic effects against human tumour cells *in vitro* (Ruggiero et al, 1987).

Approximately 30% to 40% of all tumour cell lines are inhibited by TNF-α (Ruggiero et al, 1987; Sugarman et al, 1985). Cellular activation by TNF-α is an important antitumour activity and it is a potent activator of macrophage-mediated tumour cell cytotoxicity (Urban et al, 1986). Human recombinant TNF-α also enhances the cytolytic activity of NK cells (Ostensen et al, 1987). The actions of TNF-α and TNF-β are mediated through a single high-affinity cell surface receptor present on a variety of normal and malignant cells, that binds both molecules with equel affinity (Bharat et al, 1985). IFN-γ can upregulate expression of this receptor, although IFN-γ does not bind directly to it (Tsujimoto and Vilcek, 1986).

Studies with recombinant TNF-α have proved disappointing in humans and overall response rates of less than 5% with significant toxicities have been reported in

phase I and II trials of systemic recombinant TNF-α (Jones and Selby, 1989). TNF-α given intraperitoneally for intractable ascites in patients with ovarian cancer may have a palliative role but does not have a demonstrable antitumour effect (Rath et al, 1991). The limited efficacy of TNF in humans contrasts with animal cancer models and may be due to the limiting toxicity of high dosages needed to induce *in vivo* effects. This emphasises both the practical problems of cytokine therapy over their theoretical advantages and emphasises toxicity as a major factor limiting some forms of immunotherapy (Hill et al, 1992).

2.3.4   Interleukin 2.

Interleukin 2 (IL-2) plays a pivotal role in the regulation of the immune response (Figures 1.2 and 2.1). The potential for IL-2 as a cancer treatment is based not only on the release of other cytokines that may potentially destroy neoplastic tissue but also on the activation of direct cell-mediated cytolysis against tumour cells. IL-2 as a cancer treatment is most importantly associated with an increase in LGL numbers, increase in LGL activity, enhanced IL-2R expression, enhancement of ADCC, and the release of other cytokines notably IFN-γ and TNF-α which modulate cellular cytotoxicity.

IL-2 (initially termed T-cell growth factor) was discovered by Morgan et al. in 1976. Gillis and Smith (1977) utilized IL-2 to demonstrate that murine cytolytic T cell lines could be made to proliferate indefinitely. Natural human IL-2 was initially studied using the IL-2 producing Jurkat T cell line (Welte et al, 1983). Following cloning of the IL-2 gene by Taniguchi et al, (1983) and subsequent expression in *E coli*, large quantities of recombinant IL-2 (rIL-2) became available for study. Unlike IFN and TNF, IL-2 is a group of glycoproteins of 13 - 17.5 kD, coded for by a single gene located on the long arm of chromosome 4 (4q) that is structurally similar to the genes for interleukin-4 (IL-4) and GM-CSF (Rosenberg et al, 1984). The mature IL-2 polypeptide is a 15.5 Kd glycoprotein of 133 amino acids that is derived from a 153 amino acid precursor by the removal of a single peptide sequence. It possesses a relatively restricted range of biological activity, being primarily a regulator of T-cells, from which it is derived and B cells and NK cells.

IL-2 production appears to be restricted to mature T-cells, activated through the TcR complex in the presence of interleukin 1 (IL-1) secreted by monocytes and macrophages. Neither IL-2 nor its mRNA is detectable in T-cells without mitogenic or antigenic stimulation and expression is probably controlled at the level of transcription. IL-2 secreted by activated T_h cells acts in an autocrine manner on T_h cell proliferation and in a paracrine manner on adjacent cytotoxic T cells and other T_h cells. A complex cascade of events regulates both IL-2 production and the IL-2 cellular response depending upon the interaction with a specific cell surface receptor.

Cellular recognition of IL-2 is mediated in humans by a bimolecular receptor complex (Farrar et al, 1990) (Figure 2.2). A 55Kd component (Tac, α chain, or p55)

was identified first (Uchiyama et al 1981a and 1981b) and subsequently identified as the low-affinity IL-2Rα chain (CD25) (Robb and Greene, 1983). It is recognized by an mAb termed anti-Tac (Waldmann, 1986). Normal baseline CD25 expression is found on less than 10% of lymphocytes although CD25 is readily detected on *in vitro* activated T and B cells and has been intensively studied as an activation marker in many clinical situations (Ohashi et al, 1989). With the development of techniques with greater sensitivity CD25 has been detected at low density on approximately 50% of CD4$^+$ T cells and 30% of all lymphocytes in healthy donors (Jackson et al, 1990). Caution must therefore be exercised when anti-CD25 mAb are used in clinical research because the sensitivity of the technique used may vary considerably. Quantitative measurement of antigen levels rather than enumeration of positive cells may be important in clinical situations. The shedding of the α chain results in a soluble IL-2Rα (sIL-2Rα) and correlates with the activation of lymphocytes and the expression of cellular IL-2Rα. It thus provides a serum marker of lymphocyte activation (Rubin et al. 1985; Rubin and Nelson, 1990; Windsor et al, 1991). The α chain is unstable with a half life of only 2-3 hours. However, continual synthesis and rapid breakdown is responsible for continued expression (Sayer et al, 1990).

It was subsequently recognized that certain cells which do not normally express the IL-2Rα including NK cells can respond to IL-2 and subsequently synthesize and express this molecule on their surface (Siegel et al, 1987). This led to the identification of a 75 kD molecule (β chain) that also functioned as an IL-2 receptor (Hori et al, 1988). Only the β chain is constitutively expressed by the majority of large granular lymphocytes (LGL) purified from normal subjects (Dukovich et al, 1987; Phillips et al, 1989) and less than 5% of B and T-lymphocytes (Tsudo et al, 1986). It is not expressed by resting monocytes and granulocytes.

Affinity-binding analysis revealed that the IL-2Rα binds IL-2 rapidly but weakly, whereas the β chain binds IL-2 slowly but with intermediate affinity. In combination the bimolecular α/β IL-2R complex is able to bind IL-2 rapidly and with high affinity (Lowenthal and Greene, 1987). Activated T and B cells express the α/β complex where the α chains are thought to promote presentation of IL-2 molecules to the β chains (Kamio et al, 1990) which are crucial for the signal transduction of IL-2 effects (Ferris et al, 1989). The β chains are then responsible for IL-2 internalization (Robb and Greene, 1987). Once IL-2 is bound to its receptors it is internalized and degraded in lysosomes (Peters and Norback, 1990). IL-2 mRNA can be detected 1 hour following stimulation of the TcR complex and once T cells have been stimulated for 2-3 hours by primary activation signal further synthesis is no longer required for IL-2 dependent proliferation (Lowenthal et al, 1985).

An overall picture of the role of the IL-2R can therefore be presented. Resting antigen-specific T-lymphocytes in the presence of IL-1, synthesize and secrete IL-2 and

Figure 2.2 - Schematic representation of the components of the interleukin 2
receptor.

Bimolecular complex

Low affinity   Intermediate affinity   High affinity
$Kd = 10^{-8}$ M $Kd = 10^{-9}$ M $Kd = 10^{-11}$ M
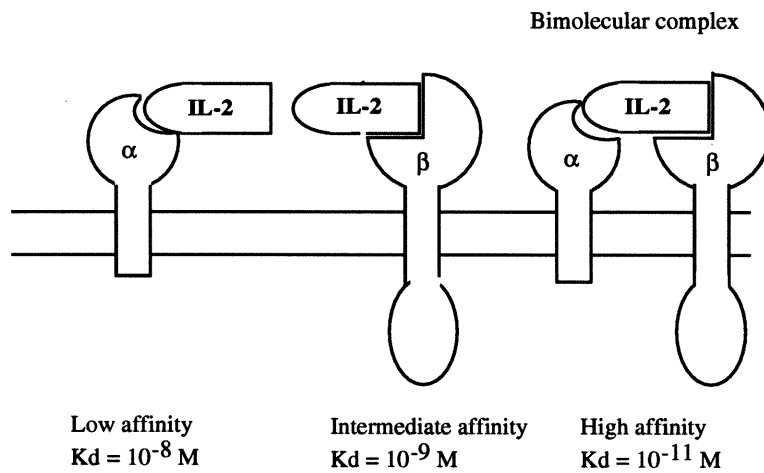
Adapted from Farrar et al, (1991)

IL-2Rα following their activation by antigens presented in association with molecules of the MHC. This enables those cells with the αβ IL-2 receptors to rapidly proliferate in the presence of low concentrations of IL-2. Some of these cells mediate direct cell-mediated destruction of the antigen-bearing target cell. However, as concentrations of IL-2 rise in the vicinity of the immune response, lymphocytes that have not been activated by antigen and expressing only the β chain of the IL-2 receptor (NK cells) are activated and proliferate. Some of these cells mediate direct destruction of transformed cells and others release lymphokines and cytokines including IL-4, TNF and IFN-γ which in turn activate a cascade of antitumour responses.

2.4   IL-2 GENERATED CELLS WITH ANTI-TUMOUR EFFICACY.

The development of immunotherapy with IL-2 was based on experiments *in vitro* showing that effector cells could be generated with enhanced cytolytic activity against tumour cells. Although NK cells can destroy some highly susceptible tumour cell lines *in vitro* without prior sensitization or MHC restriction, most fresh tumour preparations are resistant to resting NK cells. Grimm et al, (1982) demonstrated that following short-term (2-3 day) culture of PBMC from cancer patients with IL-2, a population of cells became activated that were capable of killing fresh primary and metastatic tumours. This reaction was designated lymphokine-activated killer (LAK) cell activity.

The majority of activity can be accounted for by activated NK cells, the progenitors of which are mainly LGL with the CD3$^-$CD16$^+$CD56$^+$ phenotype (Phillips and Lanier, 1986a; Ortaldo and Longo, 1988). LAK should be considered a phenomenon, rather than a new or distinct cell type, with most of the activity attributable to NK cells (Phillips and Lanier, 1986a). The molecules that enable NK cells to bind to and destroy tumour cells have been identified as molecules important in cell adhesion (Sanders et al, 1988) and the same molecules are probably utilized by LAK cells. This is supported by evidence showing that in some situations LAK cells are toxic to normal cells (Damle et al, 1987). These reactions against normal tissues *in vitro* may also account for some of the systemic immune-mediated toxicity that has been observed with prolonged high-dose treatment with IL-2.

A variety of *in vivo* approaches have successfully utilized IL-2 to treat mice bearing immunogenic or nonimmunogenic tumours and have played a critical role in the development of adoptive immunotherapy with IL-2 in humans (Ettinghausen et al, 1986). A summary of this work (Borden and Sondel, 1989) has shown that the greatest anti-tumour effects (prolonged survival or increased cure rates) have been obtained when; IL-2 treatment was given at a time of lower tumour burden; treatment was prolonged over several days or weeks; higher doses of IL-2 were used; IL-2 was administered by a route or schedule to maintain prolonged *in vivo* IL-2 levels.

Tumour infiltrating lymphocytes (TIL) are lymphoid cells infiltrating solid tumours, which can be grown *in vitro* under the influence of IL-2 and offer an alternative

form of adoptive immunotherapy. They comprise expanded populations of CTL rather than NK cells and have been shown to be 50-100 times more potent than LAK cells in destroying murine metastases from sarcoma or colon adenocarcinoma (Rosenberg et al 1986).

2.5   CLINICAL TRIALS WITH IL-2.

Phase 1 clinical trials using high-dose recombinant IL-2 (rIL-2) administered by intravenous bolus injection demonstrated partial and complete responses in a minority of patients with advanced tumours when using IL-2 alone or in combination with adoptively transferred cells (TIL or LAK) (Rosenberg et al. 1987 and 1989). Patients developed 'flu-like' symptoms due both to IL-2 and secondary cytokine release. Patients treated with these high-dose regimens also developed severe and at times life-threatening systemic toxicity due to a vascular leak syndrome and required monitoring in intensive care (Lotze et al, 1986).

Evaluation of these high-dose intravenous bolus regimens has now confirmed that approximately 20% of patients with renal cell carcinoma or metastatic melanoma show objective tumour responses (Rosenberg et al, 1989). These remissions are usually partial and incomplete, but occasionally are prolonged and this latter response may be the true advantage of treatment with IL-2 over conventional therapy (Malik and Waxman, 1992). Toxicity of these high dose bolus regimens remains a significant problem and led to attempts to reduce toxicity by employing IL-2 as a constant intravenous infusion (West et al, 1987). These studies demonstrated similar tumour responses to those with intermittent intravenous bolus therapy and allowed the management of patients on general wards. Attempts to further minimise rIL-2-induced toxicity have included low-dose subcutaneous (sc) therapy (Sleijfer et al, 1992) and exploration of synergy with interferon (Atzpodien et al, 1990) and cytotoxic agents such as dacarbazine (Flaherty et al, 1990). These regimens have demonstrated similar clinical efficacy to high dose administration of IL-2 alone.

The advantages of adoptive immunotherapy with LAK cells and IL-2 over the use of IL-2 alone remains unproven as similar rates of response are seen with both approaches (Rosenberg et al, 1987). LAK cells do not home to tumour sites but sequestrate in the liver and lungs which suggests that they do not play a direct role in tumour regression (Lee et al, 1989). In contrast to LAK cells, TIL do localize to tumour sites when reinfused (Fisher et al, 1989) and 55% response rates have been reported in selected patients with malignant melanoma (Rosenberg et al, 1988). Such an approach may be more suitable for melanoma because the principle effector cell appears to be the CTL rather than the NK cell or LAK cell (Belldegrun et al, 1988; Darrow et al, 1989; Melief, 1992).

The mechanism by which rIL-2 therapy results in tumour regression is poorly understood. Studies have failed to demonstrate a correlation between the therapeutic

efficacy of systemic rIL-2 in advanced cancer and biologic response to rIL-2 as defined
by *ex vivo* cell-mediated cytotoxicity (including LAK cell activity) and expansion of
peripheral blood lymphocytes (Boldt et al, 1988). Factors favouring a clinical response
include a low-tumour burden, high performance status, a baseline lymphocyte count
above $1.4 \times 10^9/L$ and an rIL-2-induced lymphocyte count of at least $6 \times 10^9/L$ when
rIL-2 is administered by intravenous infusion (Moertel, 1986). The limited efficacy of
rIL-2 therapy may be due to the poor penetration of activated NK cells into the tumour
site and the inability of IL-2 to activate endogenous T-cells *in vivo* (Hank et al, 1990;
Weil-Hillman et al, 1991). The value of IL-2 in cancer treatment remains unproven and
the true tumour response rates have undoubtedly proved lower than the 40 - 60% initially
reported (Barr et al, 1990).

It has become clear that cytokines are part of a complex, highly interactive,
intercellular regulator network, most of which induce production of other cytokines. The
prospect of using combinations of cytokines, monoclonal antibodies and conventional
cytotoxics rather than single agents presents a future challenge.

2.6   ANTIBODIES AND CANCER THERAPY.
2.6.1   Basic Antibody Structure and Function.

The basic protein structure of immunoglobulins was established over 30 years
ago. Immunoglobulins in all species have the same basic structure and consist of a light
and heavy polypeptide chain (Figure 2.3). The light chain has approximately 215 amino
acids with a relatively invariant carboxy-terminal half designated the constant region of
which two types have been identified (termed κ and λ), and an NH_2-terminal portion,
designated the variable region. The latter is highly diverse and contains the site for
antigen binding. The heavy chain also has a variable NH_2-terminal portion of 110 amino
acids which is complementary to that of the light chain, and a number of heavy-chain
constant regions (domains), which define the various immunoglobulin classes.
Intrachain disulphide bonds found in both the variable and constant regions of the light
and heavy chains maintain a compact antibody structure.

A short stretch between the first two homologous domains forms a hinge region
that is amenable to cleavage by a number of proteases (Porter, 1959). Papain digestion
results in 3 fractions consisting of two identical fragments designated Fab (fraction
antigen binding) and containing the variable regions, and a single constant
('crystalizable') fragment termed Fc. The Fab fragment is responsible for binding
specificity and the Fc for effector functions. Five classes of antibody IgG, IgM, IgA,
IgD, and IgE have been identified based on structural differences in the constant regions
and are identified by the corresponding Greek letters γ, μ, α, δ, and ϵ. In addition four
subclasses of IgG (IgG1, IgG2, IgG3, and IgG4) have been identified that possess
structurally differing Fc regions and these subclasses have varying biological effector
functions.

Figure 2.3 - Basic antibody structure.

Antigen binding sites

V_H V_L V_L V_H C_H^1 C_L C_H^1 C_L

Disulphide bonds

C_H^2 C_H^2 C_H^3 C_H^3

Fc region (effector functions)
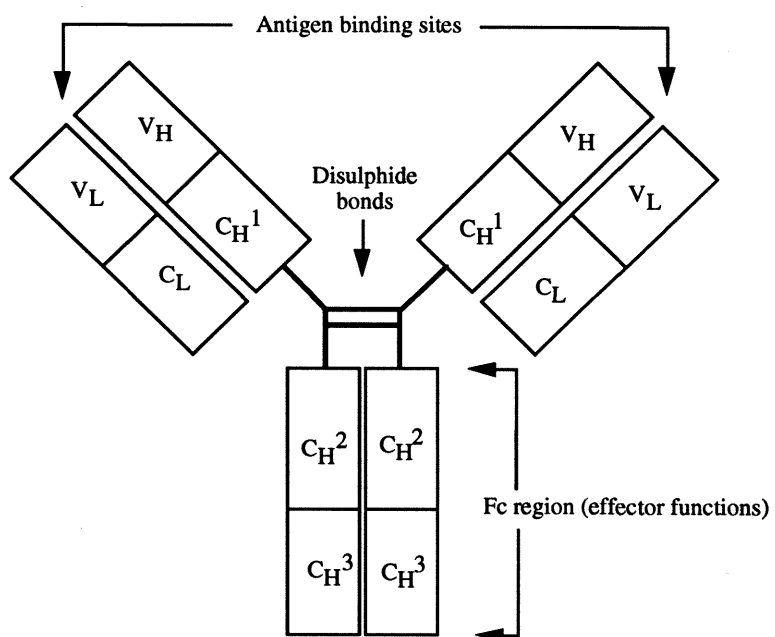
V = variable regions
C = constant regions
H = heavy chain
L = light chain

(From Llewelyn et al, 1992)

2.6.2 Monoclonal Antibodies and Cancer Therapy.

Paul Ehrlich is generally attributed with the concept that tumours express antigens which may provide targets for therapeutic agents ("magic bullets") at the turn of the century (Ehrlich 1909). However, it was the pioneering work of Pressman and Korngold (1953) that conclusively demonstrated that antisera from immunized rabbits contained antibodies with specificity for mouse tumour cells. The antibody response to a typical antigen is highly heterogenous. When an animal is immunized with an agent that elicits an immune response, diverse antibody molecules are produced that recognize different epitopes on a single antigen. Conventional antiserums therefore contain mixtures of antibodies, although each antibody is made from a single line of lymphocyte and its derived plasma cell. Myeloma cells demonstrate immortality and secrete an immunoglobulin of one type although the specificity cannot be manipulated. George Kohler and Cesar Milstein's discovery in 1975 centered on the fusion of mouse myeloma cells with spleen cells from an immunized mouse to produce a hybrid-myeloma or "hybridoma" cell which expressed the myelomas property of immortality and the lymphocytes property of specific-antibody production.

Clones that secrete a single molecular species of the desired antibody can be maintained in culture or frozen and stored for future use. The clone can be injected into animals of the same strain, which develop tumours secreting the specific monoclonal antibody, which is then present in the serum in high concentrations; usually more than 10 milligrams of antibody per ml of serum may be retrieved. Alternatively a sample of the clone can be grown in a mass culture and the antibody harvested from the medium.

The use of monoclonal antibodies in the immunotherapy of cancer has so far proved disappointing. The first example of successful serotherapy using an mAb was reported by Miller (1981) who treated a patient with an aggressive, poorly differentiated B-cell lymphoma using an anti-idiotype Mab which resulted in a durable complete response. The most extensively studied mAb used for the treatment of a visceral malignancy is the mouse anti-colon-cancer Mab CO17-1A and early trials have resulted in an occasional clinical response (Verrill et al, 1986). However, there were only 23 partial and 3 complete remissions reported among the initial 185 patients included in 25 clinical trials incorporating mAb (Catane et al, 1988). Only one monoclonal antibody, OKT3, has been licensed for clinical use. The mechanisms now considered to be most important in mAb therapy, involve the targeting of cytolytic effector cells to tumour cells or, alternatively the use of antibodies conjugated with drugs and toxins to deliver specifically these agents to the tumour site.

A combination of factors are probably responsible for the low therapeutic efficacy observed in clinical trials and include, immunogenicity of the antibody, lack of participation in human effector functions, lack of specificity for tumour targets (most human tumours are polyclonal, notably those that are poorly differentiated), and restricted

movement between the intravascular compartment and penetration of large tumour masses
(Russell et al, 1992a).

The following factors must be considered when antibody therapy is undertaken :

- Toxicity
- Binding to target cells
- Antitumour effect
- *In vivo* specificity
- Mechanism of cellular effects
- Bioavailability
- Free antigen
- Antigenic modulation/immunoselection
- Endogenous anti-mouse antibody
- Clinical Efficacy

Infused antibodies are diluted almost immediately in plasma and subseqently
diffuse more slowly across the walls of small blood vessels into the interstitial fluid
(distribution phase). The half life of circulating antibody is determined by the rate at
which it is metabolised and excreted (elimination phase). The degree to which the target
antigen is bound varies with the exposure time, the concentration, and the kinetic
properties of the mAb.

The percolation of antibodies into tumours is a complex yet critical issue
(McCready et al, 1988). The percentage of specific delivery of antibody to antigen-
bearing tumour sites versus "nonspecific" antibody retention in normal tissues following
intravenous injection has been calculated at only 0.0007% to 0.01% (Carrasquillo, 1988).
Systemic delivery of antibody which results in its percolation at a significant
concentration throughout a poorly vascularized tumour nodule remains a major problem
in treating patients with metastatic carcinoma (Ong and Mattes, 1989). MAb have good
access to the tumour surface but poor access to the tumour parenchyma where blood
vessels are less permeable (Dvorak et al, 1991). Other physiological barriers responsible
for poor localization include a heterogenous blood supply, elevated interstitial pressure,
and large transport distances in the interstitium.

Patients treated with murine monoclonal antibodies have been shown to develop
human antimouse antibody (HAMA) responses in the majority of cases which potentially
limits therapy with murine mAb (Sears et al, 1985). HAMA may be directed against the
mAb idiotype as well as the isotype (Koprowski et al, 1984). The issue is complicated
by the fact that up to 60% of HAMA may represent anti-idiotype (Koprowski et al,1984).
Anti-anti-idiotype antibodies have been postulated as having potential antitumour activity
in their own right (Herlyn et al, 1986). Idiotypic monoclonal antibodies to IgG have been

used for treatment of patients with B cell lymphomas (Miller et al, 1982; Mecker et al, 1985) with encouraging results.

These problems limit the therapeutic potential of unmodified mAb. Advances in biotechnology have however, resulted in genetically engineered antibodies with enhanced therapeutic properties as well as new effector functions through conjugation with drugs, toxins, and radioisotopes and have improved the potential of mAb therapy (Winter and Milstein, 1991). The success of mAb in clinical practice is dependent on good design (Russell et al, 1992a) as described below.

2.6.3 Designer Antibodies.

For therapeutic applications, antibodies derived from human lymphocytes would be preferred. Unfortunately attempts to immortalize antibody-producing human cells by fusing them with mouse or rat myeloma cells results in unstable interspecific hybrid cells with preferential loss of human chromosomes. Immortalization of human cells by Epstein-Barr virus has therefore been attempted, but leads to unstable lines which are low producers of antibody (Roder et al, 1986). Human lymphocytes have been used to populate severe combined immunodeficient (SCID) mice which are then immunized to make human antibodies (McCune et al, 1988; Mosier et al, 1988), although so far such antibodies have not been used in human clinical trials. Designer antibodies are aimed at reducing immunogenicity, prolonging survival, and increasing the capacity to recruit effector functions.

Attempts to improve the effector functions of antibodies and reduce immunogenicity have focussed on the 'humanization' of rodent antibodies by linking human constant regions to rodent variable regions (Neuberger et al, 1985), to produce a so called chimaeric antibody. A murine human chimaeric IgG1 antibody specific for a gastrointestinal tumour antigen elicited an antibody response in only 10% of patients (LoBuglio et al, 1989). A 'humanized' monvalent anti-CD3 mAb has also been developed which can activate homologous complement using the same technique (Routledge et al, 1991). Unfortunately, residual immunogenicity is at least partly retained by virtue of the variable regions (Bruggemann et al, 1989). Attempts to overcome the problem of residual immunity due to the foreign V-region framework have involved the 'humanization' of mouse antibodies by grafting only the antigen-binding loops to human antibody to produce a reshaped antibody (Verhoeyen et al, 1988). Prolonged survival of 'humanized' antibodies has also been reported by LoBuglio et al, (1989). In the future bacteriophage clones may be used to produce human mAb of sufficiently high affinity for clinical studies (Russell et al, 1992b).

Effector functions can be recruited by generating bispecific antibodies whose antigen-binding sites have dual specificity for a target cell and host cytotoxic cells. This can be achieved by making a "hybrid hybridoma" by fusing hybridomas of two-different specificities (Suresh et al, 1986). For example bispecific mAb specific for antigen and

also specific for the CD3 antigen on cytotoxic cells or the FcγRIII (CD16) receptor on natural killer cells may recruit these effectors to the tumour site. Such bispecific antibodies with specifity between these receptors and tumour targets are effective in mediating the killing of tumour targets *in vitro* and *in vivo* and a universal bispecific antibody for retargeting CD3+ effectors to a range of tumour cells, each coated in rat monoclonal antibodies has been generated (Gilliland et al, 1988).

An alternative to producing bispecific antibodies using hybrid-hybridomas is to manufacture bifunctional antibodies by combining two monoclonal antibodies at their isotype using a disulphide bond. An antibody heteroconjugate can then be produced with specificity for a tumour target and an antigen on the surface of the CTL. Jung and Eberhard, (1988) have demonstrated that by incubating two such antibody heteroconjugates (with specificity for a tumour antigen and also specificity to CD3 and CD28 on the effector cell), with a culture of tumour cells and CTL, tumour cell killing is enhanced. Both antibodies are required to stabilise the conjugate and activate the T cell. Human T cells retargeted by bifunctional antibodies have been successfully used to treat established human ovarian carcinoma in a nude mouse model (Garrido et al, 1990).

2.6.4 MAb Therapy and Fc Receptor Interactions.

Binding of the Fc fragment of immunoglobulin to the surface of haematopietic cells and tumour cells has important consequences for directing killing, phagocytosis and lymphocyte activation. Fc receptors also have considerable importance in immunoglobulin therapy because they affect antibody kinetics and have important consequences for antibody design. It is now known that there are three distinct FcγR designated FcγRI (CD64), FcγRII (CDw32), and FcγRIII (CD16) which belong to a heterogenous group of glycoproteins (Ravetch and Kinet, 1991; Schreiber et al. 1992). FcγRI and FcγRII are found on freshly isolated monocytes whereas FcγRIII is also present on tissue macrophages, *in vitro* cultured monocytes, neutrophils and NK cells (Schreiber et al, 1992). Looney et al, (1986) isolated the 40kDa FcγRII IgG membrane binding protein from the erythroblastoid cell line K562 and also from the B cell line Daudi and Raji.

A characteristic of human Fc receptors which has considerable experimental and therapeutic importance is their subclass specificity for mouse IgG. The binding affinity of FcγR varies with IgG subclass both for human and murine IgG. FcγRI binds human IgG1 and IgG3 with higher affinity than subclasses IgG2 and IgG4 and murine IgG2a = IgG3 > IgG1 > IgG2b (Alegre et al, 1992). This accounts for variation in the mitogenic properties of anti-CD3 mAb. Murine IgG2a and IgG3 are mitogenic for all individuals whereas IgG1 and IgG3 are mitogenic for only 70% and 5 to 10% respectively (Alegre et al, 1992). Murine IgG2a is more effective in directing ADCC by human monocytes and macrophages (Steplewski et al, 1983; Imai et al, 1982). Kipps et al, (1985) demonstrated, using switch variant hybridoma cells to produce antibodies of identical

idiotype but variable isotype, that the IgG2a isotype was more effective than IgG2b and that IgG1 was completely ineffective in directing ADCC by human NK cells against a B lymphoblastoid target. This is because IgG2a binds more readily to FcγRIII than IgG2b,and IgG1 has little or no affinity.

The FcγRII has greater affinity for mouse IgG1 than Mouse IgG2b as evidenced by the finding that this 40kDa molecule mediates rosette formation with erythrocytes bearing mIgG1 as well as T cell stimulation by mIgG1 anti-T3 (Looney et al, 1986). An antibody raised against this 40kDa molecule blocked mIgG1 responses to mIgG1 OKT3 but not mIgG2a OKT3. FcγRII has an important role in the human T-cell proliferative response to mouse IgG1 or IgG2a anti-CD3 mAb (Anderson et al, 1987). The high affinity FcγRI on monocytes is probably responsible for the IgG2a response whereas monocyte FcγRII mediates the IgGI response (Looney et al, 1986). A variable expression of FcγRII is present on human monocytes. In 30% of normal individuals and IgG1 anti-CD3 mAb fails to stimulate T-cell proliferation (Tax et al, 1984). Racial factors are also important since although the majority of caucasions mediate IgG1 anti-T3 responses only a small minority of Asians do so (Abo et al, 1984).

Small substitutions in amino acid sequences of mAb isotypes have significant consequences for *in vivo* effects. The CH2 domain of IgG selectively binds FcγRI and FcγRII and a "humanized" OKT3 mAb that retains its' immunosuppressive properties with greatly reduced lymphocyte activation following substitution of only a single amino acid has been developed (Alegre et al, 1992). 'Designer antibodies' may therefore be developed to either optimise lymphocyte activation which is associated with cytokine release or alternatively reduce activation and lessen toxicity depending upon the clinical situation. Anti-CD3 mAb illustrate the potential diversity of function.

2.7   OKT3 AND ANTI-CD3 MONOCLONAL ANTIBODY THERAPY.
2.7.1   Properties of Anti-CD3 mAb.

Following the development of the hybridoma technique Kung et al, (1979) generated several monoclonal antibodies (designated OKT1, OKT2, OKT3, and OKT4) against cell membrane determinants of human peripheral T lymphocytes. OKT3 is a murine mAb of IgG2a subclass that reacts with the CD3 complex of the TcR through which it exerts its effects. The OKT series of mAb was initially used as markers to differentiate T cell subsets and T cells from other peripheral blood cells by virtue of the restricted expression of their respective antigens. It was only later that the potential role of OKT3 as a modulator of T cell function became apparent. Other mAb reactive with CD3 include Leu 4, and UCHT-1, and all anti-CD3 mAb have diverse effects on T cell function (Tsoukas et al, 1984). Apart from T cells, only Purkinje cells in the cerebellum are known to specifically bind OKT3 (Garson et al, 1982).

The majority of anti-CD3 mAb react with the CD3 ε subunit (Transy et al, 1989). The biologic effects of anti-CD3 mAb include the induction of proliferation and activation

of resting T cells and inhibition of the response of activated T cell clones to specific antigen (Tsoukas 1984). The effect of anti-CD3 mAb via the TcR-CD3 complex on mediating T cell proliferation, may be agonistic or antagonistic depending upon the experimental conditions. These seemingly paradoxical effects are dependent upon complex biological variables including the type of antibody used, its concentration, the *in vitro* situation and the nature of both target and effector cells. The agonistic effects require the immobilization of antibody, whereas the antagonistic effects are mediated by soluble monomeric antibody. Immobilization may be achieved either by the addition of monocytes (Van Wauwe et al, 1980; Chang et al, 1982; Ceuppens et al, 1985), or by immobilising the antibody on sepharose beads or on the culture plate surface (Piehler et al, 1987).

Anti-CD3 mAb may inhibit T cell functions as demonstrated by CTL-mediated cell lysis, and *in vitro* generation of CTL following allogeneic stimulation in mixed lymphocyte culture (Chang et al, 1981). This effect occurs at a similar concentration to that inducing non-specific activation (1000 ng ml^{-1}). Anti-CD3 mAb block antigen-specific T cell recognition by modulation of the TcR antigen interaction (Reinherz et al, 1980).

Soon after its discovery OKT3 was also shown to be a potent mitogen at subnanomolar concentrations (Van Wauwe et al, 1980) and to induce cytokine secretion (Chang et al, 1982). IL-2, IFN, and TNF are all released by T cells *in vitro* upon activation by anti-CD3 mAb (Weiss et al, 1986; Turner et al, 1987; Sung et al, 1988; Steffen et al, 1988; Stankova et al, 1989). Stabilization of mAb for anti-CD3 antibody-dependent T cell activation is acheived by the presence of monocytes or other substrate to facilitate crosslinking of antibody (Van Wauwe et al, 1980; Ceuppens et al, 1985; Rinnooy et al, 1986; Piehler et al, 1987; Stankova et al, 1989). As has been discussed mitogenic potencies of anti-CD3 mAbs *in vivo,* vary with the mAb isotype as murine mAbs of different isotypes possess varying affinities for human FcγR (Ravetch and Kinet, 1991) and it is the distinctive preferences of FcγRI for murine IgG subclasses that underlies the capacity of monocytes to mediate IgG2a anti-CD3 lymphocyte activation. Antibody binding to Fc receptors is also inhibited by competition with other antibodies present in human serum which may modulate the process of OKT3 presentation *in vivo* (Van Wauwe et al, 1980).

Analysis of early T cell activation, proliferation and lymphokine production (TNF-α, IFN-γ, and GM-CSF) have also confirmed that mAb isotype exerts a profound effect on activation potency (IgG2a> IgG1>IgG2b) and that epitope specificity is of only minor influence (Woodle et al, 1991a). With respect to the development of chimaeric antibodies the human γ1 (IgG1) isotype is highly effective in both complement and cell-mediated killing, and is the most suitable class of antibody for therapeutic use against tumour cells (Bruggemann et al, 1987; Riechemann et al, 1988; Steplewski et al, 1988).

Differences in early activation events have also been found between antigen and anti-CD3 antibodies (Sussman, 1988). Resting T cells differ in their activation requirements from T cells that have been previously stimulated. Resting T cells require immobilized anti-CD3 antibodies and additional signals, notably IL-1 (Manger et al, 1985) or antibodies to other cell surface determinants (CD2, CD5, or CD28) (Weiss and Imboden, 1987). Previously activated T cells require only immobilized anti-CD3 antibodies (Meuer et al, 1983; Manger et al, 1985).

Anti-CD3 mAb do not have a direct effect on NK cells which do not express the CD3 antigen. Stankova et al, (1989) demonstrated in the mouse that spleen lymphocytes can develop non-specific cytolytic activity after preincubation with soluble anti-CD3 antibody that is not dependent on the continued presence of antibody. This may occur because of direct activation of CTL capable of non-specific cytotoxicity and also secondary activation of NK cells by lymphokines produced by anti-CD3-stimulated T cells which include IL-2 and TNF-α and β. They do however enhance non-specific cytotoxicity against cultured tumour targets whilst blocking specific cytotoxicity in antigen specific T cell clones (Spits et al, 1985; van Seventer et al, 1987). Activated CTL or *in vitro* propagated CTL lines can be triggered to lyse "inappropriate targets" in the presence of anti-CD3 antibody (Leeuwenberg et al, 1985; Spits et al, 1985; Mentzer et al, 1985). The mechanism involves direct antibody mediated linkage of the T effector cell and Fc receptors on the target cell. Fc receptor negative targets are not killed (Van de Griend et al, 1987).

These mechanisms are shown in Figure 2.4 and are compared with the mechanism of tumour cell killing using antibody heteroconjugates targeted against CD3 and tumour antigens (Jung and Eberhard, 1988). Similarities exist between the mechanism involved in redirected killing by anti-CD3 and heteroconjugates. Both depend upon activation of the T cell brought about by a stable anti-CD3 interaction and the conjugation of effector cells and target cells. The presence of FcIIγR on K562 and Daudi targets allows crossbridging of effectors and targets through Fc interactions (Staerz and Bevan, 1985). Anti-CD3 mAb-mediated cytotoxicity has however, been reported against targets that do not express Fc receptors (Loughran et al, 1987; Suthanthiran et al, 1984) therefore other mechanisms must also be involved.

Reports from our department have also shown that preincubation of freshly isolated PBMC with anti-CD3 mAb for 30 minutes prior to the addition of K562 targets, results in enhancement of cytotoxicity in four hour ^{51}Cr-release assays (Ubhi et al, 1991). Antibody was present throughout the assay and the mechanism is again likely to be due to crosslinking of CD3$^+$ cells to targets bearing Fc receptors.

Figure 2.4 - Comparison between the mechanisms by which anti-CD3 mAb enhance cytotoxicity and the mechanism of activation and redirected killing using combinations of antibody heteroconjugates.
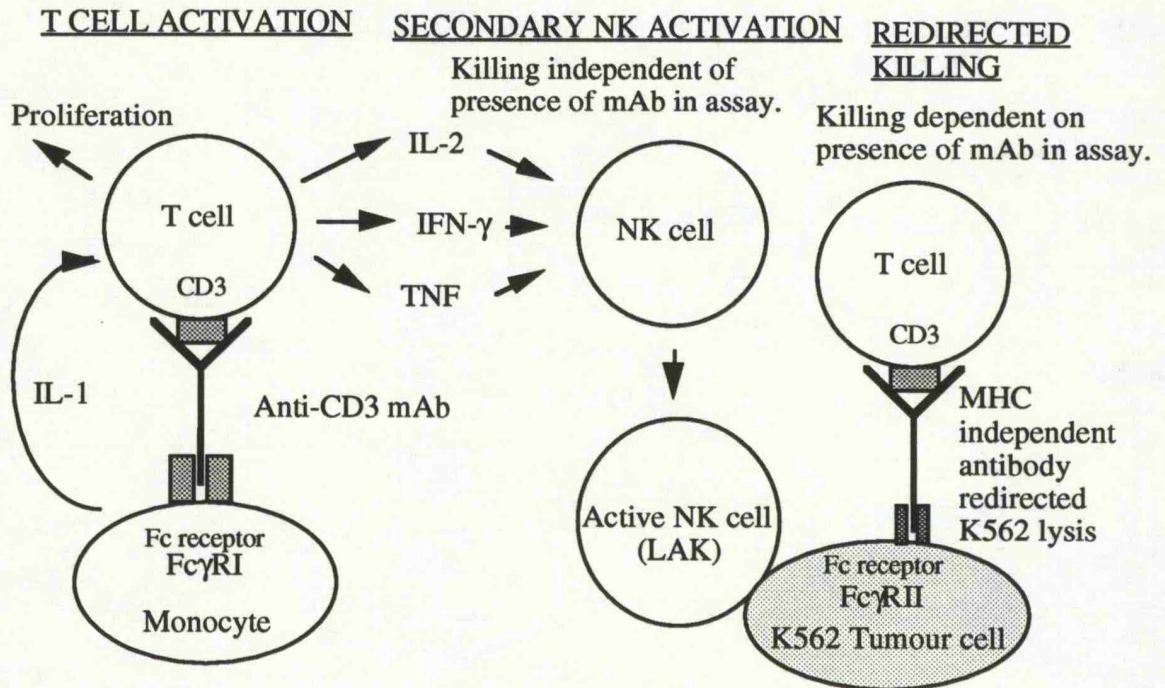
MONOCYTE-INDEPENDENT T-CELL ACTIVATION USING COMBINED ANTI-TARGET- ANTI-CD3 AND ANTI-CD28 HETEROCONJUGATES
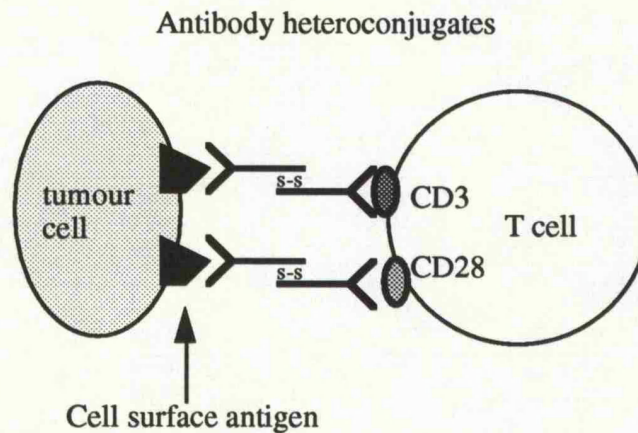
From Jung and Eberhard, 1988

2.7.2 *In Vivo* Effects of Anti-CD3 mAb.

The *in vivo* effects of anti-CD3 mAb are likely to be extremely complex and not easily predictable from *in vitro* studies. Initially OKT3 was successfully used in the treatment of renal allograft rejection (Cosimi et al, 1981). It has subsequently been confirmed as a potent immunosuppressive agent in the treatment of corticosteroid-resistant renal, hepatic, pancreas, and cardiac allograft rejection (Ortho Multi-Center Transplant Study Group, 1985). The majority of the present data regarding the *in vivo* effects of OKT3 therefore relate to its use in regimens involving 5 mg intravenous bolus doses administered for between 10 and 14 days in transplant recipients where OKT3 successfully reverses rejection in 94% of cases (Ortho Multi-center Transplant Study Group, 1985).

OKT3 24-hour trough plasma levels were first reported by Goldstein et al, (1986). One hour following 5 mg of OKT3 levels were 1002 ng ml^{-1} which dropped to 104 ng ml^{-1} at 24 hours. This is due to the rapid binding of OKT3 to its target (CD3) on T cells and subsequent removal from the circulation. OKT3 levels thereafter increase steadily during the first three days to reach a steady state level of 902 ($\pm$ 71 ng ml^{-1}) which was maintained for the duration of therapy (usually 10 to 14 days). Levels then fell rapidly within three days of discontinuation. *In vitro* studies and clinical findings support the concept that the dose required to maintain immunosuppression is of the order of 1000 ng ml^{-1} (Chang et al, 1981).

Considerable data is now available regarding the immunological effects of high doses of OKT3 used in the treatment of allograft rejection. Neutrophilia and a profound lymphopaenia occurs within two hours of OKT3 administration to humans (Gaston et al, 1991). This finding is identical to that following infusion of recombinant TNF-α to rats although lymphocyte counts returned to normal by 24 hours (Ulich et al, 1989). TNF-α is typically produced by stimulated monocytes and macrophages (Halloran et al, 1989) although T cells are also capable of TNF-α release (Sung et al, 1988). *In vivo* administration of OKT3 induces a dramatic decrease in the number of circulating CD3$^+$ cells (Cosimi et al, 1981; Chatenoud et al, 1982). Binding of OKT3 may result in shedding or endocytosis of the TcR into the cells involved (Reinherz et al, 1982). Cells expressing CD2, CD4, and CD8 are depleted in similar fashion but re-expression of these antigens occurs after several days of therapy (Hooks et al, 1991). OKT3 is also associated with a decrease in the density of expression of CD4 and CD8 antigens despite its specificity for CD3.

The mechanism underlying this rapid initial lymphodepletion has not been deciphered but the massive lymphocyte lysis that was originally reported seems not to occur. It may involve margination due to the induction of adhesion molecules upon TcR-mediated activation (Dustin and Springer, 1989) and because OKT3 also initially opsonizes circulating T cells they may also be removed by the reticuloendothelial system (Miller et al, 1982). Released cytokines such as TNF-α may also contribute to

lymphocyte depletion as discussed above. Lymphocyte depletion is not a major contributory mechanism in immunosuppression, since mAb that induce removal of T cells from the circulation do not induce similar rates of reversal of rejection (Goldstein et al, 1986). Modulation of the TcR complex together with TcR blockade probably plays a major role in the mechanism of inhibition of cell-mediated cytotoxicity by anti-CD3 mAbs (Reinherz et al, 1982; Chatenoud et al, 1982). Studies with OKT3 F(ab')$_2$ fragments of anti-human CD3 mAb (Woodle et al, 1991b) and anti-mouse CD3 mAb (Hirsch et al, 1990) demonstrated that minimal T cell proliferation occurs in the absence of multivalent TcR crosslinking by macrophage FcR . It has been shown that whole mAb and F(ab)$_2$ fragments both effectively coat the TcR. However, whole mAb was more efficient at modulating the TcR confirming that modulation is enhanced by FcR interactions (Woodle et al, 1991a). Experiments in mice suggest that immunosuppression by F(ab')$_2$ fragments does not relate to the degree of T cell depletion which is similar to that seen with the whole mAb (Hirsch et al, 1990). However, treatment with whole mAb induces a profound and prolonged immunosuppressive state during which CTL dysfunction persists even following reexpression of the TcR (Hirsch et al, 1988). It is possible that T cell activation may play a role in this subsequent T cell dysfunction, since there is evidence suggesting that activation of T cells can induce a prolonged dysfunctional state (Jenkins and Schwartz, 1987).

The administration of the initial dose of OKT3 to transplant recipients is characteristically accompanied by significant side-effects in 50 to 80% of patients (Ortho Multicenter Study Group, 1985; Thistlethwaite et al, 1988; Hooks et al, 1991). These typically involve some combination of fever, chills, dyspnoea, wheezing, tachycardia, hypotension, and nausea and vomiting ('influenza-like' symptoms). Typical symptoms usually occur within 1 to 2 hours of injection and are restricted to the first or second dose. They are similar to the side-effects observed with administration of IL-2 (Rosenberg et al, 1985). This is supported by the finding that high serum levels of TNF-α are detectable, peaking at one hour and IL-2 and IFN-γ peaking at two hours are also detectable following OKT3 administration (Chatenoud et al, 1989; Abramowicz et al, 1989). Cytokines are undetectable following subsequent doses when symptoms are less pronounced. Some investigators have correlated the levels of TNF-α at two hours following the first dose of OKT3 with severity of symptoms (Gaston et al, 1991). The first-dose reaction may therefore, be more appropriately referred to as a 'cytokine release syndrome'. Occassionally patients develop a meningitis-like symptom complex involving fever, severe headache and neck stiffness which resolves spontaneously in two to three days regardless of continuing therapy (Emmons et al, 1986).

The development of host antibodies to both the OKT3 isotype and idiotype have the potential to neutralize the therapeutic effect of OKT3 (Jaffers et al, 1986). The frequency of the HAMA response is related to concomitant immunosuppressive therapy (Goldstein et al, 1986). One approach toward optimization of anti-CD3 mAb therapy

involves the use of anti-CD3 mAb of defined idiotype, isotype and epitope specificity, so that the desired T cell activation and suppression properties are obtained and neutralization by anti-idiotypic antibody is avoided.

In vivo murine studies with whole anti-murine CD3 mAb and F(ab')$_2$ fragments have demonstrated a marked decrease in activation potency relative to whole mAb and also a decrease in the humoral response to the xenogeneic anti-CD3 mAb while maintaining their immunosuppressive effects in vivo (Colonna et al, 1987; Hirsch et al, 1990). Selection of anti-CD3 mAbs with defined T cell activation potencies and defined idiotype may also provide a viable means for enhancing anti-CD3 mAb therapy for immunopotentiation in tumour therapy.

2.7.3   Potential of Anti-CD3 mAb in Cancer Immunotherapy.

Significant problems are associated with IL-2 therapy which include toxicity and a failure of most tumours to respond. The reason for the poor tumour response rates may be due to poor penetration and localisation of effector cells at the tumour site and a failure of IL-2 to specifically activate CTL with anti-tumour activity. The concept that LAK cells are responsible for direct in vivo antitumour effects is difficult to reconcile with their inability to home to tumour sites and the finding that TIL contain relatively few NK cells which are regarded to be LAK cell precursors. LAK cells derived from both PBL and TIL have been used for adoptive immunotherapy in mice and humans. Because large numbers of lymphocytes are required for adoptive transfer in immunotherapy protocols it is frequently difficult to obtain sufficient cells from cancer patients (particularly TIL).

With regard to adoptive immunotherapy, the mitogenic properties of anti-CD3 mAb have been investigated as both capable of inducing lymphocyte proliferation and influencing the cytotoxic potential of LAK cell cultures. Anderson et al, (1988 and 1989b) used anti-CD3 to generate large numbers of TIL with anti-tumour efficacy in mice. Ohta et al, (1991) have studied the in vitro effect of OKT3 on PBL, TIL, and regional lymph node lymphocytes. OKT3 induced significant and rapid proliferation of lymphocytes from all three sources. The disappearance of tumour cells in the TIL preparations at a faster rate than in unstimulated cultures suggested that cytotoxicity was enhanced in this population.

However, the role of anti-CD3 in the generation of LAK activity remains controversial. Most reports suggest that when cytotoxicity is expressed as LU per culture OKT3 enhances cytotoxicity of PBL derived LAK cells (Anderson et al, 1988 and 1989b). Percentage cytotoxicity of PBL, TIL, and RLNL was noted to decrease against K562 and Daudi targets compared with unstimulated groups (Ohta et al, 1991). However these workers also demonstrated that OKT3 enhanced NK activity in terms of total LU per culture and this may be more important with respect to adoptive immunotherapy. The reason for these discrepancies is because OKT3 culture leads to preferential expansion of CD3+ CTL and decreases the percentage of CD16+ and CD56+ NK cells. Moreover the

problems associated with adoptive immunotherapy namely expense, technological expertise, and toxicity, are still highly relevant in the routine clinical setting.

Evidence suggests that T cells may be important in mediating anti-tumour effects, particularly in melanoma (Griffith et al, 1989; Balch et al, 1990; Itoh et al, 1988). This is supported by the findings that CTL are the predominant cell population in melanoma TIL and when TIL are adoptively transferred to melanoma patients they migrate to the tumour site. Prior to and following therapy with IL-2 and *in vitro* expanded populations of TIL, T cells rather than NK cells are predominantly located at the tumour site (Griffith et al, 1989; Balch et al, 1990; Melief, 1992). Class I MHC restricted cytotoxic T lymphocytes (CTL) can play an important role in tumour eradication and it is from IL-2 responsive tumours, including melanoma, that it has been possible to cultivate both autologous tumour-specific CTL from peripheral blood and tumour-infiltrating lymphocytes (TIL). The CTL among TIL, may be the mediators of responses seen among TIL and rIL-2 therapy of melanoma (Melief and Kast, 1991a and 1991b; Melief 1992). In contrast non-MHC-restricted PBL may be the more important effector cell in other tumours such as those of gastrointestinal origin which frequently lack MHC class I antigens (Durrant et al, 1987).

However, IL-2 appears to be unable to activate effectively endogenous T cells *in vivo* since circulating T cells obtained from patients following IL-2 therapy have been shown not to respond to further IL-2 *in vitro* (Weil-Hillman et al, 1989 and 1990) and are incapable of lysing tumour targets in a non-MHC-restricted manner (McMannis et al, 1988; Weil-Hillman et al. 1989 and 1990). These T cells also demonstrate decreased responses to mitogens and alloantigens following IL-2 therapy (Hank et al, 1990).

The development of protocols that activate cytolytic effector cell populations *in vivo* is desirable because of the expense and technical expertise which is required for adoptive transfer of large numbers of *ex vivo* activated cells. Evidence that anti-CD3 mAb activate T lymphocytes *in vivo* has only recently been presented (Ellenhorn et al, 1990a and1990b). Hirsch et al, (1989) demonstrated increased IL-2R expression on T cells and production of colony stimulating factor within two to three hours of injection of anti-CD3 into mice. Recent evidence also suggests that T cells can be activated *in vitro* by anti-CD3 mAb together with IL-2, which results in upregulation of the IL-2Rα chain and subsequent proliferation (Weil-Hillman et al, 1991). These studies demonstrated that T cell are not irreversibly inactivated by IL-2 *in vivo*. These findings taken together with the previous human and murine studies suggest that activation *in vivo* with anti-CD3 and IL-2 might enhance both T cell and NK cell function especially if anti-CD3 is administered prior to IL-2.

Despite the numerous reports of *in vitro* activation of lymphocytes by anti-CD3 mAb, relatively little work has been undertaken into the therapeutic potential of these effects *in vivo* against cancer. Ellenhorn et al, (1988) used weakly immunogenic fibrosarcoma (PRO4L) mouse model to demonstrate that intraperitoneal injection of low

doses of the anti-CD3 mAb 145-2C11 protected against the development of implanted skin tumours. This effect was very specific for the dose of anti-CD3 administered. Whereas 4 μg was protective, 40 μg and 400 μg had no effect and tumour growth was potentiated by the 400 μg dose. Interestingly animals treated with 4 μg also developed specific lasting immunity to subsequent challenge with PRO4L. This suggested that T cells are involved in tumour rejection. Although anti-CD3 induced IL-2Rα expression on lymphocytes retrieved from lymph nodes in all animals, and these lymphocytes demonstrated enhanced proliferation *in vivo* to IL-2, the overall effect of higher doses of anti-CD3 was immunosuppressive. It was concluded that the optimal dose of anti-CD3 required to induce protection against tumour cells was one which was high enough to induce lymphocyte activation without the immunosuppressive properties caused by TcR modulation and blockade.

Subsequent studies using *in vivo* T cell depletion have demonstrated that the effector cells responsible for tumour protection in this model are indeed classical CD4^{+} helper and CD8^{+} cytotoxic T cells with αβ TcR (Ellenhorn et al, 1990a). Although non-specific cytolytic effector cells from the spleens of treated mice showed enhanced cytolytic acticivity *in vitro* they were not primarily responsible for mediating the antitumour effect *in vivo* since their depletion did not adversely affect rejection. It could not be determined whether anti-CD3 activated CTL directly or through the secondary release of lymphokines.

Similar studies have demonstrated that intravenous and intraperitoneal injections of 5 μg of 145-2C11 mAb induce at least three populations of effector cells in mice *in vivo* which are MHC-restricted CTL, activated NK cells, and LAK cells (Hoskin et al, 1989). Treatment was associated with a reduction in lung metastases from a *ras*-transfected syngeneic fibroblast cell line. The intravenous route was more effective than the intraperitoneal route. Although anti-CD3 induced T cells as well as activated NK and LAK cells these studies did not confirm which cells were predominantly responsible for the anti-tumour effect. These workers suggested that anti-CD3 treatment may have advantages over IL-2 alone which activates principally non-specifc effector cells. Anti-CD3-induced natural killer cells undoubtedly have an important role in protecting against viral infections in mice (Kast et al, 1990) although their cytolytic effect against tumours in these models remains to be fully defined. NK cell activity may be enhanced by the release of TNF-α from anti-CD3-activated T cells. This is particularly relevant to humans as TNF-α is known to be a potent activator of human NK cells (Ostensen et al, 1987).

In contrast to high dose OKT3 transplant rejection therapy little information is available regarding the optimal dose or mode of administration of anti-CD3 mAb that might result in immune enhancement in humans. Only one study has been reported to date using anti-CD3 mAb as a potential immunostimulant. A single 50 μg dose of OKT3 mAb followed 24 hours by 300 mg/m^2 of cyclophosphamide was shown to increase lymphocyte numbers in human cancer patients after 4 weeks (Wiseman et al, 1991). No

difference was seen between different subsets of lymphocytes. Although this study demonstrated the safety of low-dose OKT3, it was not possible to assess the contribution of the cyclophosphamide in this regimen. Nevertheless, it served to emphasise the substantial present interest in oncology to evaluate OKT3 as a potential immunostimulant.

2.8 SUMMARY.

Advances in biotechnology, most notably genetic engineering and hybridoma technology have enabled the production of large quantities of highly pure substances. The therapeutic potential of these biological agents is considerable. The effectors responsible for cytolysis of cancer cells are extremely heterogenous and different cell types are probably important in mediating effects against different types of tumours. IL-2 activates effector cells capable of non-MHC-restricted killing *in vitro* and *in vivo* although the importance of this effect in regression of human cancers remains to be proven. Impairment of T cell function that accompanies IL-2 therapy may be detrimental in the therapy of tumours expressing MHC class I antigens and TADA. IL-2 therapy is also associated with considerable toxicity.

In the 1990s biological therapy is in a position comparable to that of chemotherapy in the 1940s (Perren and Selby, 1992). Although a small number of active reagents exist, a minority of patients with any particular disease experience objective responses. The responses are usually of short duration with occasional durable remissions or cures. In the three decades after the 1940s chemotherapy developed rapidly, with new drugs, combination chemotherapy, high dose treatments, and adjuvant chemotherapy which resulted in impressive advances in treatment of paediatric cancers, haemopoietic cancers, and germ cell tumours, culminating in the cure of many patients with these cancers by the early 1980s (Perren and Selby, 1992). Too often small poorly controlled clinical trials using biological materials have suggested therapeutic benefit only to be rejected following the results of larger studies.

Specific activation of T cells using anti-CD3 mAb has been demonstrated *in vitro*. *In vivo* mechanisms of mAb may be very different to those seen *in vitro* and are complicated by the numerous interactions that occur as a consequence of cytokine release. In order to optimize immunotherapy it is important to activate all cells *in vivo* with anti-tumour activity. Caution must be exercised however, in view of the lack of information regarding T cell stimulation by anti-CD3 mAb and its consequences for long-term T cell function. A delicate balance exists between the immunosuppressive properties of anti-CD3 and their immunostimulatory effects.

2.9   AIMS OF THE FOLLOWING STUDIES.

The previous introductory chapters have defined the immune response as important in regulating tumour growth. Enhancement of cellular immunity by both NK cells and CTL is associated with regression of established tumours. Evidence suggests that NK cells are important in tumour development (Trinchieri, 1989). Previous studies from our department have suggested an association between the enhancement of NK activity *in vitro* and clinical responsiveness to IL-2 immunotherapy *in vivo* (Ubhi et al, 1991). Patients with gastrointestinal cancer frequently present to General surgeons and Physicians and gastrointestinal tumours are known to be relatively unresponsive to IL-2 in comparison to renal carcinomas and melanoma (Rosenberg et al, 1989).

Gastrointestinal tumours frequently fail to express MHC class I antigens (Durrant et al, 1987) which may make them less susceptible to CTL responses. On the other hand a failure of MHC expression may make tumours more susceptible to killing by NK cells Ljunggren and Karre, 1990). The cytolytic activity of lymphocytes is enhanced by both cytokines and mAb to the CD3 antigen. The principle aim of this study was to investigate cellular cytotoxicity *in vitro* in healthy individuals and assess its modulation using both mAb and IL-2. The mechanism of *in vitro* enhancement of cytotoxicity by mAb was also investigated. Further studies were then undertaken using PBMC from cancer patients to determine whether comparable levels of cytotoxicity and response to mAb are seen. A study was then undertaken in which different doses of OKT3 were administered to patients with cancer to determine its safety and immunomodulating properties and also the capacity for further enhancement by anti-CD3 mAb *in vitro*.

CHAPTER THREE

METHODS

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3.1 INTRODUCTION.

3.1.1 Choice of experimental methods.

We have previously established techniques to assess peripheral blood NK activity in the laboratory and have reported a method for assessing the effect of mAb *in vitro* using a standard four hour ^{51}Cr-release assay and K562 target cells (Ubhi et al, 1991). NK activity was studied because of our previous experience with this assay and the probable involvement of NK cells in regulating human tumour growth. Patients with gastrointestinal cancer were mostly studied because the variable degree of MHC expression by these tumours may make them more susceptible to NK cell lysis and also because patients with these tumours frequently presented for surgical treatment.

In the clinical studies, OKT3 was administered to patients who required analysis of NK activity in fresh venous blood samples using techniques which could be completed within 12 hours. Assessment of NK activity depends upon measurement of both numbers of effectors and their functional ability to kill tumour cells. Reproducible methods needed to be adopted to study changes in NK activity measuring these two parameters. PBMC were separated from whole blood by density gradient centrifugation and NK activity measured using the standard 4-hour ^{51}Cr-release assay and K562 tumour targets. Flow cytometry incorporating fluorescein-conjugated mAb was used to analyse peripheral blood cells in both whole blood and PBMC preparations. Because serum could be stored at -70°C and subsequently thawed, these specimens were batch assayed for cytokines or assessed for their influence on *in vitro* cytotoxicity. Cytokine and IL-2Rα levels were assessed using commercially available kits incorporating ELISA techniques. Measurement of serum OKT3 levels was undertaken using a technique recently developed in our laboratory (manuscript in preparation).

3.1.2 Subjects and investigations.

Figure 3.1 outlines the subjects studied, the investigations undertaken, and the investigator responsible for performing the assay. Subjects comprised healthy donors and cancer patients. Healthy donors were recruited according to subjects aged < 50 years (young healthy donors) or ≥ 50 years (elderly healthy donors). Cancer patients were either recruited into the OKT3 trial or underwent a single venesection. Not all subjects underwent the same investigations and not all investigations were undertaken by the same investigator. Flow cytometry was performed on both PBMC preparations or whole blood by either Dr. T. Horsburgh (Principal Clinical Scientist, Department of Surgery Laboratories, TH) or Mr. R. Catterick (Temporary Research Assistant, Department of Surgery, RC).

TH undertook the flow cytometric analyses on blood on all the cancer patients in the OKT3 trial and 24 young healthy donors. In 8 cases PBMC preparations from young healthy donors were also analysed by TH. RC undertook flow cytometry analyses of blood on the 15 cancer patients who were not in the OKT3 trial, some of the young

Figure 3.1 - Subjects, investigations, and investigator performing cytotoxicity assays and
flow cytometry.

		ALL (154)		
	HEALTHY DONORS (103)		CANCER PATIENTS (51)	
	< 50 Years (75)	≥ 50 years (28)	OKT3 Trial (36)	Single venesection (15)
Flow cytometry				
Blood				
TH	24	0	36	0
RC	15	20	0	15
PBMC TH	8	0	15	0
RC	21	20	0	15
Cytotoxicity assays				
JH	55	8	36	0
RC	20	20	0	15

healthy donors (15), and all of the elderly healthy donors (20). PBMC analyses by RC were performed in an identical way to those performed by TH. However, results were stored and subsequently calculated in such a way that it was not possible to determine the proportions of lymphocytes, neutrophils, and monocytes in PBMC preparations. The proportion of NK cells as a percentage of lymphocytes in the PBMC preparations was determined.

Cytotoxicity assays were undertaken by the principal investigator (JH) or RC. All assays on the cancer patients in the OKT3 trial were performed by JH. RC undertook the assays on the cancer patients who were not in the OKT3 trial (15) and the majority (20 of 28) of the assays on the elderly healthy donors. The majority of the assays on the young healthy donors in which the effect of mAb and IL-2 were assessed were undertaken by JH (55). All the cancer patients who were not in the OKT3 trial underwent analysis of blood and PBMC by flow cytotometry simultaneously by RC with the measurement of K562 cytotoxicity. This was also the case for the 20 elderly healthy donors analysed by RC. However, among the young healthy donors, although flow cytometry was frequently performed, simultaneous measurement of K562 cytotoxicity was not always successful. This was because of a failure of some early assays performed by both JH and RC to fulfill the inclusion criteria for acceptance into the final analysis (ie. SR < 10%, MR < 4000 CPM, correlation coefficient < 0.95). Therefore not all flow cytometry data of PBMC and blood described in Figure 3.1 in the young healthy donors corresponds with a simultaneous measurement of cytotoxicity. Therefore for some of the young healthy donors in which PBMC analysis and blood analysis by flow cytometry are cited, cytotoxicity data were retrieved in that individual from an assay performed on a different occassion. When cell data is related to cytotoxicity data the results are always cited from simultaneous experiments.

An assay was undertaken by JH and RC simultaneously to establish that results were comparable. Except for measurement of OKT3 levels (undertaken by Mr S Weston, Technician, Dept. of Surgery Laboratories) all other experimental techniques and clinical studies were performed by the principal investigator (JH). Chapter 3 outlines the techniques used and when relevant, their validation. Details of materials and the precise technique are outlined in the appropriate sections of the appendices A to F.

3.1.3 Studies on Healthy Donors.

103 healthy volunteers were recruited from nursing, laboratory and other staff employed in the University and Hospitals of Leicester. Subjects donated a single blood sample between 0700 and 0900 hours. Nurses who had just finished working a night shift were excluded. The majority of these subjects were young (median age 34 years, range 19 - 84). They did however include an older population from the Womens Royal Voluntary Service (WRVS) and ancillary workers at LGH who were mostly recruited towards the completion of the studies. Subjects were arbitrarily separated into those aged

< 50 years (young healthy donors) and ≥ 50 years (elderly healthy donors). This was undertaken both to assess the effect of age on cellular cytotoxicity and provide a more closely matched age group for comparison with the cancer patients.

Inclusion criteria are shown in Table 3.1 and sex and age distributions within individual groups are described in Table 3.2.

Donors comprised 48 males (median age 33 years, range 19 - 84) and 55 females (median age 43 years, range 20 - 66). With the exception of the subjects ≥ 50 years which included 18 females and 10 males, the sex distribution was similar in all groups.

Between August 1991 and March 1993 experiments were performed using PBMC from these healthy donors over the same time period as the clinical studies reported in chapters 5 and 6. The regular study of healthy donors served to ensure consistency of technique, minimise error due to experimental drift, and identify problems in the cytotoxicity assay at an early stage without jeopardizing the clinical studies.

Cellular cytotoxicity was assessed in all individuals using the K562 cell line and all experiments included the analysis of at least one mAb or IL-2. Details of the mAb studied are described in Table C1 in appendix C. No more than 4 donors were studied on one day and this number was usually reduced when larger experiments were undertaken. It was usually possible to complete the assay within a 7 hour period beginning with obtaining a blood sample to commencing measurement of emitted radiation in the gamma counter.

35 of the donors aged < 50 years and 20 of the donors aged ≥ 50 years also underwent simultaneous flow cytometry of whole blood and measurement of cytotoxicity. PBMC preparations were also simultaneously assessed on 8 occassions by TH and also on 15 of the 20 occassions in which RC measured cytotoxicity among the young healthy donors. The elderly subjects were also mostly recruited towards the completion of studies along with the 20 young healthy donors in whom cytotoxicity was measured by RC. Most individuals underwent measurement of cytotoxicity on one occasion only. Some more easily accessible individuals were tested more than once and provided information on the variability of cytotoxicity over time. One individual (JH) was frequently tested. The first assay cited was undertaken on 23.8.91 and this date was used as a reference point when describing variation in cytotoxicity over time (days). Approximately 2 months were spent establishing the necessary laboratory techniques prior to this date.

The data used to define the range of K562 cytotoxicity in all 103 individuals fulfilled the criteria for inclusion in analysis and were selected to include individuals who also had a simultaneous assessment of blood by flow cytometry.

Table 3.1 - Inclusion criteria for healthy donors.

1.   No previous history of drug ingestion for one month (except for the oral contraceptive pill and simple analgesia).

2.   No evidence of viral infection within previous month.

3.   Not working night shifts within the previous week.

4.   No history of alcohol ingestion within the previous 48 hours.

5.   Active apparently healthy individual who presently feels in good health.

Table 3.2 - Sex and age distribution of healthy donors.

Group	Sex	n	Median age (range)
	Males	48	33 (19 - 84)
	Females	55	43 (20 - 66)
Total		103	34 (19 - 84)
< 50 years	Males	38	29.5 (19 - 45)
	Females	37	31 (20 - 49)
Total		75	30 (19 - 49)
≥ 50 years	Males	10	62 (50 - 84)
	Females	18	57 (50 - 66)
Total		28	58.5 (50 - 84)

3.1.4 Studies on cancer patients.

A   Subjects.

All patients were under the care of Consultant General surgeons and Consultant Physicians at Leicester General Hospital. No patient underwent a change from routine clinical management because of recruitment into the studies. Inclusion and exclusion criteria are described in Table 3.3. All patients had a positive histological diagnosis of cancer at the time of study.

All patients had full haematological and biochemical investigations including serum creatinine and liver function tests performed by the hospital pathology department within the week prior to study. Abnormal liver function tests (LFTs) did not exclude patients from study. Routine radiological assessment included a chest X-ray. An abdominal ultrasound scan was also undertaken when clinically indicated. Computerized tomography was undertaken in many patients to assess operability and was also useful in defining the presence of liver metastases (Plate 1). A minimum expected survival period was not a criteria for inclusion. The measurement of performance status using the ten-point scale developed by Karnofsky (1948) was used to exclude patients who were particularly debilitated (Table 3.4). All patients achieved a performance status of $\geq 60\%$. Signed informed consent was obtained from all patients.

Details of individual cancer patients are described in Tables 3.7 to 3.9 in Appendix H. An overview of their types of tumours is described in Table 3.5. 51 cancer patients were studied. 55 of the healthy donors presented in Chapter four underwent analysis of peripheral blood cells by flow cytometry. These comprised 20 donors aged < 50 years of age and 35 donors aged ≥ 50 years of age. The age and sex distribution in cancer patients is compared with these healthy donors undergoing flow cytometry in Table 3.5. Comparison of cytotoxicity between cancer patients and healthy donors is undertaken using data from all 103 healthy donors. Comparisons were therefore possible between peripheral blood cells in 51 cancer patients and 55 of the healthy donors in whom flow cytometry was performed and patient details are outlined in Table 3.6.

A total of fifty-one patients comprising 36 males and 15 females with a median age of 70 years (range 38 - 87 years) were studied. The cancer patients were significantly older than the ≥ 50 years group of healthy donors (median age 58.5 years, range 50 to 84 years). Cancer patients were separated into those < 70 years of age and those ≥ 70 years of age. Of the 51 patients studied there were a total of 47 adenocarcinomas, 3 squamous carcinomas and 1 malignant melanoma. There were 24 upper gastrointestinal tumours (17 oesophagus, 7 stomach), 18 lower gastrointestinal tumours (13 rectum, 5 colon), and 6 tumours arising from the biliary tract or pancreas (2 biliary, 4 pancreas). It was in 4 of the biliary/pancreatic cases that bilirubin levels were elevated or had previously been elevated. Two patients were clinically jaundiced at the time of study (GW - bilirubin 85 μmol/L, and ABT - bilirubin 111 μmol/L). In both

Table 3.3 - <u>Inclusion and exclusion criteria of cancer patients.</u>

<u>Eligibility.</u>

1.   Histologically documented evidence of malignancy.
2.   No chemotherapy, radiotherapy, immunotherapy or surgery in
      the previous four weeks.
3.   No prior blood transfusion within four weeks.
4.   Anticipated life expectancy > 3 months.
5.   Ambulatory performance status > 60% (Karnofsky Grading)
6.   White cell count $\geq$ 4 X 10^6/ml; Platelet Count $\geq$ 120 X 10^6/ml;
      Haemoglobin $\geq$ 11 g/dl
7.   Urea and creatinine levels within the normal range.

<u>Exclusions</u>

1.   If any of the above criteria are not met.
2.   Significant history or current evidence of cardiovascular disease (eg.
      congestive cardiac failure; uncontrolled hypertension; coronary artery
      disease) serious arrhythmias or evidence of previous myocardial infarction.
3.   Evidence of serious active infection requiring antibiotic therapy.
4.   Patients with major organ allografts.
5.   Patients taking corticosteroid therapy.
6.   Patients with concurrent second primary malignancies.
7.   Patients with central nervous system metastases.
8.   Patients with diabetes mellitus and thyroid disorders.
9.   Patients unable to give informed consent.

Plate 1 - Computerized tomographic abdominal scan in a patient (FB) with malignant melanoma liver metastases (arrows).
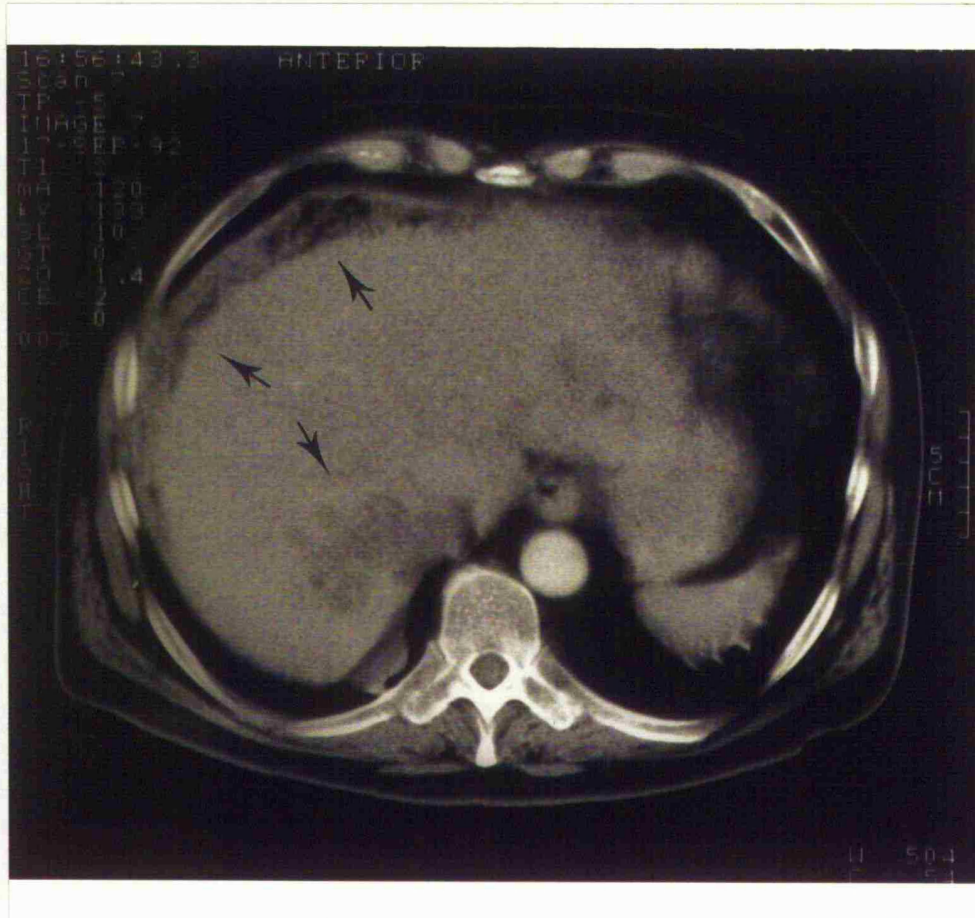
Table 3.4 - <u>Karnofsky Grading (Karnofsky et al. 1948)</u>

Description	Scale (%)
Normal; no complaints.	100
Able to carry on normal activity; minor signs or symptoms of disease.	90
Normal activity with effort.	80
Cares for self. Unable to carry on normal activity or to do active work.	70
Requires occasional assistance but able to care for most of his/her needs.	60
Requires considerable assistance and frequent medical care.	50
Disabled; requires special care and assistance.	40
Severely disabled; hospitalisation indicated though death not imminent.	30
Very sick; hospitalisation and active supportive treatment necessary.	20
Moribund.	10
Dead.	0

Table 3.5 - Distribution of cancer patients in groups defined by age, sex, tumour type, and a previously or presently elevated bilirubin.

Group	Number	males	Females	Median age (Range)
All	51	36	15	70 (38 - 87)
Males	36	-	-	69 (38 - 87)
Females	15	-	-	71 (53 - 87)
< 70 years	25	19	6	61 (38 - 69)
≥ 70 years	26	17	9	76 (70 - 87)
Upper GIT	24	16	8	70 (38 86)
Lower GIT	18	15	3	69.5 (38 - 87)
Biliary/pancreas	6	3	3	70 (53 - 72)
Unknown primary	3	2	1	72 (65 - 84)
Elevated bilirubin	4	3	1	70.5 (67 - 72)
Liver mets	20	11	9	69 (38 - 85)
No liver mets	31	25	6	71 (38 - 87)

Table 3.6 - Characteristics of cancer patients compared with healthy donors on whom flow cytometry of blood and cellular cytotoxicity was measured.

Subjects	Group	n	median (range) age	males	females
Cancer patients	Liver mets	20	69 (38 - 85)	11	9
	No liver mets	31	71 (38 - 87)	25	6
	TOTAL	51	70 (38 - 87)	36	15
Healthy donors	≥ 50 years	20	58 (50 - 66)	7	13
	< 50 years	35	28 (19 - 44)	24	11
	TOTAL	55	40 (19 - 66)	31	24

cases this was due to an obstructed common bile duct and in neither case was there evidence of liver involvement by tumour. Two other patients (AG and WW) had been previously jaundiced but bilirubin levels had returned to normal following endoscopic stenting of the common bile duct and choledochoduodenostomy performed one month previously. One of these patients also had liver metastases (AG).

Three patients did not have an identifiable primary tumour. HW and JR presented with liver metastases which were diagnosed on Tru-cut needle liver biopsy and proved to be adenocarcinomas. FB presented with malignant melanoma deposits in the liver and an unknown primary site. These were diagnosed from biopsies obtained at laparoscopy.

Thirty-six of the patients received varying doses of OKT3 and then underwent sequential venesections to monitor cellular immunity. These patients are also presented in Chapter 6. The remaining 15 patients underwent a single venesection only and were recruited during the completion of the studies. In all of these 15 cases, cytotoxicity was assessed by RC. These patients were not otherwise knowingly selected in any way differently to the OKT3 treated patients.

Patients comprised a highly heterogenous population with regard to tumour type and extent. Previous treatment and subsequent management was also very variable. Following a diagnosis of cancer, patients usually underwent surgery or medical treatment during the same admission. 14 patients had undergone a previous surgical procedure which required a general anaesthetic and in 11 patients this was undertaken to excise the primary tumour. The remaining 3 patients had undergone diagnostic surgery or a procedure to alleviate symptoms. This included the relief of jaundice by choledochoduodenostomy (AG), relief of ascites by the placement of a Denver shunt (ABT), and a laparoscopy to obtain biopsies which led to the diagnosis of melanoma (FB). The tumour present at the time of study in the patients who had undergone a previous procedure repesented a recurrence in 6 cases, residual tumour in 5 cases and irresectable primary or secondary tumours in the remaining 3 cases. One patient with two liver metastases (RH) had these successfully resected. However the remaining 13 patients had tumours that were not amenable to curative resection.

The remaining 37 patients who had not undergone a previous procedure were studied shortly after their cancer (primary, residual, or recurrence) was first diagnosed. 17 of these patients subsequently underwent resections which excised the primary tumour and left no obvious residual tumour. 6 patients underwent laparotomy at which the tumour was found to be inoperable either because it was fixed to other structures or because the presence of metastases precluded undertaking resection. The remaining 20 patients underwent either endoscopic palliative intubation of oesophageal strictures or received palliative medical relief of symptoms. In all, out of the 51 patients, 33 had tumours for which curative surgery was not possible.

Duration of symptoms prior to diagnosis and survival time following diagnosis is also shown in months in Table 3.7 (Appendix H). Of the 50 patients who complained of

symptoms these had been present for a median time of 4 months (range 1 to 12). Only RH was asymptomatic and was diagnosed as having liver metastases on routine follow up. Data on survival following diagnosis was estimated retrospectively by either contacting the patients General Practitioner or from hospital records. Patients were documented as being alive up to 1.6.93. Patient outcome was obtained for 48 patients. Of these 20 patients were still alive and these were mostly from the recent recruits. The median survival post diagnosis was only 2.5 (range 1 - 12) months in the 28 patients who perished. This is consistent with the advanced nature of the tumours.

B   Tumour Staging.

Tumours were staged on the basis of operative findings and clinical assessment. Dukes staging was used for the colorectal neoplasms when the primary tumour was resected (Dukes stage A, B, or C). Dukes stage D which is sometimes used to describe the presence of liver metastases was not used. Patients who underwent primary resection of their neoplasms were staged according to the TNM (UICC) classification. Patients whose tumours were found to be irresectable at laparotomy were not formally staged and are described as 'fixed' to other structures in Table 3.9 (appendix H). Grading of tumours was retrieved retrospectively from routine pathology reports and was defined as well, moderate, or poorly differentiated tumour, or grade not stated (N.S.). Grading depended upon that part of the tumour with the poorest degree of differentiation. In 13 cases the grade was not stated. 15 tumours were poorly differentiated, 18 tumours were moderately differentiated and 5 were well differentiated.

Because of difficulties with accurately staging tumours, patients were also separated into those with liver metastases (n = 20) and those without liver involvement (n = 31). Liver metastases were positively identified by radiology (ultrasound or CT scanning) or at surgery. An attempt was not made to measure the volume of liver metastsases. Most of the patients with liver involvement did not have these tumours histologically confirmed. It was not practical to assess the volume of tumour present and liver involvement is simply described as being present or absent.

In summary a heterogenous group of cancer patients with respect to stage and subsequent treatment were studied. This was principally due to practical limitations with the timing of the laboratory studies and patient availability.

C   Laboratory Studies.

The methods are outlined in appendices A to D. All patients underwent an assessment of cytotoxicity against K562 targets and cellular cytotoxicity was measured against the Daudi cell line in 10 patients. The effect of preincubating PBMC with OKT3 was assessed in all patients except one (EY) and with T3 in 43 patients. The effect of anti-CD16 was assessed in 14 cancer patients. Some patients were also studied using OKT3, IL-2, and IL-2 in combination with OKT3. Flow cytometry was used to measure

the differential white blood cell count and NK cells including those cells expressing NK markers and CD3 in all patients. Expression of the IL-2Rα chain in association with CD3, CD4, CD8 and CD2 was also assessed in some patients.

3.1.5 Studies on Patients Receiving OKT3.

A   Introduction.

Local ethical committee approval was obtained for these studies and a Doctors and Dentists Exemption (DDX) certificate was approved through the Department of Health. A clinical trial was undertaken in which cancer patients received either 50 μg, 20 μg, 10 μg, 5 μg, 0.5 μg, or normal saline by intravenous bolus injection and then underwent clinical monitoring and sequential venesections. Patients were studied in two groups undergoing similar investigations but at different times. Initially six patients receiving 50 μg OKT3 were studied during the same two month period as four patients receiving normal saline. The four patients (controls, n = 4) who received normal saline were studied on different days to the patients receiving OKT3, but underwent venesections at the same times. The second group, in whom the timing of investigations was modified, comprised six patients each receiving 20 μg, 10 μg, and 5 μg, and two patients receiving 0.5 μg OKT3. These OKT3 treated patients were studied over the same 6 month period but on different days to six patients who received only normal saline (controls, n = 6). A 50μg dose of OKT3 was chosen initially based on the report by Wiseman et al, (1991).

The reason for the two groups differing in timing of investigations was as follows. Initially it was planned to investigate modulation of cellular immunity in the first 8 hours following 50 μg OKT3 and then also at approximately 7 to 10 days and 4 to 6 weeks. However, it proved difficult to retrieve samples following patient disharge. Also, only small numbers of patients were to be studied and subsequent clinical management was so variable that it was felt that interpretation of results at 7 to 10 days and 4 to 6 weeks would be unlikely to yield meaningful results. Early results also raised concerns that the 50 μg dose of OKT3 may be too high, and possibly even immunosuppressive. Therefore subsequent studies were directed toward investigating the immunomodulating properties of lower doses of OKT3 over a 24 hour period.

The cancer patients decribed here are included in those described in Chapter 5 and details of the individual cancer patients are described in Tables 3.7 to 3.9 in Appendix H. Thirty-six patients, comprising 25 males and 11 females with a median age of 69 years (range 38 - 84) were studied. Signed informed consent was obtained from all patients. Inclusion criteria are outlined in Table 3.3. All patients had full haematological and biochemical investigations including serum creatinine and liver function tests performed by the hospital pathology department within the week prior to study. Patients underwent full clinical assessment immediately prior to receiving OKT3.

Most patients received OKT3 during admission for routine treatment although some patients were primarily admitted for study. OKT3 administration did not alter patient management which was otherwise undertaken in accordance with standard clinical practice.

B Assessment of Toxicity.

Patients were monitored for development of toxicity following OKT3 administration using a modification of the criteria defined by the World Health Organization (Table 3.10). A routine blood test following OKT3 was not undertaken therefore changes in biochemical and haematological criteria of toxicity were not formally assessed. Toxicities are expressed as maximum grade/patient. In particular the development of pyrexia, vomiting, and hypotension were noted. Blood pressure and pulse rate were assessed at 30 minute intervals using a Dinamap (Critikon) monitor. An oral temperature was recorded prior to OKT3 and hourly for the first four hours then four-hourly and at identical times in the control patients. Patients were also directly questioned about 'flu-like' symptoms including flushing, light-headedness, and nausea.

C Reconstitution of OKT3.

Two preparations of 50μg OKT3 were simultaneously reconstituted in a 10 mls solution of 5% glucose and 0.2% human serum albumin for each patient. This was undertaken by either the staff of the pharmacy department at Leicester General Hospital or at Leicester Royal Infirmary who followed a standard protocol (appendix G). Different members of staff were involved in OKT3 reconstitution. One syringe of OKT3 was administered to the patient. The quantity of OKT3 in the duplicate syringe was measured in 10 cases using the method described in appendix F (Table 3.16 in appendix H). The mean (± SEM) OKT3 content of the 10 syringes was 74.6 ± 6.1 μg and varied between 58.8 μg and 103.8 μg.

The quantity of OKT3 in the syringes was therefore consistently greater than predicted and varied up to two fold. OKT3 was always reconstituted and administered within 48 hours and was stored at 4°C prior to use. For administration of doses of OKT3 of less than 50μg, the appropriate quantity of OKT3 was removed from the above solution and diluted to a total volume of 10 mls in 0.9% saline immediately prior to injection. OKT3 was given by rapid bolus injection between 7 am and 8 am into an 18 gauge venous cannula (Venflon, Viggo-Spectramed, Helsingborg, Sweden) sited in a peripheral forearm or antecubital fossa vein.

Table 3.10 - Grading of Toxicities (modified from WHO recommendations for grading of acute and subacute toxic effects).

WHO Grade	0	1	II	III	IV
Gastrointestinal					
Nausea/vomiting	None	Nausea alone	1 - 5 episodes of emesis/day	6 - 10 episodes of emesis/day	Intractable vomiting need iv fluids
Respiratory	No change	Breathless on exertion	Breathless at rest	Breathless hospitalised	Requires intubation
Fever (^oC)	None	< 38	38 - 40	> 40	Fever with hypotension
Allergic	None	Oedema	Bronchospasm	Bronchospasm needs parenteral feeding	Anaphylaxis
Blood Pressure Change	None	>20mmHg systolic No therapy	>30mmHg orthostatic symptoms no therapy	Requires iv fluids for$\leq$ 8 hours	Requires iv fluids for$\geq$ 8 hours or pressors
Central Neurological	None	Mild difficulty with concentration	Moderate difficulty with concentration	Confusion, disorientation	Coma, seizures

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D <u>Method For Obtaining Blood Samples and Laboratory Investigations.</u>

Venous blood samples were collected prior to OKT3 and then at regular intervals following OKT3. A standard procedure was followed. A tourniquet was applied to the upper arm and an 18G intravenous cannula (Venflon) was inserted into either a forearm vein or an antecubital fossa vein and immediately used to obtain a venous sample. The tourniquet was removed and either OKT3 or 10 mls of normal saline was administered through the cannula by bolus injection over approximately two minutes. 5 mls of 0.9% saline was then injected into the cannula. Subsequent blood samples were obtained from the cannula by injecting another 1 ml of 0.9% saline, waiting for 5 minutes, applying the tourniquet, and withdrawing a 10 mls sample which was discarded prior to collecting the final specimen. A further 5 mls 0.9% saline was injected into the cannula. Patients were encouraged not to flex the elbow to avoid excessive angulation of the cannula, and in some cases the elbow was splinted. Heparin was not used to flush the cannula at any stage. Using this technique it was usually possible to obtain venous samples over the 24 hours study period without subjecting patients to the discomfort of repeated venepuncture. Occassionally direct venepuncture was necessary when a 21G needle was used.

Blood samples were collected for flow cytometric assessment of peripheral blood cells, and assessment of cellular cytotoxicity. Samples were also collected for serum separation which was then stored at -70°C for later assessment of OKT3, cytokine, and sIL-2Rα levels. The effect of serum on K562 cytotoxicity mediated by PBMC from one healthy donor was also assessed. Methods are described in the relevant sections of the appendices A to F.

An outline of the schedule of investigations is described in Figure 3.2. As has been discussed the schedule for obtaining samples differed in the first ten patients studied (six patients treated with 50μg OKT3 and four with normal saline). From these 10 patients a 5 mls sample was collected into EDTA for flow cytometry along with a 10 mls clotted sample (for serum separation) immediately pre-OKT3/normal saline then, at 15 minutes, 30 minutes, 60 minutes, 240 minutes, and 480 minutes. The WBC differential, NK cells and CD3+ NK cells were determined using Leucogate (BD) and Simultest preparations (anti-CD16/anti-CD56) in all patients as described in appendix D. Expression of CD2, CD3, CD4, CD8, and CD25 were also measured in some patients. OKT3 binding to lymphocytes was determined using an anti-mouse IgG preparation. 20 mls of blood was collected into heparin for assessment of cytotoxicity pre-OKT3/normal saline, then at 30 minutes, 60 minutes, and 240 minutes. Cellular cytotoxicity was measured against K562 and Daudi cells in the 50 μg patients and the initial 4 control patients and *in vitro* modulation by OKT3 and T3 assessed.

A final blood sample was obtained from the patients receiving 20μg to 0.5μg OKT3 and also six patients receiving normal saline at 24 hours post-OKT3/normal saline. The 15 minute and 30 minute samples were omitted in these patients. Cytotoxicity was

Figure 3.2 - Schedule of investigations for patients in the OKT3 trial.

	Minutes				
	0 15 30 60	240		480	1440
Dose and subjects					
OKT3 (50 µg) (n = 6) N. saline (n = 4)					
K562/Daudi cytotoxicity	* * *	*			
Flow cytometry	* * * *	*		*	
Serum	* * * *	*		*	
OKT3 (20 µg) (n = 6) **OKT3 (10 µg)** (n = 6) **OKT3 (5 µg)** (n = 6) **OKT3 (0.5 µg)** (n = 2) N. saline (n = 6)					
K562 cytotoxicity	* *	*			*
Flow cytometry	* *	*		*	*
Serum	* *	*		*	*
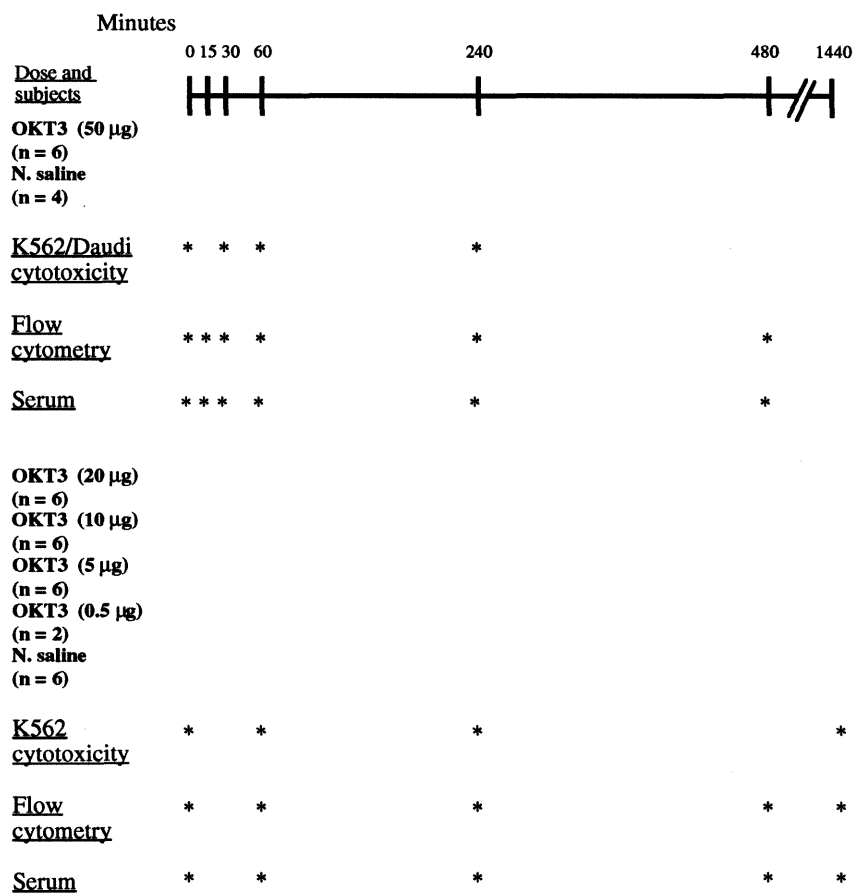
assessed against the K562 cell line only. OKT3 and T3 modulation *in vitro* was also assessed in these patients except for three in the 5 μg group. In some patients modulation with IL-2 was also assessed. Flow cytometry was undertaken on blood although assessment of expression of CD4 and CD8 was ommitted. All 6 patients receiving normal saline and 3 each from the 20 μg to 5 μg groups patients also underwent analysis of PBMC preparations.

The effect of serum on K562 cytotoxicity from the 50μg and 20μg patients (and the 6 controls studied with the 20 μg group) was assessed using PBMC from one healthy donor (JH) (appendix C). CRP levels were measured by the LRI immunology service on samples obtained at 0 minutes and 480 minutes in the 50 μg patients , and also at 1440 minutes in the 20 μg patients and their respective 6 control patients.

Serum was assessed for IL-2, TNFα, IFNγ and sIL-2R levels pre-OKT3/normal saline at 30 minutes, 60 minutes, 240 minutes, and 480 minutes in the 50 μg and control groups (n = 4) only. OKT3 levels in serum were measured in two of the patients receiving 50μg. The total volume of blood obtained from each patient during study was approximately 185 mls.

3.2   PBMC PREPARATION.
3.2.1   Introduction.

The method for preparing effector cells has important consequences for measuring NK activity. Most investigators prepare lymphocytes from heparinised blood by flotation on Ficoll-Hypaque (F-H) gradients based on the method described by Bøyum (1968). Bøyum showed that by centrifuging heparinised or defibrinated whole blood over a F-H gradient, a band of cells clearly separated between the plasma and F-H regions which consisted predominantly of mononuclear cells (mainly lymphocytes and monocytes). The principle of density gradient centrifugation depends upon the differential migration of different cell types due to differences in their density.

F-H is commercially available as an aqueous solution containing in each 100 ml, 5.7 g of high density sucrose epichlorohydrin polymer (Ficoll-400) and 9 g sodium diatrizoate (Lymphoprep, Pharmacia). Because the density of lymphocytes is less than that of F-H (1.099), they are found at the interface between the plasma and the F-H with other slowly sedimenting particles (platelets and monocytes). The denser red cells and granulocytes form a pellet at the bottom of the tube (Plate 2). The proportion of monocytes in the PBMC layer is variable but is usually of the order of 20%, and contamination by neutrophils, eosinophils and basophils approximately 10% (Bøyum, 1968).

Separated PBMC are washed by repeated centrifugation to remove F-H which is toxic to cells. The main disadvantage with this technique is that it is relatively time consuming to perform if large numbers of samples are being processed. Variation in

Plate 2 - Separation of PBMC (arrow) following centrifugation of whole blood on ficoll-hypaque.
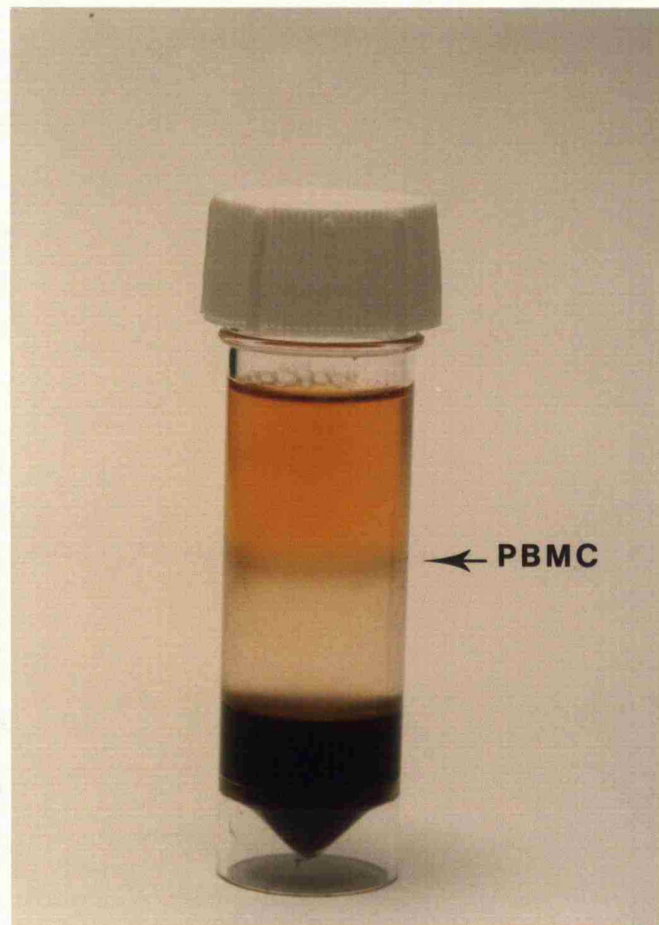
technique may also produce different yields of lymphoid and nonlymphoid cells (Bean et al, 1975).

Alternative methods include the use of defibrinated blood, sedimentation of erythrocytes with gelatin or passage of leucocytes through nylon columns to remove adherent cells. All routine procedures tend to yield heterogenous mixtures of mononuclear cells, composed of varying proportions of T, B, and other cells. Methods have been described for obtaining purified preparations of NK cells from whole blood using density gradient centrifugation (Timonen and Saksela, 1980; Timonen et al, 1981). These methods were not used here because they are lengthy to perform and although they provide significant numbers of NK cells for *in vitro* study preparations, do not reflect changes in NK cell numbers in peripheral blood. Alternatively some workers have described methods for measuring NK cytotoxicity using whole blood (Rees and Platts, 1983). This avoids the problem of effector preparation altogether and may be more representative of the *in vivo* situation. This method was not adopted in these studies because it has not been validated in the assessment of mAb on cytotoxicity in our laboratory.

Any method which provides cells for use in cytotoxicity assays requires that the separation process does not affect their viability. For this reason it was necesary to assess the viability of cells in the PBMC preparations. This was undertaken by adding a solution of acridine orange (AO) and propidium iodide (PI) to PBMC and examining cells using fluorescence microscopy (method described in Appendix A-iii). AO is a small molecule which crosses the membrane of viable and non-viable cells. Cells incorporate AO into their nuclei which when visualised using fluorescence microscopy appear green when excited at a wavelength of 450 nm. Only non-viable cells with highly permeable cell membranes incorporate PI and appear red when excited at 490 nm. This method was used to assess viability of both PBMC preparations (Plate 3) and target cell preparations (Plate 7) and results were expressed as the percentage of viable cells present in these suspensions.

The materials used and the precise methods for preparing PBMC are described in appendix A.

3.2.2 Yields and viability of PBMC separated from whole blood.

Sufficient numbers of viable PBMC were required for experiments involving assessment of cytotoxicity in healthy donors and cancer patients. The method for separating PBMC and assessing viability is described in detail in appendix A. The method for assessment of PBMC preparations and whole blood using flow cytometry is described in appendix D. PBMC were separated from whole blood obtained from 41 healthy donors by RC in which total yields and viability were also determined. Flow cytometry was performed on the blood and the PBMC preparations. The mean ($\pm$ SEM) yield of PBMC was 0.96×10^6 ml^{-1} of blood processed. Absolute numbers of cell

Plate 3 - Fluorescence microscopy (X 100 magnification) following incubation with
acridine orange and propidium iodide showing viable (green) and non-viable
(red) PBMC.
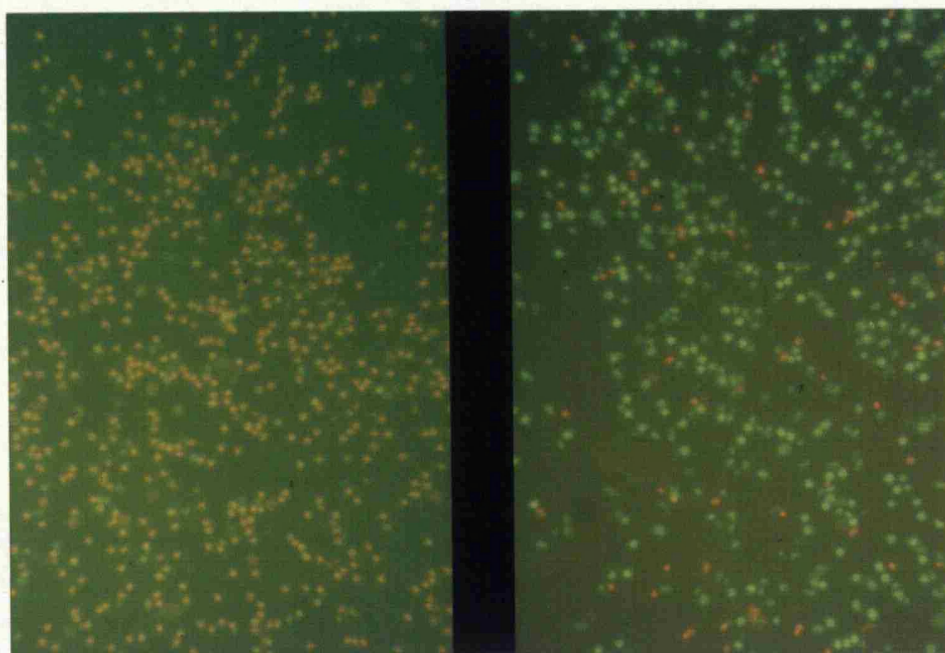
subsets in PBMC could not be calculated. Yields of PBMC were proportional to the combined monocyte and lymphocyte count (1.67 ($\pm$ 0.1) X 10^6/ml, mean $\pm$ SEM) in whole blood (p < 0.001, r = 0.53). Approximately 57% of available lymphocytes and monocytes were harvested. Mean ($\pm$ SEM) % viability of PBMC was 98% ($\pm$ 0.22). Results were similar among 30 cancer patients in whom PBMC were assessed by flow cytometry (15 by RC and 15 by JH) in which approximately 57% of available lymphocytes and monocytes were harvested and mean ($\pm$ SEM) viability was 96.2% ($\pm$ 0.39).

2.4 X 10^6 PBMC were required per assay (performed with the highest E:T ratio of 40:1), therefore processing 20 mls of blood provided on average, enough PBMC to perform 8 assays. The technique described for the separation of PBMC from 20 mls of heparinised blood therefore provided sufficient numbers of viable mononuclear cells.

3.3   MEASUREMENT OF NK ACTIVITY.

3.3.1   Principles of cytotoxicity assays.

Although the cytotoxic capacity of cell preparations depends in part upon the number of NK cells present, a functional assay which measures the ability of NK cells to kill a sufficiently sensitive tumour target is required. Such an assay depends upon the association of NK cells and target cells for a period of time sufficient to ensure killing of some of the target cells. A significant fraction of human PBL form conjugates with NK target cells (Plate 4) yet only a small fraction of the conjugate-forming cells are lytic (Rubin et al, 1982). Therefore, the frequency of conjugate formation with K562 cells does not closely correlate with the cytolytic activity of a given lymphocyte population (Radcliff et al, 1991). The microscopic enumeration of effector-target interactions accompanied by the determination of the frequency of nonviable target cells bound to effector cells forms the basis of the so-called 'single cell' cytotoxicity assay (Bonavida et al, 1983). This method is tedious to perform because a large number of cells and conjugates need to be counted to ensure accuracy.

NK activity is usually measured using short-term (4 hours) *in vitro* cytotoxicity assays based on the chromium-51 (^{51}Cr) release assay first described by Brunner et al, (1968). The NK sensitive erythroleukemic cell line K562 described by Lozio and Lozio (1973) remains the prototype target. The assay depends on the observation that the radioactive chromate ion (^{51}Cr$_2$O$_4{}^{2-}$), following diffusion through the cell membrane is retained in the cytoplasm for a relatively long period of time. Chromium release from a labelled target cell does not occur unless the cell membrane is sufficiently damaged to allow the efflux of intracellular molecules resulting in cell death. The released isotope is not subsequently reincorporated into undamaged cells and therefore reflects cell lysis. A constant number of target cells is usually mixed with graded numbers of effector cells in triplicate wells of a microtitre culture plate (Plate 5). The assay is usually performed at four effector (E) to target (T) cell ratios. Killing is quantitated by measuring the amount

Plate 4 - Peripheral blood mononuclear cells and K562 target cell conjugates following 15 minutes incubation (trypan blue X 400 magnification).
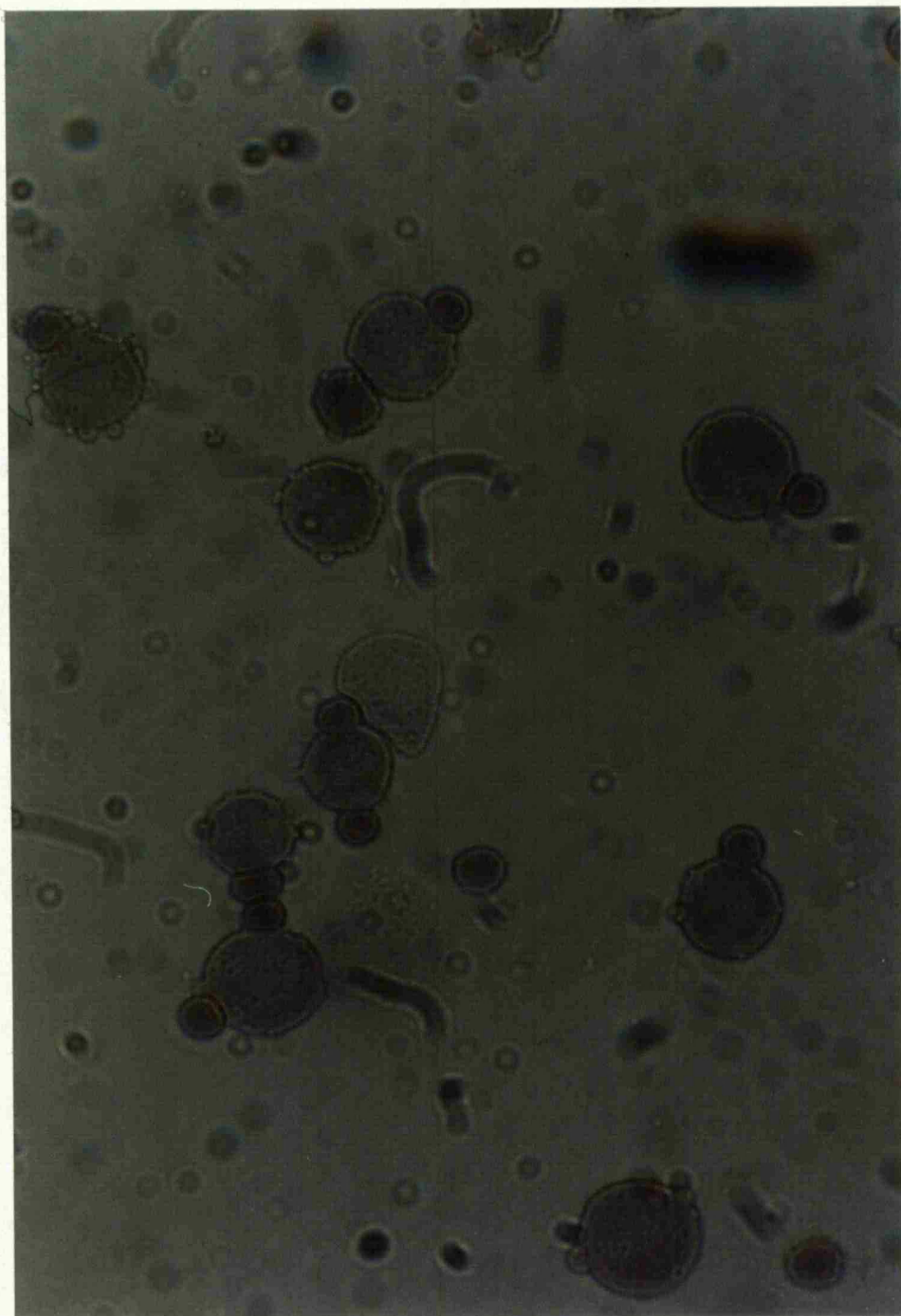
Plate 5 - 96 well round-bottomed microtitre culture plate (Nunc) (without lid).
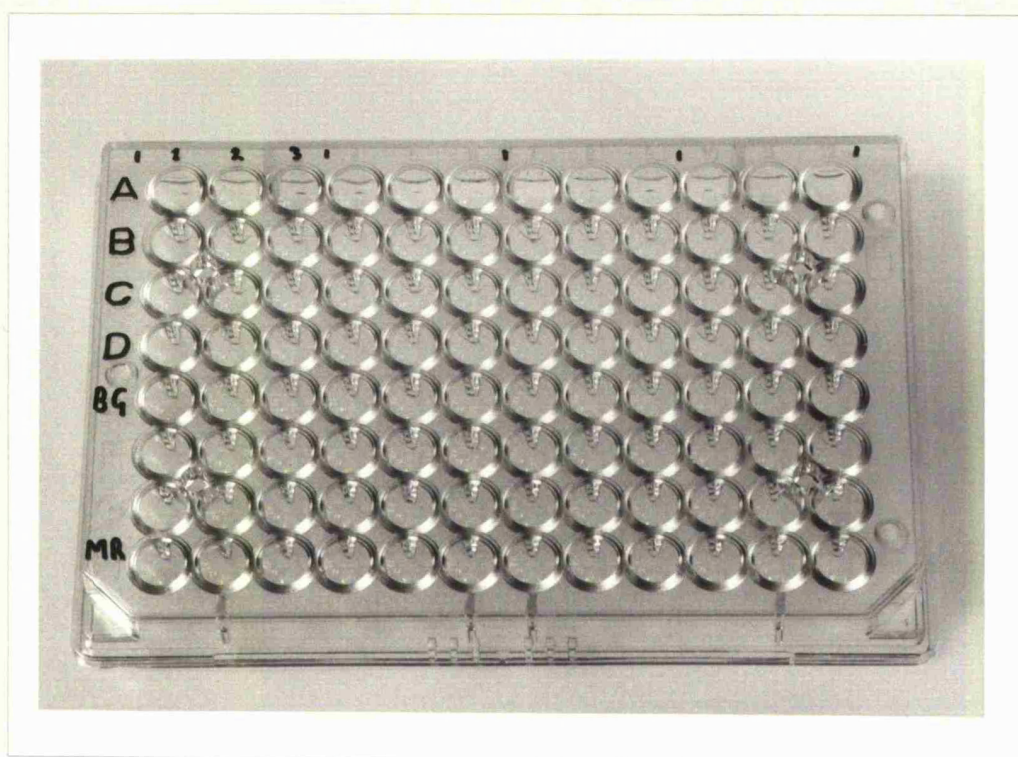
of ^{51}Cr released into the supernatant following 4 hours incubation in a gamma counter (Plate 6). The higher the cytolytic activity of effectors the greater the amount of ^{51}Cr released from killed target cells. An overview of the assay is described in Figure 3.3.

Specific percentage cell-mediated cytotoxicity (% lysis) can be calculated using the formula

$$\% \text{ Lysis} = \frac{\text{Cpm Experimental Release - Cpm Spontaneous Release}}{\text{Cpm Maximal Release - Cpm Spontaneous Release}} \times 100$$

where Cpm is the released radioactivity expressed as the number of counts measured per minute. Maximal release may be obtained by adding detergent to target cells and spontaneous release occurs from targets incubated in tissue culture medium due to diffusion of ^{51}Cr from damaged and dying cells.

The MR is usually less than the total incorporation (TI) of isotope because a fraction of the chromate ion becomes incorporated into insoluble intracellular constituents. The assay is limited to those targets that have a relatively low spontaneous isotope release and this factor largely precludes their use in more long-term assays.

Although the short-term ^{51}Cr-release assay incorporating the NK sensitive tumour cell line K562, has been extensively used to measure NK activity in peripheral blood it is not without significant problems. It may be difficult to determine between daily variation in assay technique and actual variation in NK activity. This problem is compounded by the finding that NK activity shows considerable variation between different individuals (Whiteside and Herberman, 1989). Heppner et al, (1975) found considerable variability in NK activity of the same normal donors tested repeatedly. Other workers have found good reproducibility when assays are run several days in a row with the same normal individual, but wide divergence when run at more widely spaced intervals (Oldham et al, 1973). More extensive studies have shown that the consistency of normal donors is sufficient, that the rank order of donors is usually maintained (Pross and Maroun, 1984). This enables healthy donors to be subdivided into consistently high or consistently low responders and in any one experiment it is usually possible to predict the relative cytotoxicity of a normal donor who has been tested several times previously (Pross and Baines, 1982).

These levels may be influenced by age, sex, exercise and a variety of health factors (Kay, 1986). It is therefore important to obtain samples at the same time of day when serial measurements are performed as circadian variations in NK activity have been demonstrated with the maximum activity occurring in the morning or early afternoon (Gatti et al, 1987). A normal range of levels of NK activity needs to be established in the laboratory by testing a large population of normal volunteers.

Variation in NK activity over time is important when serial measurements of NK activity are needed. Any method which relates cytotoxicity to control donors also

Plate 6 - 1280 ultragamma gammacounter (LKB).
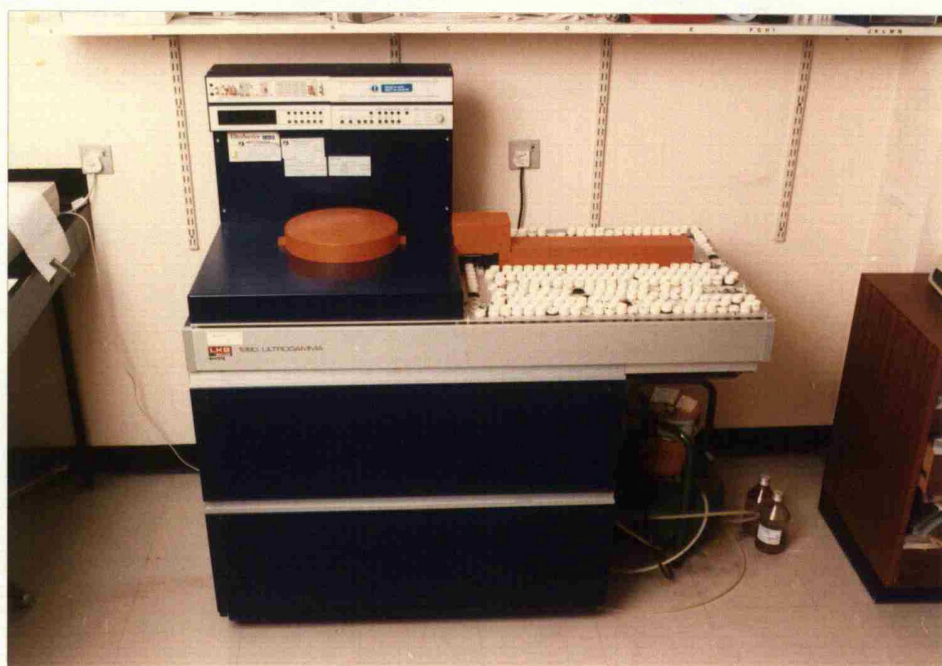
Figure 3.3 - Overview of the 4-hour chromium-^{51}-release assay used in these studies.

PBMC separation by density gradient centrifugation

Target cell preparation

Plasma

PBMC

Ficoll-hypaque

RBC

K562 incubated with 10MBq ^{51}Cr

PBMC resuspended to 8 X 10^6 ml^{-1}

K562 resuspended to 1 X 10^5 ml^{-1}

0.1ml added in triplicate to first 2 rows of a 96 well U bottomed microtitre culture plate and 2nd row serially diluted. PBMC preincubated with mAb or medium for 30 minutes

40 : 1
20 : 1
10 : 1
5 : 1

BG

MR

0.1ml added to each well

Plate incubated for 4 hours

Centrifuged for 5 minutes. 0.1ml supernatant harvested and counted in gamma counter

depends on these donors being consistent in their activity. Pross and Maroun (1984), advocated a method based on the selection of normal control donors from a group of individuals who have been tested repeatedly over several months or years and whose NK activity relative to normal donors as a whole can be stated with reasonable confidence. These values can then be used as correction factors which are applied to the relevant control donors' cytotoxicity every time that person is used. Therefore, in each experiment, the mean of the corrected control values may be used as the denominator for the calculation of patient NK activity relative to normal. Cryopreserved lymphocytes have been examined as an alternative to using fresh blood as controls. Pross and Maroun, (1984) reported that cryopreservation may result in full recovery of NK function provided careful attention is directed to the thawing procedure. It is important to assess each batch of cryopreserved cells prior to use because of the chance that cryopreservation has altered control donor function.

It must be emphasised that most studies are performed on separated peripheral blood lymphocytes which may not reflect events in whole blood or at tumour level or in tumour draining lymph nodes. Rees and Platts, (1983) reported methods using whole blood. This method eliminated the laborious and time consuming lymphocyte isolation procedures and permits studies of cytotoxicity under conditions considered more similar to the *in vivo* situation. These studies demonstrated comparable NK activity in whole blood, washed blood cells and separated PBMC from normal donors. Before a conclusion can be drawn as to the immune status of any tumour-bearing host, it is important to investigate whether immune effectors can also be demonstrated *in situ* in the tumour (Manson, 1991). The study of *in vitro* non-MHC-restricted cytotoxicity may be complicated by secondary events following lymphocyte activation. For example, following the lengthy *in vitro* incubation of triggered CTL and target cells, TNF-β can accumulate and mediate non-specific cytotoxicity of target cells (Paul and Ruddle, 1988).

In conclusion, all reports involving the *in vitro* measurement of cytotoxicity must be interpreted with caution. Normalization of cytotoxicity data was suggested as long ago as 1978 (Herberman et al,) yet the majority of published reports do not use such a method as that described above. This is because the use of numerous controls is time consuming, costly and requires the frequent use of volunteers (often graduate students and technicians) particularly if only 1 or 2 patients are being evaluated as is often the case.

3.3.2   Expression of cytotoxicity data

There are two types of data reduction. The most common is comparison of % lysis (Experimental ^{51}Cr-release - spontaneous release) at a single effector to target (E/T) ratio, in the mid range of lysis for a normal effector population (Brunner et al, 1968). Chromium release data directly measuring percentage cell lysis although frequently quoted, is the least quantitative means of data expression. It is primarily useful in demonstrating dose response curves, or gross differences or similarities between groups.

This may be useful in respect to demonstrating the relative sensitivities of various targets to lysis. Typical experimental ^{51}Cr release data (ER) is shown and compared with spontaneous release (SR), maximal release (MR), and total incorporation (TI) (means ± SEM) in Figure 3.4, for one individual on whom an assay was performed in triplicate at 4 E:T ratios. Mean (± SEM) maximal release (MR_{CPM}) of 6 samples in this experiment was 17024 (± 186.2). MR was 88.4% of total incorporation (TI) (TI_{CPM} = 19266 ± 175.5) and SR_{CPM} was 537.0 (± 16.3) (3.2% of MR).

Alternatively, comparison of the E/T ratio at which a certain fraction of target cells (eg., 20% or 30%) are lysed in a given time period may be calculated from a linear regression analysis (Pross et al, 1981). This is obtained by measuring percentage cytotoxicity at different E:T ratios and plotting % cytotoxicity against the E:T ratio. A logarithmic transformation of the E:T is then performed. This method is demonstrated in Figure 3.5 using the same data as in figure 3.4 over four E:T ratios from a maximum of 40:1. It forms the basis of the lytic unit (LU). The percentage of target cells lysed is arbitarily taken as 30% in these calculations. A lytic unit is therefore defined as the number of effectors required to kill 30% of target cells. Results are expressed /10^7 PBMC. When it is necessary to undertake meaningful statistical analysis to show that sets of data are different, and to what extent, lytic unit data are more appropriate (Pross et al, 1981).

A close linear relationship must be obtained in this calculation and unfortunately many factors affect the shape as well as the position of the curve (Thoma et al, 1978; Bryant et al, 1992). In such cases this type of analysis may be misleading (Bryant et al, 1992).

The ^{51}Cr-release assay although widely used has other disadvantages which include the use of a radioactive isotope, relatively high and potentially variable spontaneous release of ^{51}Cr, ^{51}Cr influence on target cells, problems with labelling certain cells, high cost, and prolonged assay time. For these reasons Radosevic et al, (1990) described a simplified flow cytometric assay for the determination of human natural killer cell activity whereby K562 target cells were labelled with a fluorochrome-conjugated monoclonal antibody (F-18). Good correlations were noted between this method and a standard ^{51}Cr-release assay. Methods such as this may be useful when measuring basal NK activity on large numbers of samples. However, their acceptance has been slow and the 4-hour radioisotope-release assay has remained the technique most widely used. We have established the ^{51}Cr-release assay as a predictable technique and the precise method is described in appendix B.

Figure 3.4 - ^{51}Cr-release (experimental release - ER) for one individual
(CPM ± SEM) at different E:T ratios compared with
spontaneous release (SR) against K562.
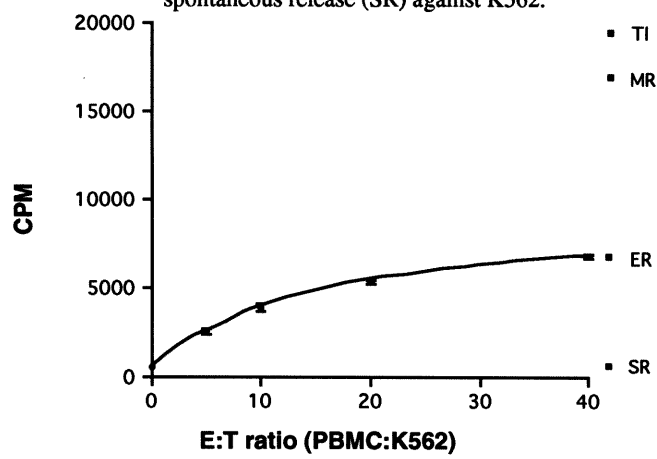
Figure 3.5 - Method for calculation of LU/10^7 PBMC for 30% lysis.
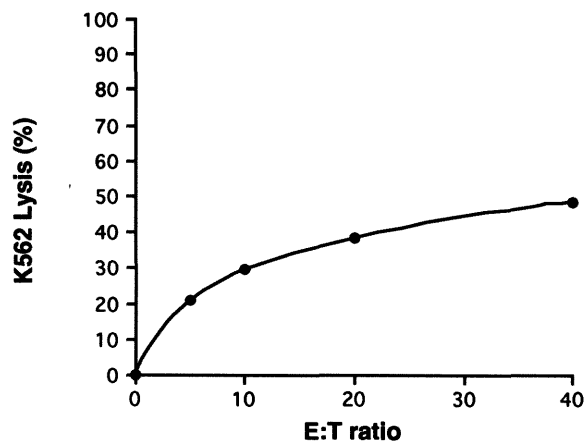
Linear regression analysis.
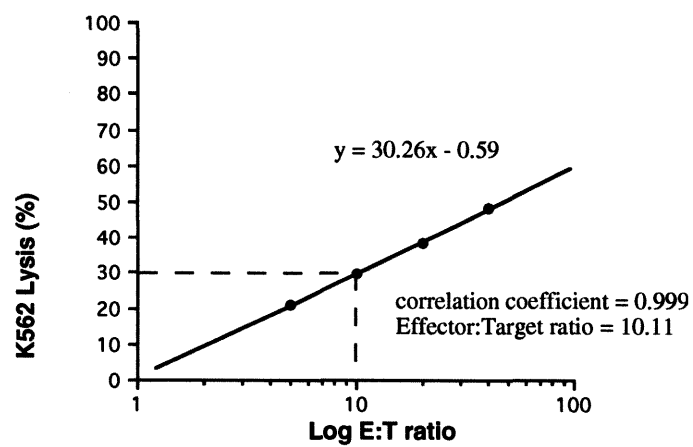
$$LU/10^7 \text{ PBMC} = \frac{10^7}{T \times Xp} = \frac{10^7}{10^4 \times 10.11} = \mathbf{98.9}$$

where T = No. of targets (10^4)
         Xp = the effector:target ratio at which
         30% of target cells are lysed.

3.3.3 Variation in conditions for ^{51}Cr-Labelling of K562 cells.

The maintenance of consistently healthy cell lines that readily incorporate and retain ^{51}CrO$_4$$^{2-}$ (^{51}Cr) is an essential prerequisite for use in cytotoxicity assays. Day to day variation of culture conditions in which K562 cells are maintained may result in variation in ^{51}Cr incorporation and susceptibility to lysis by effector cells. The different target cells studied, their culture conditions and their preparation prior to use in assays are described in appendix B (i). Both adherent cells and cells which grow in suspension were used. The former required detachment prior to study. Provisional experiments demonstrated that with the exception of K562, other targets were not susceptible to lysis by fresh PBMC. An important part of this study was therefore to achieve a reliable preparation of K562 cells for use in assays.

The precise mechanism by which cells incorporate the chromate ion are unknown. Most studies label cells by incubation with ^{51}Cr for only a few hours. For these studies sodium ^{51}chromate (Amersham Plc.) was purchased on a monthly basis and reconstituted to a volume of 1.85 ml using normal saline to give an initial activity of 10MBq per 100 μl. The half-life of ^{51}Cr is 27.8 days and therefore decay results in the need to add increasing volumes of isotope.

Initial studies were undertaken to assess optimal conditions for isotope incorporation (maximal release-MR) and retention (spontaneous release-SR) by K562 cells. This was performed by measuring the MR (following incubation of K562 in NP40) and the SR (following incubation in tissue culture medium) of six aliquots of 5000 K562 cells. Radiactivity in the supernatants were then counted in a gamma counter over one minute and results expressed as mean ($\pm$SEM) counts per minute (CPM). The percentage SR of the MR is also stated. Viability of K562 cells was assessed using the same technique as described for assessing viability of PBMC (Section 3.2.2 and appendix A)

A. Effect of varying the number of K562 cells labelled.

Figure 3.6 shows the mean ($\pm$ SEM) CPM obtained when incubating varying numbers (between 0.25 and 3 million) of K562 cells in 1 ml of complete tissue culture medium with 5MBq ^{51}Cr for 1 hour. Incorporation of isotope was inversely related to the number of cells labelled. SR also increased from 4.6% to 8.3% with the number of cells labelled.

Figure 3.6 - Variation in maximal ^{51}Cr-release (MR) and spontaneous release (SR) (% SR also shown) with number of K562 labelled.
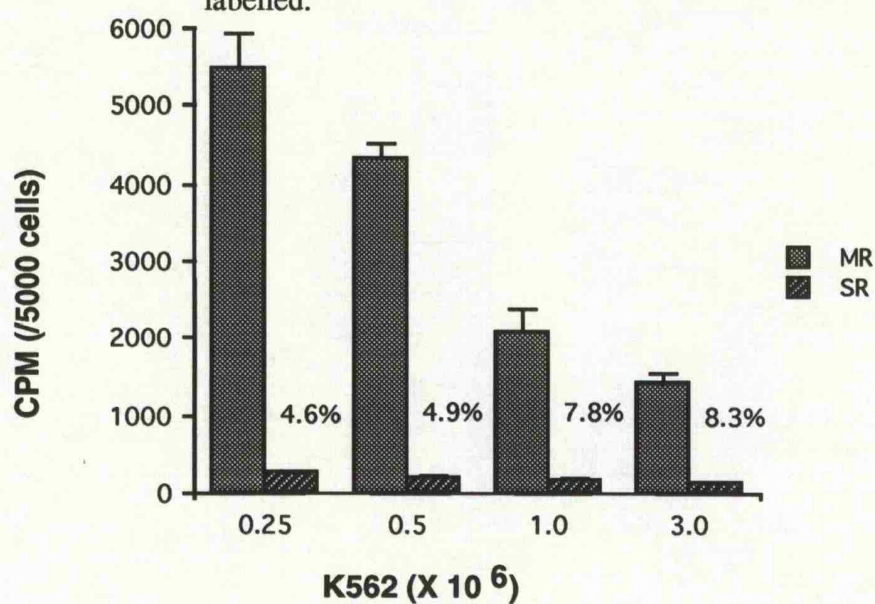
Figure 3.7 - Variation in MR and SR (% also shown) with volume of tissue culture medium in which K562 are suspended (mean ± SEM).
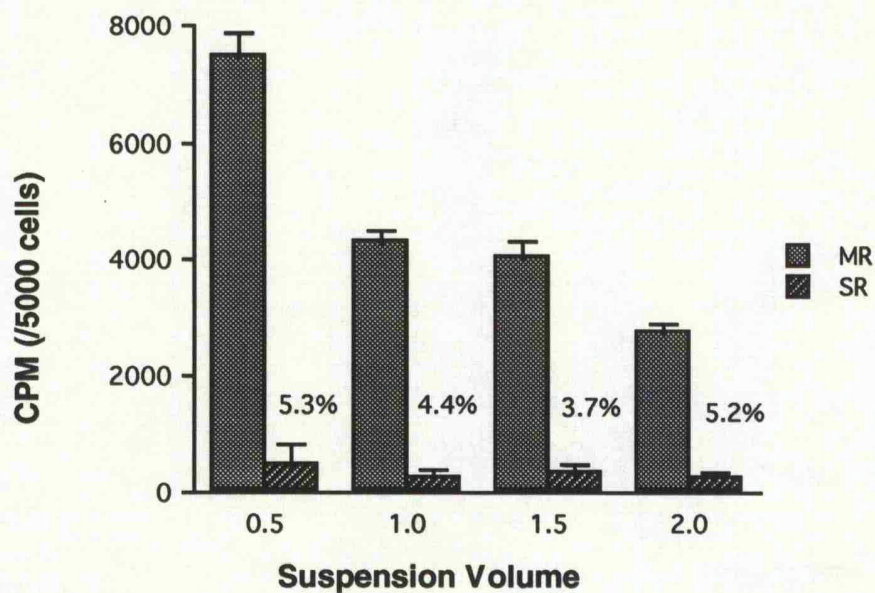
B.   Effect of varying the volume of tissue culture medium in which K562 cells are labelled.

2 million K562 were incubated with 5MBq ^{51}Cr in varying volumes of tissue culture medium for 1 hour and CPM assessed for 5000 cells. Results are described in Figure 3.7. ^{51}Cr incorporation was inversely related to the volume of medium in which cells were suspended during labelling. Variation in volume of medium had no consistent effect on SR.

C.   Effect of varying the culture conditions of K562 cells prior to labelling.

^{51}Cr incorporation and retention by K562 vary from day to day even when the same amount of ^{51}Cr is incubated with the same number of K562 in the same volume of tissue culture medium. To investigate the influence of culture conditions on K562 labelling the following experiment was performed. Three preparations of K562 were incubated at 0.5 million cells ml^{-1} in 10 mls of tissue culture medium. Cells were passaged (centrifuged at 200 X g for 5 minutes and replaced with fresh medium) at 12 hours, 24 hours and 48 hours prior to labelling 2 million cells from each flask with 5 MBq ^{51}Cr for 2 hours in 1 ml complete tissue culture medium.

Figure 3.8 describes the results (means ± SEM) of ^{51}Cr incorporation for the three preparations. Cells that had not been passaged for 48 hours incorporated only slightly less ^{51}Cr than cells which had been most recently passaged. However, levels of spontaneous release were substantially greater (13.9%) in the cells that had been maintained for the longest time in continuous culture.

D.   Effect of varying exposure time to ^{51}Cr.

The cost of isotope and the quantity of isotope that could be stored in the Department of Surgery (for reasons of safety) were important limiting factors to the amount of isotope that could be used for labelling. Therefore, it was essential to obtain efficient labelling of target cells with the smallest posible quantity of isotope. Experiments were performed to investigate the effect of varying the length of exposure of K562 to ^{51}Cr on incorporation and retention of isotope.

2 million K562 were incubated with 10MBq ^{51}Cr in 1 ml of tissue culture medium for 30 minutes, 2 hours, and 18 hours. Results are described in Figure 3.9. Incorporation and retention of ^{51}Cr varied with the duration of exposure to ^{51}Cr. MR was greatest and associated with the lowest % SR following incubation with ^{51}Cr for 18 hours (overnight). Viability of K562 assessed by fluorescence microscopy and AO/PI labelling (Plate 7) prior to all experiments and following ^{51}Cr-labelling and was > 95%.

E.   Influence of the duration of exposure of K562 to ^{51}Cr on susceptibility to lysis.

Most reports do not label K562 cells by incubation with ^{51}Cr for more than 2 hours. It was therefore important to substantiate that this method of target cell

Figure 3.8 - Variation in MR and SR (% also shown) with variation in length of continuous culture prior to ^{51}Cr labelling (means ± SEM).
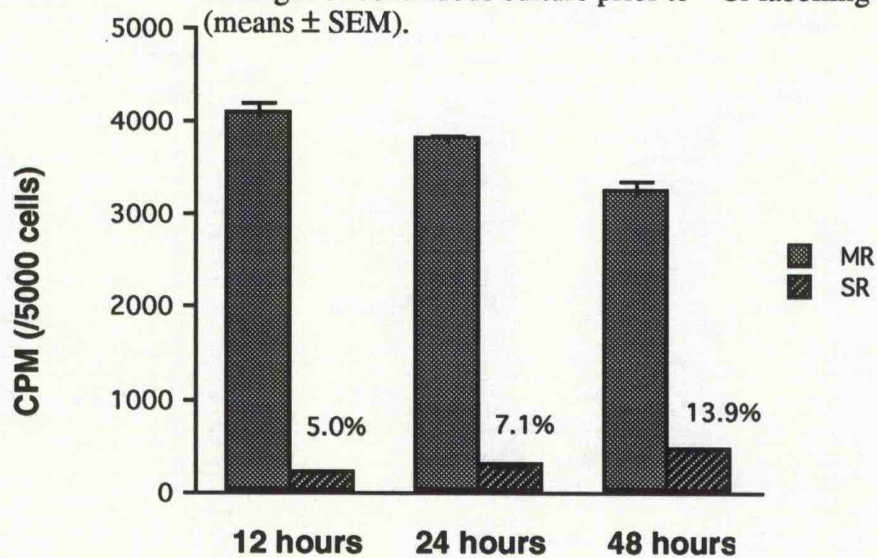
Figure 3.9 - Variation in MR and SR (% also shown) following incubation of K562 with ^{51}Cr for varying lengths of time (means ± SEM).
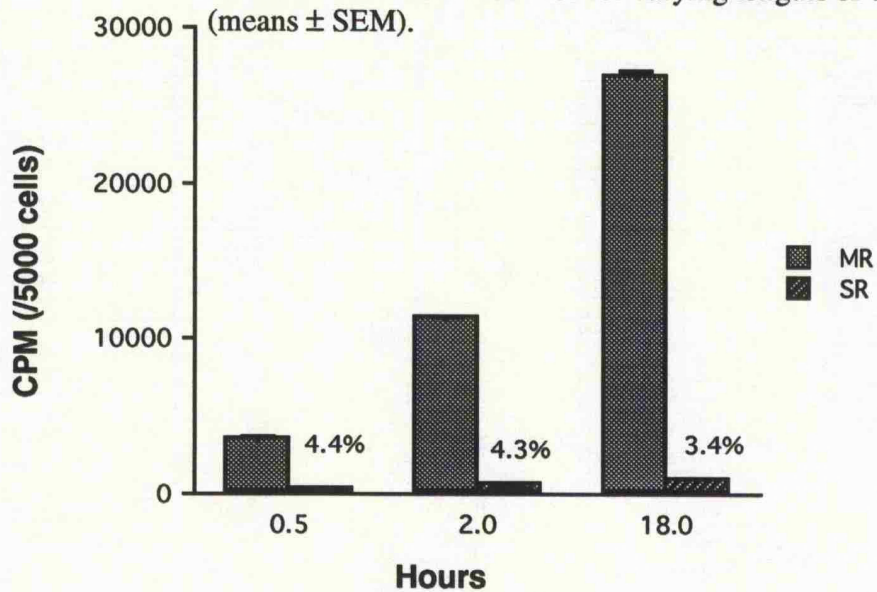
Plate 7 - Fluorescence microscopy (X 100 magnification) following incubation with acridine orange and propidium iodide showing viable (green) and non-viable (red) K562 cells.
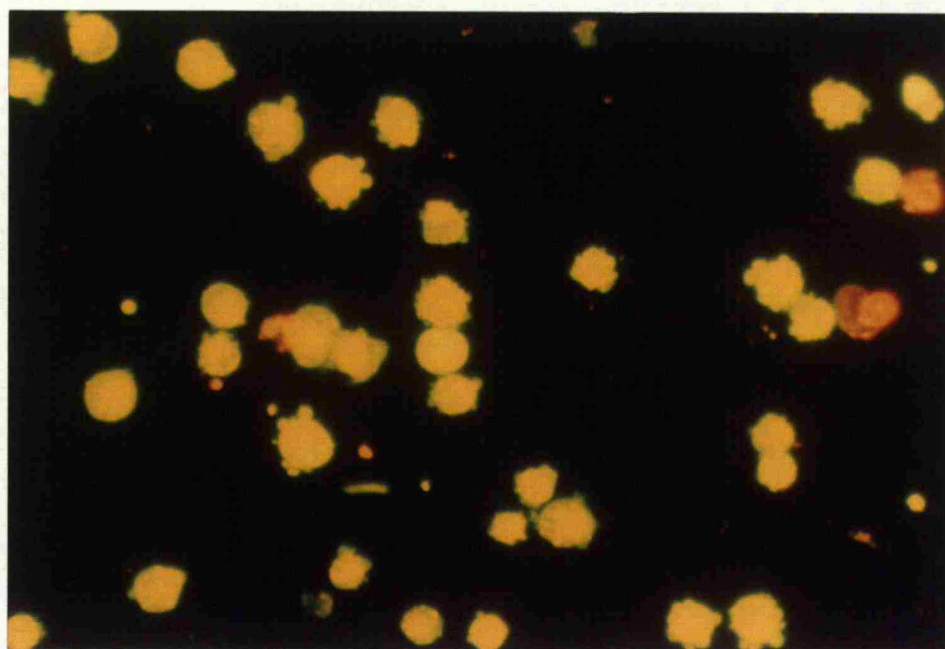
preparation did not alter their susceptibility to lysis before adopting it in these studies. Cytotoxicity using PBMC from 2 healthy donors (JH and MH) was assessed against K562 cells labelled with ^{51}Cr for 2 hours and 18 hours.

Results are described in Table 3.11 which also shows the effect of preincubation of PBMC with 1 μg ml^{-1} OKT3, T3, and anti-CD16 mAb. No difference in either baseline cytotoxicity (in medium) or cytotoxicity following preincubation in various mAb was seen, demonstrating that 18 hours incubation in ^{51}Cr does not affect K562 susceptibility to lysis.

Addition of 10^4 target cells per well requires that an individual assay performed in triplicate requires 1.2 ml of target cells at a concentration of 10^5 ml^{-1}. Therefore 5 million cells provides sufficient target cells for 50 assays. From the above experiments it was concluded that for satisfactory incorporation of isotope into K562 target cells (maximal release > 5000 counts min^{-1}, spontaneous release was < 10% of maximal release, and viability > 95%) was obtained with incubation of between 3 and 5 million K562 in 0.5 ml tissue culture medium with between 5 and 10MBq ^{51}Cr for 18 hours. Furthermore, incubation with ^{51}Cr for 18 hours did not influence the susceptibility of K562 to lysis compared with incubation for 2 hours. The method routinely adopted for labelling of K562 cells is described in detail appendix B (i).

3.3.4   Validation of lytic units for expression of cytotoxicity data.

The validity of using a linear regressional analysis to express results of cellular cytotoxicity obtained from different experiments depends upon a close and consistent linear correlation between a plot of percentage specific cytotoxicity and the E:T ratio. Differences in the shape of the curves may result in variation when comparing results of different experiments expressed in LU. When high levels of killing are obtained eg. at high E:T ratios or when killing is enhanced to high levels by BRM the curve begins to plateau because maximal killing has been achieved. Similarly, at low E:T ratios the curve also flattens. When levels of killing are at the extremes of the curve inacuracies may therefore occur because a linear relationship is lost between percentage ^{51}Cr-release and cytotoxicity. This type of experimental variation is referred to as heteroscidicity. Variation in the E:T ratio and differences in the quantity of enhancement by BRM between different experiments, is an important consideration in comparing results expressed as LU.

A.   Variation in E:T ratio and effect on results expressed in LU.

For experiments when large numbers of PBMC were required it was sometimes necessary to perform assays at E:T ratios of 20:1. In the clinical studies using the higher doses of OKT3, because the effect of OKT3 was to deplete circulating lymphocytes, insufficient PBMC were frequently obtained to perform assays at the highest E:T ratio of

Table 3.11 - K562 cytotoxicity (LU/10^{7}PBMC) against K562 targets labelled with ^{51}Cr for varying incubation times.

		K562 labelled with ^{51}Cr and Cytotoxicity LU/10^7 PBMC	
Donor	Medium/mAb	2 hours	18 hours
JH	medium	25.6	25.1
MH		3.9	2.8
JH	OKT3	54.9	54.2
MH		4.1	2.8
JH	T3	207.9	197.9
MH		2.9	1.5
JH	anti-CD16	64.6	50.4
MH		10.5	7.8

40:1. It was therefore important to ascertain the effect that variation in the E:T ratio had on results expressed in Lytic Units. PBMC were separated from one healthy individual (JH) and cytotoxicity against K562 cells measured at seven E:T ratios varying from 1.25:1 to 80:1. Each assay was performed in triplicate and three experiments were performed at each E:T ratio. PBMC were also preincubated in the mAb T3.

Results are described in Figure 3.10. Although the correlation coefficient was always > 0.98 a linear relationship was most closely maintained over the four E:T ratios ranging from 40:1 to 5:1. Results at the extremes of the curve were most divergent from it. In this experiment cytotoxicity was 20.4 LU/10^7 PBMC for PBMC incubated in medium at a maximum E:T of 40:1 (calculated from intercept a). The effect of reducing the maximum E:T on cytotoxicity expressed in LU is described in Figure 3.11. Cytotoxicity was calculated at 4 maximum E:T ratios varying from 80:1 to 10:1 using the same data expressed in Figure 3.17. Although the calculation of LU takes the E:T into consideration this fails to correct the decline in measured cytotoxicity seen at lower E:T ratios.

B.   Effect of mAb on cytotoxicity expressed in LU at different E:T ratios.

Different mAb enhance cytotoxicity by different amounts. T3 consistently enhanced K562 cytotoxicity to a greater extent than other mAb. Enhancement of cytotoxicity by BRM may exceed the capacity for the assay to measure further killing in the same way that increasing the E:T ratio does. Figure 3.10 demonstrates a plateau developing in the T3 curve at an E:T ratio of 80:1 (r = 0.98) where K562 lysis was 57.5%. At an E:T ratio of 40:1 a linear relationship was re-established (r = 0.99). K562 cytotoxicity was substantially enhanced to 262.5 LU/10^7 PBMC following preincubation with T3 (intercept b) calculated over four E:T ratios at a maximum of 40:1 in this individual. Experiments using mAb to enhance cytotoxicity seldom exceeded 60% lysis at the highest E:T ratio which approximately represents the maximal killing capacity of the system.

In conclusion the value of a given method of expressing cytotoxicity data varies with the situation being assessed. Variation in the correlation coefficient during linear regression analysis is less important if an experimental manipulation (eg. the addition of an antibody) is assessed within the same experiment. The pretreatment value then provides a baseline to which the post-treatment value can be compared and the experiment has an internal control. In these circumstances all experimental conditions are the same except for the addition of antibody. However, when experiments are performed during the serial monitoring of patients, variation in experimental conditions are sometimes unavoidable. This occurred when insufficient PBMC were obtained to perform all experiments at a maximum E:T of 40:1. A decline in E:T ratio does reduce cytotoxicity for both PBMC incubated in tissue culture medium and similarly with mAb enhancement and this must be considered in the interpretation of results expressed in LU.

Figure 3.10 - K562 cytotoxicity for PBMC preincubated in medium or
T3 over a range of E:T ratios varying from 1.25:1 to 80:1.
(one healthy donor).
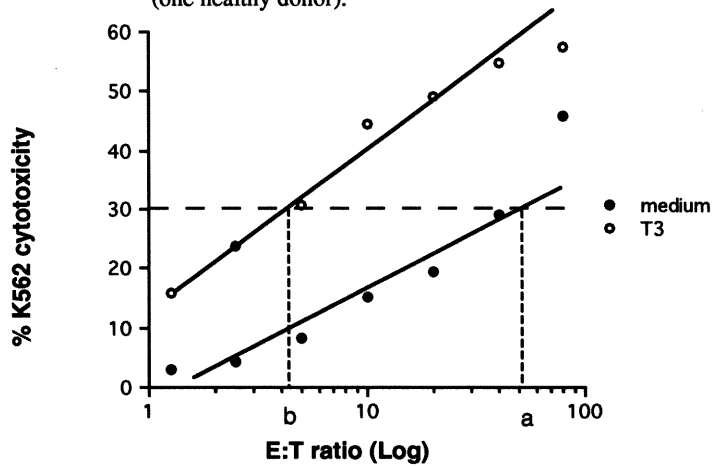
Figure 3.11 - Variation in K562 cytotoxicity calculated in LU at
different maximal E:T ratios for one individual.
(mean ± SEM of 3 assays).
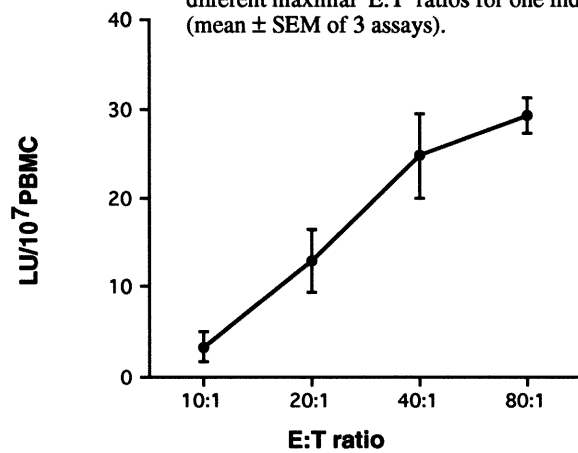
These experiments demonstrated that it was valid to compare results of mAb enhancement in LU/10^7 PBMC when an E:T ratio of 40:1 to 5:1 was used over 4 dilutions in different experiments. Assays were routinely discarded if the correlation coefficient (r) was $\leq$ 0.95.

3.3.5 Inter-plate and intra-plate variation in K562 cytotoxicity.

Assessing assay variation becomes increasingly important with the study of small numbers of patients and when the influence of BRM is relatively small. It was important to identify technical errors particularly with respect to PBMC preparation that may lead to variation in cytotoxicity. Intra-plate variation refers here to the variation observed between assays when one PBMC preparation was incubated with the same K562 preparation. Such variation is mostly due to variation in pipetting technique between individual wells of the culture plate during serial dilutions, and ultimately when supernatant was collected for measurement of ^{51}Cr-release. It was important to document this variation because it was the principal cause of experimental variation in cytotoxicity obtained between PBMC incubated in medium and PBMC incubated in mAb for one individual.

Inter-plate variation refers here to the variation in results obtained between PBMC prepared separately from the same whole blood sample and then incubated with the same target cells. It was important to document this variation because it was particularly relevant to the sequential monitoring of cytotoxicity in the patient studies.

140 mls of blood was collected from one healthy donor (JH) and separated into a 40 mls aliquot and a 100 ml aliquot. 10 mls of blood from the 40 mls sample were layered onto Ficoll-Hypaque in four universal containers and following a single centrifugation and collection of PBMC from the surface of the F-H, cells were combined, washed, counted and resuspended at 8 X 10^6 ml^{-1}. Yields of PBMC from this sample were 0.96 X 10^6 ml^{-1} blood processed. PBMC were separated from the 100 mls sample using the same technique although PBMC were not combined prior to washing and were subsequently manipulated separately. Mean ($\pm$ SEM) PBMC yields were 0.73 ($\pm$ 0.10) X 10^6 ml^{-1} blood processed. 10 assays were then performed using the combined PBMC preparation and the 10 individual PBMC preparations. Individual results are described in Figure 3.12. Mean ($\pm$ SEM) cytotoxicity was 109.5 ($\pm$ 2.1) LU/10^{7}PBMC in the combined preparation and for the 10 individual preparations it was 87.3 ($\pm$ 6.0) LU/10^{7}PBMC. The variation in results was relatively small for the combined preparation (range 87.5 to 121.3 LU/10^7 PBMC). Greater variation was seen for the individual PBMC preparations (range 58.4 to 121.5 LU/10^7 PBMC).

Figure 3.12 Variation in K562 cytotoxicity between PBMC prepared separately or combined after the first centrifugation (bars = means).
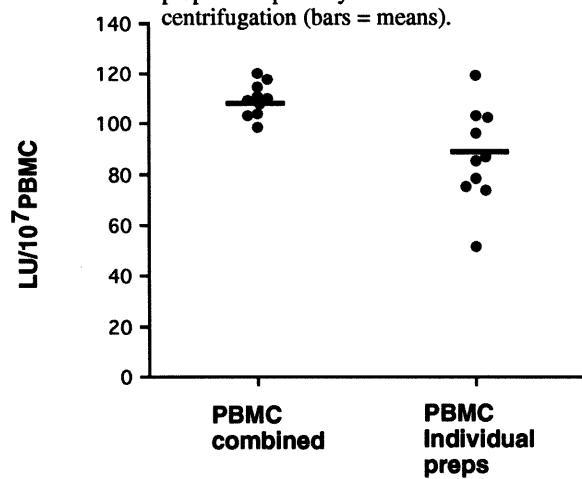
Table 3.12 - Variation in cytotoxicity and yields of PBMC during standing of whole blood.

	MINUTES				
	0	30	60	90	120
Cytotoxicity (LU/10^7 PBMC)	35.24	29.36	36.81	41.07	25.19
PBMC yields ml^{-1} blood	1.10	0.82	0.65	0.62	0.58

3.3.6 Effect of prolonged standing of blood prior to PBMC separation on K562 cytotoxicity.

During the course of the OKT3 trial three patients usually underwent sequential monitoring of cytotoxicity simultaneously. It was not possible to begin separating PBMC from whole blood prior to obtaining the 1 hour sample which resulted in the first sample being left to stand at room temperature for at least one hour. PBMC were separated from the sample obtained at 4 hours within 10 minutes of venesection as were all the samples obtained from the healthy donors. Aggregation of cells during prolonged standing of blood samples may influence the constituents of PBMC preparations. Alternatively alterations in metabolism of cells during standing (due to acidosis) may occur and affect their cytotoxic capacity.

It was therefore important to eliminate standing time of whole blood, prior to commencing PBMC separation as a potential source of variation in cytotoxicity. 50 ml of blood was obtained from one healthy donor JH. This was maintained at room temperature for up to 2 hours and 10 ml aliquots removed after gentle mixing, at 30 minute intervals following venesection. Results of PBMC separation are shown in Table 3.12. A decline in the yields of PBMC per ml of blood was observed with duration of standing of blood. Variation in cytotoxicity was seen between samples but there was no consistent effect on cytotoxicity.

3.3.7 Comparison of results between different investigators.

Toward the latter period of these studies cytotoxicity assays were performed by a temporary research fellow (RC) under the supervision of the principle investigator (JH). Although the same assay protocol was used by both investigators, it was important to demonstrate that comparable levels of cytotoxicity were being measured. 120 mls of blood was obtained from one healthy donor (JAH) and separated into six aliquots of 20 mls labeled A to F. One batch of K562 cells was prepared in the usual way. K562 cytotoxicity was measured simultaneously by JH (samples A, C, E) and RH (samples B, D, F) in which PBMC were prepared separately from each sample. RC obtained higher mean ($\pm$ SEM) PBMC yields at $1.32 \pm 0.16 \times 10^6$ ml^{-1} of blood processed than JH at 0.87×10^6 ml^{-1} blood. K562 cytotoxicity is described in Table 3.13.

Despite differences in the number of PBMC harvested, comparable results were obtained between RC and JH both with respect to baseline levels of cytotoxicity and also following preincubation in OKT3 and T3.

In conclusion, these experiments demonstrate that greater variation in cytotoxicity occurs with PBMC are prepared separately from the same blood sample compared with PBMC prepared and combined prior to addition to assays. The former variation is relevant to sequential monitoring of subjects and the latter to *in vitro* modulation of PBMC preparations with mAb. Prolonged standing of blood has the effect of reducing yields of PBMC but is not associated with a consistent decline in cytotoxicity.

Comparable results were obtained between JH and RC following the same protocol for measurement of K562 cytotoxicity and also for enhancement with mAb.

Table 3.13 - Comparison of K562 cytotoxicity (LU/10^7 PBMC) and enhancement by OKT3 and T3 for assays performed simultaneously by JH and RC.

	JH				RC		
Sample	medium	OKT3	T3	Sample	medium	OKT3	T3
A	0.2	2.5	40.0	B	0.1	1.9	46.7
C	0.1	1.2	35.2	D	0.1	2.1	25.0
E	0.2	2.0	48.6	F	0.3	3.9	54.1
mean	0.2	1.9	41.3		0.1	2.7	42.2
Median	0.2	2.0	40.0		0.1	2.1	40.0
SEM	0.0	0.4	3.9		0.1	0.6	8.9

3.4 IN VITRO MANIPULATION OF CELL-MEDIATED CYTOTOXICITY.

3.4.1 Introduction.

The standard ^{51}Cr-release assay was modified to study the effect of various mAb, rIL-2, and cancer patients serum on cytotoxicity mediated by fresh PBMC. The precise technique is described in Appendix C and has been previously reported (Ubhi et al, 1991). All antibodies were stored at either 4°C or -70°C according to the manufacturers instructions and were used within the stated expiry time from purchase. Some antibodies did not have a known isotype concentration in which case an estimate was made based on product information from the manufacturer. Sodium Azide was present as the preservative in some antibody preparations at a maximum concentration of 0.1% . Further Sodium Azide was not added during reconstitution. Properties of all antibodies studied are described in Table C1 in appendix C.

Recombinant IL-2 (Proleukin, Eurocetus Ltd.) was purchased in vials containing 1 mg (18 X 10^6 IU) rIL-2. This was reconstituted in tissue culture medium to a stock solution of 20 000 IU ml^{-1} and stored in 1 ml aliquots at -70°C. These were then thawed at room temperature, stored at 4°C for up to 1 month, and reconstituted in tissue culture medium on the day of assay.

Serum samples were prepared and stored as described in Appendix E and were added to assays undiluted in place of mAb or rIL-2.

3.4.2 Validation.

A. Effect of Sodium Azide on K562 cytotoxicity.

Sodium Azide may influence cytotoxicity by being directly toxic to target cells and PBMC. The maximum concentration of Sodium Azide initially present in any of the antibodies was 0.1% (1 g L^{-1}) at an antibody concentration of 1 mg ml^{-1}. However, reconstitution of antibodies to a concentration of 2 μg ml^{-1} prior to use in assays resulted in this being diluted to a final concentration of only 2 X 10^{-8}%. A 0.1% Sodium Azide solution was prepared by dissolving 1 mg of Sodium Azide in 10 mls tissue culture medium and serially diluting this stock solution provided solutions of varying concentrations. The effect on K562 cytotoxicity following preincubation of PBMC in different concentrations of Sodium Azide was then assessed in 3 healthy donors. Results are described in Table 3.14. Although a 0.1% solution markedly impaired K562 cytotoxicity no consistent effect was seen below a concentration of 0.001%.

B. Effect of non-specific antibody preparations on K562 cytotoxicity.

To exclude the possibility that antibodies may influence K562 cytotoxicity through a non-specific binding effect, both a polyclonal mouse IgG preparation (PmIgG) and mouse and human polyclonal antibodies of known isotype (mIgG1, mIgG2a, and hIgG1) were studied at varying concentrations in the standard K562 assay. Results of individual experiments are decribed in Table 3.15 in appendix H.

Table 3.14 - Effect of Sodium Azide on K562 cytotoxicity (LU/10^7PBMC) in three
healthy donors.

Conc (%)	0	0.1	0.01	0.001	0.0001	0.00001
JH	37.4	0.2	34.1	36.3	32.8	28.0
TH	29.9	0.0	17.7	26.5	24.6	25.8
PSV	36.2	0.3	22.8	37.0	49.8	50.8
mean	34.5	0.2	24.9	33.3	35.8	34.9
median	36.2	0.2	22.8	36.3	32.8	28.0
SEM	2.3	0.1	4.9	3.4	7.4	8.0

There was no significant effect on K562 cytotoxicity of preincubating PBMC from healthy donors in polyclonal antibodies of either variable isotype or specific isotype at a concentration of 1 µg ml^{-1}. Antibodies did not have a direct effect on spontaneous ^{51}Cr release. Following this finding experiments were not routinely undertaken which incorporated a non-specific control antibody.

3.5   FLOW CYTOMETRY

The development of flow cytometry has enabled the routine and rapid analysis of cells by their variable expression of surface (CD) antigens. This may enable both the enumeration of cells defined by their expression of surface antigens and analysis of changes in antigen expression following activation by BRM.

In these studies cells were analysed using a fluorescence-activated cell analyser equiped with a 488 nm, 15mW, air cooled, argon ion laser (FACScan, Becton Dickinson) (Plate 8). The principle of this method is that cells can be identified by their size and granularity into distinct populations. They can be further distiguished using fluorochrome-conjugated monoclonal antibodies antibodies specific for cell membrane antigens which are analysed by spectral emissions. Cells are introduced into a flow chamber and then forced into a fluid stream of cells under pressure in a quartz cuvette. The stream is illuminated by the argon laser and individual cells deflect the light through varying angles which is detected by photodiodes and photomultiplier tubes. In this way reproducible analysis of large numbers of cells (>1000 sec^{-1}) is possible.

The characteristic scatter pattern of subpopulations of mononuclear cells is created by comparing foreward angle versus orthogonal light scatter signals of individual cells (Thompson et al, 1985). The forward light scatter (FSC) is a measure of cell size, is detected with a photodiode, and is plotted on the x-axis. The side scatter (SSC) is a measure of granularity, is detected by photomultiplier tubes and is plotted on the y-axis. Mononuclear cells can therefore be distinguished ("gated") into different populations. By setting the threshold in the gate, particles with a smaller scatter signal, representing platelets, erythrocytes, and cellular debris can be excluded.

Fluorochrome-conjugated mAb are commercially available conjugated to fluorescein isothiocyanate (FITC) (green emission) or phycoerythrin (PE) (red emission). Antibodies used in these studies are described in Table D1 in appendix D. By combining antibodies labelled with one or the other fluorochrome and undertaking a two-colour analysis it is possible to distinguish multiple cell populations simultaneously. This reduces the time taken for analysis, requires fewer cells and provides added information regarding double-stained cell populations. Simultest products (BD) utilise this concept and were used to identify lymphocytes bearing both NK markers (CD16 and/or CD56) and also CD3. Emission signals are detected by the SCC. For two-colour analysis a dichroic mirror is used to separate the FITC and PE spectral emissions. All signals were

recorded and analysed on a Hewlett Packard computer incorporating Simulset software (Becton Dickinson).

Two parameter correlated isometric displays (dot-plots) were used to show the percentages of neutrophils, monocytes, and lymphocytes after counting 10 000 events using a Simultest Leukogate antibody (FITC-Leu-1 (CD45) and PE-Leu-M3 (CD14), Becton Dickinson) and a typical result is described in Plate 9. Based on this isometric display the lymphocyte population was specifically gated and subsequently used to analyse lymphocyte subpopulations. Single and double fluorescence parameters were also displayed on a dot plot with green fluorescence (FITC) on the x axis and red fluorescence (PE) on the y-axis. A typical display is shown in Plate 10 for enumeration of NK cells following labelling with the PE-Leu 11c (CD16) and/or PE-Leu 19 (CD56) and PE-Leu 4 (CD3) Simultest antibody (Becton Dickinson).

It was possible to calculate absolute numbers of cell subsets in the original sample from the WBC count which was determined manually. The precise method for undertaking flow cytometry is described in appendix D.

Plate 8 - Becton Dickinson flow cytometer (Fluorescence activated cell analyser - FACscan).

Plate 9 - Isometric (dot plot) display of whole blood labelled with leucogate (BD)
demonstrating lymphocyte (L), neutrophil (N), and monocyte (M) populations.
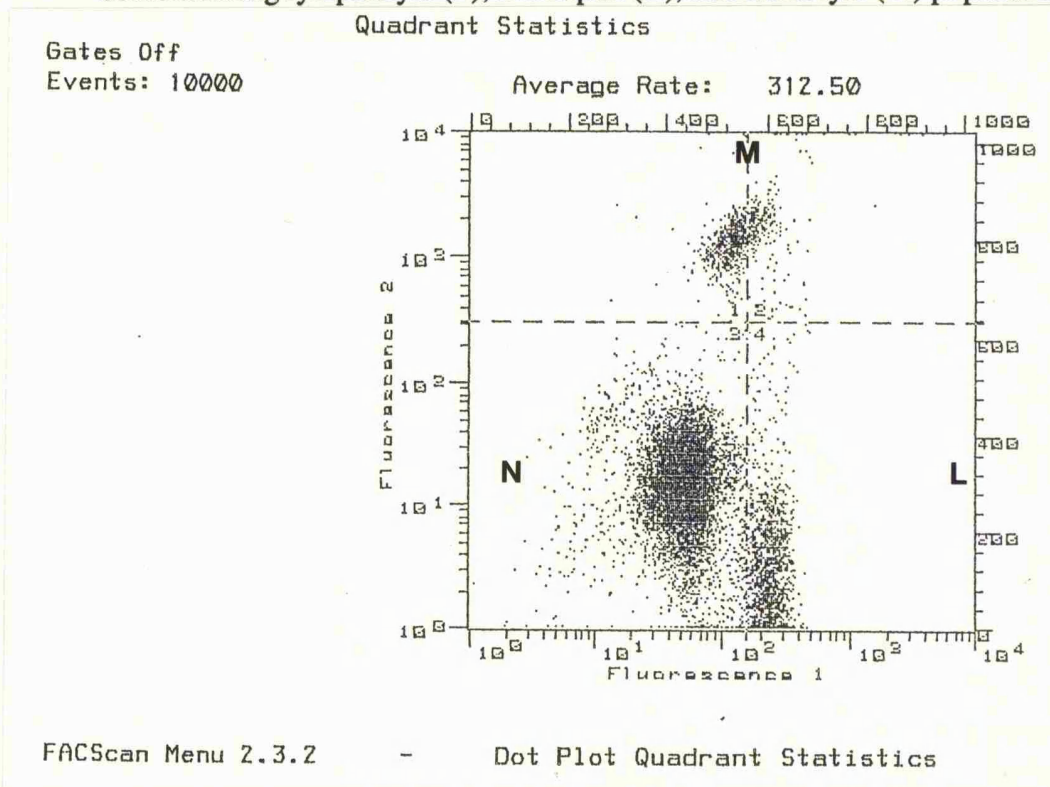
Plate 10 - Isometric (dot-plot) display of whole blood labelled with simultest preparation
(Leu-4, Leu-11c/Leu-19(BD)) demonstrating natural killer (NK) cells, CD3+
lymphocytes (CD3+L), and CD3+ cells co-expressing NK markers (CD3+NK).
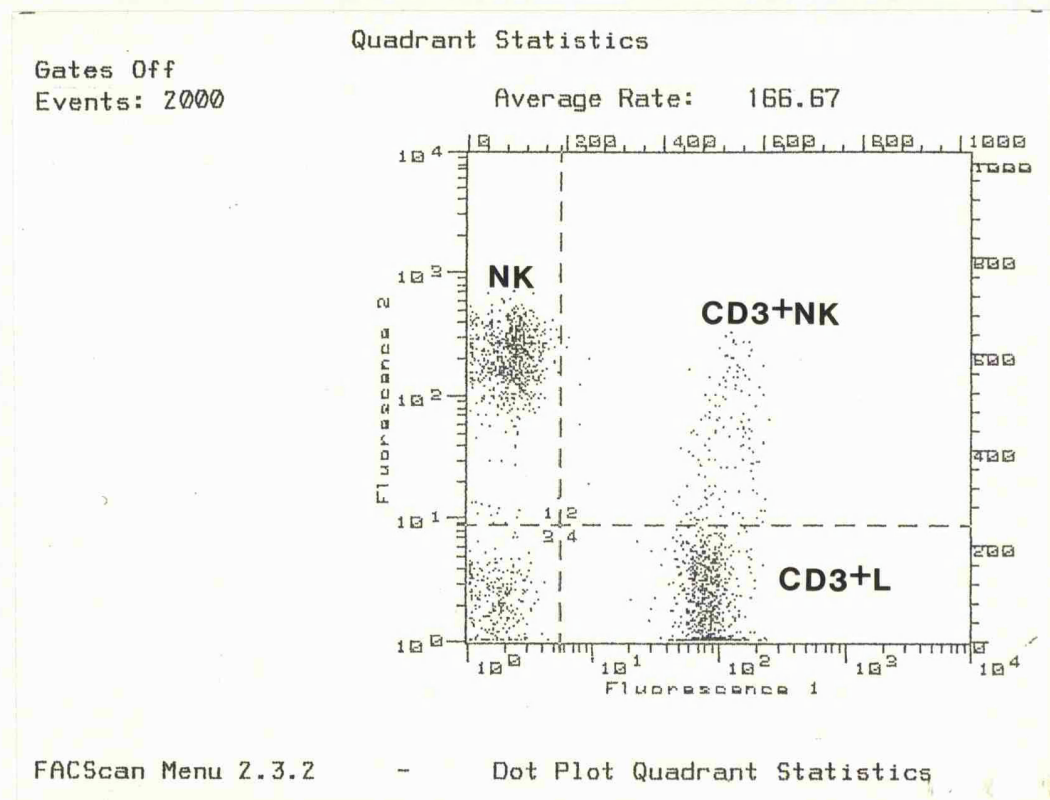
3.6   MEASUREMENT OF IL-2, IFN-γ, TNF-α, AND sIL-2Rα LEVELS.

Cytokines in common with most peptides are transported in serum both bound to proteins and free in solution. Unbound cytokines represent the biologically active component which is freely available to interact with its receptor. IL-2, IFNγ, TNFα, and sIL-2Rα levels were measured in order to assess the effect of OKT3 in the clinical studies. Commercially available kits were purchased which utilize the principle of an enzyme-linked immunosorbent assay (ELISA) technique. This method was chosen because the technique is rapid and simple to undertake, and is a sensitive method of assessing these substances. The principle disadvantage of the kits was their cost. Details of the kits are described in appendix E.

The principle depends on a 'sandwich' enzyme immunoassay technique. An antibody to the substance (cytokine or sIL-2Rα) to be detected is adsorbed onto polystyrene microtitre wells (kits are supplied in this form by the manufacturer). Standards of known concentration and the samples to be analysed are then added to the wells and following a short incubation period the wells are washed. Compounds which exhibit specific binding to the wells are retained and unreacted sample components are thus removed. The amount of bound compound is then detected by completing the sandwich by adding a further enzyme conjugated anti-component antibody to the wells. Excess enzyme conjugated antibody is removed by washing, a substrate for the enzyme is added to the wells, and this results in formation of a coloured product. The intensity of this product is proportional to the amount of the component present. The reaction is terminated by the addition of a stop solution and absorbance at 490 nm is measured using an automatic plate reader. Standard curves were generated (Figures 3.13 to 3.16) by plotting average corrected absorbance at 490 nm (measured - background) against the absorbance from the standard cytokine. Cytokine levels in experimental samples were then measured by plotting corrected mean (of 2 wells) absorbance on the standard curve.

Assays were performed according to the manufacturers instructions on a limited number of samples.

Figure 3.13 - Standard curve for IL-2.
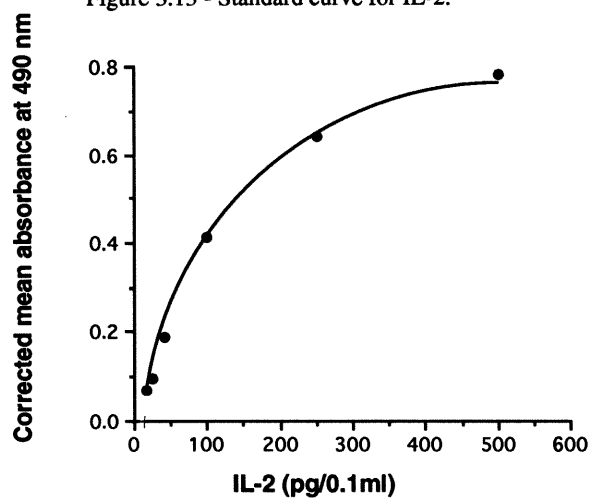
Figure 3.14 - Standard curve for IFNγ.
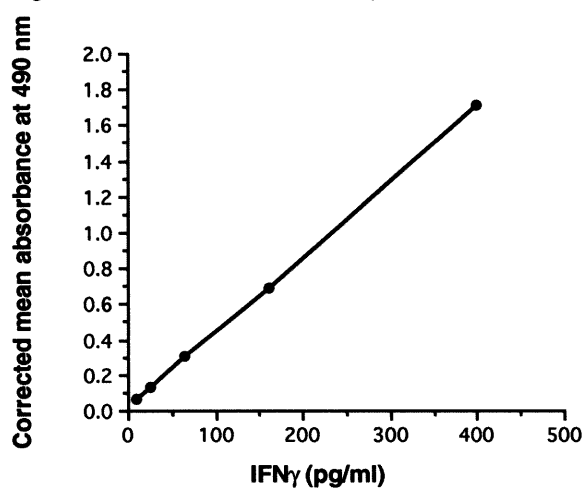
Figure 3.15 - Standard curve for TNFα.

WW corrected mean absorbance at 490 nm = 0.142
TNFα = 75 pg ml $^{-1}$.
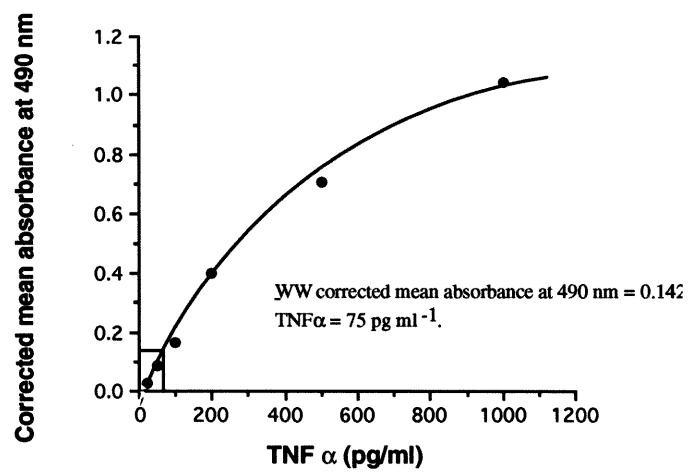
Figure 3.16 - Standard curve for IL-2R.

$r = 0.99$
$y = 5.28^{-4} x$
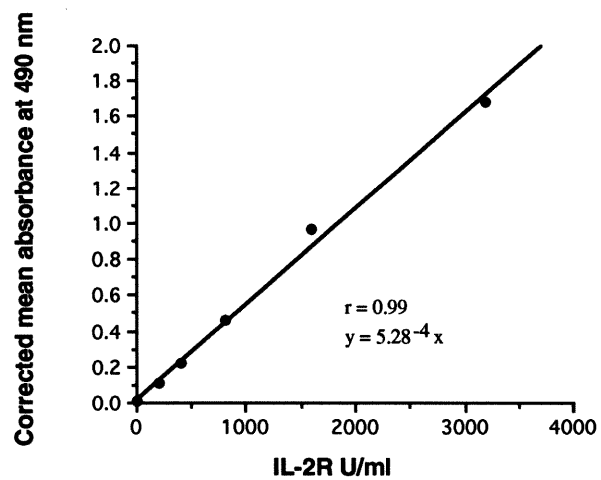
3.7   MEASUREMENT OF OKT3 LEVELS.

OKT3 is only available in glass ampoules containing 5 mg of isotype in 5 mls of solution. Syringes were reconstituted according to the method described in appendix F. Different technicians were responsible for this process and so operator variability may be expected. It was important to verify that the amount of OKT3 actually administered to patients approximated closely to the quantity stated by the pharmacy department.

The method for measuring OKT3 levels was developed in the Department of Surgery by Mr. S. Weston (manuscript in preparation) who also performed the assays on serum and in the duplicate syringes and is described in detail in appendix G. The principle of the method depends upon the detection of OKT3 binding to the surface membrane of a T cell line (HUT 78) using a fluorochrome conjugated mAb specific for mouse IgG. The amount of binding of the conjugated mAb is determined by measuring the intensity of fluorescence using a flow cytometer. A standard curve is generated using samples of known OKT3 concentration (Figure 3.17). OKT3 is measured in the unknown sample assessed at a number of dilutions by comparing the mean value of their intensity of fluorescence with the standard curve.

Figure 3.17 - Standard curve for OKT3 levels.

$y = 139.0 (\log x) - 11.8$

Median Channel Fluorescence

OKT3 (ng/ml)

- ● OKT3 Standards
- ○ Ab Serum
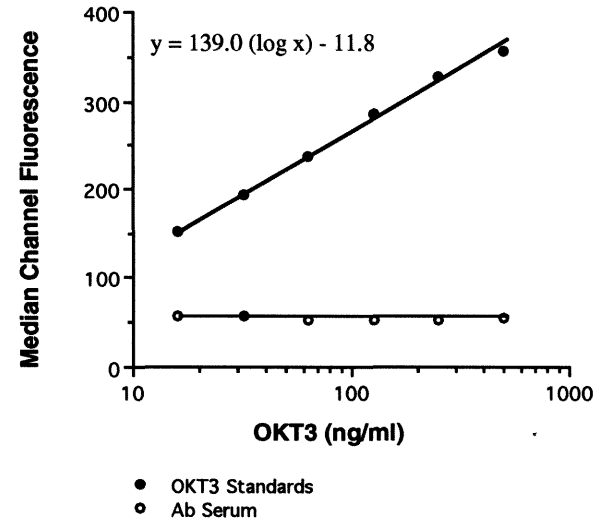
3.8   STATISTICAL ANALYSIS.

The InStat statistical computer package (version 1.12, GraphPad Software, Inc. 10855 Sorrento Valley Road, # 204 B San Diego, CA 92121, USA.) developed in The Department of Pharmacology at the University of California, San Diego was used throughout this study. Advice regarding the statistical analysis of data was kindly provided by Professor D Jones of The Department of Epidemiology at Leicester University

Data regarding cell numbers in both blood and PBMC data was usually normally distributed (Gaussian curve) and parametric tests were applied to compare results in different groups (paired and unpaired T tests). Cellular cytotoxicity was positively skewed in distribution with the majority of subjects having low levels of killing and a few subjects having high levels. Cytotoxicity data is always expressed with both the mean ($\pm$ SEM) and median values stated. Non-parametric tests were used to analyse cytotoxicity data. The Mann-Whitney U-test was used to analyse differences in cytotoxicity between different groups. The Wilcoxon signed rank test was used to analyse paired observations on cytotoxicity within a group. The relationship between cytotoxicity and NK cell numbers was analysed using the Spearman Rank correlation coefficient.

All experiments involving the addition of mAb or IL-2 resulted in paired data values. Cytotoxicity measured with incubation in tissue culture medium is referred to as baseline cytotoxicity. Cytotoxicity with incubation in mAb/IL-2 is experimental cytotoxicity. The level of cytotoxicity following incubation with mAb or IL-2 minus the baseline level was equal to the quantity of enhancement by mAb. Because baseline cytotoxicity varied so greatly between different individuals, it was not valid to calculate the amount of enhancement in one individual as a percentage of baseline values and then compare this with another individual.

Cancer patients were separated into males and females and those aged < 70 years and those aged $\geq$ 70 years. For a comparison of the effect of tumour type on cytotoxicity patients were separated into two groups. Oesophageal and gastric tumours were combined (upper GIT group), and colonic and rectal tumours were combined (lower GIT group). Patients with either a recent history of an elevated bilirubin or who had an elevated bilirubin at the time of study were also studied separately. Additionally patients were separated according to the presence or absence of liver metastases. Patients were also analysed with regard to tumour grade.

Changes in peripheral blood cells were expressed both as means ($\pm$ SEM) of absolute values (X 10^9 L^{-1}) and as percentage values as a fraction of the pre-treatment (baseline) value.

Percentage value   = $\dfrac{\text{No. of cells pre-OKT3/normal saline}}{\text{No. of cells at time x.}}$

Absolute values are usually expressed since these relate more closely to the *in vivo* situation with respect to numbers of circulating effectors. However, expressing cells as a percentage enables the effect of different doses of OKT3 on relative change in a single subset to be more easily compared, as well as comparing changes between different subsets regardless of their absolute baseline values.

Advice regarding data analysis was obtained from Professor D. Jones in the Department of epidemiology at Leicester University. Statistical significance was always defined as $p \leq 0.05$.

CHAPTER FOUR

IN VITRO MODULATION OF CELLULAR CYTOTOXICITY IN HEALTHY
DONORS

4.1 INTRODUCTION

Previous studies within our department have shown that freshly isolated PBMC from healthy individuals and cancer patients demonstrate variable levels of cellular cytotoxicity against the NK sensitive target K562 (Ubhi et al, 1991). These studies also demonstrated that cytotoxicity was enhanced by the anti-CD3 mAb T3 (Dako), and that optimal enhancement followed preincubation of PBMC for 30 minutes at a concentration of 0.1 µg ml^{-1} prior to the addition of K562 cells. The mechanism by which anti-CD3 mAb enhance cytotoxicity remained unproven. The failure of T3 to enhance cytotoxicity of PBMC from cancer patients who subsequently failed to respond to rIL-2 therapy suggested a deficiency in the effectors responsible for K562 cytotoxicity *in vitro* and tumour regressions *in vivo*. The data presented in this chapter was obtained from studies using PBMC from healthy donors. These were primarily undertaken to investigate *in vitro* modulation of cytotoxicity by various mAb and IL-2 on cellular cytotoxicity against cultured tumour targets.

4.2 AIMS

The aims of the studies presented in this chapter were to confirm our previous findings and provide preliminary data for comparison with the results of the clinical studies presented in chapter 5 and 6. These experiments were therefore undertaken to define the parameters for the clinical studies and in particular to;

1. Define a normal range for K562 cytotoxicity among healthy donors.
2. Investigate cytotoxicity against different target cells.
3. Investigate the effect of various mAb on cellular cytotoxicity and confirm optimal conditions for mAb enhancement of cytotoxicity *in vitro*.
4. Investigate the effect of combinations of antibodies on cellular cytotoxicity.
5. Investigate the effect of anti-CD3 mAb and IL-2 on cytotoxicity.

4.3 RESULTS.

4.3.1 Range of K562 cytotoxicity in healthy donors.

Levels of K562 cytotoxicity in the 103 healthy individuals are described in Table 4.1 in appendix H. Mean (±SEM) and median K562 cytotoxicity is described in Table 4.2 and individual results illustrated in Figure 4.1. Subjects were analysed according to those aged < 50 years and those aged ≥ 50 years and also by sex distribution.

K562 cytotoxicity demonstrated considerable individual variation (Figure 4.1) and varied between 0 and 110.0 LU/10^{7}PBMC. The majority of individuals demonstrated relatively low levels of cytotoxicity mean 15.8 (± 2.5) (median 3.30) LU/10^7 PBMC. When all donors were analysed (Figure 4.2) no significant difference in cytotoxicity was detected between males and females (p = 0.57). Similarly no significant correlation was seen between cytotoxicity and age (r = 0.004).

Figure 4.1 - K562 cytotoxicity (means ± SEM) for 103 healthy
donors analysed by age and sex (lines = medians).
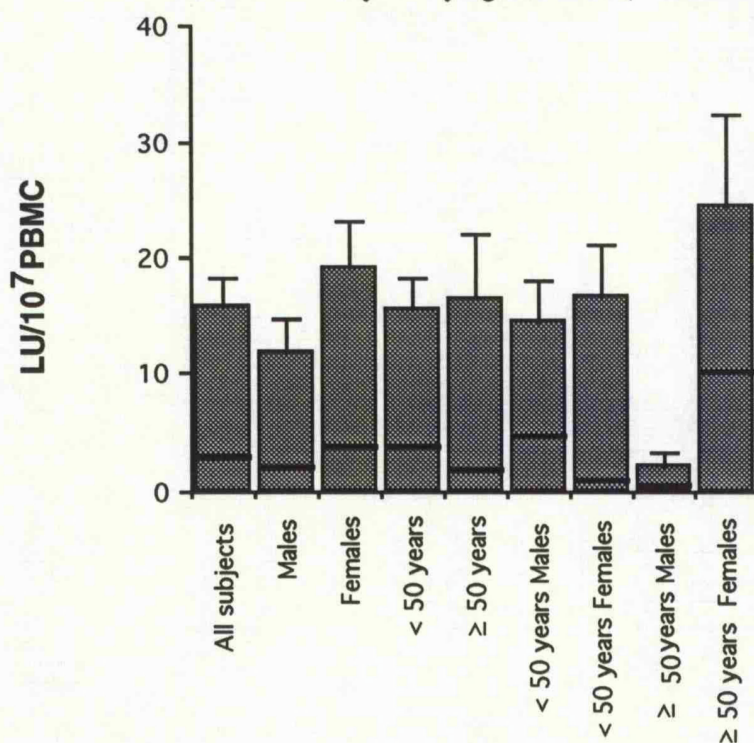
Table 4.2 - K562 cytotoxicity (LU/10^7PBMC) in 103 healthy donors.

Group	Sex	n	Mean K562 cytotoxicity	SEM	Median
	Males	48	11.8	2.8	2.7
	Females	55	19.2	3.9	3.8
Total		103	15.8	2.5	3.3
< 50 years	Males	38	14.5	3.4	4.8
	Females	37	16.7	4.4	1.5
Total		75	15.6	2.8	3.8
≥ 50 years	Males	10	2.2	1.2	0.7
	Females	18	24.4	7.9	10.7
Total		28	16.5	5.5	2.0

Cytotoxicity was not significantly different between the < 50 years (15.6 ± 2.8 (median 3.8) LU/10^7 PBMC) and ≥ 50 years group (16.5 ± 2.0 (median 2.0) LU/10^7 PBMC). Mean (± SEM) cytotoxicity was not significantly different in the 40 donors assayed by RC (14.4 ± 3.9 (median 1.9) LU/10^7 PBMC) and the 63 donors assayed by JH (16.2 ± 3.3 (median 4.7) LU/10^7 PBMC).

Although cytotoxicity was similar in the < 50 years and ≥ 50 years groups, the distribution of K562 cytotoxicity in the ≥ 50 years group was markedly different in the males and females. Mean (± SEM) and median levels of cytotoxicity among the female donors was considerably higher (24.4 ± 7.9 (median 10.7) LU/10^7 PBMC) than in the male donors (2.2 ± 1.2 (median 0.7) LU/10^7 PBMC) (p = 0.05). This was influenced by the inclusion of 5 female subjects (CAH, PS, DS, MA, and GC) with unusually high levels of cytotoxicity (35.4, 38.8, 80.1, 84.1, and 110.0 LU/10^7 PBMC) but also by the generally low levels of cytotoxicity in the males. Cytotoxicity was measured by JH in 8 of the elderly subjects (3 males and 5 females) and was 17.6 ± 7.5 (median 2.1) LU/10^7 PBMC and by RC in 20 (7 males and 13 females) and was 14.1 ± 9.7 (median 1.6) LU/10^7 PBMC. There was no apparent selection bias or variation in experimental technique to explain the difference in distribution in the elderly group. The large variability in cytotoxicity results between different individuals and even within the same indivividual tested on different occasions is a general feature of K562 cytotoxicity and must be considered in the interpretation of results in small numbers of subjects.

4.3.2 Comparison of K562 cytotoxicity with cytotoxicity against other tumour cell lines.

PBMC from 11 healthy donors were investigated for cytotoxicity against K562, Daudi, a lymphoblastoid cell line (LCL), human umbilical vein endothelial cell (HUVEC) primary cultures, a prostate cell line, and the epithelial cell line A549. The method for preparing these cell lines is described in appendix B (i) and cytotoxicity was assessed as described in appendix C. Results are expressed in Table 4.3 as percentage K562 cytotoxicity at an E:T ratio of 40:1. PBMC from these donors demonstrated relatively high mean (± SEM) levels of % K562 cytotoxicity when preincubated in tissue culture medium (32.9 % ± 3.3, equivalent to 45.4 ± 13.1 LU/10^7 PBMC). In contrast percentage cytotoxicity against all the other targets studied, remained below 10%.

4.3.3 Variation in individual K562 cytotoxicity with time.

Some individuals were studied more than once and one individual (JH) was studied regularly throughout the 18 months. Figure 4.2 describes K562 cytotoxicity for JH tested on 40 occassions over 564 days.

Table 4.3 - % cytotoxicity against various tumour cell lines at an E:T ratio of 40:1 for PBMC from 11 healthy donors preincubated with medium or OKT3.

Target	K562		Daudi		LCL		HUVEC		Prostate		A549	
Donor	Med	mAb	Med	mAb	Med	mAb	Med	mAb	Med	mAb	Med	mAb
SM	29.7	28.9	7.2	6.2	3.3	4.3	-	-	-	-	-	-
SW	24.3	27.8	3.3	4.3	2.3	4.1	-	-	-	-	-	-
NM	24.9	30.7	8.2	9.4	2.1	4.8	-	-	-	-	-	-
TD	50.1	59.2	7.3	9.8	0.1	1.9	-	-	4.3	4.4	-	-
TH	44.7	68.1	8.3	12.4	2.3	3.5	-	-	6.0	12.4	-	-
JE	19.6	34.6	-	-	-	-	4.7	10.6	-	-	4.3	8.1
SH	34.0	31.3	-	-	-	-	3.4	2.3	-	-	4.6	6.5
JL	47.6	57.0	-	-	-	-	4.7	6.2	-	-	5.3	7.2
JH	39.0	51.9	5.6	8.3	-	-	-	-	-	-	-	-
DU	22.4	24.3	-	-	-	-	0.8	1.1	-	-	4.8	10.0
DW	25.7	27.2	-	-	-	-	0.3	1.4	-	-	7.3	10.8

Figure 4.2 - Variation in K562 cytotoxicity with time in one healthy donor (JH).
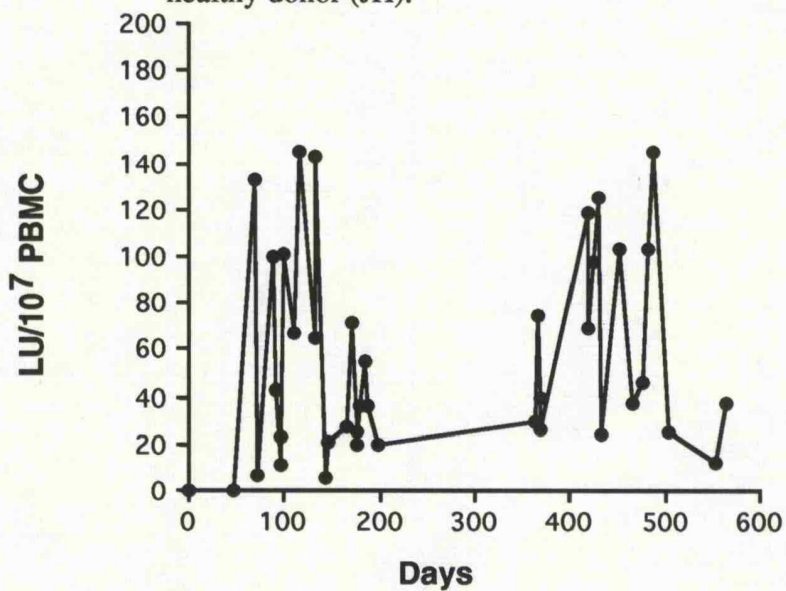
These results illustrate the considerable individual variation in K562 cytotoxicity. Mean (± SEM) cytotoxicity was 56.7 (± 7.0, median 38.0) LU/10^7 PBMC and varied from 0.0 to 144.5 LU/10^7PBMC. JH usually demonstrated relatively high levels of cytotoxicity in comparison to the other donors tested. Figure 4.3 describes variation in K562 cytotoxicity with time for 6 healthy donors also tested on more than one occassion on different days.

Despite the wide variation within individuals, the tendency was for some donors to demonstrate relatively high levels of cytotoxicity (eg. TH) and others low levels (eg. AH) supporting the concept that the rank order of K562 cytotoxicity is maintained.

4.3.4   Preliminary studies on the the stimulation of cytotoxicity by mAb.

A.   Introduction.

PBMC were preincubated with a variety of mAb according to the method described in appendix C using the mAb listed in Table C1. Initially anti-CD3 mAb were studied because these are known to be involved in T cell activation and we have previously demonstrated enhancement of K562 cytotoxicity using T3 (Ubhi et al, 1991). Initial studies were also undertaken with T3 (Dako) rather than OKT3. T3 was chosen because of our experience from its use in previous studies and because preliminary experiments demonstrated that enhancement was greater than with OKT3. Significant results were therefore obtained from a relatively small number of experiments. Two anti-CD7 mAbs (murine (mCD7) and 'humanised' (hCD7) mAb) were also initially studied. Results of these studies were used to define experimental conditions for all mAb. Initial experiments were undertaken to define the optimal conditions for enhancement of K562 cytotoxicity *in vitro*.

B   Effect of varying mAb concentration for OKT3 and T3 on K562 cytotoxicity.

PBMC were separated from one healthy donor (JH) on two different occasions and the effect of OKT3 and T3 at concentrations varying between 10^{-4} and 100 μg ml^{-1} on K562 cytotoxicity was investigated. PBMC were preincubated for 30 minutes prior to the addition of targets as described in appendix B. Results are described in Figure 4.4.

On the day of study levels of baseline cytotoxicity were significantly lower for T3, than for OKT3 (36.9 LU/10^7 PBMC compared with 128.0 LU/10^7 PBMC). Enhancement increased with increasing concentration of mAb. Both OKT3 and T3 resulted in maximal enhancement at a concentration of 10 μg ml^{-1}. At 10 μg ml^{-1} cytotoxicity was enhanced 720% by T3 compared with 407% by OKT3 over baseline cytotoxicity. Some enhancement was also seen at the lowest concentration (10^{-4} μg ml^{-1}) for both mAb.

This experiment was then repeated in 15 healthy donors using OKT3 and 5 healthy donors using T3 over a smaller range of concentrations varying between 0.01 and 10 μg ml^{-1}.

Figure 4.3 - Variation in K562 cytotoxicity with time for
6 healthy donors.
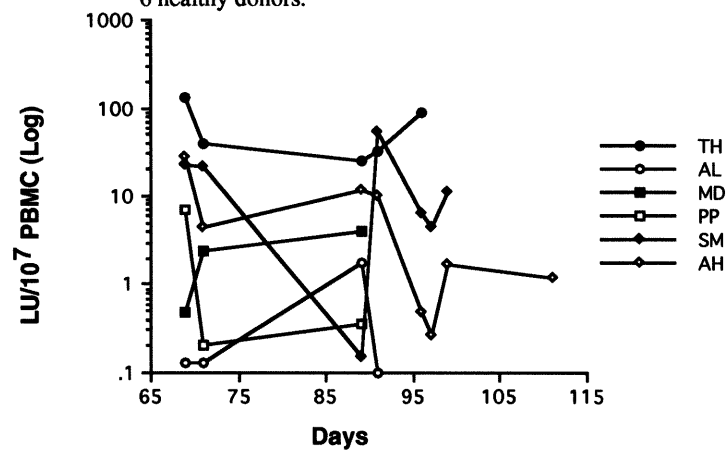
Figure 4.4 - Effect of varying concentrations of OKT3 and T3
on K562 cytotoxicity in one individual (JH).
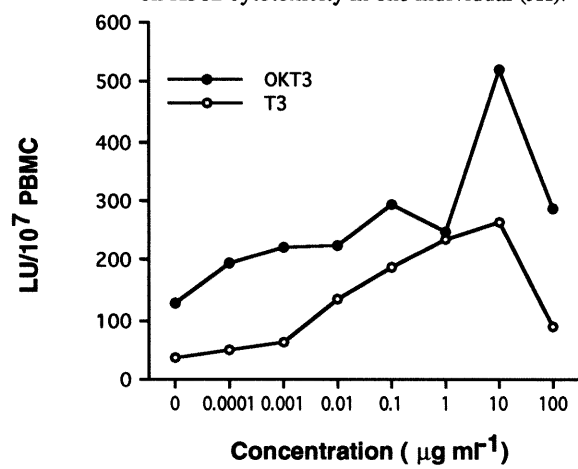
Results are described (means ± SEM) in Figure 4.5 and 4.6 for OKT3 and T3 and individual results (and median values) are shown in Table 4.4 and 4.5 in appendix H.

Mean baseline levels were substantially higher among the 15 healthy donors stimulated by OKT3 (26.1 ± 8.6 (median 15.2) LU/10^7 PBMC) than among the 5 healthy donors stimulated with T3 (3.8 ± 1.5 (median 4.7) LU/10^7 PBMC). Enhancement by both mAb was a constant feature in all individuals. A plateau was reached at 1 µg ml^{-1} for OKT3 (43.7 ± 10.8 LU/10^7 PBMC) and for T3 (34.4 ± 12.9 LU/10^7 PBMC). Mean enhancement over baseline values was considerably greater for T3 (911%) than OKT3 (167%).

C.   Effect of varying concentration of anti-CD7 on K562 cytotoxicity.

Experiments at varying antibody concentration were then undertaken using two anti-CD7 mAb. The CD7 antigen is expressed by T cells and NK cells. A mouse antibody (mCD7) and a 'humanized' antibody (hCD7) were used. Mean (± SEM) cytotoxicity is described in Figure 4.7 for 3 subjects studied with mCD7 and in Figure 4.8 for 7 subjects studied with hCD7. Individual results and medians are described in Table 4.6 and Table 4.7 in appendix H.

Mean (± SEM) baseline K562 cytotoxicity was substantially higher in the 3 subjects studied with mCD7 (15.6 ± 2.4 (median 15.5) LU/10^7 PBMC) than hCD7 (2.3 ± 1.2 (median 0.1) LU/10^7 PBMC). Enhancement was a constant feature in all individuals studied. Although hCD7 produced a plateau at only 0.01 µg ml^{-1} mCD7 resulted in an increase in cytotoxicity in a dose dependent manner up to the highest concentration studied (10 µg ml^{-1}). Mean percentage enhancement was greater for hCD7 at 409% compared with 243% for mCD7 although numbers of subjects studied were too small to draw firm conclusions regarding the relative efficacy of these two antibodies.

Based on these preliminary findings a concentration of 1 µg ml^{-1} was adopted for all mAb in subsequent studies.

D.   Effect of duration of incubation with mAb on K562 cytotoxicity.

PBMC were routinely preincubated in mAb prior to the addition of targets. PBMC were therefore exposed to the mAb throughout the duration of the assay. Kinetic factors prior to addition of K562 target cells may affect interactions between effectors and targets and influence cytotoxicity during the preincubation period. When multiple experiments were performed, small variations in length of exposure of PBMC to mAb were inevitable because of the time required for serial dilution of PBMC and the addition of targets. It was therefore important to assess how critical this initial period of mAb exposure to PBMC was and to define an optimal time for preincubation with mAb.

PBMC were separated from two healthy donors and preincubated with T3 and hCD7 for between 0 and 150 minutes prior to the addition of K562 cells at 30 minute intervals. Supernatants were then harvested as usual after 4 hours incubation.

Figure 4.5 - K562 cytotoxicity at varying OKT3 concentration in 15 healthy donors (means ± SEM).
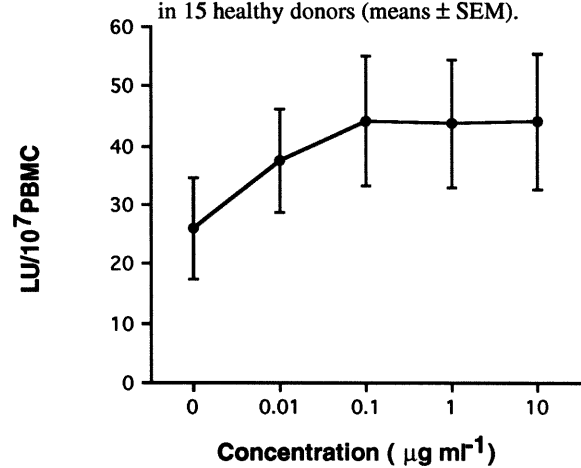
Figure 4.6 - K562 cytotoxicity at varying T3 concentration in 5 healthy donors (means ± SEM).
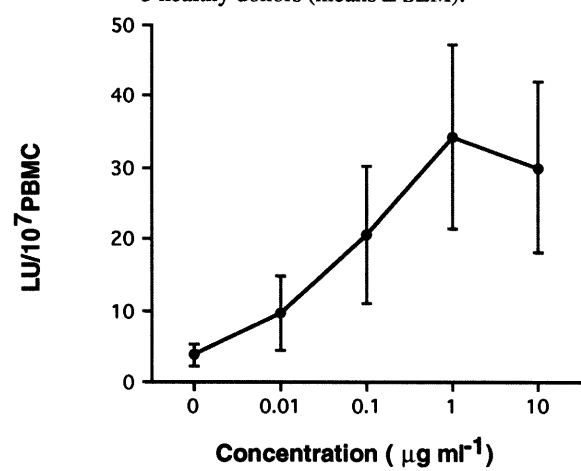
Figure 4.7 - K562 cytotoxicity at varying mCD7 concentration
in 3 healthy donors (means ±SEM).
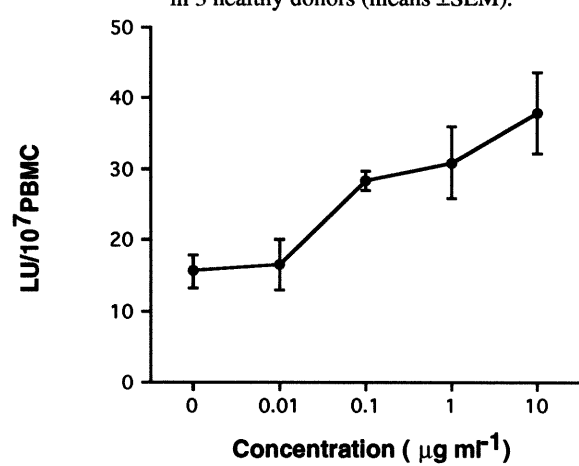
Figure 4.8 - K562 cytotoxicity at varying hCD7 concentratiol
in 7 healthy donors (means ± SEM).
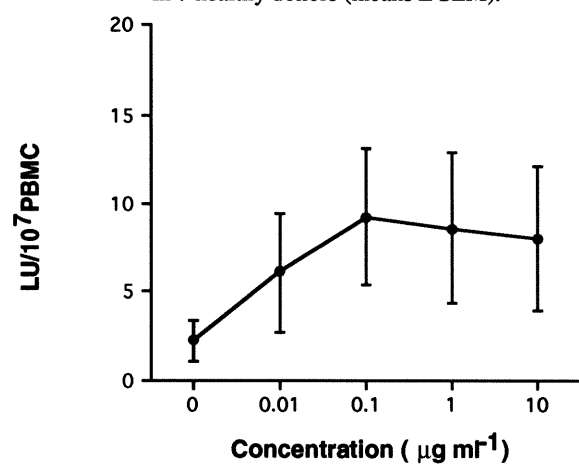
Figure 4.9 and Figure 4.10 describe the effect of varying preincubation time (JH and LA) for T3 and hCD7.

In both individuals increasing duration of preincubation in tissue culture medium resulted in an increase in baseline cytotoxicity. Preincubation of PBMC with T3, prior to addition of K562 was not required to enhance cytotoxicity in this experiment. The enhancing effect of T3 subsequently diminished with the duration of preincubation time. The immediate effect of hCD7 was not as marked as for T3. However, cytotoxicity was enhanced up to 60 minutes and then levels of enhancement declined.

Based on these results 30 minutes was routinely adopted as the preincubation time for PBMC with all mAb. This was because more prolonged preincubation with mAb did not enhance cytotoxicity and also increased the length of time required to complete the assay.

E.   Effect on K562 cytotoxicity and mAb modulation of varying the total assay time.

The effect of T3 and hCD7 on K562 cytotoxicity at different stages during incubation with K562 was assessed using PBMC separated from 6 healthy donors. Assays were performed in the usual way and incubation times varied from 1 to 6 hours by harvesting supernatants at hourly intervals. Figures 4.11 and Figure 4.12 describe the effect on mean (SEMs not shown) K562 cytotoxicity of varying the duration of the assay and modulation by T3 and hCD7 during this period. Individual results are described in Table 4.8 and Table 4.9 in appendix H.

Both antibodies enhanced cytotoxicity uniformally during the assay period. Both T3 and hCD7 were associated with a decline in both K562 cytotoxicity and enhancement of cytotoxicity at 6 hours. The reason for this was unclear. However, it could have occurred because of exhaustion of nutrients in the tissue culture medium. A standard 4 hour incubation time was considered sufficient to demonstrate reliably mAb enhancement of K562 cytotoxicity.

F.   Effect of OKT3 on cytotoxicity against other tumour cells lines.

The effect of preincubating PBMC from 11 healthy donors with OKT3 prior to the addition of a variety of other tumour cell lines was investigated. Results are described in Table 4.3. Low baseline levels of cytotoxicity were detected against all cell lines except K562 when PBMC were incubated in tissue culture medium as previously described (4.4.2). OKT3 enhanced mean ($\pm$ SEM) K562 cytotoxicity from 32.91% $\pm$ 3.27 (equivalent to 45.4 $\pm$ 13.1 LU/10^7 PBMC) to 40.09% $\pm$ 4.73 (equivalent to 62.2 LU/10^7 PBMC $\pm$ 21.6). The addition of OKT3 to PBMC prior to the addition of other tumour cell lines resulted in only marginal enhancement which was maximal at 12.4% against the Daudi cell line for TH. This individual also had relatively high levels of K562 cytotoxicity (128.9 LU/10^7 PBMC enhanced to 226.8 LU/10^7 PBMC).

Figure 4.9 - Effect of varying PBMC preincubation time
with T3 on K562 cytotoxicity in one healthy donor
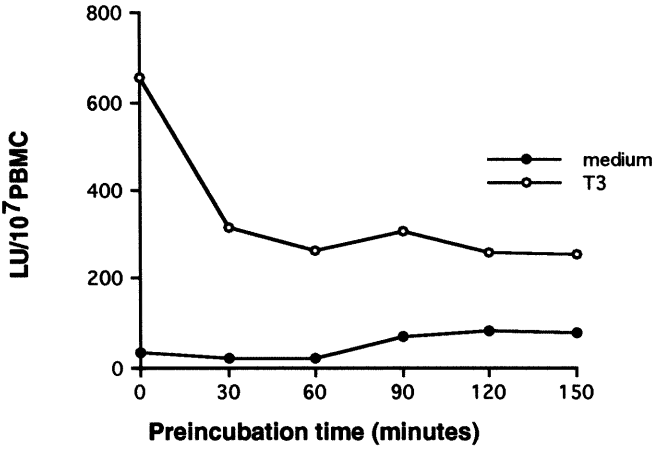
Figure 4.10 - Effect of varying PBMC preincubation time
with hCD7 in one healthy donor.
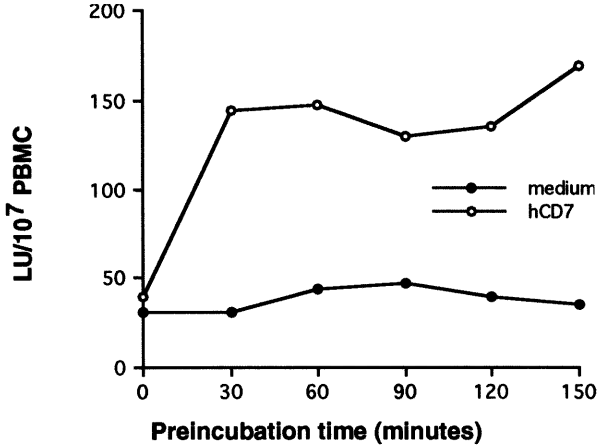
Figure 4.11 - K562 cytotoxicity (means) and T3 at varying
incubation times in 3 healthy donors.
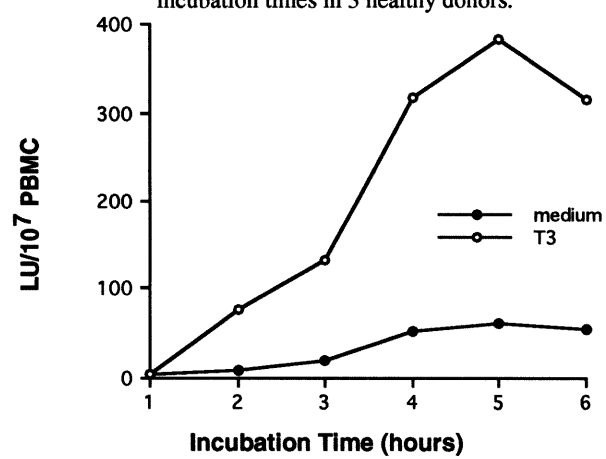
Figure 4.12 - K562 cytotoxicity (means) and hCD7 at varying
incubation times for 3 healthy donors.

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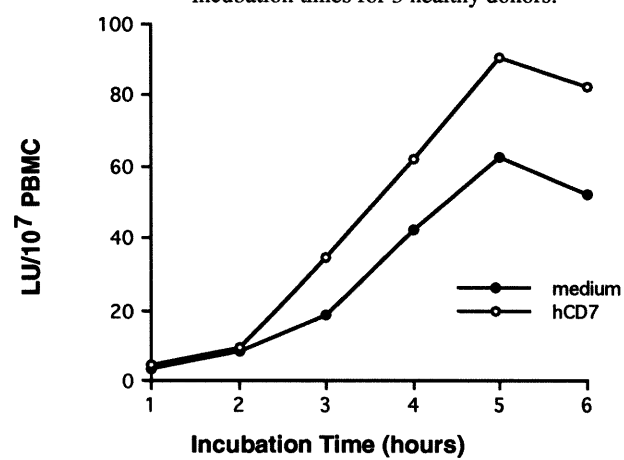
4.3.5   Influence of various mAb at 1 μg ml⁻¹ on K562 cytotoxicity and possible
         mechanisms of enhancement.

A.   Introduction.

        The above preliminary results confirmed that significant cytotoxicity was seen
against K562 tumour cells but not the other cells studied. OKT3 enhanced cytotoxicity
against K562 and the other tumour cell lines but in the latter case post-OKT3 cytotoxicity
was still very low (approximately 10% at 40:1). Therefore it was decided to investigate
the influence of various mAb on the K562 tumour cell line. A non-specific control
antibody was not routinely incorporated in assays for three reasons. Firstly, experiments
demonstrated that all polyclonal antibody preparations studied did not influence
cytotoxicity at a range of concentrations regardless of isotype specificity (section 3.4.2.
B). Secondly, routine incorporation of control antibodies reduced the number of
experiments that could be performed. Thirdly, all experiments included a cytotoxicity
result from PBMC incubated in medium alone for comparison with incubation in mAb.
Donors therefore acted as their own controls with regard to enhancement of cytotoxicity.

        Results are expressed in LU/10⁷ PBMC as individual experiments with a
cytotoxicity value obtained from PBMC incubation in medium (baseline cytotoxicity) and
a value obtained with mAb. When percentage enhancement is stated, this refers to
changes in the median values (value with mAb ÷ baseline value) and is intended to give
an overall comparison of the amount of enhancement observed between different mAb
and different groups.

        All assays were performed with a PBMC preincubation time of 30 minutes, an
assay duration of 4 hours, and an antibody concentration of 1 μg ml⁻¹ as described in
appendix B and C.

B.   Effect of OKT3 and T3 on K562 cytotoxicity.

        Of the 103 individuals described in Table 4.1 (appendix H), 81 donors underwent
assessment with OKT3, and 59 with T3. Characteristics of donors and mean and median
cytotoxicity are described in Table 4.10 in appendix H. Of the 81 donors studied with
OKT3, 34 were male and 47 female, and 53 were aged < 50 years and 28 aged ≥ 50
years. Enhancement of cytotoxicity by both OKT3 and T3 was consistently observed for
individual donors. Cytotoxicity is described in Figure 4.13 (means ± SEM, and
medians) and compared with the corresponding baseline cytotoxicity.

        OKT3 enhanced median cytotoxicity by 573% compared with 1415% by T3.
Enhancement by T3 was significantly greater than OKT3 (p < 0.01) and enhancement by
both mAb was significant over baseline values (p < 0.001).

        In 42 cases (19 males, 23 females, median age 53 years range 20 - 80 years),
OKT3 and T3 were assessed simultaneously, therefore allowing a direct comparison to be
made between the efficacy of OKT3 and T3 on enhancement of cytotoxicity. Results are
described in Table 4.11 in appendix H and Figure 4.14.

Figure 4.13 - Influence of OKT3 and T3 on K562 cytotoxicity
(means ± SEM, lines = medians).
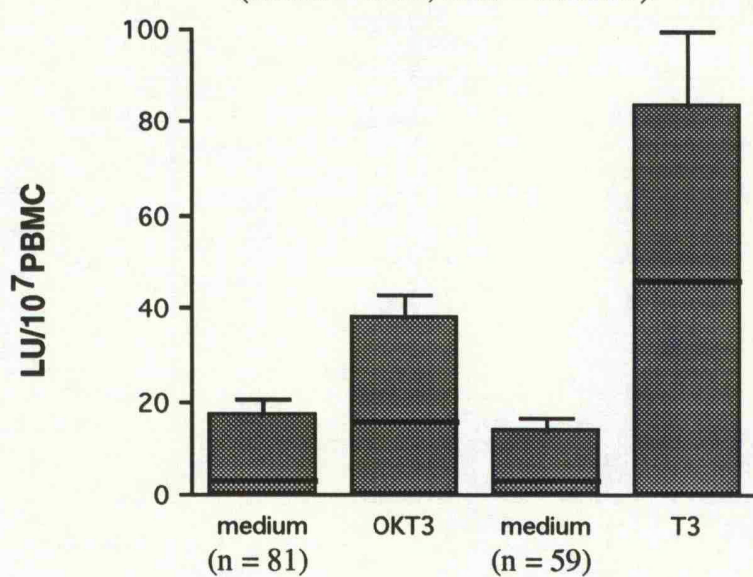
Figure 4.14 - Simultaneous assessment of OKT3 and T3 on
K562 cytotoxicity (n = 42, means ± SEM, lines =
medians).
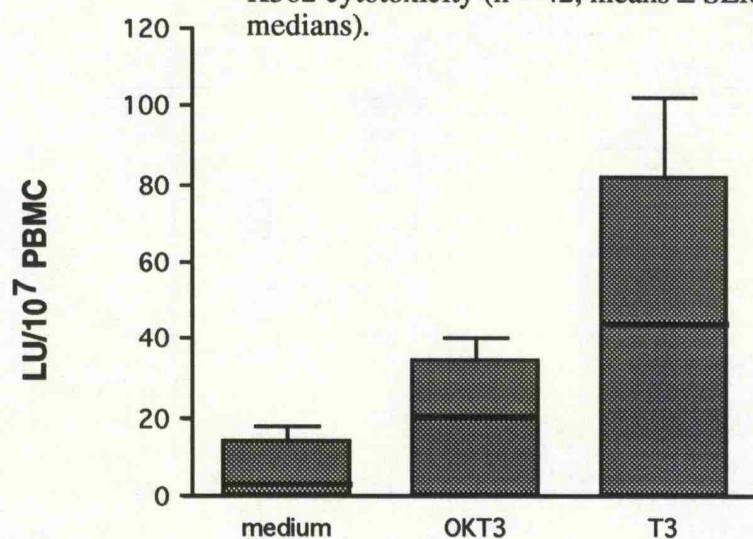
OKT3 and T3 consistently and significantly enhanced cytotoxicity over baseline values ($p < 0.001$). A direct comparison of cytotoxicity between T3 and OKT3 in these experiments confirmed that T3 resulted in significantly greater levels of cytotoxicity than OKT3 ($p < 0.01$) (1201% for OKT3 compared with 2606% for T3).

The amount of enhancement in mean ($\pm$ SEM) K562 cytotoxicity by OKT3 was not significantly different in the male and female donors (22.1 $\pm$ 6.2 compared with 19. $\pm$ 3.4 LU/10^7 PBMC) or the < 50 years and $\geq$ 50 years donors (19.6 $\pm$ 4.5 compared with 24.2 $\pm$ 4.7 LU/10^7 PBMC).

C.   Comparison between a 'humanised' monovalent OKT3 preparation, OKT3, and T3.

A 'humanised' monovalent OKT3 mAb (hOKT3) was donated as a generous gift (Dr. R. Routledge, see Table C1). Its effect on K562 cytotoxicity was studied at a concentration of 1 μg ml^{-1} in experiments using PBMC from 11 healthy donors in which OKT3 and T3 were simultaneously assessed. Individual results are described in Table 4.12 in appendix H and Figure 4.15. 'Humanised' OKT3 enhanced mean ($\pm$ SEM) cytotoxicity from 9.0 ($\pm$ 3.6, median 3.4) to 18.4 ($\pm$ 8.2, median 5.1) LU/10^7 PBMC. This just failed to reach statistical significance ($p = 0.07$). OKT3 and T3 were associated with significant enhancement of cytotoxicity ($p < 0.001$) and post-T3 cytotoxicity levels were significantly greater than either OKT3 or hOKT3 ($p < 0.01$).

D. Effect of anti-CD16 on K562 cytotoxicity.

Figure 4.16 describes the effect on K562 cytotoxicity in which PBMC from 15 healthy donors were incubated simultaneously, with OKT3, T3, or anti-CD16 mAb at 1 μg ml^{-1}. Individual results are described in Table 4.13 in appendix H. All antibodies significantly enhanced cytotoxicity over baseline values ($p < 0.001$). As previously demonstrated T3 was consistently associated with greater enhancement of cytotoxicity than OKT3 (< 0.001). Anti-CD16 also enhanced cytotoxicity in all individuals except one (CAH) and the mean level of enhancement was intermediate between OKT3 and T3 being significantly greater than OKT3 ($p < 0.01$) but not significantly different from T3.

Figure 4.15 - Comparison of hOKT3 and OKT3 and T3 on K562 cytotoxicity in 11 healthy subjects (means ± SEM, lines = medians).
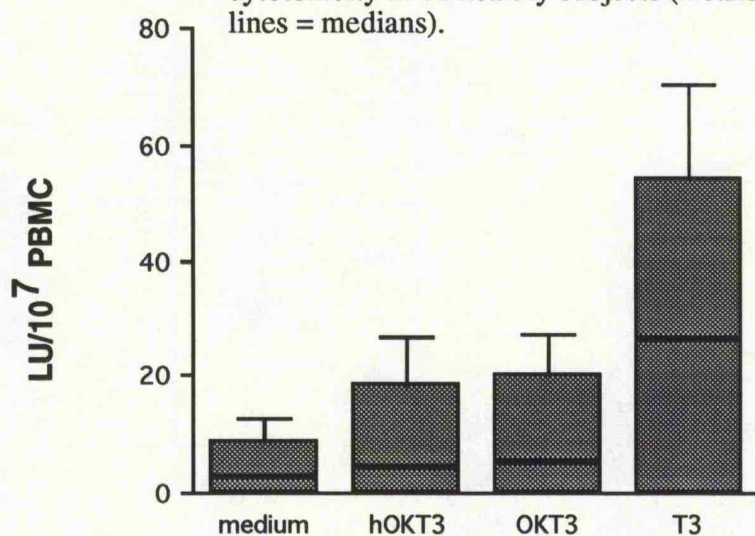
Figure 4.16 - Enhancement of K562 cytotoxicity by OKT3,T3, and anti-CD16 in simultaneous experiments performed on 15 healthy donors (means ± SEM, lines = medians).
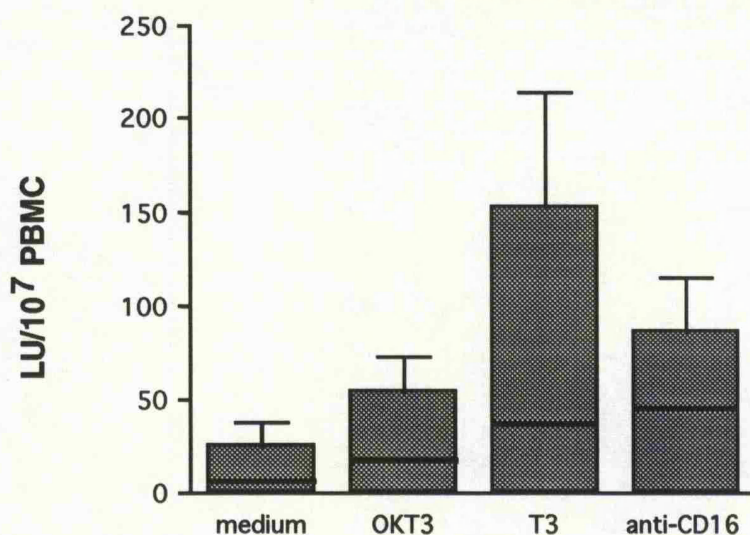
E.   Effect of anti-CD2, anti-CD4, anti-CD8, anti-CD7 and anti-ζ mAb on K562
        cytotoxicity.
        MAb specific for CD2, CD4, CD8, CD7, and the zeta (ζ) chain of the TcR
antigens expressed by peripheral blood cells were tested using PBMC from healthy
donors. Their effects are compared with their corresponding baseline levels of
cytotoxicity. Numbers of subjects were too small to achieve 95% confidence intervals
with respect to comparing the relative efficacy of one antibody over another with regard to
enhancement of cytotoxicity. The anti-ζ mAb was only tested against 3 individuals.
Individual results are described in Table 4.14 and Table 4.15 in appendix H.
Cytotoxicity for all mAb except anti-ζ is described in Figure 4.17.   All mAb except anti-
ζ were associated with some overall enhancement of cytotoxicity however this only
reached statistical significance for hCD7 (p = 0.03). The effect of anti-ζ was equivocal
with enhancement of cytotoxicity in 2 subjects and depression in the third.

F. Antibodies inhibiting K562 cytotoxicity
        PBMC were preincubated with mAb specific for surface antigens known to be
important in antigen independent cell adhesion. These included anti-LFA-1, anti-ICAM-
1, anti-VECAM, anti-PECAM, and anti-ELAM. Results are shown in Table 4.16. Anti-
LFA-1 was associated with profound impairment of cytotoxicity most marked in JH.
Anti-ICAM-1 and anti-ELAM were also associated with partial impairment of K562
cytotoxicity.

4.4.6   Effect of combinations of mAb on K562 cytotoxicity.
A.   Introduction.
        Multiple mechanisms are probably involved in the enhancement of K562
cytotoxicity described in the above experiments. MAb of differing antigen specificity
appear to have varying capacities to modulate cytotoxicity in vitro. MAb with similar
epitope specificity but differing isotype also appear to differ in their capacity to enhance
cytotoxicity. This suggests that the Fc fragment is involved in the mechanism of
enhancement. The following is a description of the results of preliminary studies to
investigate the effect of combinations of antibodies, each individually capable of
enhancing cytotoxicity. MAb were reconstituted to a concentration resulting in a final
concentration of 1 μg ml^{-1} in assays and were preincubated with PBMC for 30 minutes as
described in appendix B and C.

Figure 4.17 - Effect of mAb to CD2,CD4,CD8 and CD7 on K562 cytotoxicity (means ± SEM, lines = medians).
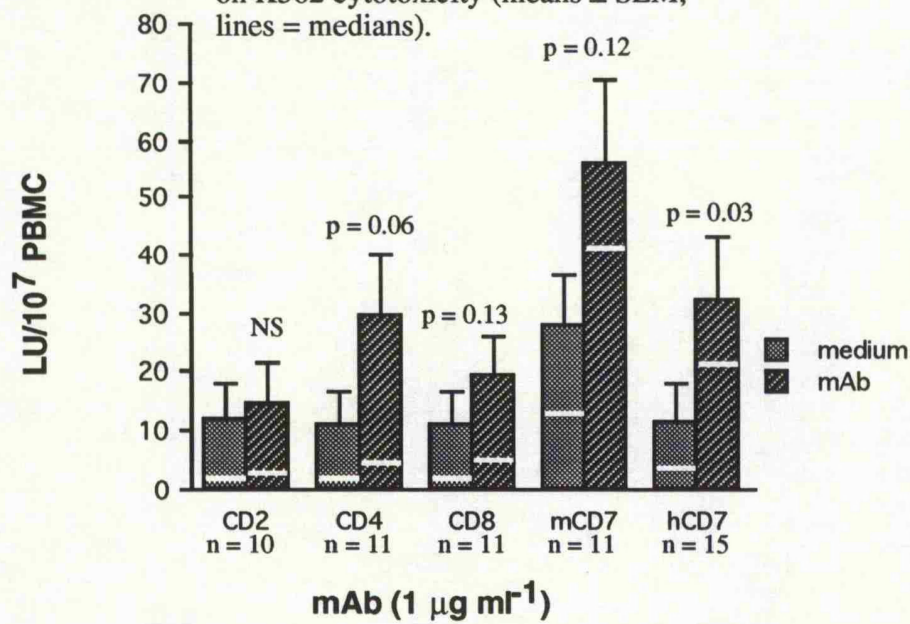
Table 4.16 - Effect on K562 cytotoxicity (LU/10^7 PBMC) of mAb specific for adhesion molecules.

Subject	medium	anti-LFA1	Subject	medium	anti-ICAM	anti-VECAM	anti-PECAM	anti-ELAM
JH	144.5	1.2	SM	11.0	8.8	6.5	4.4	2.6
CS	0.2	0.0	CE	58.5	13.7	49.6	43.3	28.7
PP	6.9	0.0	JH	46.7	9.3	67.8	31.8	34.8
Mean	50.5	0.4		38.7	10.6	41.3	26.5	22.1
Median	6.9	0.0		46.7	9.3	49.6	31.8	28.7
SEM	47.0	0.4		14.3	1.6	18.2	11.5	9.9

B.   Effect of OKT3 and hCD7 on K562 cytotoxicity.

PBMC from 6 healthy donors were preincubated with OKT3, hCD7 and both mAb prior to the addition of K562. Individual results are described in Table 4.17 in appendix H. Results are also described in Figure 4.18. OKT3 and hCD7 individually enhanced cytotoxicity. OKT3 and hCD7 in combination resulted in greater enhancement than for either mAb alone. However, the effect was only partially additive rather than synergistic.

C.   Effect of T3 and hCD7 on K562 cytotoxicity.

Table 4.18 describes the results of preincubating PBMC with T3 and hCD7 in 3 healthy donors. T3 and hCD7 had no additional effect over enhancement by T3 alone.

D.   Effect of T3 and anti-CD16 on K562 cytotoxicity.

Table 4.19 decribes the results of preincubating PBMC with T3 and CD16 in 4 healthy donors. Although T3 and anti-CD16 substantially enhanced cytotoxicity in these 4 healthy donors who had unusually low levels of cytotoxicity, anti-CD16 in combination with T3 partially inhibited enhancement by T3.

4.4.7   Effect on mAb enhancement of preincubating K562 with antibodies.
A.   Introduction.

Antibodies may bind simultaneously to both antigen on effector cells via their idiotype and to Fc receptors on target cells via their isotype. This may influence cytotoxicity by enabling activation of the effector and also redirecting killing to a target by virtue of crosslinking to the two cells. Experiments were performed to investigate the possibility that Fc interactions between mAb and Fc receptors expressed by K562 cells may influence enhancement of cytotoxicity. K562 express $Fc\gamma RII$ (CD32w) receptors with which the mouse IgG1 isotype has a relatively high affinity compared with other isotypes. The interaction between T3 (IgG1) and $Fc\gamma RII$ may be particularly significant and account for the greater efficacy of T3 with respect to enhancement of cytotoxicity.

Figure 4.18 - Combined effect of OKT3 and hCD7 on K562 cytotoxicity in 6 healthy donors (means ± SEM, lines = medians).
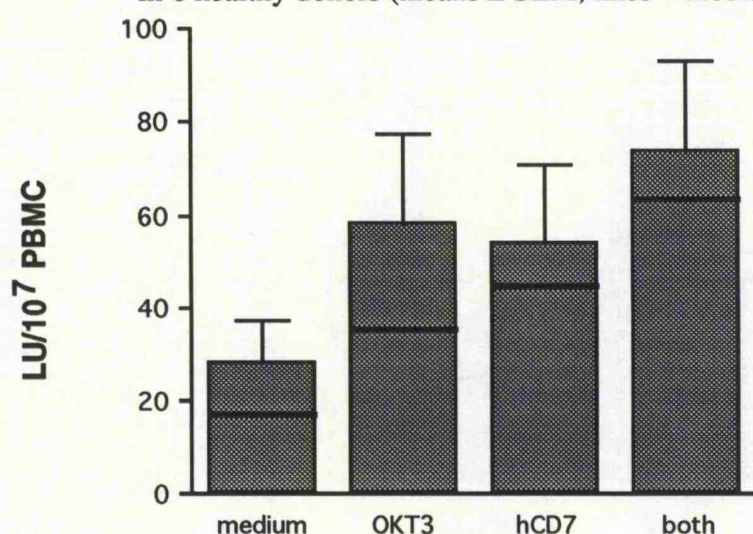
Table 4.18 - Effect on K562 cytotoxicity (LU/10^7 PBMC) of combining T3 and hCD7.

Subject	medium	T3	hCD7	T3 + hCD7
AH	9.8	64.9	25.8	62.9
JH	20.6	82.0	24.9	76.9
LA	16.8	51.0	31.8	65.8
mean	15.7	66.0	27.5	68.5
median	16.8	64.9	25.8	65.8
SEM	3.2	9.0	2.1	4.3

Table 4.19 Effect on K562 cytotoxicity (LU/10^7 PBMC) of combining T3 and anti-CD16.

Subject	medium	T3	anti-CD16	T3 + anti-CD16
AR	2.6	29.3	17.3	25.5
EB	0.0	1.5	3.4	0.6
BR	0.3	84.8	31.4	43.8
CW	6.5	42.3	53.2	20.2
mean	2.3	39.5	26.3	22.5
median	1.4	35.8	24.4	22.8
SEM	1.5	17.3	10.6	8.9

B. <u>Preincubation of K562 with polyclonal antibody preparations</u>

Non-specific antibody preparations (NSA) were incubated with K562 cells prior to their addition to a standard assay in an attempt to 'block' the availability of Fc receptors. The test antibodies studied were OKT3 and T3. The effect of NSA on T3 should be more easily detected because of its superior enhancing effect. K562 were incubated in either tissue culture medium or 10 µg ml^{-1} of pmIgG, or polyclonal preparations of mIgG1, mIgG2a, or hIgG1 ('blocking' antibody, NSA) for 30 minutes at room temperature. Each experiment was performed using PBMC from three healthy donors. MD, JH, and AH were studied twice, therefore a total of nine different individuals were studied. PBMC were also preincubated with the NSA under investigation at a concentration of 1 µg ml^{-1} to ensure that the NSA did not directly influence cytotoxicity.

Individual results are shown in Tables 4.20 and 4.21 in appendix H. OKT3 and T3 enhanced median cytotoxicity by 434% and 1472% in these experiments. There was no overall inhibition of enhancement by any of the non-specific antibodies used. The mIgG1 preparation inhibited T3 enhancement of cytotoxicity in 2 of the subjects (MD and SB) and enhanced cytotoxicity in the third (PF). Figure 4.19 describes the effect of the NSA on K562 cytotoxicity and enhancement by T3 for all 12 experiments.

The NSA did enhance baseline cytotoxicity in the 12 experiments (9.6 ± 3.5 (median 2.7) to 15.4 ± 8.7 (median 5.6) LU/10^7 PBMC). However this failed to reach statistical significance.

C. <u>Preincubation of K562 with anti-FcγRI (CD64) and anti-FcγRII (CD32w) mAb</u>

K562 were incubated in tissue culture medium, anti-FcγRII (CD32w) or anti-FcγRI (CD64) at a concentration of 10 µg ml^{-1} at room temperature for 30 minutes . These were then added to PBMC from three healthy donors which had been preincubated in 1 µg ml^{-1} anti-CD32w, anti-CD64 or T3. Individual results are described in Table 4.22.

T3 enhanced cytotoxicity in all 3 donors. Anti-CD64 had no significant effect on cytotoxicity. Anti-CD32w slightly impaired baseline cytotoxicity in all 3 donors. Preincubating K562 with anti-CD64 had no effect on cytotoxicity. However, preincubation of K562 in anti-CD32w resulted in a considerable decrease in the amount of enhancement induced by T3 in all individuals (median fall of 88%).

D. <u>Summary.</u>

Various mAb specific for CD antigens including anti-CD3, anti-CD4, anti-CD8, and anti-CD7 enhance K562 cytotoxicity *in vitro* over the effect of polyclonal preparations. Other mAb inhibit *in vitro* cytotoxicity. These are notably mAb to CD antigens involved in cell adhesion including anti-LFA1, anti-ICAM-1, and anti-ELAM. Combinations of anti-CD3 and antiCD7 mAb which individually enhance cytotoxicity

Figure 4.19 - Effect of non-specific antibody (NSA) preparations on OKT3 and T3 enhancement of K562 cytotoxicity (means ± SEM, lines = medians).
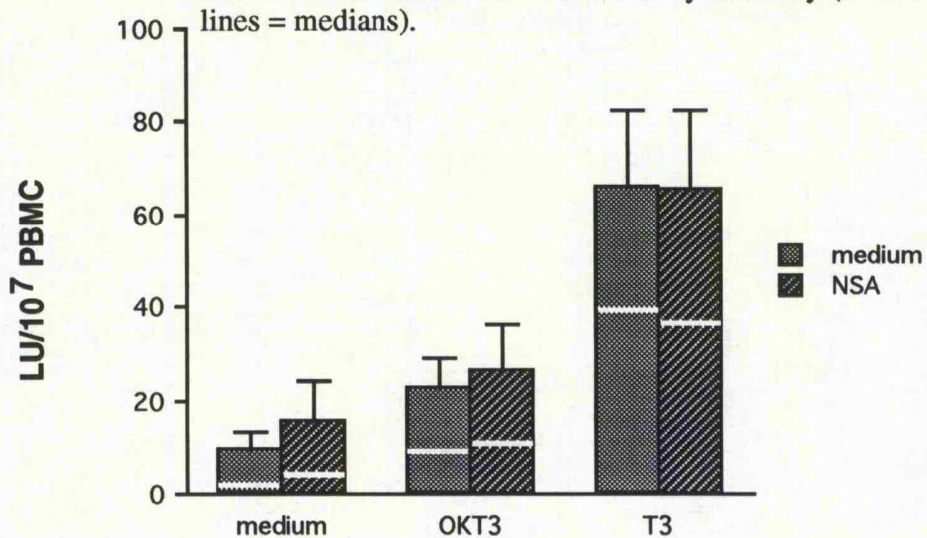
Table 4.22 - Effect of anti-CD64 and anti-CD32w on T3 enhancement of K562 cytotoxicity (LU/10^7 PBMC).

Subject	medium	CD32w	CD64	T3	K562 with CD32w T3	K562 with CD64 T3
JS	144.5	105.3	131.1	591.7	195.3	557.8
CS	0.2	0.1	0.1	8.4	3.4	9.3
PP	6.9	5.2	6.4	28.7	2.9	31.7
Mean	50.5	36.9	45.8	209.6	67.2	199.6
Median	6.9	5.2	6.4	28.7	3.4	31.7
SEM	47.0	34.2	42.7	191.2	64.1	179.2

have a greater effect than each mAb individually. However this effect is additive rather than synergistic. Anti-CD16 inhibited enhancement by T3 in the 3 individuals studied. Although preincubation of K562 with polyclonal antibody preparations failed to inhibit enhancement by mAb, incubation of K562 in the anti-CD32w mAb substantially reduced enhancement by T3.

4.3.8   Comparison of enhancement of K562 cytotoxicity by OKT3 and IL-2.

A.   Introduction.

IL-2 has the capacity to enhance substantially non-MHC-restricted cytotoxicity both *in vitro* and *in vivo*. Combination therapy using IL-2 and anti-CD3 mAb may therefore theoretically be of use in cancer immunotherapy. We have not previously studied the effect on K562 cytotoxicity of preincubating PBMC with IL-2. Preliminary experiments were undertaken to investigate the effect of varying IL-2 concentrations on K562 cytotoxicity and to explore synergy with anti-CD3 mAb. IL-2 was reconstituted as described in the methods section and appendix C. PBMC were preincubated for 30 minutes with IL-2 as for mAb prior to the addition of K562 cells. Concentrations of IL-2 were prepared by doubling dilution from a stock concentration of 5000 IU ml^{-1}.

B.   Effect of IL-2 concentration on K562 cytotoxicity.

An initial experiment was performed to define the effect of varying concentrations of IL-2 at between 78 IU ml^{-1} and 2500 IU ml^{-1} using PBMC from one healthy donor (JH). In the same experiment T3 was studied at a concentration varying between 10^{-4} μg ml^{-1} and 10 μg ml^{-1}. Results are described in Figure 4.20. Enhancement in K562 cytotoxicity by IL-2 was dose-dependent as was observed with T3.

Further experiments were then performed using PBMC from 3 healthy donors to investigate the effect of IL-2 at concentrations of 25, 50, and 100 IU ml^{-1} and OKT3 at 1 μg ml^{-1}. Results are described in Table 4.23. Enhancement by OKT3 was substantially lower in two of the three individuals than in previous experiments, although the reason for this was unclear. Because enhancement of K562 cytotoxicity by IL-2 at 50 IU ml^{-1} was comparable to that of OKT3 this concentration was chosen to explore synergy between IL-2 and OKT3.

C.   OKT3 and IL-2.

PBMC were separated from three healthy donors and incubated with OKT3 at 1 μg ml^{-1}, IL-2 at 50 IU ml^{-1} and both agents at the same concentration. Table 4.24 describes the results. 1 μg ml^{-1} OKT3 and 50 IU IL-2 induced comparable levels of enhancement of cytotoxicity. The effect of combining IL-2 and OKT3 was to enhance cytotoxicity above that achieved by either agent alone. However, the effect was only partially additive in these subjects.

Figure 4.20 - Effect of varying concentration of IL-2 on K562 cytotoxicity compared with T3 (1 donor JH).

Concentration T3 (μg ml^{-1}) and I L-2 (IU ml^{-1})
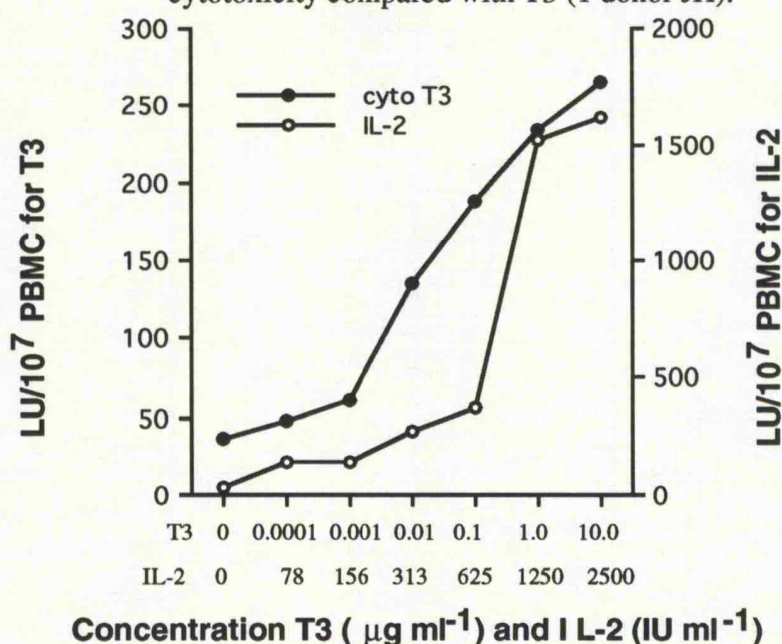
Table 4.23 - Effect of IL-2 concentration (IU ml^{-1}) on K562 cytotoxicity (LU/10^7 PBMC).

Subject	medium	25 IU IL-2	50 IU IL-2	100 IU IL-2	OKT3
TH	100.3	105.4	120.0	155.3	100.8
JH	97.3	105.4	159.5	209.2	119.2
PP	14.6	20.9	21.6	25.8	24.1
Mean	70.9	77.2	100.4	13.1	81.4
Median	97.3	105.4	120.0	155.3	100.8
SEM	28.1	28.2	41.0	54.4	29.1

Table 4.24 - Effect of combined IL-2 and OKT3 preincubation on K562 cytotoxicity (LU/10^7 PBMC) in 3 healthy donors.

Subject	medium	50 IU IL-2	OKT3	Both
JH	125.2	289.0	239.8	310.6
PS	80.1	111.5	121.7	146.2
TH	42.7	124.1	158.2	229.9
Mean	82.7	174.9	173.2	228.9
Median	80.1	124.1	158.2	229.9
SEM	23.9	57.2	34.9	47.5

D.   Summary.

IL-2 at a minimum concentration of 78 IU ml^{-1} results in substantially greater enhancement in K562 cytotoxicity than that acheived with the most potent anti-CD3 mAb studied (T3) at 1 μg ml^{-1}. IL-2 and OKT3 in combination enhance cytotoxicity to a greater extent than either agent alone but no evidence of synergy was demonstrated.

4.3.9   Analysis of peripheral blood cells.

A.   Introduction.

It is important to define the numbers of peripheral blood cells with specific phenotypes in individual subjects, in order to understand their influence on cytotoxicity *in vitro*. Analysis of peripheral blood cell subsets by correlation with K562 cytotoxicity would be anticipated to provide information regarding their role in mediating cytotoxicity. In the case of modulation of cytotoxicity by various mAb, an association might be expected to exist between the numbers of effectors expressing surface antigens for which the mAb is specific and the amount of enhancement observed. Flow cytometry was undertaken on whole blood in 35 of the < 50 year-old donors and 20 of the ≥ 50 years-old donors to determine the number of neutrophils, monocytes, lymphocytes, NK cells and cells expressing CD3 and NK markers (CD3$^+$ NK cells). NK cells were determined by expression of CD16 and/or CD56. In addition expression of CD20 (B cell marker, CD4 (T helper cells), CD8 (T helper/suppressor cells) and CD25 (IL-2Rα) was identified in some of the young healthy donors. The method is described in appendix D.

B.   Peripheral blood cell subsets.

55 healthy donors underwent flow cytometry to measure peripheral blood cell subsets and also underwent simultaneous assessment of K562 cytotoxicity. These subjects comprised 31 males and 24 females and were distributed by age into 20 of the 28 donors age ≥ 50 years compared with 35 of the 75 donors aged < 50 years. Individual results are described in Tables 4.25, 4.26, and 4.27 in appendix H. Figure 4.21 describes results in the < 50 years and the ≥ 50 years old groups.

The ≥ 50 years group had a higher WBC (p = NS) due to increased numbers of neutrophils (p = NS) and monocytes (p = 0.04). Lymphocytes and NK cell numbers were similar in both groups. However, mean (± SEM) CD3$^+$NK cells were significantly higher in the ≥ 50 years group at 0.22 X 10^9 L^{-1} than the < 50 years group at 0.15 X 10^9 L^{-1} (p = 0.03).

When the male and female donors were analysed separately there was no significant difference in any cell population. When males and females were analysed in the ≥ 50 years group NK cell numbers were also similar in the male (0.21 ± 0.05 X 10^9 L^{-1}) and female donors (0.22 X 10^9 L^{-1}).

Figure 4.21 - Peripheral blood cells in healthy donors by age (means ± SEM).
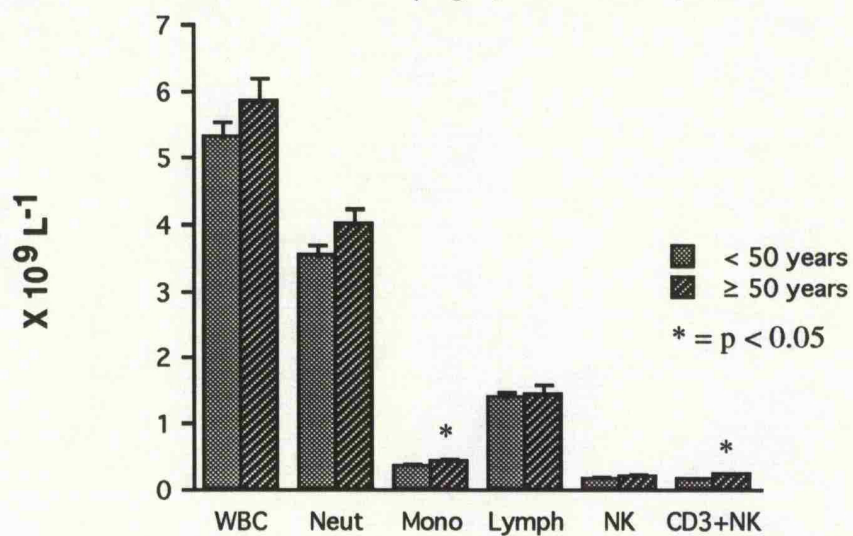
Figure 4.22 - Comparison of cellular constituents of whole blood and PBMC for 8 healthy donors (means ± SEM).
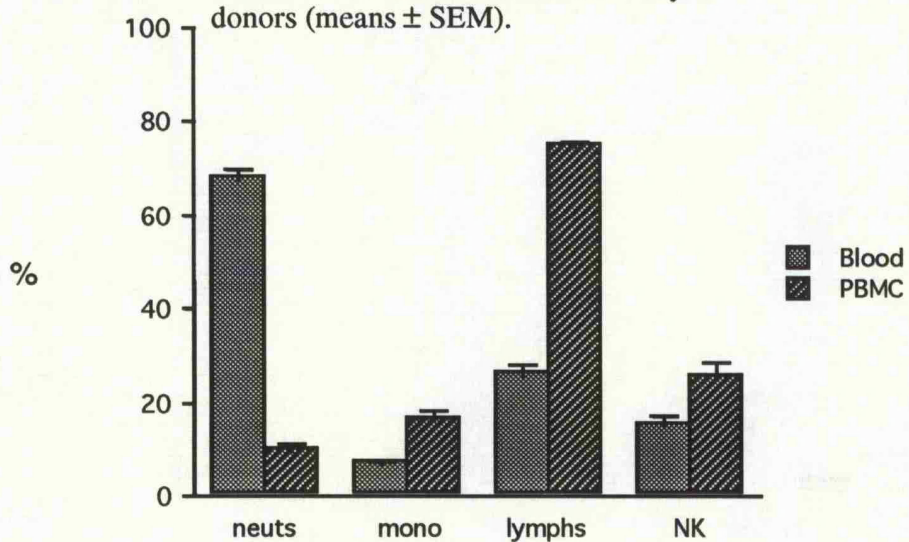
In a limited number of donors aged ≤ 50 years numbers of B cells (CD20) as well
as T cells (CD3$^+$), and cells expressing CD4 and CD8 in association with CD25 were
determined. Results are described in Table 4.27 in appendix H. Numbers of cells were
within the expected range for healthy donors. B cells constituted 25% of circulating
lymphocytes and among T cells CD4$^+$ (helper) cells predominated over CD8
(cytotoxic/suppressor) cells with a T_h:T_s ratio of 1.3:1. CD25 expression was detected
on 12% of T_h cells but only 4% of T_s cells.

C.   Constituents of PBMC preparations.

PBMC preparations and blood were simultaneously analysed by flow cytometry
by TH in 8 healthy donors. Figure 4.26 compares the mean % (± SEM) constituents of
the PBMC preparations with the % of these cells in whole blood. Individual results are
also shown in Table 5.9 in appendix H. Mean (± SEM) % contamination of PBMC
preparations by neutrophils was only 9.6 (± 1.6) % and the proportion of monocytes was
15.8 (± 2.2)% respectively. The percentage of NK cells as a fraction of lymphocytes
was 25.4% (± 3.4). This compared with 14.9% (± 2.2) in whole blood. The
preparation technique therefore resulted in PBMC preparations in which the majority of
cells were lymphocytes and there were relatively greater proportions of NK cells as a
percentage of the lymphocyte population than were present in whole blood.

NK cells constitute only a fraction of lymphocytes in whole blood and PBMC
preparations, they are however, responsible for the majority of non-MHC-restricted
cytotoxicity *in vivo* and *in vitro*.(Trinchieri, 1989). PBMC preparations contain more
NK cells as a percentage of total lymphocytes than are present in whole blood. For all 49
healthy donors undergoing simultaneous assessment of PBMC and blood by flow
cytometry, the mean (± SEM) percentage of circulating NK cells was 13.9% (± 0.9)
compared with 20.4% (± 1.3) in PBMC. The greater percentage of NK cells in PBMC
preparations is probably due to the relative difference in their density due to their
morphology as large granular lymphocytes. It is this property that allows them to be
selectively separated by density gradient centrifugation (Timonen and Saksela, 1980;
Timonen et al, 1981). It is important to determine how closely the distribution of NK
cells in PBMC preparations reflect their distribution in whole blood if *in vitro* assessment
is to relate to the *in vivo* situation. Small variatons in the separation technique may be a
source of significant variability when measuring cellular cytotoxicity between different
individuals and also the same individual undergoing sequential monitoring.

The proportions of lymphocytes in PBMC preparations and blood were measured
in 8 healthy donors by TH. The relationship between the percentage of lymphocytes in
whole blood and their percentage in PBMC is described in Figure 4.27. Although there

Figure 4.23 - Comparison of the proportion of lymphocytes in blood and the corresponding PBMC fraction in 8 healthy donors.
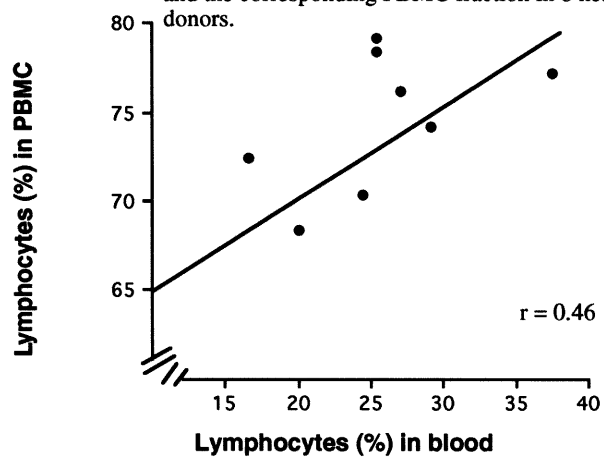
Figure 4.24 - Comparison of the proportion of NK cells in blood and corresponding PBMC preparations in 49 healthy donors.
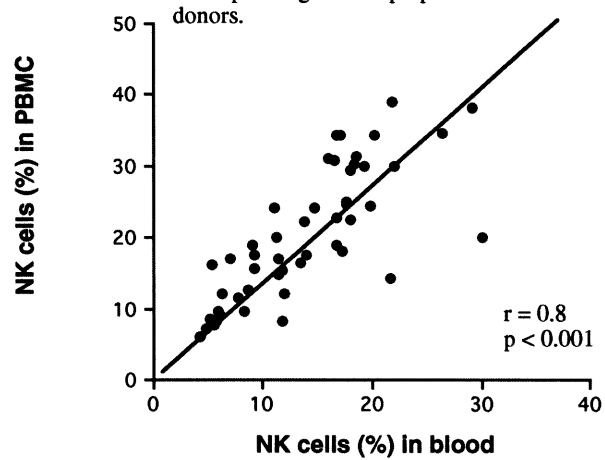
were insufficient points to calculate meaningful confidence intervals (r = 0.46) for the 8 healthy donors an association was suggested. This suggests that proportions of lymphocytes in blood are represented in PBMC. This was an important observation because meaningful analysis of % NK cells in PBMC preparations depends upon consistency of the lymphocyte fraction. The relationship of NK cells in blood is shown for 49 healthy donors in Figure 4.28. A good correlation between % NK cells in PBMC and blood was seen (r = 0.8).

D.   Relation between peripheral blood cells and K562 cytotoxicity.

Figure 4.22 descibes the relationship between % NK cells in whole blood and K562 cytotoxicity for the 55 healthy donors who underwent simultaneous measurement of K562 cytotoxicity. The percentage of circulating NK cells was analysed because this is directly related to the proportions of NK cells in the PBMC preparations which reflect *in vitro* activity. A significant positive correlation between % NK cells in whole blood and K562 cytotoxicity (r = 0.37, p = 0.005) was observed for all 55 donors.

Some individuals had high proportions of circulating NK cells but very low levels of cytotoxicity whereas other individuals had low numbers of NK cells and high levels of cytotoxicity. NK activity *in vivo* is probably more closely related to absolute NK cell numbers. A significant but poorer association was seen between numbers of circulating NK cells and K562 cytotoxicity (r = 0.3, p = 0.03). No significant correlation was seen between % CD3$^+$NK cells and cytotoxicity in all 55 healthy donors (r = 0.17, p = 0.23). This suggested that freshly isolated CD3$^+$NK cells despite expressing antigens associated with NK activity do not play a major role in mediating NK activity. Variation in the proportion of circulating NK cells was associated with corresponding variation in K562 cytotoxicity on the 6 occasions when simultaneous measurements were made in one donor (Figure 4.24).

When healthy donors were analysed according by age, a stronger association was seen between %NK cells and cytotoxicity in the < 50 years group (r = 0.56, p < 0.001) and no correlation was seen in the ≥ 50 years group (r = 0.94). Although NK cell numbers were similar in the < 50 year and ≥ 50 years groups, the elderly population had significantly more CD3$^+$ cells coexpressing NK markers (CD16 and/or CD56) than the population aged < 50 years. When % CD3$^+$ NK cells were correlated with cytotoxicity no significant association was observed (r = 0.12, p = 0.61). This suggests that this population of cells is not significantly involved in mediating K562 cytotoxicity in the ≥ 50 years group.

Although CD3$^+$ NK cells do not play a role in K562 cytotoxicity in unstimulated PBMC preparations they may be involved in cytotoxicity following incubation with OKT3 by virtue of the direct interaction between OKT3 and CD3. 40 donors (20 < 50 years and 20 ≥ 50 years) underwent simultaneous flow cytometry and OKT3 modulation

Figure 4.25 - K562 cytotoxicity and % NK cells in whole blood for
55 healthy donors.

r = 0.37
p = 0.005
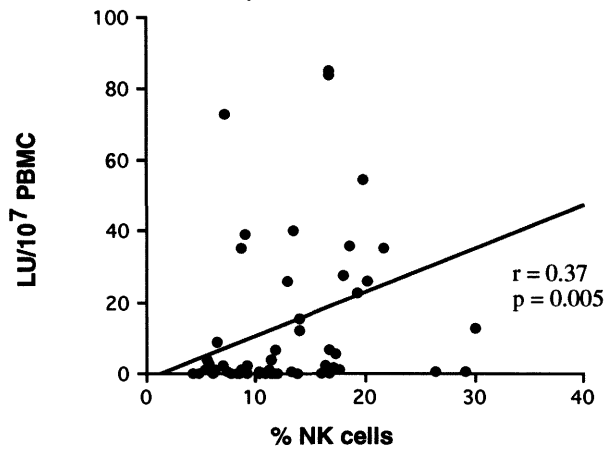
Figure 4.26 - Variation in K562 cytotoxicity and NK cells
with time for one healthy donor (JH).
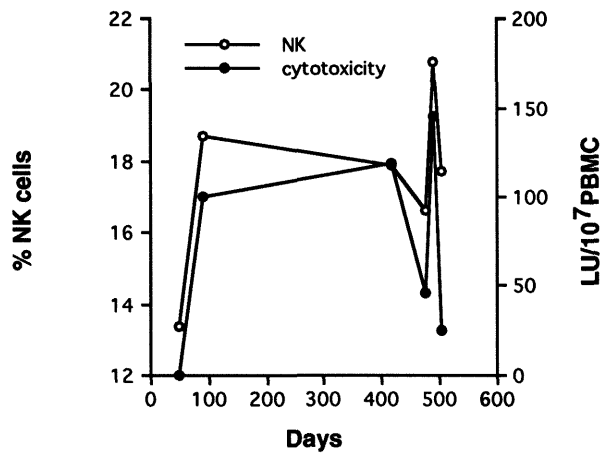
of cytotoxicity *in vitro*. The quantity of enhancement (mean ± SEM) by OKT3 in all 40 donors was 27.8 ± 5.9 LU/10^7 PBMC and when analysed according to age was 25.3 ± 5.3 and 30.2 ± 10.7 LU/10^7 PBMC in the ≥ 50 years and < 50 years groups respectively. There was no significant difference between these groups. There was no significant correlation between % CD3$^+$ NK cells and the quantity of OKT3 enhancement in either all 40 donors or the < 50 and ≥ 50 years groups. A significant correlation was seen between % NK cells and the quantity of enhancement by OKT3 for all 40 donors (r = 0.39, p = 0.01) which was maintained for the < 50 years group (r = 0.45, p = 0.04). No correlation was observed in the > 50 years group.

12 donors underwent simultaneous assessment of whole blood by flow cytometry and received anti-CD16. Anti-CD16 specifically interacts with the CD16 antigen present on NK cells which represents an activation site for NK cells. However, no significant association was observed between the amount of enhancement seen with anti-CD16 and the % of NK cells in whole blood.

4.4 SUMMARY

A wide range of K562 cytotoxicity is observed between different healthy donors. Considerable variation in K562 cytotoxicity was observed for one donor tested repeatedly over an 18 month period. Evidence suggests that the rank order of cytotoxicity is maintained and some individuals show consistently low and others high levels of cytotoxicity. Cytotoxicity against targets other than K562 was extremely low. There was no significant association between levels of K562 cytotoxicity and age or between males and females in all 103 healthy donors. However, the female subjects in the > 50 years group had higher levels of K562 cytotoxicity compared with the males. The reason for this is unclear but may be due to the small numbers of donors studied. Although RC undertook 20 of the 28 cytotoxicity assays in the elderly group it seems unlikely that this distribution was due to technical variation between operators (JH and RC). An identical protocol was followed in all cases, there was no difference in values obtained from simultaneous assays performed by RC and JH (methods), and there was no significant difference in overall cytotoxicity between results obtained by JH and RC.

PBMC preincubated with anti-CD3 mAb demonstrate enhanced K562 cytotoxicity. Significant enhancement was not observed against other tumour cell lines. The optimal concentration for this effect was 1 μg ml^{-1} and the optimal preincubation time 30 minutes. T3 (IgG1) consistently enhanced cytotoxicity to a greater extent than OKT3 (IgG2a). A 'humanised' monovalent OKT3 preparation was less effective than OKT3. Other mAb including anti-CD16 and anti-CD7 also enhanced K562 cytotoxicity. Anti-CD2, CD4, and CD8 also enhanced cytotoxicity but failed to reach statistical significance in these limited studies. Other mAb notably anti-ICAM-1 and anti-LFA-1 impaired K562 cytotoxicity presumably by inhibiting effector/target cell adhesion.

Combinations of anti-CD3 and anti-CD7 enhanced cytotoxicity to a greater extent than either mAb alone. However, this effect was additive rather than synergistic. Although T3 and anti-CD16 individually enhanced cytotoxicity, anti-CD16 appeared to inhibit T3 enhancement of K562 cytotoxicity. The mechanism of enhancement remains unclear. Incubating K562 in polyclonal preparations of antibodies failed to impair OKT3 or T3 enhancement of cytotoxicity. However, anti-FcγRII did significantly decrease T3 enhancement in three donors. This suggests that Fc receptors may play a role in enhancement by T3.

Il-2 enhanced cytotoxicity in a dose dependent manner. 50 IU IL-2 was adopted for exploration of synergy with 1 μg ml^{-1} OKT3. An additive effect was seen when both agents were combined but no evidence of synergy.

The effectors responsible for mediating the majority of K562 cytotoxicity are probably NK cells. A significant correlation was observed between NK cell numbers and cytotoxicity for 55 donors. However, in the ≥ 50 years-old donors a significant correlation was not observed between NK cells and K562 cytotoxicity. The finding that cytotoxicity was not correlated with NK cells in these donors may be due to the small number of subjects studied or possibly that another factor relating to the activity of individual NK cells may be involved. Freshly isolated CD3$^+$ lymphocytes expressing NK markers do not appear to significantly contribute to K562 cytotoxicity as evidenced by a lack of correlation between CD3$^+$ NK and cytotoxicity. They were not associated with the baseline levels of K562 cytotoxicity observed in the ≥ 50 years donors. The quantity of enhancement by OKT3 was similar in males and females and was unrelated to age. It was also unrelated to the number of CD3$^+$NK cells in whole blood. Enhancement was significantly correlated to the number of NK cells in whole blood for the 40 healthy donors. This suggests that NK cells mediate K562 cytotoxicity and enhancement by OKT3 is also related to baseline NK cell activity.

CHAPTER FIVE

IN VITRO MODULATION OF CELLULAR CYTOTOXICITY IN CANCER
PATIENTS

5.1 INTRODUCTION.

Cellular cytotoxicity was examined in patients with advanced cancer during the studies on healthy donors described in Chapter 4. These subjects underwent similar investigations of cellular cytotoxicity and peripheral blood cells as the healthy donors. A clinical trial was undertaken in some of the cancer patients to investigate the immunomodulating properties of OKT3 *in vivo*. The effects of OKT3 administration are discussed in Chapter 6. Ubhi et al, (1991) demonstrated that freshly isolated PBMC from healthy individuals and cancer patients demonstrate variable levels of cellular cytotoxicity against the NK sensitive target K562 which was enhanced by their preincubation in the anti-CD3 mAb T3 (Dako). The failure of T3 to enhance cytotoxicity of PBMC from four cancer patients who subsequently failed to respond to rIL-2 therapy suggested a deficiency in the effectors responsible for tumour cell killing both *in vitro* and *in vivo*.

Three of the four patients who failed to respond to IL-2 had gastrointestinal cancers. It was therefore unproven from these studies whether a failure to respond to IL-2 and lack of enhancement of cytotoxicity *in vitro* was a specific property of gastrointestinal cancer or a general property of unresponsive tumours. This report does however emphasise the potential relevance of *in vitro* studies in understanding the mechanisms of tumour regressions *in vivo* during cancer immunotherapy and prompted the studies on cancer patients undertaken here.

5.2 AIMS.

The studies reported here were undertaken to compare peripheral blood cell subsets and cellular cytotoxicity in cancer patients (all gastrointestinal except 1) and healthy donors. The principal aims were to define in cancer patients and compare with healthy donors;

1. A normal range for K562 cytotoxicity .
2. Investigate cytotoxicity against Daudi target cells.
3. Investigate the effect of OKT3, T3 and anti-CD16 mAb on cellular cytotoxicity.
4. Investigate the effect of OKT3 mAb and IL-2 on cytotoxicity.
5. Define peripheral blood cells in cancer patients which mediate K562 cytotoxicity.

5.3 RESULTS

5.3.1 Cellular cytotoxicity.

A.   Range of K562 cytotoxicity and relation to disease status, age, and sex.

The distribution of K562 cytotoxicity in individual cancer patients is described in Table 5.1 in appendix H. K562 cytotoxicity among the cancer patients is compared with healthy donors in Figure 5.1. As was observed for the healthy donors considerable individual variation in K562 cytotoxicity was detected. For all 51 cancer patients median cytotoxicity was 4.4 LU/10^7 PBMC and varied between 0.0 and 197.2 LU/10^7 PBMC (mean ± SEM, 20.9 ± 4.8 LU/10^7 PBMC). There was no statistically significant difference between levels among the cancer patients and the 103 healthy donors (3.3 LU/10^7 PBMC). Among the cancer patients there was no statistically significant difference in median levels of K562 cytotoxicity between males 4.1 LU/10^7 PBMC and females (7.3 LU/10^7 PBMC) or between patients aged < 70 years (4.4 LU/10^7 PBMC) and ≥ 70 years (5.6 LU/10^7 PBMC). Median levels of K562 cytotoxicity were greater in the patients with upper GIT tumours (8.3 LU/10^7 PBMC) than those with lower GIT tumours (2.1 LU/10^7 PBMC) but this did not reach statistical significance. Median cytotoxicity was actually higher but not significantly different in the patients with liver metastases (8.0 LU/10^7 PBMC) compared with patients without liver involvement (3.3 LU/10^7 PBMC).

Median levels of K562 cytotoxicity were higher in the cancer patients compared with the healthy donors aged ≥ 50 years (2.0 LU/10^7 PBMC) although this failed to reach statistical significance. However, when male cancer patients were compared with male healthy donors the male cancer patients had higher median levels of cytotoxicity (4.1 LU/10^7 PBMC compared with 0.7 LU/10^7 PBMC) which also failed to reach significance (p = 0.1). A statistically significant difference in median K562 cytotoxicity was not observed between female cancer patients (7.3 LU/10^7 PBMC) and ≥ 50 years-old female healthy donors (10.7 LU/10^7 PBMC). As was noted in Chapter 4 the ≥ 50 years-old healthy donors demonstrated an unusual distribution of K562 cytotoxicity with regard to sex and the study of only small numbers of subjects casts doubt on the significance of any comparison between the ≥ 50 years healthy donors and the cancer patients. RC performed most of the assays on the elderly healthy donors. However, there was no significant difference between K562 cytotoxicity measured by JH and RC in the elderly healthy donors. Similarly there was no significant difference in median levels of K562 cytotoxicity measured by JH and RC among the cancer patients, although levels were higher in the assays performed by JH (9.0 LU/10^7 PBMC compared with 3.3 LU/10^7 PBMC).

The presence of an elevated bilirubin at the time of study was not associated with particularly low levels of K562 cytotoxicity (ABT = 53.9 and GW = 21.9 LU/10^7 PBMC). The two patients who had been previously jaundiced both had unusually low levels of K562 cytotoxicity (WW = 0.0 and AG = 0.0 LU/10^7 PBMC).

Figure 5.1 - K562 cytotoxicity (means ± SEM)in 51 cancer patients compared with 103 healthy donors (lines = medians).
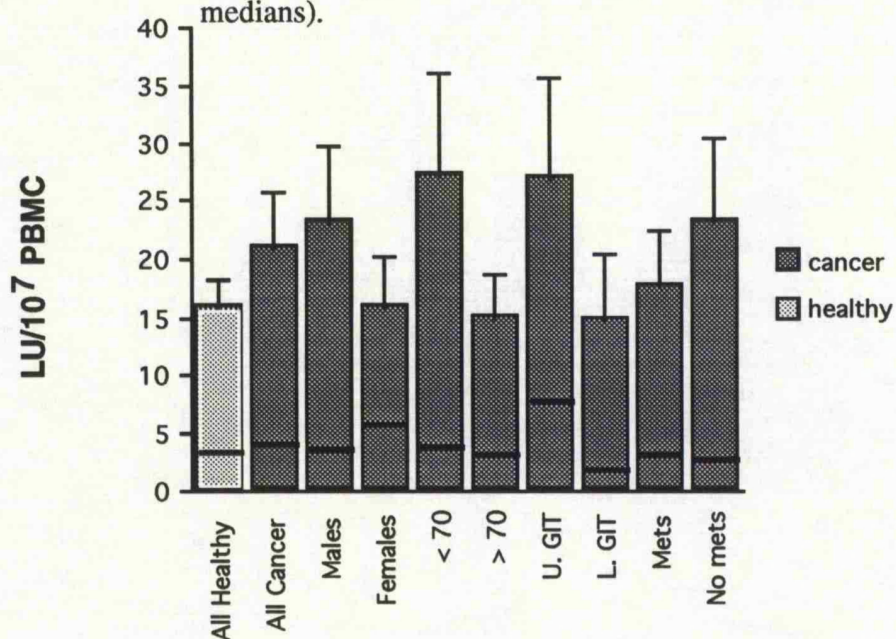
Table 5.2 - K562 cytotoxicity (LU/10^7 PBMC) and tumour grade (NS = not stated).

Grade	Mean	SEM	Median
Well	12.9	11.1	3.3
Moderate	15.1	4.7	6.5
Poor	34.1	14.0	4.7
NS	16.8	5.3	7.3

There was no statistically significant association between tumour grade and levels of K562 cytotoxicity (Table 5.2).

B.   Comparison of K562 cytotoxicity and Daudi cytotoxicity.

10 cancer patients (DH, AP, IB, WD, ME, WW, MD, PW, GG, and HC) had cytotoxicity assessed against the Daudi tumour cell line as well as K562. These 10 patients were also studied in the OKT3 trial and are discussed further in chapter 6. Individual results are expressed in LU in Tables 6.25 to 6.33 in the appendix. Results are also expressed as % cytotoxicity at an E:T ratio of 20:1 in Table 6.34 in appendix H. This form of data expression was used because assays were performed in some of these patients at an E:T of 20:1. Conversion to lytic units also resulted in low values (< 0.1) which fail to demonstrate relative cytotoxicity between K562 and Daudi. Figure 5.2 compares mean % cytotoxicity at an E:T of 20:1 for the 10 cancer patients and also describes the effect of OKT3 and T3 on cytotoxicity.

As was demonstrated in the healthy donors mean ($\pm$ SEM) levels of cytotoxicity were consistently lower against Daudi cells ($7.9 \pm 3.0\%$) than K562 cells ($23.4 \pm 4.9\%$). However, two individuals demonstrated relatively high levels of K562 cytotoxicity (ME at 42.8 LU/10^7 PBMC (27.6%) and GG at 197.2 LU/10^7 PBMC (56.0%)). These individuals also demonstrated relatively high levels of Daudi cytotoxicity (ME at 10.4 LU/10^7 PBMC (20.8%) and GG at 38.4 LU/10^7 PBMC (28.7%)).

C.   Modulation of K562 cytotoxicity by OKT3 and T3.

In vitro modulation by mAb was assessed in all but one of the cancer patients according to the method described in appendix C. PBMC from 50 patients were preincubated with OKT3, from 43 patients with T3, and from 14 patients with anti-CD16. Results for OKT3 and T3 for individual patients are described in Table 5.1 in appendix H. Post-OKT3 levels of K562 cytotoxicity together with the quantity of enhancement by OKT3 (post-OKT3 - baseline value) are shown in Table 5.3 in appendix H and compared with the healthy donors.

OKT3 and T3 enhanced cytotoxicity in most patients. OKT3 significantly enhanced K562 cytotoxicity compared with baseline levels in all groups (p < 0.05). The amount of enhancement by OKT3 also was also similar in all groups.

Figure 5.3 describes cytotoxicity for cancer patients PBMC with and without preincubation with OKT3 and compares them with the 81 healthy donors presented in chapter 4 who underwent *in vitro* modulation with OKT3. Median post-OKT3 levels of cytotoxicity were not significantly different in the cancer patients (4.5 LU/10^7 PBMC pre-OKT3 to 20.0 LU/10^7 PBMC post-OKT3), to those observed in the healthy donors, either when compared with all healthy donors (3.3 LU/10^7 PBMC pre-OKT3 to 18.9 LU/10^7 PBMC post-OKT3) or the elderly ($\geq$ 50 years) group (2.0 LU/10^7 PBMC pre-OKT3 to 24.0 post-OKT3). There was therefore, no significant difference in the amount of enhancement of K562 cytotoxicity by OKT3 between any of the groups of cancer patients and the healthy donors.

Figure 5.2 - K562 and Daudi cytotoxicity with *in vitro* modulation by OKT3 and T3 in cancer patients (means ± SEM).
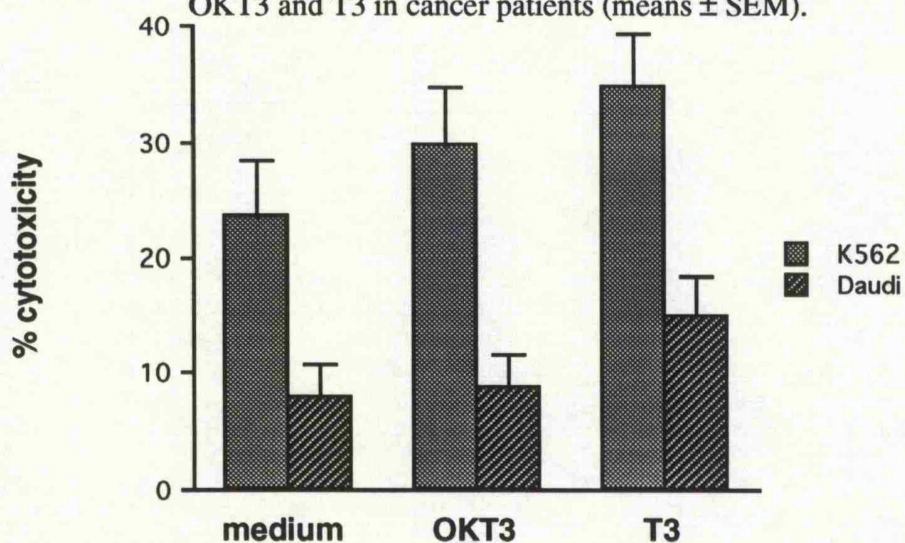
Figure 5.3 - OKT3 modulation of K562 cytotoxicity *in vitro* in 50 cancer patients compared with 81 healthy donors (means ± SEM, lines = medians).
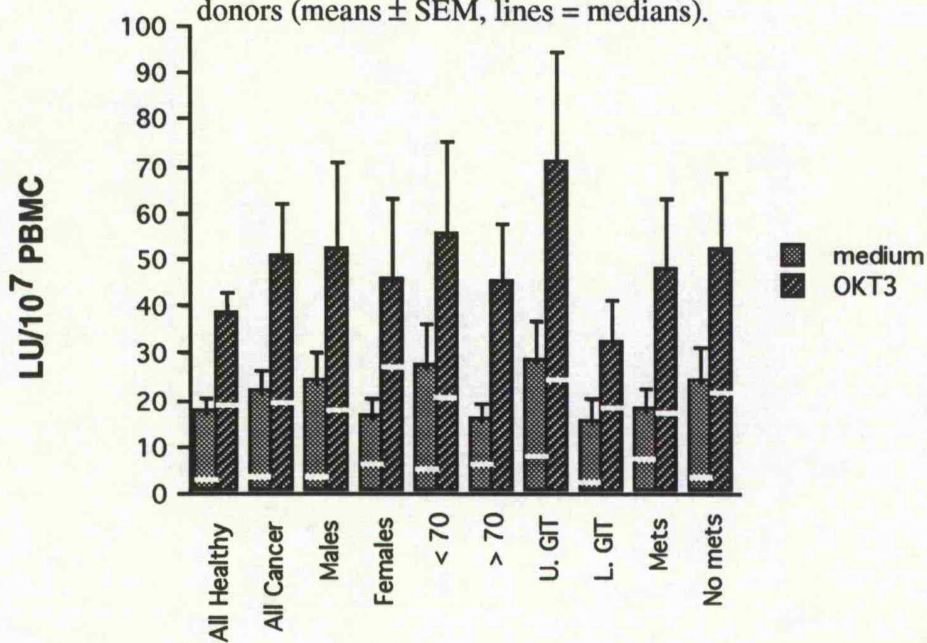
Figure 5.4 describes the effect of T3 on cytotoxicity in 43 cancer patients and compares the results with 59 healthy donors. Pre and post-T3 levels are also shown in Table 5.4 in appendix H for cancer patients and are compared with 59 healthy donors including 24 of the 28 elderly healthy donors who underwent *in vitro* modulation with T3.

Enhancement by T3 was not significantly different between any of the groups of cancer patients or between cancer patients and healthy donors or between the cancer patients and the elderly healthy donors. T3 enhanced cytotoxicity to a significantly greater extent than OKT3 in the healthy donors and cancer patients ($p < 0.05$). Percentage enhancement of median cytotoxicity by OKT3 was similar in the cancer patients and healthy donors (444% and 573% respectively) and this was also true for T3 in the cancer patients and healthy donors (1370% and 1946% respectively).

Confirmation of the superior ability of T3 to enhance cytotoxicity over OKT3 was confirmed when T3 was assessed simultaneously with OKT3 in 43 of the cancer patients ($p = 0.01$) (Figure 5.5). Again in these experiments there was no significant difference between the cancer patients and the healthy donors. Median levels of cytotoxicity in tissue culture medium, OKT3 and T3 were 1.8, 21.0, and 45.6 $LU/10^7$ PBMC in the healthy donors, and 3.3, 19.0, and 45.2 $LU/10^7$ PBMC in the cancer patients respectively.

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Figure 5.4 - *In vitro* modulation of K562 cytotoxicity by T3
in 43 cancer patients compared with 59 healthy
donors (means ± SEM, lines = medians).
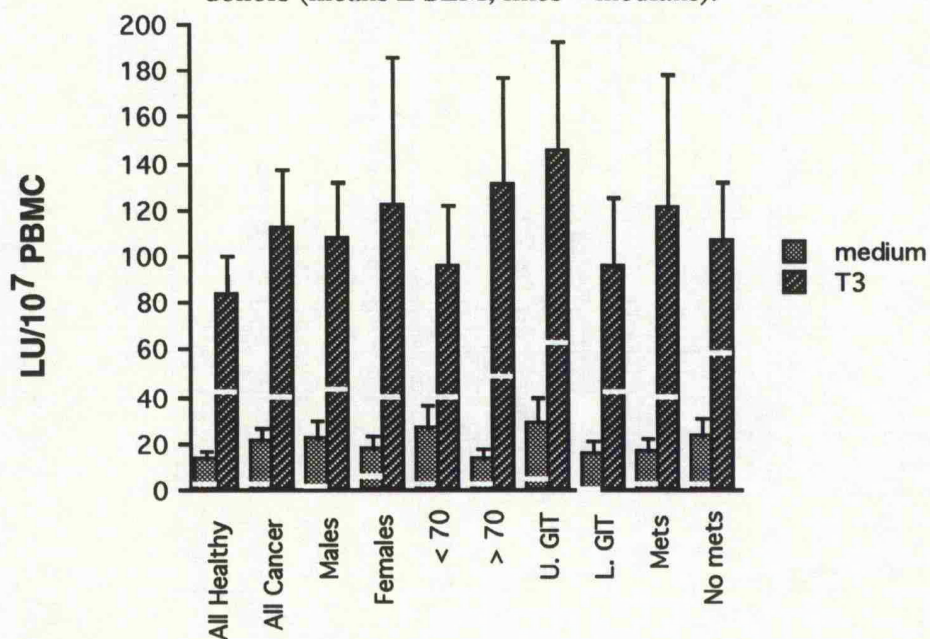
Figure 5.5 - Simultaneous enhancement of K562 cytotoxicity
by OKT3 and T3 in 42 healthy donors and 43 cancer
patients (means ± SEM, lines = medians).
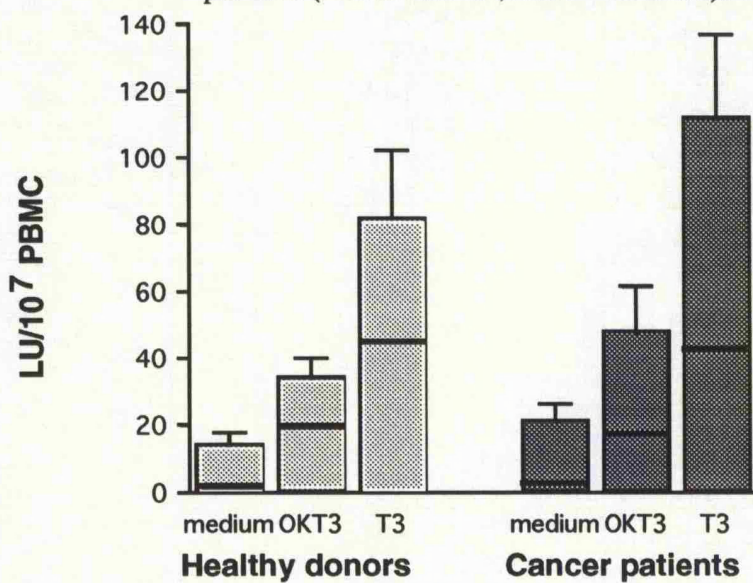
D. Modulation of K562 cytotoxicity with anti-CD16.

In vitro modulation of K562 cytotoxicity was assessed using anti-CD16 mAb in 14 of the cancer patients and 13 of these were also assessed with OKT3 (Table 5.5 in appendix H). Anti-CD16 enhanced median cytotoxicity over baseline levels (0.74 to 17.53 LU/10^7 PBMC) which just failed to reach statistical significance (p = 0.07). The effect of anti-CD16 was extremely variable and one individual (AJR) demonstrated considerable enhancement (33.05 to 1292.54 LU/10^7 PBMC). Although AJR also demonstrated significant enhancement with OKT3 (182.60 LU/10^7 PBMC), suggesting that effectors were particularly responsive to both mAb, a strong association between OKT3 and anti-CD16 enhancement was seen in the other patients.

E. Modulation of Daudi cytotoxicity by OKT3 and T3.

As demonstrated in the healthy donors, preincubation of PBMC with OKT3 and T3 resulted in enhancement of Daudi cytotoxicity (Figure 5.3 and Table 6.25 to 6.33 appendix H) as well as K562 cytotoxicity. Mean ± SEM percentage specific cytotoxicity was enhanced from baseline levels of 7.9 ± 3.0% to 8.78 ± 3.0% with OKT3 and to 15.0 ± 3.4% with T3 in 10 cancer patients. Enhancement of Daudi cytotoxicity with OKT3 was therefore very low, but for T3 substantially greater in these patients. In the same 10 cancer patients, OKT3 resulted in an increase in mean K562 cytotoxicity from 23.4 ± 4.9% to 29.8 ± 5.0% with OKT3 compared with 34.8 ± 4.6% for T3. One individual (AP) who had above average baseline K562 reactivity (34.4 LU/10^7 PBMC (26.2%)) demonstrated more considerable enhancement with T3 (125.5 LU/10^7 PBMC (45.7%)) and this was reflected in significant levels of cytotoxicity against Daudi cells when enhanced by T3 (27.0 LU/10^7 PBMC (30.1%)) although baseline Daudi cytotoxicity in this individual was low (6.6%).

F. Modulation of K562 cytotoxicity by IL-2 and OKT3.

In 15 cancer patients who were also in the OKT3 trial the effect of IL-2 at a concentration of 50 IU ml^{-1} was assessed simultaneously with OKT3 at 1 μg ml^{-1} against K562 cells. In 11 patients OKT3 and IL-2 were combined to investigate the possibility of synergy. The dose of IL-2 was based on the findings presented in chapter 4. Individual results in lytic units are described in Tables 6.25 to 6.33 and overall results in Table 5.6.

As was the case with the healthy donors, IL-2 at a concentration of 1 μg ml^{-1} resulted in comparable levels of enhancement of cytotoxicity to that achieved with 1 μg ml^{-1} OKT3. The effect of combining OKT3 and IL-2 showed individual variability. Although median enhancement was slightly greater than seen with either agent alone the additional effect was only partially additive and not statistically significant.

Table 5.6 - Effect of combining OKT3 and IL-2 on K562 cytotoxicity (LU/10^7 PBMC).
(Figures in parentheses are corresponding levels in medium alone).

PBMC in	Mean	Median	SEM
Medium (n= 15)	15.6	4.4	5.5
OKT3 (n = 15)	34.5	21.5	9.1
IL-2 (n = 15)	36.6	20.6	11.7
OKT3 and IL-2 (n = 12)	45.7 (17.2)	28.7 (4.4)	14.6 (7.2)

5.3.2   Analysis of peripheral blood cells.
A.   Peripheral blood cell subsets in cancer patients compared with healthy donors.

Analysis of peripheral blood cells both with respect to the WBC differential and lymphocyte subsets was undertaken by flow cytometry as described in appendix D. Results are presented graphically as absolute numbers ($X \ 10^9 \ L^{-1}$) of circulating cells. NK cells and $CD3^+$ NK cells are also referred to in the text as percentages of circulating lymphocytes. Correlations between cytotoxicity and NK cells and $CD3^+$ NK cells are also expressed as their percentage of lymphocytes. Percentages were generally used because the proportion of cells bearing NK markers is more closely related to the measurement of cellular cytotoxicity *in vitro* than absolute numbers.

Figure 5.6 shows peripheral blood cells determined by flow cytometry in the 51 cancer patients which are compared with the 55 healthy donors described in Chapter 4 (individual results for cancer patients are described in Table 5.7 in appendix H and for healthy donors in Tables 4.25, 4.26, and 4.27 in appendix H).

Cancer patients had a significantly higher WBC than healthy donors due to greater numbers of circulating neutrophils and monocytes. Circulating lymphocyte numbers were not significantly different. Cancer patients also had significantly higher mean ($\pm$ SEM) NK cells ($0.22 \pm 0.02 \ X \ 10^9 \ L^{-1}$) than all 55 healthy donors ($0.18 \pm 0.02 \ X \ 10^9$ L^{-1}) (p = 0.02, unpaired T test). Numbers of $CD3^+NK$ cells were also higher in the cancer patients ($0.24 \pm 0.03 \ X \ 10^9 \ L^{-1}$) compared with the healthy donors (0.17 ± 0.01 $X \ 10^9 \ L^{-1}$) although this failed to reach statistical significance (Mann-Whitney U-test = NS). The (mean $\pm$ SEM) proportions of NK cells as a percentage of lymphocytes was also significantly higher in the cancer patients ($16.4 \pm 1.1\%$) than the healthy donors ($12.9 \pm 0.8\%$) (p = 0.02). $CD3^+$ NK cells were not significantly different between the cancer patients ($15.7 \pm 1.6\%$) and healthy donors ($12.9 \pm 0.8\%$). When cancer patients were compared with the donors aged $\geq$ 50 years there was no significant difference between numbers of WBC, neutrophils, NK cells, or $CD3^+$ NK cells. Monocyte numbers were significantly higher in the cancer patients compared with the elderly healthy donors (p = 0.02).

Figure 5.7 compares peripheral blood cells in the patients with liver metastases and those without liver involvement. The presence of liver metastases was associated with higher numbers of circulating neutrophils and lymphocytes than in the patients without liver involvement (p = NS). Numbers of $CD3^+NK$ cells were substantially higher in patients with liver metastases ($0.34 \pm 0.05 \ X \ 10^9 \ L^{-1}$) than those without liver involvement ($0.17 \pm 0.03 \ X \ 10^9 \ L^{-1}$) (p = 0.003, Mann-Whitney U-test). This contributed to the observation that cancer patients with liver metastases had greater numbers of mean ($\pm$ SEM) lymphocytes ($1.55 \pm 0.12 \ X \ 10^9 \ L^{-1}$) than patients without liver metastases ($1.28 \pm 0.08 \ X \ 10^9 \ L^{-1}$) which was not significant (p = 0.07).

Figure 5.6 - Peripheral blood cells (means ± SEM) in 51 cancer patients and 55 healthy donors.
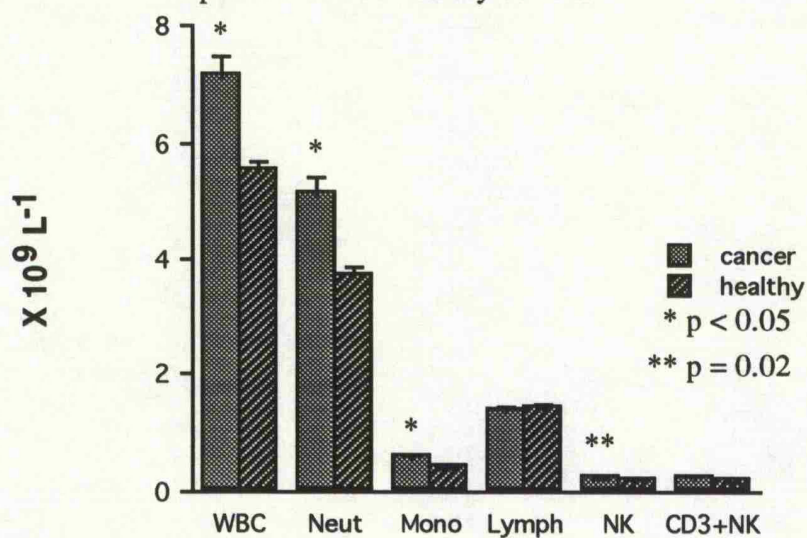
Figure 5.7 - Peripheral blood cells (means ± SEM) in patients with liver metastases and without liver involvement.
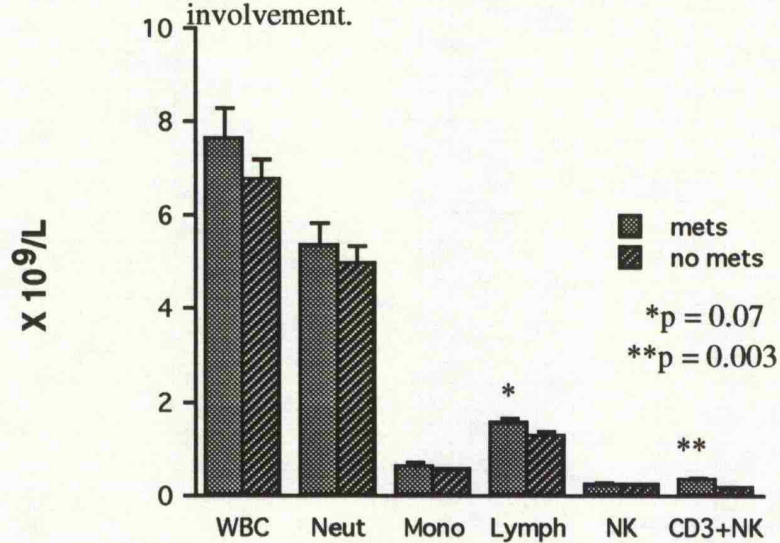
Proportions of CD3$^+$ NK cells were also significantly greater in patients with metastases (21.9 ± 3.34%) compared to those without metastases (11.7 ± 1.2%) p = 0.004). NK cell numbers were similar in both cancer patients with metastases (0.22 ± 0.03 X 10^9 L^{-1}) and patients without metastases (0.22 ± 0.02 X 10^9 L^{-1}) as were proportions of NK cells (15.3 ± 1.7% and 17.1 ± 1.4% respectively).

Lymphocyte subsets determined by the expression of CD4 and CD8 in association with CD25 are presented in Table 4.27 in appendix H for the healthy donors and Table 5.8 in appendix H for the cancer patients. Of the cancer patients, 16% and 6% of CD4 and CD8 cells expressed CD25 respectively compared with 12% and 4% in the healthy donors. Numbers were too small to enable a meaningful statistical comparison between cancer patients (n = 10) and healthy donors (n = 15). There was no significant difference between the T$_h$ (CD4): T$_{s/c}$ (CD8) ratio in the healthy donors (1.3) and the cancer patients (1.6).

B. Constituents of PBMC preparations.

PBMC preparations and blood were simultaneously analysed by flow cytometry by TH in 15 cancer patients. Figure 5.8 compares the mean % (± SEM) constituents of the PBMC preparations with the % of these cells in whole blood. Individual results are also shown in Table 5.9 in appendix H. Mean (± SEM) % contamination of PBMC preparations by neutrophils was 11.6 (± 1.0) % (compared with 9.6 (± 1.6) % in the healthy donors). The proportion of monocytes was 17.4 (± 1.7)% (compared with 15.8 (± 2.2) % in the healthy donors. The percentage of NK cells as a fraction of lymphocytes was 27.0% (± 2.9) compared with 16.8% (± 1.32) in the cancer patients whole blood. As was observed in the healthy donors the preparation technique therefore resulted in PBMC preparations in which the majority of cells were lymphocytes and there were relatively greater proportions of NK cells as a percentage of the lymphocyte population than were present in whole blood.

For all 30 cancer patients studied by both RC and TH NK cells comprised 17.1% (± 1.4) in blood and 25.2% (± 2.2) of lymphocytes in PBMC. The greater percentage of NK cells in PBMC preparations is probably due to the relative difference in their density due to their morphology as large granular lymphocytes. It is this property that allows them to be selectively separated by density gradient centrifugation (Timonen and Saksela, 1980; Timonen et al, 1981). It is important to determine how closely the distribution of NK cells in PBMC preparations reflect their distribution in whole blood if *in vitro* assessment is to relate to the *in vivo* situation. Small variatons in the separation technique may be a source of significant variability when measuring cellular cytotoxicity between different individuals and also the same individual undergoing sequential monitoring.

The relationship between the percentage of lymphocytes in whole blood and their percentage in PBMC is described in Figure 5.9. The correlation coefficient was 0.48 (p = 0.08) for cancer patients.

Figure 5.8 - Comparison of cellular constituents of whole blood and PBMC for 15 cancer patients (means ± SEM).
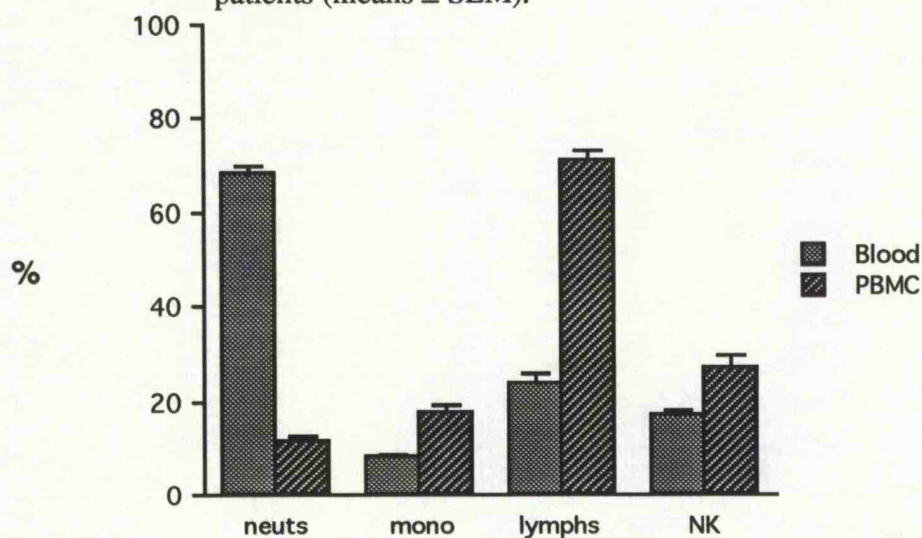
Figure 5.9 - Comparison of the proportion of lymphocytes in blood and the corresponding PBMC fraction in 15 cancer patients.

r = 0.48
p = 0.08
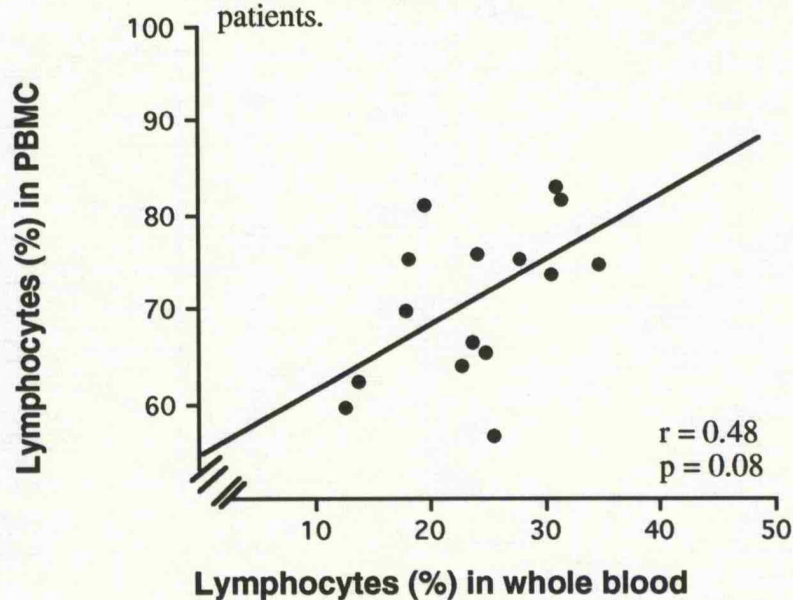
The relationship of NK cells in blood is shown for 30 cancer patients in Figure 5.10. A good correlation between % NK cells in PBMC and blood is seen for both healthy donors (r = 0.8) and cancer patients (r = 0.8).

C.   Relationship between effectors expressing NK markers and K562 cytotoxicity.

As was undertaken for the healthy donors, the percentage of circulating lymphocytes expressing the NK markers (CD16 and/or CD56) in the absence of CD3 coexpression (NK cells) or with CD3 expression (CD3+ NK) was correlated with K562 cytotoxicity to explore their role in mediating lysis. A significant correlation was seen between K562 cytotoxicity and % NK cells for all 55 cancer patients (r = 0.47, p < 0.001) as was observed among the healthy donors. Results are described in Figure 5.11. No correlation was seen between CD3+ NK cells and cytotoxicity.

The number of circulating CD3+NK cells was significantly greater in the cancer patients with metastases compared with patients without liver involvement although the number of NK cells was similar in these groups. When these two groups were analysed separately and a different association was observed between percentage of NK cells and CD3+ NK cells and K562 cytotoxicity. A significant correlation was maintained between the proportion of circulating NK cells and K562 cytotoxicity for patients without liver metastases (r = 0.52, p < 0.01). No correlation was observed between CD3+ NK cells and cytotoxicity (r = 0.18, p = 0.34). The amount of enhancement of cytotoxicity by OKT3 and proportions of circulating NK cells was also significantly correlated (r = 0.48, p < 0.01) but no association was seen between enhancement and CD3+ NK cells.

In contrast, patients with liver metastases did not have a significant correlation between proportions of NK cells and cytotoxicity (r = 0.34, p = 0.14) and a stronger and significant correlation was observed between CD3+ NK cells and K562 cytotoxicity (r = 0.54, p = 0.01). A significant correlation was observed both between proportions of CD3+ NK cells and NK cells and the quantity of enhancement by OKT3 (r = 0.52, p = 0.02 and r = 0.45, p = 0.05 respectively).

Figure 5.10 - Comparison of the proportion of NK cells in blood and corresponding PBMC preparations in 30 cancer patients.

r = 0.81
p < 0.001
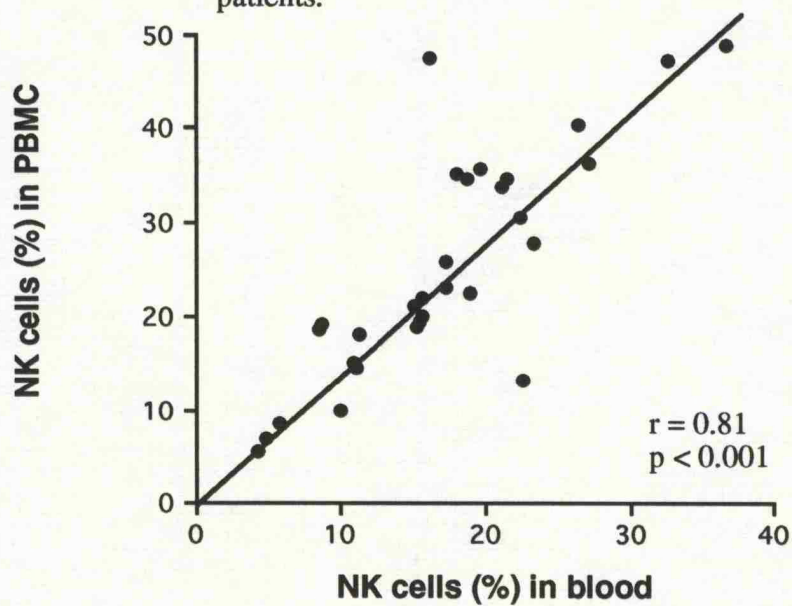
Figure 5.11 - K562 cytotoxicity and %NK cells in 51 cancer patients.

r = 0.47
p < 0.001
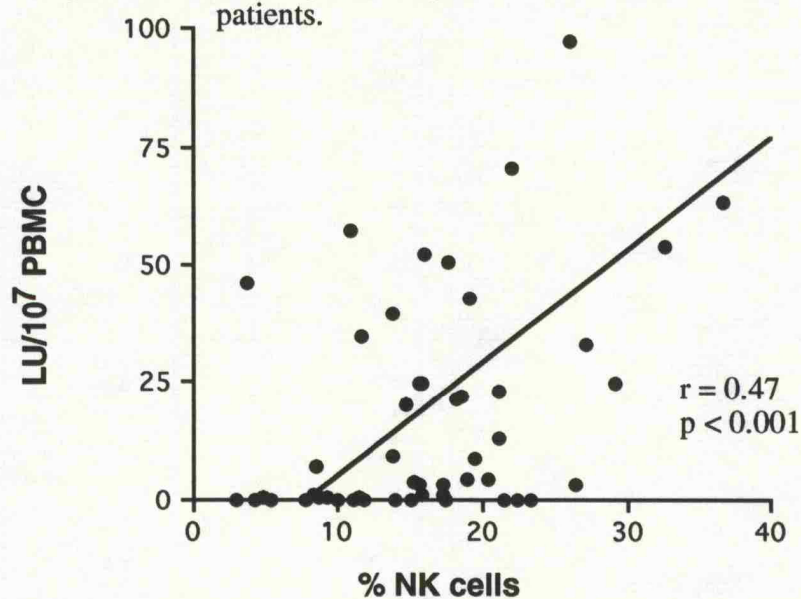
5.4   SUMMARY.

As for the healthy donors, K562 cytotoxicity was not significantly influenced by age or sex in the cancer patients. Neither did tumour stage or grade significantly influence cellular cytotoxicity. Patients with liver metastases actually had higher median levels of K562 cytotoxicity than patients without liver involvement. There was no significant difference between the levels of cytotoxicity in the cancer patients compared with the healthy donors. Male cancer patients had higher levels of cytotoxicity which approached statistical significance ($p = 0.07$) compared with the male healthy donors aged ≥ 50 years. However, the actual significance of this finding is uncertain in view of the distribution of cytotoxicity in the ≥ 50 years group. Levels of Daudi cytotoxicity were low in comparison to K562 cytotoxicity.

OKT3 and T3 significantly enhanced K562 cytotoxicity over baseline levels and the effect of T3 was significantly greater than that of OKT3. Levels of enhancement were comparable to those seen with the healthy donors. OKT3 and T3 marginally enhanced Daudi cytotoxicity in keeping with the low baseline levels against this target. Non-MHC-restricted cytotoxicity was a general phenomenon against both K562 and Daudi targets. An individuals cytotoxicity against Daudi targets although lower than K562 reflected K562 reactivity. The post-OKT3 and T3 enhancement tended to reflect baseline levels of cytotoxicity for K562. The same phenomenon is also true with regard to enhancement seen against Daudi.

Anti-CD16 variably enhanced K562 cytotoxicity over levels in tissue culture medium but this was not statistically significant ($p = 0.07$) in contrast to anti-CD3 mAb. The effect of IL-2 in combination with OKT3 was to produce only a partially additive effect on enhancement of cytotoxicity.

Cancer patients had higher levels of circulating neutrophils and monocytes than the healthy donors. Numbers of NK cells were significantly greater in the cancer patients compared with the healthy donors although lymphocyte numbers were similar as were numbers of CD3+ NK cells. When cancer patients with liver metastases were compared with those without liver involvement numbers of NK cells were similar. However, patients with liver metastases had significantly greater numbers of CD3+ NK cells ($p = 0.003$). A correlation between proportions of circulating lymphocytes bearing NK markers and K562 cytotoxicity was investigated using the Spearman Rank statistic for non-parametric data, to investigate the possible association of NK cells and CD3+ NK cells with K562 cytotoxicity. Levels of K562 cytotoxicity were significantly correlated with proportions of circulating NK cells but not CD3+ NK in all 51 cancer patients. A stronger association was observed between CD3+ NK cells both with respect to baseline K562 cytotoxicity and the amount of enhancement following incubation in OKT3 for patients with liver metastases compared to those without liver involvement.

Although there are dangers in drawing conclusions from this type of analysis with regard to the role of lymphocytes bearing NK markers, CD3+ NK cells may have a more

significant role in mediating K562 cytotoxicity and its subsequent enhancement by OKT3 *in vitro,* in patients with liver metastases than in those without metastases.

CHAPTER SIX

IN VIVO MODULATION OF CELLULAR CYTOTOXICITY IN CANCER
PATIENTS BY OKT3

6.1   INTRODUCTION.

6.2   AIMS.

6.3   RESULTS.

      6.3.1 Toxicity.

      6.3.2. Serum analysis.

          A. Serum OKT3 levels and interaction with lymphocytes.

          B. IL-2, TNFα, and IFNγ, levels.

          C. sIL-2R levels.

          D. Influence of patient serum following OKT3 on K562
             cytotoxicity.

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          B. Effect of 50 μg OKT3 compared with controls (n = 4).

          C. Effect of 20 μg to 0.5 μg OKT3 compared with 50 μg
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          E. Effect of OKT3 on IL-2Rα expression.

      6.3.4 Effect of OKT3 on cellular cytotoxicity.

          A. K562 cytotoxicity and Daudi cytotoxicity in the 50 μg group
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             patients (n = 6).

      6.3.5 Association between changes in NK cells and
          cytotoxicity.

          A. Introduction.

          B. Association between variation in NK cells in blood and
             cytotoxicity following OKT3.

          C. Variation in NK cells in PBMC preparations following OKT3.

6.4   SUMMARY.

6.1 INTRODUCTION.

The *in vivo* effects of high-dose OKT3 immunosuppressive therapy for major organ allograft rejection are well documented. Anti-CD3 mAb also have the paradoxical effect of stimulating lymphocytes *in vitro*. However, in contrast to the considerable data available from these *in vitro* studies very little is known about either the optimal dose or mode of administration that might enhance cellular immunity *in vivo*. Experimental evidence and theoretical postulates predict that low doses of anti-CD3 mAb may enhance lymphocyte-mediated cytotoxicity in contrast to the immunosuppressive effect seen at high doses. Our preliminary studies demonstrated enhancement of non-MHC-restricted cytotoxicity by the anti-CD3 mAb OKT3 and T3 *in vitro*. The *in vivo* situation is likely to be extremely complex and effects are not easily predictable from *in vitro* studies where experimental conditions may limit the multitude of interactions that are observed in the clinical setting.

Only one report (Wiseman et al, 1990) has been published describing the effects of administering a 50µg dose of OKT3 to human cancer patients and this did not provide detailed descriptions regarding its toxicity or early effects on circulating peripheral blood cells. Moreover it was not possible to distinguish the effects of OKT3 from those of the cyclophosphamide concomitantly administered in this regimen. The effect of OKT3 on cellular immunity was primarily assessed to investigate whether low doses of OKT3 enhance cellular cytotoxicity *in vivo* and therefore have potential in cancer immunotherapy.

6.2 AIMS.

A a clinical trial was undertaken to investigate the immunomodulating properties of OKT3 on cancer patients *in vivo*. This was not intended to be a study with long-term patient follow-up. It was performed to provide preliminary data regarding toxicity and to determine the effect of low doses of OKT3 on peripheral blood cells and cellular immunity.

The aims of the studies were;

1. To assess toxicity following low-dose OKT3.
2. To determine serum levels of OKT3 following intravenous injection.
3. To investigate the interaction of OKT3 with peripheral blood lymphocytes.
4. To assess levels of cytokines (IL-2, TNF-α, and IFN-γ) following OKT3.
5. To assess the influence of serum obtained from patients following OKT3 on K562 cytotoxicity .
6. To assess circulating levels of the IL-2Rα (CD25) receptor.
7. To examine the effect of OKT3 on circulating peripheral blood cells.
8. To assess cellular cytotoxicity following OKT3 administration and its further modulation *in vitro* by anti-CD3 mAb and IL-2.

6.3   RESULTS.

6.3.1   Toxicity.

Side effects following OKT3 were qualitatively similar to those described
following the 5 mg doses used to treat transplant rejection but were considerably
attenuated. Side effects are described in Table 6.3. Toxicity was dose-related and most
prominant following 50μg OKT3 when all patients developed some evidence of side
effects. Only two patients receiving 20μg, and one patient each receiving 10μg and 5μg
groups developed any side effects. No toxicity was observed in the 0.5μg group or the
controls. Symptoms comprised a "flu-like" complex which developed within 30 to 60
minutes of OKT3 injection and lasted between one hour and four hours. The most
frequent symptom was nausea. Some patients reported symptoms of flushing and light-
headedness shortly after injection. Only one patient (IB) required paracetamol to control
rigors. All patients receiving 50μg OKT3 developed a fall in both systolic and diastolic
blood pressure (mean 20 mmHg) within 30 minutes of OKT3 injection, which was
associated with a tachycardia. Blood pressure had returned to pre-treatment levels by
four hours. Changes were not as marked at lower doses and active intervention was not
required for hypotension in any patients. There were no allergic, respiratory, or
neurological symptoms.

Table 6.3 - Toxicity (WHO Grade) following OKT3.

Patient	OKT3 (μg)	Nausea/ vomiting	Fever	Blood pressure changes	Symptoms
WW	50	0	0	I	Yes
ME	50	I	I	I	Yes
WD	50	0	0	I	Yes
IB	50	II	II	II	Yes
AP	50	0	0	0	Yes
DH	50	I	II	I	Yes
DC	20	I	II	I	Yes
BJ	20	0	0	0	No
GR	20	0	0	0	No
DD	20	0	0	0	No
AE	20	I	I	0	Yes
NM	20	0	0	0	No
WG	10	0	0	0	No
WL	10	0	0	0	No
IT	10	II	0	I	Yes
HB	10	0	0	0	No
KC	10	0	0	0	No
RW	10	0	0	0	Yes
DS	5	0	0	0	No
RH	5	0	0	0	No
AB	5	0	0	0	No
DB	5	0	0	0	No
RD	5	0	0	0	No
FB	5	I	0	I	Yes
TH	0.5	0	0	0	No
DV	0.5	0	0	0	No

6.3.2　Serum analysis.

A.　　Serum OKT3 levels and interaction with lymphocytes.

Following 50 μg OKT3, serum was assessed for levels of OKT3 in ME and DH at 15 minutes, 30 minutes, 60 minutes, 240 minutes, and 480 minutes. No OKT3 was detected in any samples. Assessment of OKT3 binding to lymphocytes was also studied. Figure 6.1 describes the % (means, SEM not shown) of lymphocytes with membrane bound IgG following all doses of OKT3 (n = 6 except for 0.5 μg where n = 2). Individual results are described in Table 6.2 in appendix H.

The percentage of lymphocytes with detectable OKT3 was similar (60%) for the 50 μg, 20 μg, 10μg, and 5 μg groups at 60 minutes. The mean percentage of lymphocytes coated with OKT3 then progressively declined and at 24 hours only approximately 20% of lymphocytes had detectable surface bound OKT3. In the patients receiving 0.5 μg OKT3 (TH and DV), only 16.7% and 10.3% of lymphocytes had detectable OKT3 at 60 minutes. Background levels of binding by the fluorochrome-conjugated anti-mouse antibody was always < 10% for both treated patients receiving OKT3 and also the control patients receiving normal saline.

The patients receiving 50 μg OKT3 best illustrate the association between changes in circulating lymphocytes and OKT3 binding. Results are described in Figure 6.2 (individual results shown in Table 6.37 in appendix H). CD3 and CD2 expression as well as OKT3 binding to lymphocytes was measured to assess the degree of modulation of expression of CD3 or competition for binding of the fluorochrome-conjugated anti-CD3 by OKT3.

50 μg OKT3 induced a progressive decline in CD3$^+$ lymphocytes. This was due to their physical removal from the circulation as evidenced by an equal rate loss of cells also expressing CD2. Although lymphocytes were not simultaneously duel stained for CD3 and anti-mouse IgG the number of lymphocytes coated with OKT3 approximated to the number expressing CD3. This suggests that non-specific binding to cells not expressing CD3 is low. OKT3 presumably interacts rapidly and specifically with the CD3 antigen and free OKT3 in serum is a transient phenomenon.

B.　　IL-2, TNF-α and IFN-γ levels.

Cytokines were measured pre-OKT3, and at 30 minutes, 60 minutes and 480 minutes in the 50 μg group and the four control patients. The standard curves (described in Figures 3.13 to 3.16 in chapter 3) were generated by plotting average corrected absorbance at 490 nm (measured - background) against the absorbance from the standard cytokine. Only one sample contained any detectable cytokine and this was for TNFα (75 pg ml^{-1}) at 60 minutes following 50 μg OKT3 for WW (corrected mean absorbance of 0.14). This patient did not experience significantly more side effects than the other patients. IL-2 and IFNγ were undetectable in all samples. In view of these findings further cytokine assays were not performed.

Figure 6.1 - % lymphocytes (means) with membrane bound OKT3 following varying doses of OKT3.
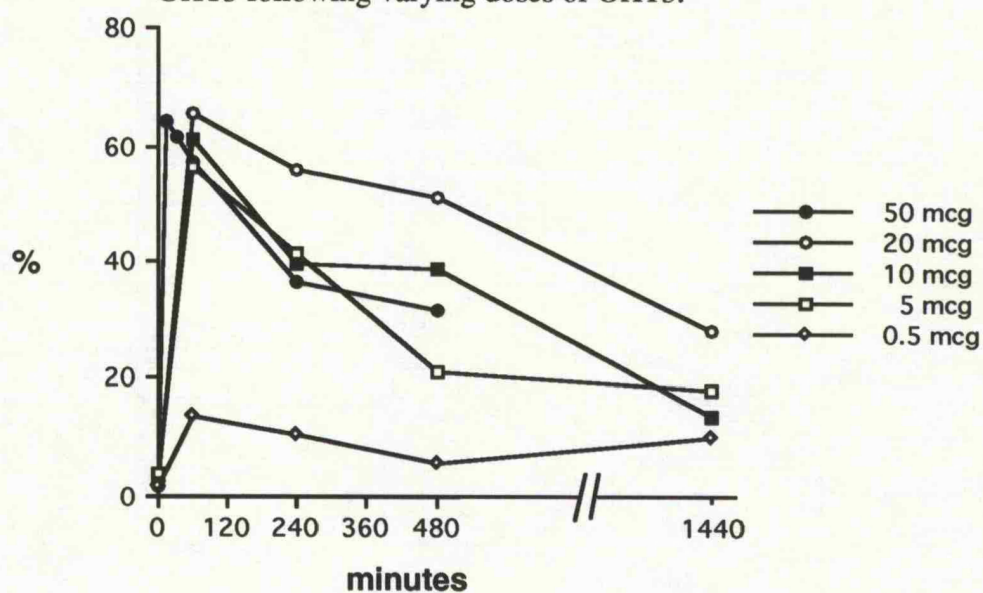
Figure 6.2 - Lymphocytes expressing CD2, CD3, and OKT3 following 50μg OKT3 (means ± SEM).
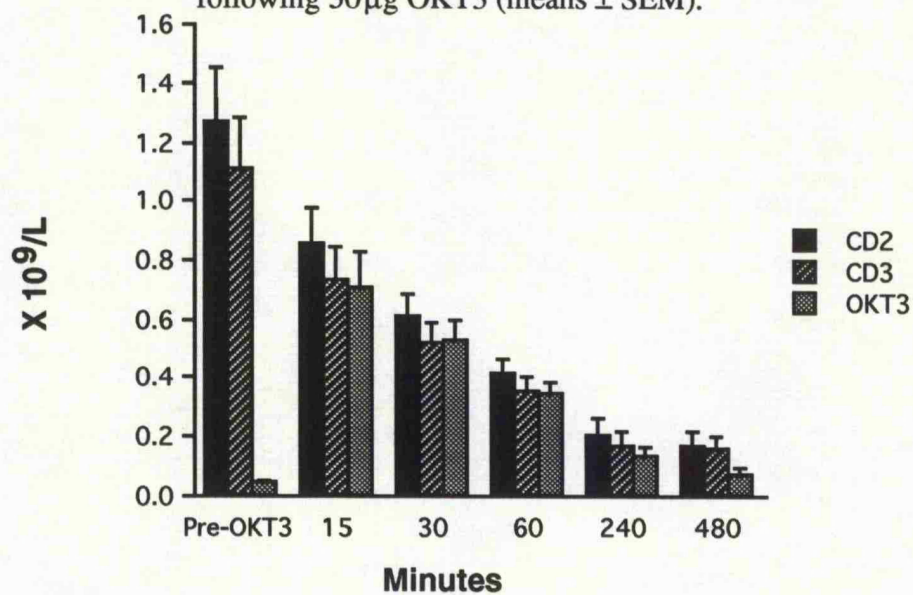
C.   sIL-2Rα levels.

    Levels of sIL-2R were measured in the same patients and at the same times as the above cytokines. Individual results are described in Table 6.3 in appendix H and were calculated from the standard curve described in Figure 3.16. Mean ($\pm$ SEM) sIL-2Rα levels pre-normal saline were similar in the 4 control patients (690.4 $\pm$ 133.8 U ml^{-1}) to the pre-OKT3 levels in the 50 µg group (535.0 $\pm$ 134.6 U ml^{-1}). There was no increase in sIL-2Rα levels at 8 hours following 50 µg OKT3.

D.   Influence of patient serum following OKT3 on K562 cytotoxicity following OKT3.

    An experiment was performed to investigate whether factors released into serum following OKT3 administration may influence K562 cytotoxicity mediated by freshly isolated PBMC from a healthy donor. PBMC from one healthy donor (JH) were preincubated with serum from patients receiving 50 µg, 20 µg OKT3, or normal saline (n = 6) prior to the addition of K562 targets as described in appendix C. Samples from patients treated with 50 µg were assessed on a different day to those from the control (6) and 20 µg groups which were studied simultaneously. Samples were assayed at an E:T ratio of 40:1 in quadruplate from which mean percentage cytotoxicity was calculated. Spontaneous ^{51}Cr release was also measured for K562 incubated in tissue culture medium alone and PBMC were incubated in human AB serum to obtain a value for direct comparison with the experimental samples. Duplicate plates were set up for all samples, in which incubation of K562 cells with serum alone enabled any direct effect of serum on K562 cytotoxicity to be assessed. Percentage cytotoxicity was calculated for each sample using the spontaneous ^{51}Cr release following incubation of K562 in tissue culture medium. Cytotoxicity was calculated using the SR in tissue culture medium for all experimental samples.

    Individual results are described in Table 6.4 in appendix H. Results for the 50 µg patients and controls are described in Figure 6.3.

Figure 6.3 - Influence of serum on K562 cytotoxicity following 50 µg and 20 µg OKT3 compared with controls.
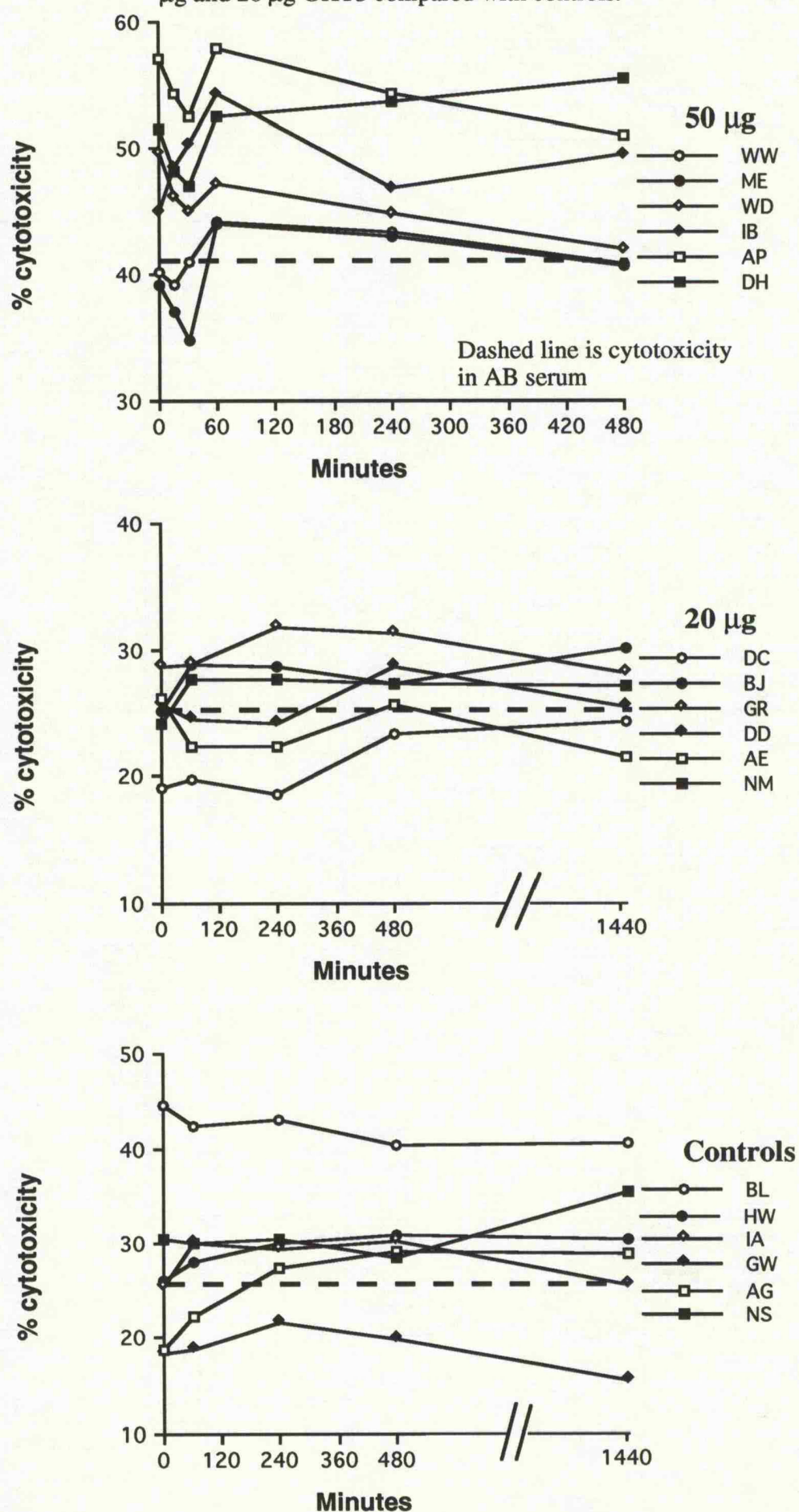
K562 cytotoxicity by serum alone was not significantly greater than that with incubation in tissue culture medium. Mean (± SEM) percentage cytotoxicity attributable to the direct effect of incubating K562 in serum for the 18 cancer patients at 0 minutes was only 0.41% (± 0.12) above that seen following incubation in tissue culture medium. No change was seen for serum samples following OKT3 therefore, cancer patients serum was not directly toxic to K562 cells. In particular serum from GW which contained significant quantities of bilirubin did not affect spontaneous ^{51}Cr-release.

Percentage cytotoxicity of PBMC incubated in AB serum was 42.1% for the 50 µg studies and 26.5% for the 20 µg studies and their 6 controls. Serum from individual cancer patients obtained pre-OKT3/normal saline did however have a variable influence on PBMC cytotoxicity. Mean (±SEM) cytotoxicity in the 6 patients receiving 50 µg OKT3 was 47.1% (± 2.8) and varied from 39.3% to 57.0%. Mean (±SEM) K562 cytotoxicity was 26.0% (± 2.0) and varied between 18.4% and 44.6% for the 6 control patients and 6 patients in the 20 µg group. Therefore factors in serum variably affected cytotoxicity mediated by PBMC from one healthy donor and both enhancement and depression of cytotoxicity was observed. There was no association between tumour stage or histological type and influence of serum on cellular cytotoxicity.

Results obtained from the patients receiving 20 µg OKT3 showed no consistent pattern of variation in effect on cytotoxicity and were similar to those obtained in the control patients. Four of the 6 patients receiving 50 µg OKT3 showed a slight fall in cytotoxicity up to 30 minutes following OKT3 which had recovered by 60 minutes. Serum from WW and IB was not associated with an early suppressive effect on cellular cytotoxicity, and these samples demonstrated an increase in cytotoxicity up to 60 minutes post-OKT3. WW was also the only individual in this group to have detectable TNF-α levels (75 pg ml^{-1} at 60 minutes). Although IB did not have detectable cytokine levels this patient did develop more pronounced symptoms than other patients following OKT3 (required paracetamol to control rigors). No consistent effect over pretherapy levels of cytotoxicity was observed at 240 minutes, 480 and 1440 minutes in any of the 12 treated patients.

6.3.3 Effect of OKT3 on peripheral blood cells.
A. Introduction.

Peripheral blood cells were measured for all patients. Baseline values of PBC subsets for all cancer patients are presented in Table 5.7 and Table 5.8 in appendix H and have been discussed in Chapter 5. Changes in peripheral blood cells in individual cancer patients following OKT3/normal saline are shown in Tables 6.5 to 6.24 in appendix H.

In the following description the patients receiving 50 µg OKT3 are directly compared with the 4 control patients undergoing investigations at similar times. The patients receiving the 20 µg to 5 µg doses of OKT3 are directly compared with both the 6 control patients undergoing investigations at similar times, the patients receiving the 50

µg dose, and their respective 4 control patients. The 2 patients who received 0.5 µg OKT3 are considered separately.

B. Effect of 50 µg OKT3 compared with controls (n = 4).

Figure 6.4 describes the effect of 50 µg OKT3 on the mean (± SEM) differential WBC compared with the control patients receiving normal saline. During the initial 60 minutes following 50 µg OKT3, the WBC fell due to a decrease in neutrophils (4.14 ± 0.8 to 3.26 ± 0.8 X 10^9 L^{-1}), lymphocytes (1.58 ± 0.23 to 0.56 ± 0.09 X 10^9 L^{-1}) and monocytes (0.52 ± 0.08 to 0.30 ± 0.05 X 10^9 L^{-1}) (means ± SEM). Although the decline in lymphocytes and monocytes continued to 480 minutes (0.31 ± 0.07 and 0.25 ± 0.04 X 10^9 L^{-1}), neutrophils increased and peaked at 240 minutes (7.83 ± 1.1 X 10^9 L^{-1}) and remained elevated at 480 minutes over pretherapy levels (5.97 ± 0.9 X 10^9 L^{-1}). Lymphocyte numbers also fell slightly in the control patients during the initial 60 minutes following normal saline (1.32 ± 0.19 to 1.15 ± 0.21 X 10^9 L^{-1}) and remained below pretherapy levels at 1.19 ± 0.27 X 10^9 L^{-1} at 240 minutes. However, lymphocyte numbers had recovered by 480 minutes (1.34 ± 0.32 X 10^9 L^{-1}). A modest increase in neutrophils from pretherapy values (5.11 ± 1.01 X 10^9 L^{-1}) was also seen in the control patients at 240 minutes (6.80 ± 0.50 X 10^9 L^{-1}).

Figure 6.5 describes the changes in cells expressing CD3, CD4, and CD8 antigens. 50 µg OKT3 was associated with a similar decline in numbers of these T cell subsets. Cells expressing CD2 declined at a similar rate to those expressing CD3 (Figure 6.2). NK cells also declined following 50 µg OKT3 at a similar rate to CD3$^+$ cells (Figure 6.6).

C. Effect of 20 µg to 0.5 µg OKT3 compared with 50 µg and controls (n = 10).

Variation in percentage NK cells and CD3$^+$ lymphocytes in individual patients following different doses of OKT3 is described in Figure 6.7 and 6.8. The amount of depletion of both mean NK cells and CD3$^+$ lymphocytes was related to the dose of OKT3. Gradual recovery with time following OKT3 was seen for both CD3$^+$ and NK cells. In the case of the 5 µg dose a rebound mean lymphocytosis was seen for NK cells but not for CD3$^+$ lymphocytes. The 10 µg group demonstrated mean NK cell numbers at 24 hours which remained lower than pretherapy values.

Figure 6.9 compares the number of lymphocytes expressing CD2 and CD3 following 20 µg (n = 6), 10 µg (n = 6), and 5 µg (n = 3) OKT3. As has been demonstrated for patients receiving 50 µg OKT3 (Figure 6.2), no evidence of modulation of expression of CD3 was seen at these lower doses of OKT3. OKT3 therefore only influenced the number of circulating lymphocytes but not their expression of CD3.

Figure 6.4 - Changes in mean (± SEM) WBC differential count following 50µg OKT3 compared with controls (n = 4).
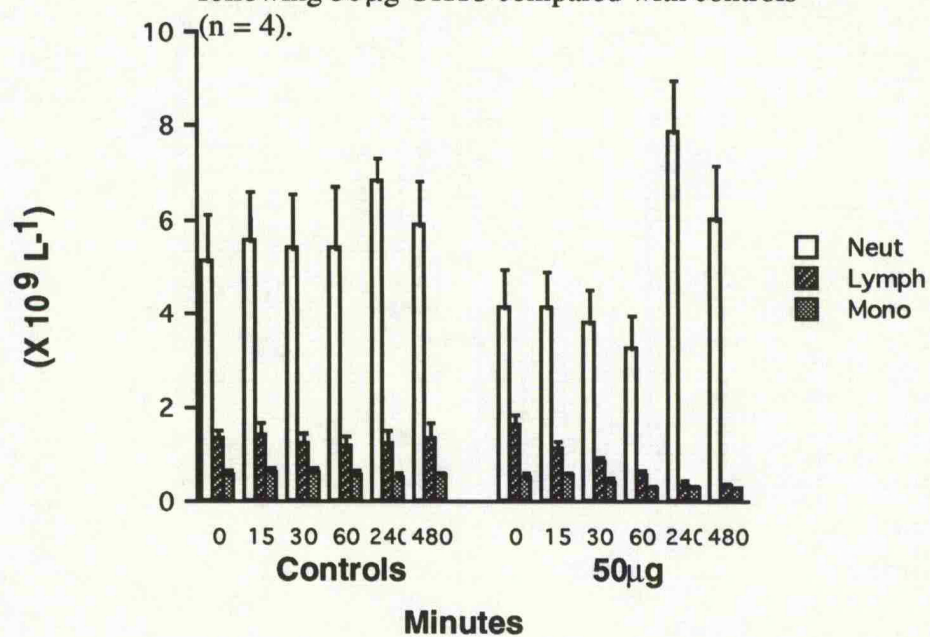
Figure 6.5 - Changes in mean (± SEM) PBL expressing CD3, CD4, and CD8 following 50µg OKT3 compared with controls (n = 4).
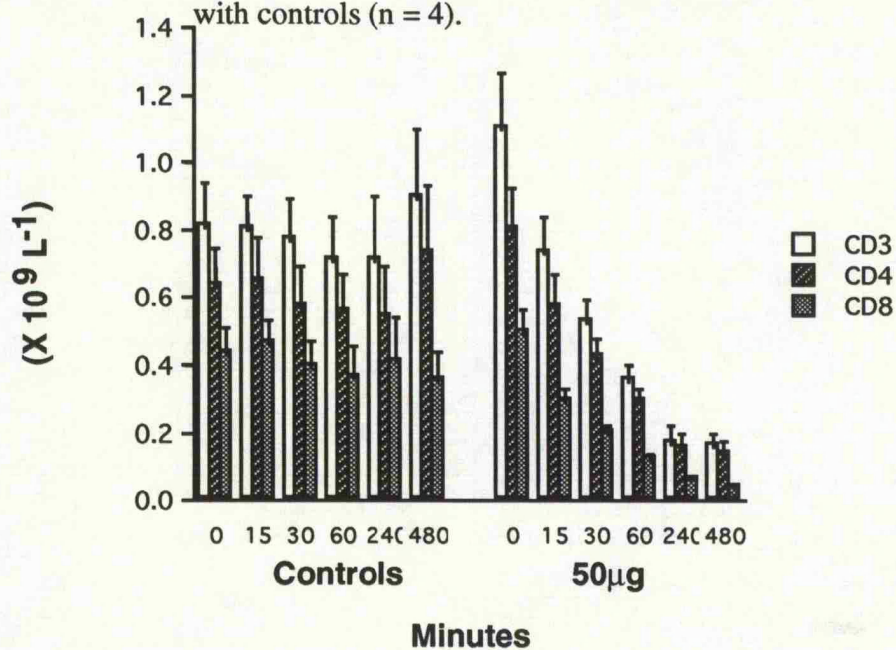
Figure 6.6 - % change in mean CD3$^+$ lymphocytes and NK cells
            following 50µg OKT3 compared with controls
            (n = 4).
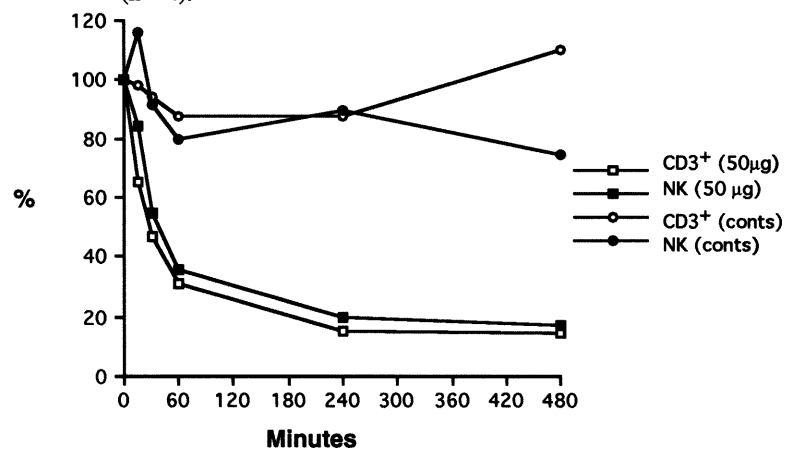
Figure 6.7 - Variation in CD3⁺ lymphocytes following OKT3 (bars = means).
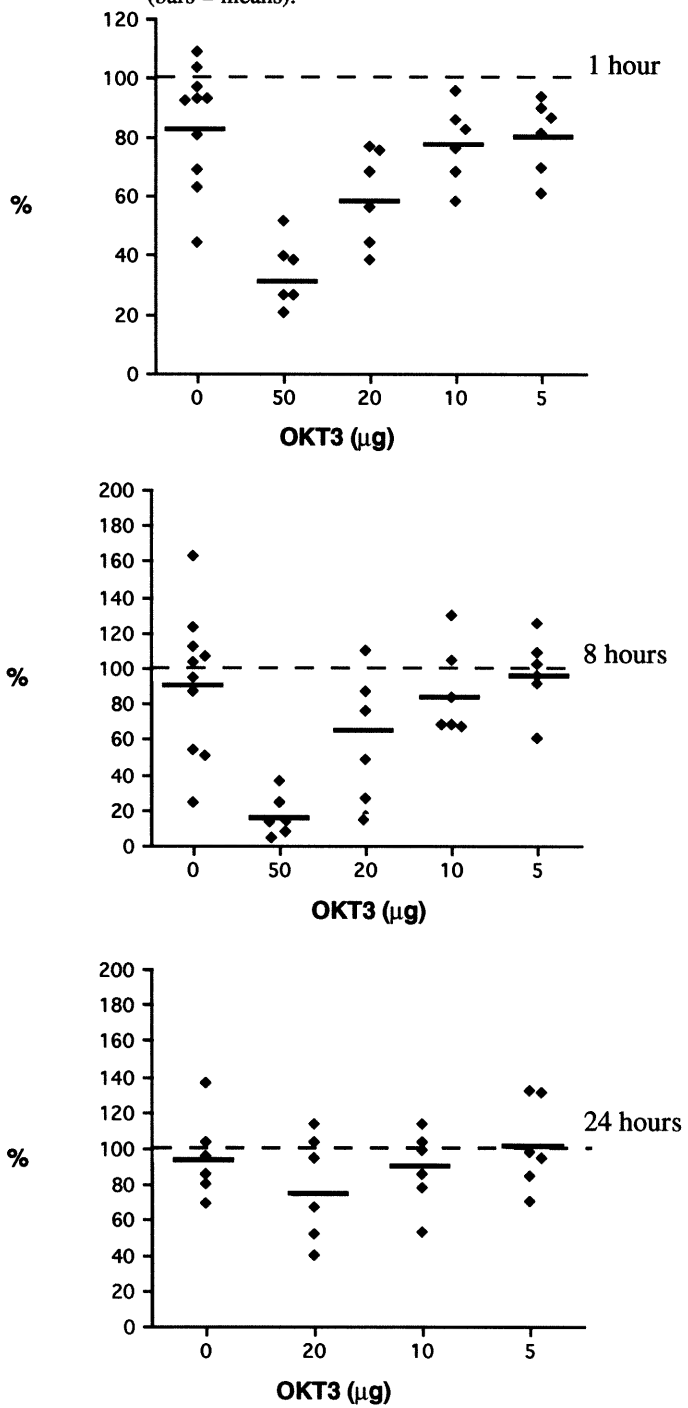
Figure 6.8 - Variation in NK cells following OKT3 (Bars = means).
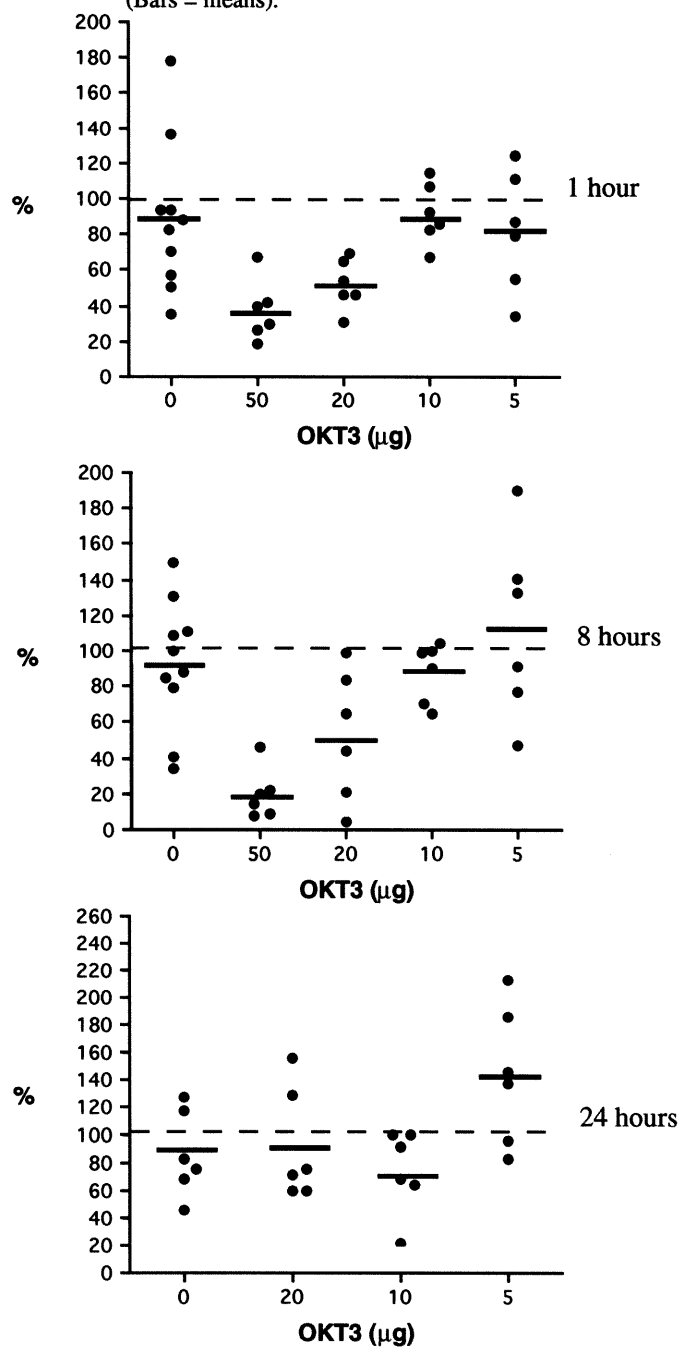
Figure 6.9 - Comparison of mean (± SEM) lymphocytes expressing CD2 and CD3 following varying doses of OKT3.
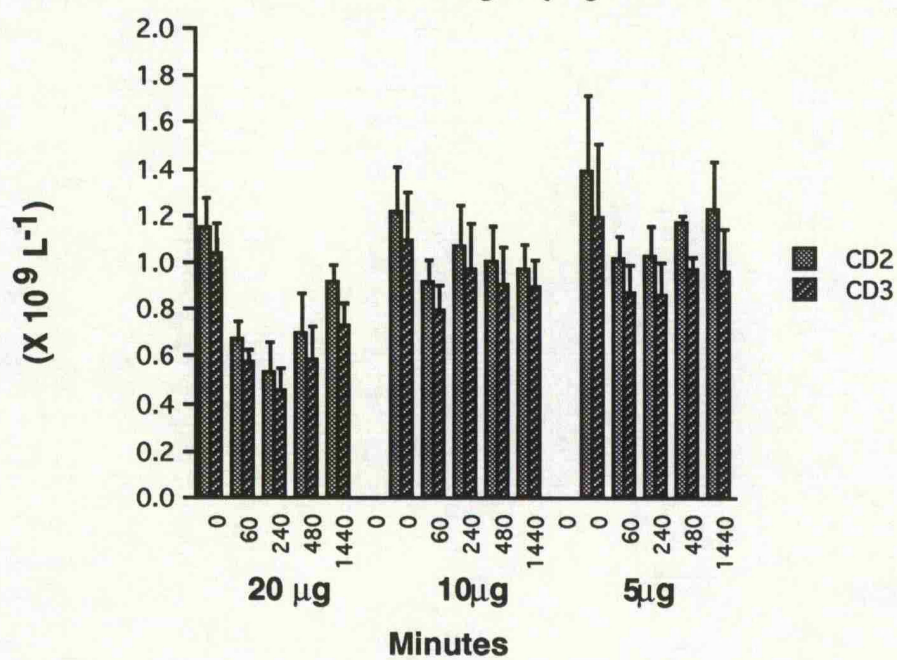
D.   Effect of 0.5 μg OKT3.

Only two patients (TH and DV) were treated with 0.5 μg OKT3. OKT3 was detected on 16.7% and 10.3 % of lymphocytes at one hour respectively. $CD3^+$ lymphocytes declined in both patients over pretherapy values at 1 hour (0.54 to 0.37 X 10^9 L^{-1} and 0.86 to 0.51 X 10^9 L^{-1} respectively). At 24 hours lymphocyte numbers had recovered (0.67 and 0.70 X 10^9 L^{-1}). NK cell numbers demonstrated a similar fall from 0.06 to 0.03 X 10^9 L^{-1} for TH at 1 hour and 0.26 to 0.10 X 10^9 L^{-1} for DV at 1 hour. Although recovery of NK cells to pretherapy levels was seen for TH at 24 hours, NK cells in DV remained reduced (0.14 X 10^9 L^{-1}). The reason for the pronounced fall in NK cells in DV was uncertain.

E.   Effect of OKT3 on IL-2Rα expression.

IL-2Rα expression was assessed to investigate evidence of T cell activation by OKT3 in the 50 μg and 4 of the patients in the 10 μg group. 50 μg OKT3 failed to induce significant mean (± SEM) IL-2Rα expression by lymphocytes at 480 minutes (15.1 ± 1.2%) over pre-therapy values (16.75 ± 2.6%). Mean (± SEM) levels of IL-2Rα also remained unchanged in the four control patients (15.1± 2.4%). Lymphocytes coexpressing IL-2Rα assessed at 0 minutes and 1440 minutes in 4 patients following 10 μg OKT3 demonstrated that mean (± SEM) pretherapy levels of IL-2Rα expression (7.9 ± 3.6%) were not significantly different to levels at 24 hours (6.7 ± 1.8%).

6.3.4   Effect of OKT3 on cellular cytotoxicity.

Individual results are described in Tables 6.25 to 6.33 in appendix H (assays undertaken at E:T ratios of < 40:1 are marked with an asterisk).

A.   K562 cytotoxicity and Daudi cytotoxicity in the 50 μg and control patients (n = 4).

K562 cytotoxicity following administration of 50 μg OKT3 is described for individual patients and compared with the four control patients in Figure 6.10. An increase in cytotoxicity over pre-therapy levels was seen in AP, WD, and ME at 30 minutes, despite a progressive fall in circulating lymphocytes. Cytotoxicity remained elevated at 60 minutes in WD. However, OKT3 resulted in a significant reduction in median cytotoxicity at 4 hours (0.1 LU/10^7 PBMC) over pre-therapy levels (20.8 LU/10^7 PBMC) (p = 0.03). Only ME had detectable levels of cytotoxicity at 4 hours (8.81 LU/10^7 PBMC). In the control patients, median levels of K562 cytotoxicity were also lower than pre-normal saline levels (60.9 LU/10^7 PBMC) at 30 minutes (43.5 LU/10^7 PBMC) and 60 minutes (28.5 LU/10^7 PBMC) (only one individual (MD) exhibited a slight increase at 30 minutes and 60 minutes). However, by 240 minutes median levels of K562 cytotoxicity had recovered to their original values (58.4 LU/10^7 PBMC) in contrast to the 50 μg treated patients.

Figure 6.11 describes *in vitro* modulation by OKT3 and T3 of K562 cytotoxicity in the 50 μg and control patients. Both OKT3 and T3 enhanced cytotoxicity over baseline values. Enhancement by T3 was also consistently greater than OKT3. OKT3 and T3 enhanced cytotoxicity as predicted prior to administration of 50 μg OKT3. However, at 30 minutes the amount of enhancement was reduced for both mAb *in vitro* in association with the increase in baseline levels at this time. No significant enhancement was observed at 4 hours post-OKT3 administration. ME still exhibited some enhancement from 8.8 to 14.6 LU/10^7 PBMC by OKT3 and to 12.8 LU/10^7 PBMC by T3 at 4 hours.

Figure 6.12 compares mean (±SEM) levels of K562 cytotoxicity with Daudi cytotoxicity expressed at an E:T ratio of 20:1 for the 50 μg and 4 control patients. Individual results are described in Table 6.37 in appendix H. *In vitro* modulation by OKT3 and T3 is also described. Daudi cytotoxicity was considerably lower than K562 cytotoxicity in all patients at all times. There was no increase in Daudi cytotoxicity following 50 μg OKT3 administration. Levels of Daudi cytotoxicity also declined in both the control patients following normal saline and the treated patients following 50 μg OKT3. In contrast to K562 cytotoxicity which demonstrated an initial decrease in the controls followed by a recovery at 4 hours to baseline levels, Daudi cytotoxicity declined progressively to 4 hours in the controls as well as the 50 μg treated patients.

Figure 6.10 - K562 cytotoxicity following 50µg OKT3 compared
with 4 controls receiving normal saline.
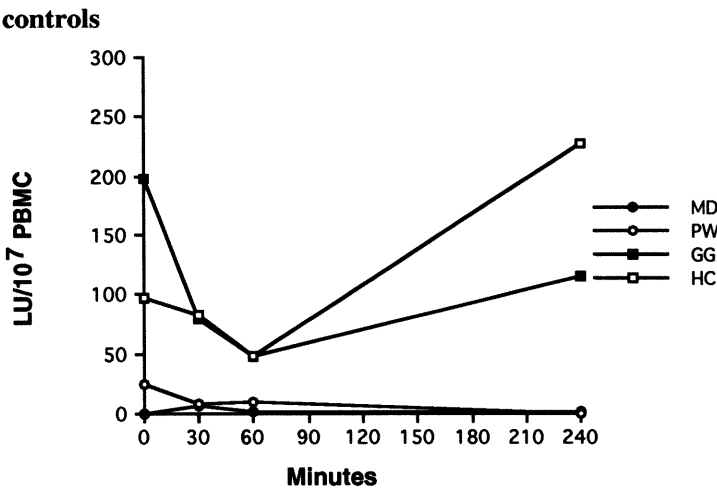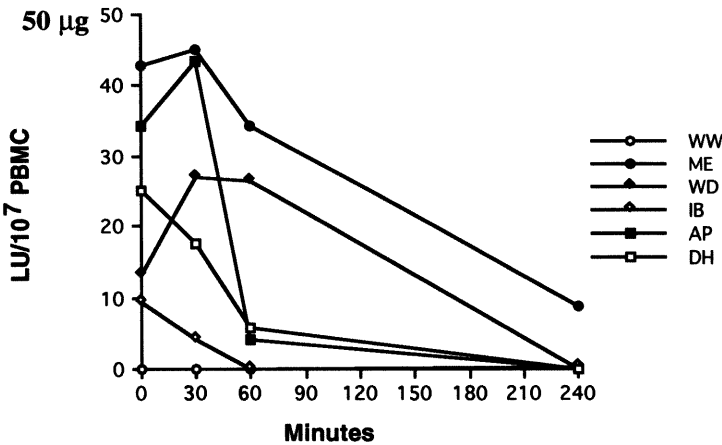
Figure 6.11 - K562 cytotoxicity (medians) and *in vitro* enhancement by OKT3 and T3 following 50µg OKT3 compared with 4 controls.

204
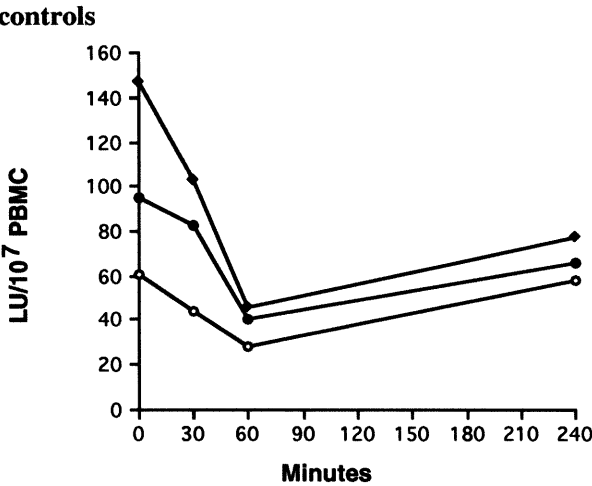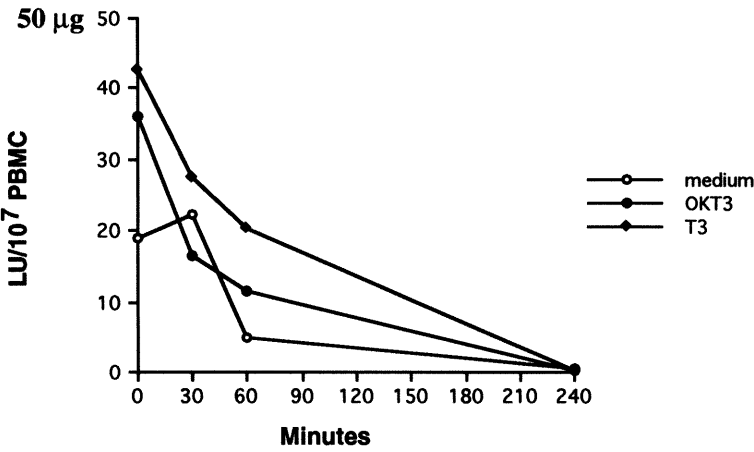
Figure 6.12 - Comparison between % K562 cytotoxicity and Daudi
cytotoxicity with OKT3 and T3 modulation *in vitro*
for 4 controls and 6 patients receiving 50μg
OKT3(means ± SEM).
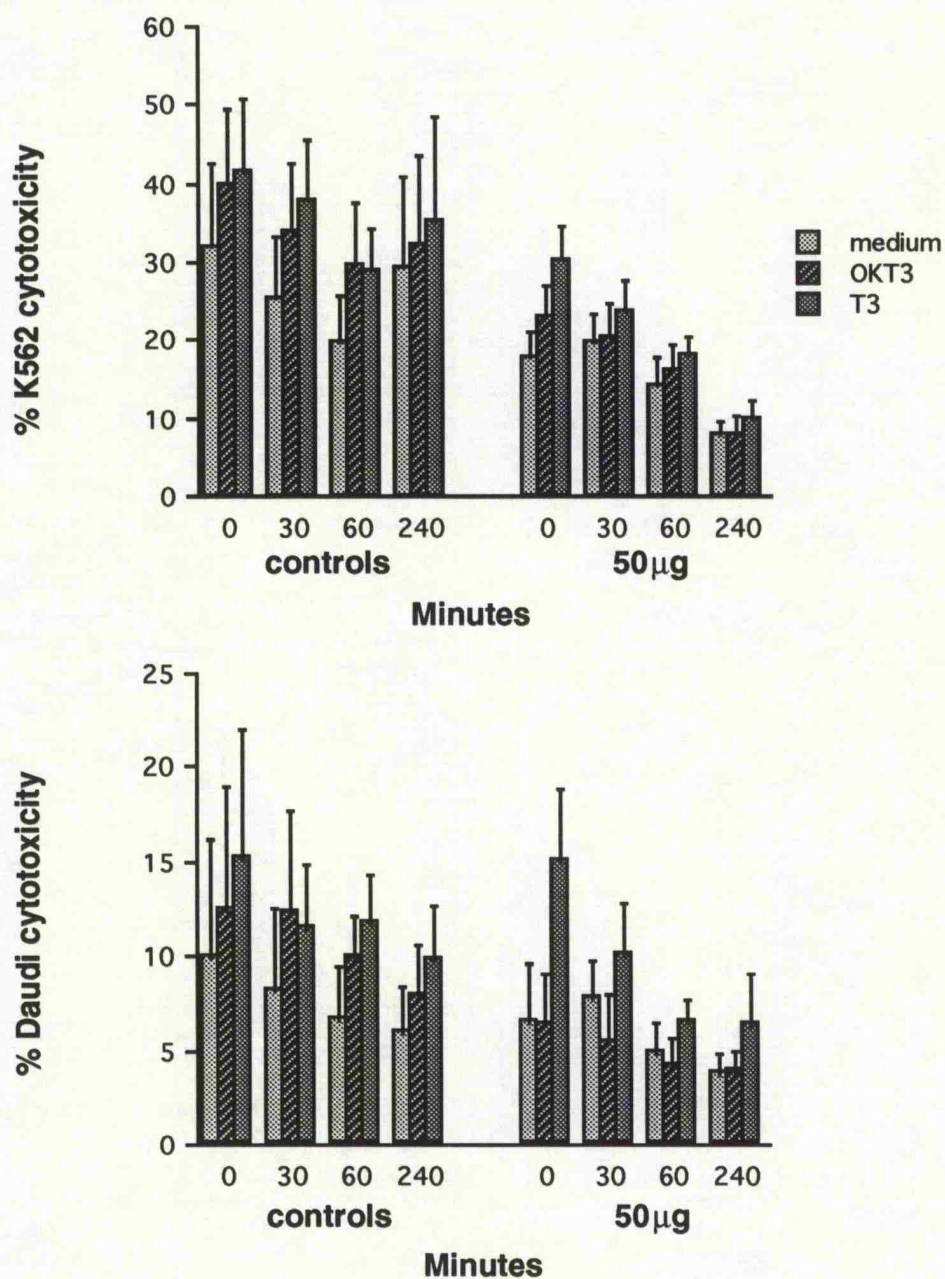
B. <u>K562 cytotoxicity in the 20 µg to 5 µg and control patients (n = 6).</u>

Individual results for K562 cytotoxicity following between 20 µg and 5 µg OKT3 are described in Figure 6.13. Figure 6.14 also describes K562 cytotoxicity for the 6 control patients given normal saline. The patients receiving 20 µg to 0.5 µg OKT3 were also characterized by considerable individual variation in K562 cytotoxicity. Following 5 µg OKT3 median (range) K562 cytotoxicity was significantly enhanced at 24 hours (43.5 LU/10^7 PBMC (1.6 to 138.7) over pretherapy levels (14.6 LU/10^7 PBMC (0.1 to 52.4)) (p < 0.03). Enhancement was also observed over pretherapy levels in the 20 µg group at 24 hours (4.5 LU/10^7 PBMC (0.3 to 50.3)) enhanced to 38.5 LU/10^7 PBMC (4.6 to 86.4)) but this failed to reach significance. Median levels of K562 cytotoxicity were reduced in the 10 µg group at 24 hours (0.1 LU/10^7 PBMC (0.15 to 70.3)) over pretherapy levels (3.2 LU/10^7 PBMC (0.0 to 238.1)). The individual levels of cytotoxicity were extremely variable in the 10 µg group of patients. 4 patients had low levels of cytotoxicity (< 5 LU/10^7 PBMC) and two patients had relatively high levels (70.4 LU/10^7 PBMC (HB) and 46.3 LU/10^7 PBMC (IT)). Cytotoxicity was increased at 24 hours to 238.1 LU/10^7 PBMC in HB.

Cytotoxicity was also reduced in the six control patients from pre-therapy levels (11.0 LU/10^7 PBMC (0.0 to 24.7)) at 24 hours (4.3 LU/10^7 PBMC (0.0 to 36.7). The two patients who received 0.5 µg OKT3 (TH and DV) had very low levels of K562 cytotoxicity prior to OKT3 (0.0 and 0.9 LU/10^7 PBMC respectively). TH exhibited enhancement of cytotoxicity at 24 hours (0.0 to 20.1 LU/10^7 PBMC) whereas cytotoxicity was unaffected in DV (0.92 to 0.41LU/10^7 PBMC).

All patients underwent *in vitro* modulation by OKT3. All patients except FB, DB, and RD also underwent *in vitro* modulation with T3. The effect of IL-2 was assessed in 3 of the 6 controls and 3 patients each in the 20 µg and 10 µg groups. The effect of combined OKT3 and IL-2 was also assessed for 3 patients each in the 20 µg, 10 µg, and 5 µg groups and in 2 of the 6 patients in the control group.

OKT3 and T3 consistently enhanced cytotoxicity and the effect of T3 was superior to that of OKT3. Treatment with OKT3 did not have a marked effect on the capacity of OKT3 and T3 to induce further *in vitro* enhancement. Modulation by OKT3, and T3, is shown in Figure 6.15 for patients receiving 20 µg and 10 µg OKT3 (T3 not shown for 5 µg). The results in the10 µg group were largely influenced by two patients who demonstrated extremely high levels of cytotoxicity following incubation with OKT3 and T3 and were responsible for the high median cytotoxicity described for the 10 µg group in Figure 6.20. Individual values were 342.5 LU/10^7 PBMC with OKT3 at 4 hours and 943.5 LU/10^7 PBMC with T3 at this time. For HB cytotoxicity was 212.8 LU/10^7 PBMC at 4 hours and 442.5 LU/10^7 PBMC at 24 hours, and for T3 420.2 LU/10^7 PBMC at 4 hours and 980.4 LU/10^7 PBMC at 24 hours.

Figure 6.13 - K562 cytotoxicity following 20μg to 5 μg OKT3.
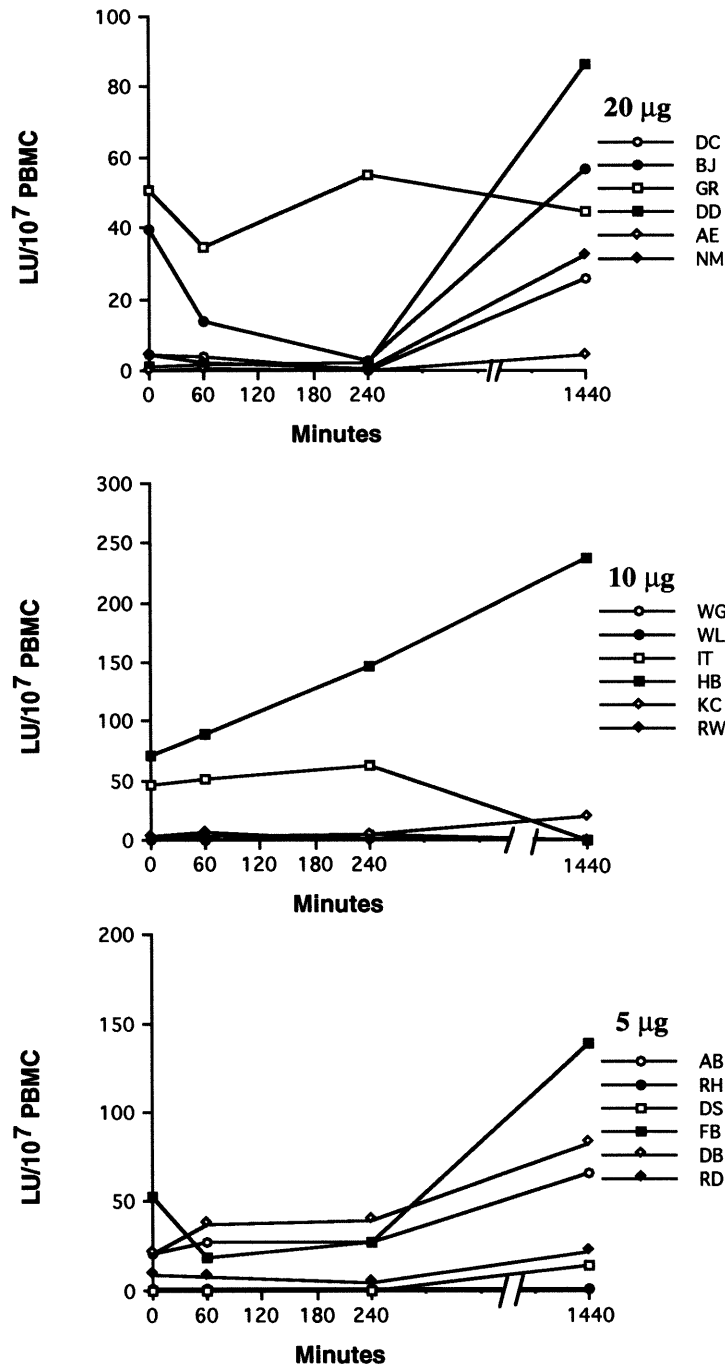
Figure 6.14 - K562 cytotoxicity in 6 control patients
following normal saline.
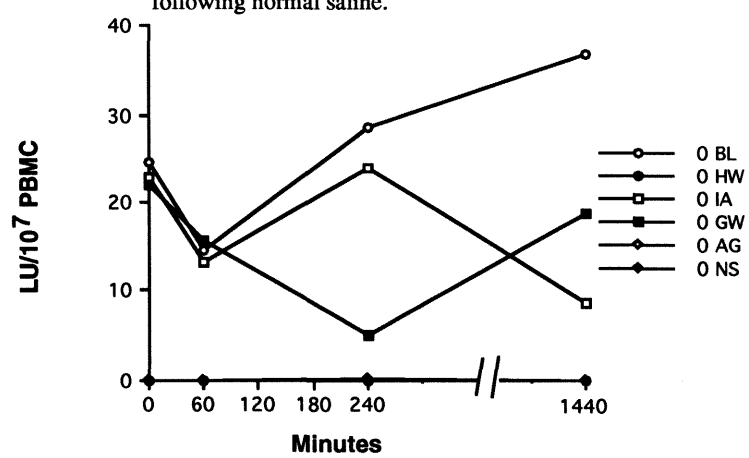
Figure 6.15 - *In vitro* modulation of K562 cytotoxicity (medians) by OKT3 and T3 following 20 μg to 5 μg OKT3.
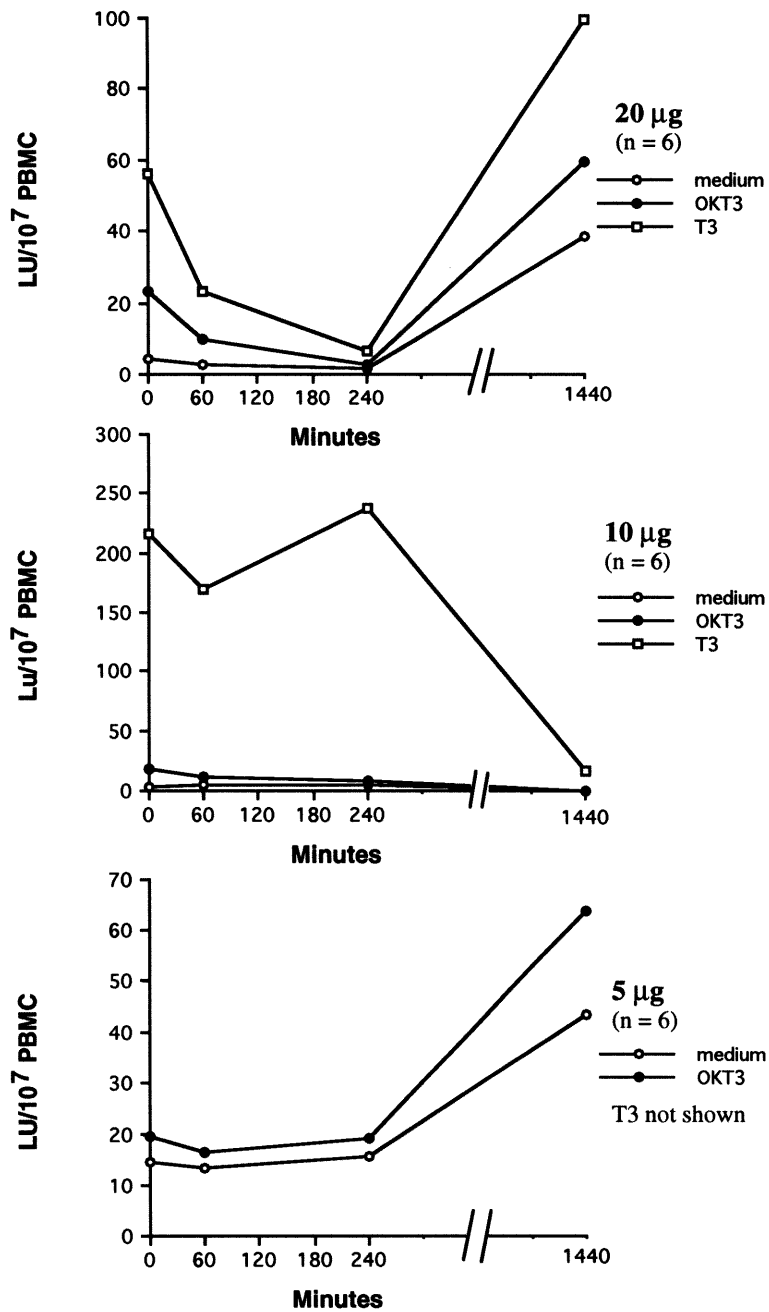
Figure 6.16 describes compares the effect of IL-2 on K562 cytotoxicity with OKT3 and both agents together in the patients receiving 20 μg to 5 μg OKT3. The effect of IL-2 was small and less than OKT3. Moreover there was no evidence that IL-2 and OKT3 in combination had a greater effect than OKT3 alone. There was no evidence that enhancement with IL-2 *in vitro* increased with time following *in vivo* OKT3 therapy. OKT3 did not sensitize lymphocytes to the effect of IL-2. This is consistent with the lack of induction of IL-2Rα levels by OKT3. *In vitro* modulation by OKT3, T3, and IL-2 is described for the 6 control patients in Figure 6.17 and demonstrates the poor levels of cytotoxicity also achieved with IL-2 in these patients. Enhancement by OKT3 and T3 was greater than IL-2 and that of T3 superior to OKT3.

Figure 6.16 - *In vitro* modulation of K562 cytotoxicity (medians)
by IL-2 following 20μg to 5 μg OKT3.
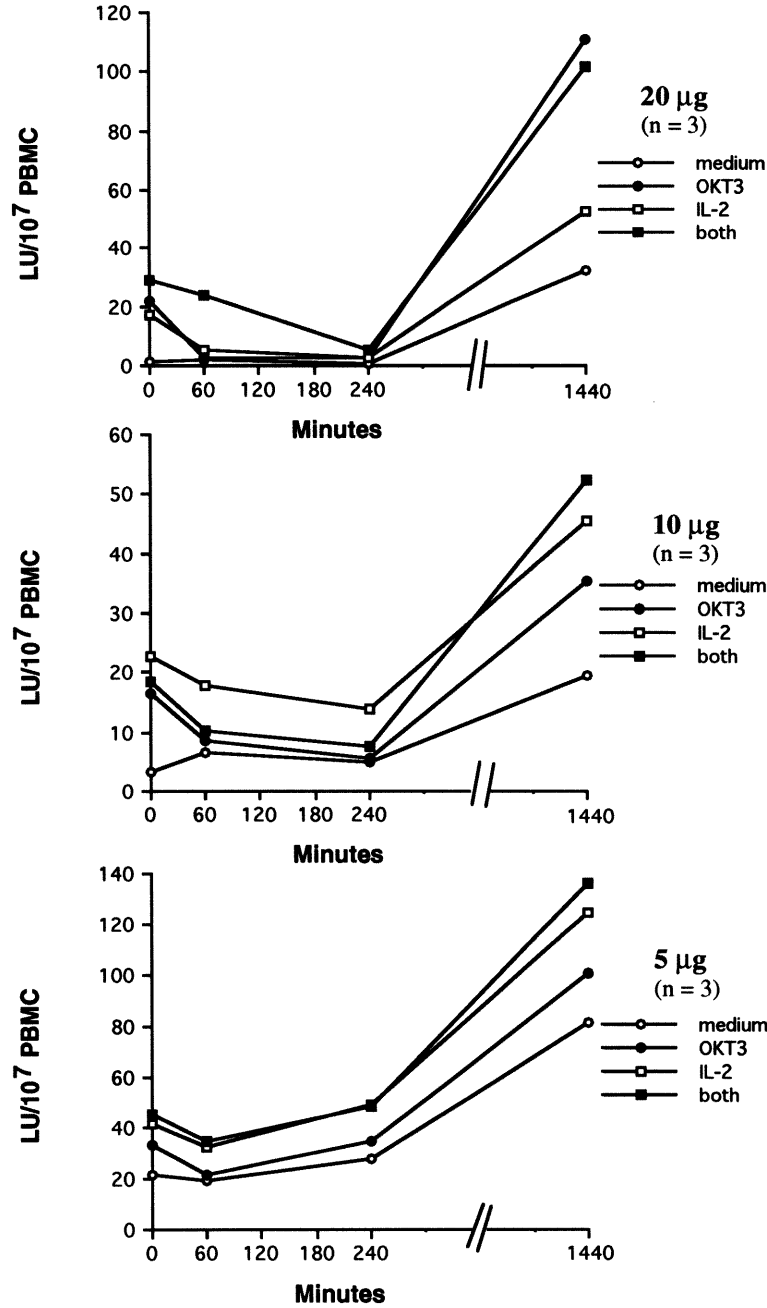
Figure 6.17 - *In vitro* modulation of K562 cytotoxicity (medians) by
OKT3, T3, and IL-2 in 6 control patients following
normal saline.
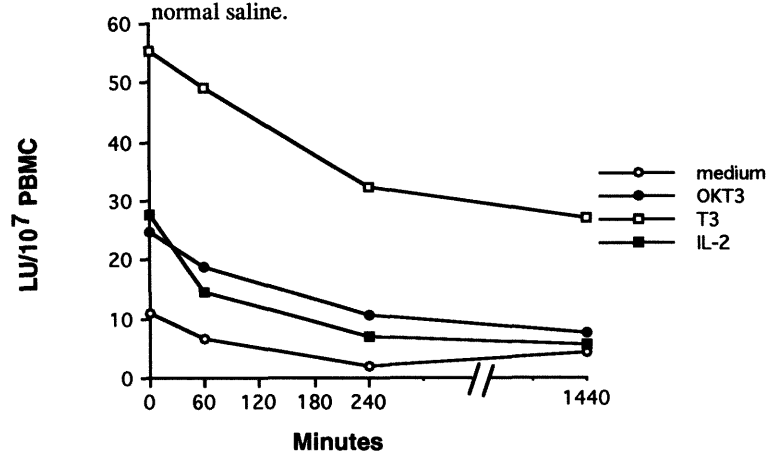
6.3.5   Association between changes in NK cells and cytotoxicity.

A.   Introduction.

BRM may influence cellular cytotoxicity *in vivo* by either modifying the numbers of effector cells present or their state of activation or both. The use of heterogenous mixtures of cells as effectors is another factor which is a relevant variable in measuring cytotoxicity. For a cellular cytotoxicity assay to be representative of *in vivo* cytotoxicity it is important that the proportions of effectors in PBMC preparations vary directly with the changes that occur following administration of BRM *in vivo*. PBMC were always corrected to 8×10^6 ml^{-1} prior to addition of 4×10^5 cells to each well in cytotoxicity assays. It has already been demonstrated in the methods section (chapter 3) that proportions of circulating NK cells are directly related to their proportions in PBMC preparations when measured on a single occassion. Also demonstrated in chapters 4 and 5 is the finding that proportions of NK cells in blood significantly correlate with levels of K562 cytotoxicity.

B.   Variation in NK cells in PBMC preparations following OKT3.

Cytotoxicity may also be influenced by variation in both the number of effectors harvested from blood and their relative proportions in PBMC preparations. In the patients receiving 50 μg OKT3, in whom insufficient PBMC were obtained to undertake assays at a maximum E:T ratio of 40:1, the decline in cytotoxicity at 240 minutes may be partly explained by the necessity to use lower maximum E:T ratios. Only one patient (ME) had sufficient PBMC at 240 minutes to enable these assays to be performed at a maximum E:T of 40:1. This patient maintained significantly higher levels of K562 cytotoxicity (8.8 LU/10^7 PBMC) compared with the other 5 patients at 240 minutes (although a significant decline was still observed from 42.8 LU/10^7 PBMC). This factor was not a prominant variable in the patients receiving the other doses of OKT3, in whom sufficient PBMC were generally obtained to perform assays at the same E:T ratio.

OKT3 may also influence cytotoxicity by affecting the proportions of cells separated from blood in the PBMC preparations. OKT3 was associated with a neutrophilia in the 50 μg patients at 4 hours. Increasing the proportion of neutrophils or monocytes in PBMC preparations would therefore have the effect of decreasing the proportion of NK cells and thereby decreasing levels of cytotoxicity *in vitro*.

Flow cytometry of PBMC preparations was therefore undertaken to investigate the possibility that OKT3 administration may influence levels of K562 cytotoxicity by changing the proportions of NK cells retrieved from blood. PBMC were assessed in 3 patients each in the 20 μg 10 μg, and 5 μg groups and also the 6 control patients receiving normal saline. Individual results are described in Tables 3.35 and 6.36 in appendix H. The numbers of different cell types in PBMC preparations was then calculated from the percentage of different subsets determined by flow cytometry. Median NK cell numbers had increased from 2.9×10^6 ml^{-1} of PBMC pretherapy to 3.4

$X\ 10^6\ ml^{-1}$ of PBMC at 24 hours in the 3 patients receiving 5 µg OKT3 and this
corresponded with an increase in median cytotoxicity from 21.2 to 81.9 $LU/10^7$ PBMC.
In the three patients receiving 20 µg OKT3 NK cells increased from 1.81 to 2.46 $X\ 10^6$
ml^{-1} and corresponding median cytotoxicity increased from 1.2 to 32.4 $LU/10^7$ PBMC.
Median cytotoxicity increased in the 3 patients receiving 10 µg OKT3 over the 24 hours
following administration (3.3 to 19.5 $LU/10^7$ PBMC). This was in contrast to the
decline in median cytotoxicity seen in for all 6 patients receiving 10 µg OKT3 over this
time and was due to the inclusion of data from HB in the PBMC calculation in whom an
increase to 238.1 $LU/10^7$ PBMC was observed. Median NK cells remained unchanged
(2.4 $X\ 10^6\ ml^{-1}$ at time 0 and 2.4 $X\ 10^6\ ml^{-1}$ at 24 hours) during this time. Variation in
the number of neutrophils and monocytes in PBMC preparations was relatively small.

C.   Association between variation in NK cells in blood and K562 cytotoxicity
        following OKT3.

        Mean (± SEM) percentage NK cells in blood are compared with median levels of
K562 cytotoxicity for the 6 patients receiving 50 µg OKT3 in Figure 6.18 (data in Table
6.37 appendix H). Despite a marked decline in median cytotoxicity from pre-OKT3
levels of 20.8 $LU/10^7$ PBMC to 0.1 $LU/10^7$ PBMC at 240 minutes, the percentage of
circulating NK cells remained constant (15.5 ± 1.6% pretherapy to 15.4 ± 2.2% at 240
minutes). The patients receiving 5 µg OKT3 developed a significant increase in median
K562 cytotoxicity at 24 hours (43.5 $LU/10^7$ PBMC) compared with pretherapy values
(14.6 $LU/10^7$ PBMC) and also demonstrated an increase in mean (± SEM) % NK cells in
blood from 15.7% (± 1.2) to 20.1% (± 1.8) (Figure 6.8). Therefore, in this group
enhancement of cytotoxicity could at least be partially accounted for by an increase in
proportions of circulating NK cells.

        Among the patients receiving 10 µg OKT3 in whom median cytotoxicity had
declined at 24 hours (0.12 $LU/10^7$ PBMC) compared with pretherapy values (3.21
$LU/10^7$ PBMC), mean (± SEM) % NK cells had fallen by only 1% from 13.2% (± 3.4)
to 12.1%. Among the patients receiving 20 µg OKT3 in whom median cytotoxicity was
also enhanced from pretherapy levels of 4.5 $LU/10^7$ PBMC to 38.5 $LU/10^7$ PBMC at 24
hours, % NK cells only increased from 17.3% (± 1.0) to only 17.8% (± 1.6).

        A better association was observed between NK cell numbers in blood and K562
cytotoxicity. For example the three patients in the 10 µg group (IT, WL, and WG) who
demonstrated a fall in cytotoxicity at 24 hours to < 0.2 $LU/10^7$ PBMC demonstrated a
corresponding decline in NK cells (0.07 to 0.05 $X\ 10^9\ L^{-1}$, 0.18 to 0.04 $X\ 10^9\ L^{-1}$, and
0.04 to 0.02 $X\ 10^9\ L^{-1}$).

Figure 6.18 - %NK cells (means ± SEM) in blood and K562 cytotoxicity (medians) following 50μg OKT3.
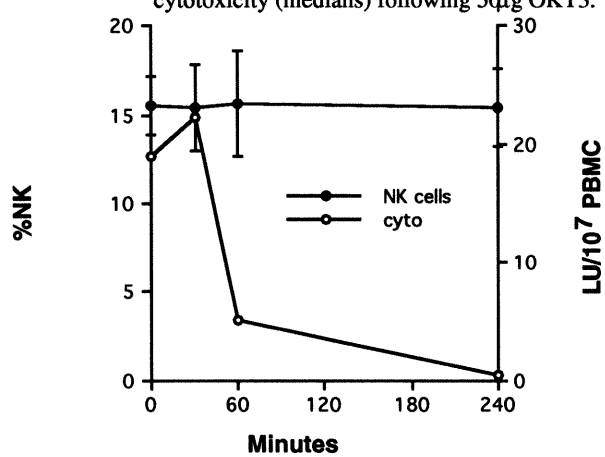
When cytotoxicity was compared with the number of NK cells per ml of blood rather than their percentages as a fraction of lymphocytes, an association was generally seen between changes in cytotoxicity and similar changes in NK cells. A typical result for one patient receiving normal saline (IA) and one patient treated with 5 µg OKT3 is described in Figure 6.19.

When patients receiving 50 µg OKT3 were analysed with their respective 4 controls and results expressed as mean (± SEM) NK cells/ml blood compared with median levels of K562 cytotoxicity an association between NK cells and cytotoxicity was observed up to 4 hours (Figure 6.20).

Variation in cytotoxicity between individuals in the remaining groups was too great and changes in cytotoxicity too small to make this form af analysis meaningful for the patients receiving lower doses of OKT3 and their respective 6 controls. A graph similar to those presented in Figure 6.19 was generated for every patient (not shown) and a qualitative analysis was therefore performed.. The association between changes in numbers of circulating NK cells and K562 cytotoxicity was noted between times 0 and 4 hours and 4 hours and 24 hours. The association could therefore be determined as a positive one (increase in NK cells and increase in cytotoxicity) or a negative one (fall in NK cells associated with an increase in cytotoxicity or an increase in NK cells associated with a fall in cytotoxicity). This avoided the need to take into account either absolute numbers of NK cells or levels of cytotoxicity.

It was anticipated that a weaker association would be observed between 4 hours and 24 hours for two reasons. Firstly, different target cell preparations were used on the second day which could be a source of error in the assay. Secondly biological factors such as the release of cytokines from lymphocytes following activation by OKT3 or other changes in the patients clinical status may be expected to have more influence when samples were collected at longer intervals. When low levels of cytotoxicity were present a poorer association was anticipated because of the relatively larger degree of assay variability.

Results of this qualitative analysis are described in Table 6.38. 5 patients had unusually low levels of cytotoxicity which precluded an accurate association with NK cells. One patient (HB) had unusually high levels of cytotoxicity with which changes in NK cells did not correspond. In 5 patients (*) an increase in cytotoxicity between 4 and 24 hours was seen to levels above those observed at time 0 with an increase in NK cells from 4 hours to 24 hours. However NK cells had not recovered to pre-OKT3/normal saline levels in these 5 patients. In 26 of the patients changes in cytotoxicity corresponded with similar NK cell changes. The association was strongest in the control patients and the 50 µg group.

Figure 6.19 - Variation in NK cells and K562 cytotoxicity in one patient
receiving normal saline (IA) and one patient treated with
5 µg OKT3 (AB).
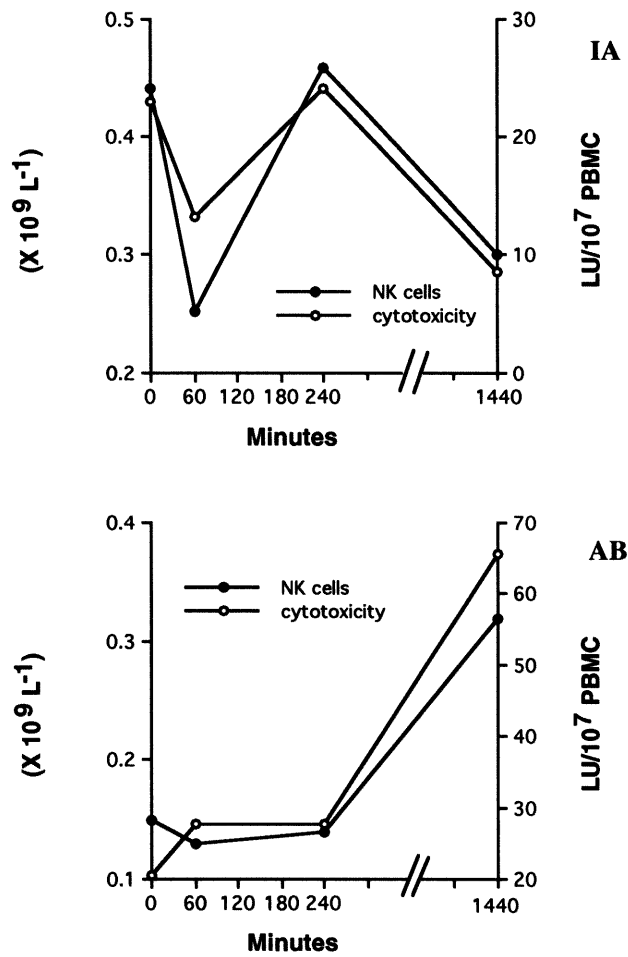
In conclusion changes in NK cell numbers were associated with changes in K562 cytotoxicity in the same direction for individual patients, both following OKT3 and normal saline. Only the 50 μg group and respective 4 control patients maintained this association when analysed as a group (median values of cytotoxicity corresponded to mean NK cell numbers) (Figure 6.20). It was not appropriate to combine the patients data within the 20 μg to 5 μg groups and their respective 6 control patients. This was because of the large variability in cytotoxicity between these patients probably arising from variability in killing capacity of NK cells between individuals. Changes in cytotoxicity were aften also small. These individuals were analysed with the 50 μg and control patients in a qualitative way. It was then observed that changes in NK cells usually followed the same trend as changes in cytotoxicity.

Figure 6.20 - Association between changes in NK cells (means ± SEM)
and K562 cytotoxicity (median) in 6 patients receiving 50 μg
OKT3 and 4 controls.
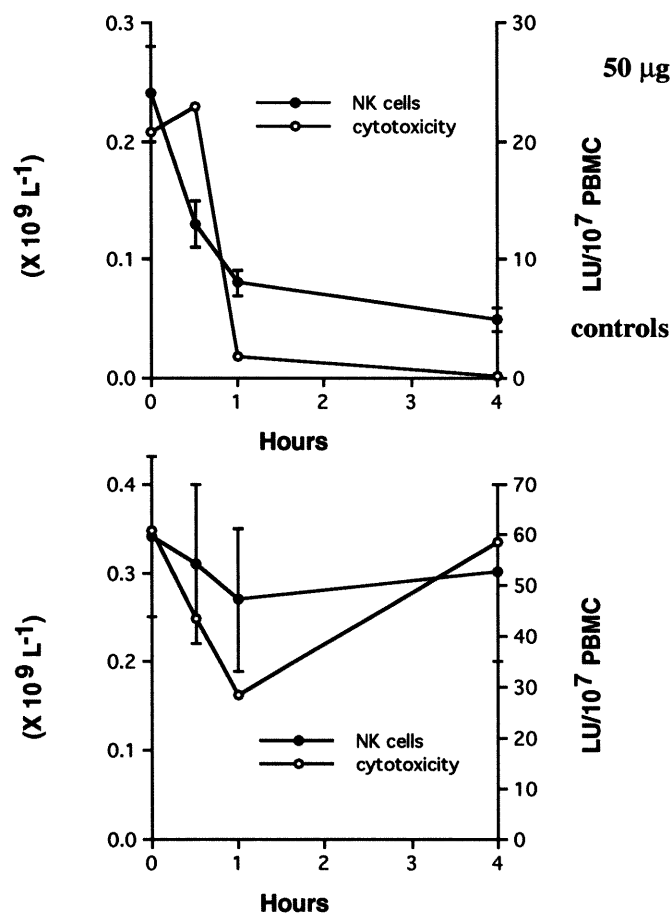
Table 6.38 - Association between changes in NK cells and K562 cytotoxicity between 0 and 4 hours and 4 and 24 hours.

Patient	OKT3 (μg)	0 to 4	4 to 24	Comment
IB	50	+	ND	
WD	50	+	ND	Rise in cyto 30 mins
ME	50	+	ND	Rise in cyto 30 mins
DH	50	+	ND	
AP	50	+	ND	Rise in cyto 30 mins
WW	50	+	ND	Cyto very low
HC	0	+	ND	Rise in cyto 30 mins
MD	0	+	ND	Rise in cyto 30 mins
GG	0	+	ND	
PW	0	+	ND	
BL	0	+	+	
HW	0	-	-	Cyto 0.0
IA	0	+	+	
GW	0	+	+	
AG	0	+	+	
NS	0	+	+	Cyto < 1 LU/10^7 PBMC
GR	20	-	+	
DC	20	+	+	*
BJ	20	+	+	*
AE	20	+	+	*
DD	20	+	+	
NM	20	+	+	
IT	10	+	+	
WG	10	+	+	
WL	10	-	+	Cyto < 1 LU/10^7 PBMC
KC	10	-	+	*
HB	10	-	-	Cyto high
RW	10	-	-	
AB	5	-	+	
DS	5	+	+	
RD	5	+	+	
RH	5	+	+	
DB	5	+	-	
FB	5	+	+	*
DV	0.5	-	+	Cyto < 1 LU/10^7 PBMC
TH	0.5	+	-	NK unchanged cyto increased

* Cytotoxicity increased above levels at time 0, associated with only partial recovery in NK cell numbers at 24 hours compared with time 0.

6.4   SUMMARY.

Following low doses (50 µg to 5 µg) of OKT3 side effects were qualitatively similar to those observed following transplant (5 mg) doses. They were however attenuated, dose-related and rapidly resolving. Only one patient required treatment for rigors (paracetamol).

Following administration of 50 µg to 5 µg doses, OKT3 was detectable (within 15 minutes) on the majority of lymphocytes. Following 0.5 µg, OKT3 was detectable on less than 20% of lymphocytes. By 24 hours most OKT3 had disappeared from the cell surface for the 20 µg to 0.5 µg doses. OKT3 did not induce any modulation of expression of the CD3 antigen.

Only one patient developed elevated cytokine levels (TNF-α 75 pg ml^{-1} in WW) at 60 minutes following 50 µg OKT3 which were undetectable at 4 hours. This patient did not experience significantly more toxicity than the other patients. Levels of sIL-2Rα did not change following OKT3 in either the patients receiving 50 µg or the 4 controls.

Serum from individual patients receiving 50 µg and 20 µg OKT3 had a variable influence on K562 cytotoxicity mediated by PBMC from one healthy donor. In 5 of the 50 µg OKT3 treated patients the effect of serum at 30 minutes was to suppress cytotoxicity. Cytotoxicity then increased in all patients to 240 minutes. However cytotoxicity was enhanced in two patients from 30 minutes and one of these (WW) had elevated TNF levels while the other (IB) had pronounced symptoms. This suggested that TNF-α or another cytokine released by OKT3 may be the cause of this enhancement

Between 50 µg and 5 µg doses of OKT3 were associated with a decline in numbers of all circulating lymphocytes in a dose dependent manner irrespective of CD3 surface antigen expression. NK cells were depleted at the same rate as T cells and CD4 and CD8 cells were also depleted at a similar rate. There was no evidence of lymphocyte activation as determined by increased expression of IL-2Rα. Although 0.5 µg OKT3 was detected on only a minor fraction (< 20%) of circulating lymphocytes at 60 minutes following administration although 0.5 µg was associated with some initial lymphodepletion. One patient (DV) developed a decline in NK cells up to 24 hours following 0.5 µg OKT3 which seemed disproportionate to the dose. Control patients also demonstrated a fall in lymphocytes at 30 and 60 minutes, However, in contrast to the OKT3 treated patients recovery was seen by 240 minutes.

Recovery of lymphocytes and NK cells was also dose related and the 5 µg group demonstrated a rebound lymphocytosis of NK cells over pretherapy levels.

50 µg OKT3 administration was associated with an increase in median K562 cytotoxicity at 30 minutes followed by a progressive decline in cytotoxicity to 240 minutes in all patients. There was no increase in Daudi cytotoxicity up to 4 hours following 50 µg. These findings contrasted with the 4 control patients receiving normal saline in whom an initial decline in K562 cytotoxicity was seen up to 60 minutes followed by recovery to original levels at 240 minutes.

The effect on K562 cytotoxicity in the patients receiving 20 µg to 5 µg OKT3 demonstrated individual variation over the initial 4 hours although the marked decline seen with 50 µg was not seen. The effect on median K562 cytotoxicity at 24 hours was of enhancement in the 20 µg and 5 µg groups which reached significance in the latter group ($p = 0.03$). Cytotoxicity in the 10 µg group behaved differently to the other groups. Median levels of cytotoxicity in tissue culture medium were relatively low in 4 of the 6 patients (although significant enhancement with T3 was observed). Median cytotoxicity in the 10 µg patients was reduced at 24 hours in comparison with pretherapy levels. A modest reduction was also seen at 24 hours in the 6 control patients.

OKT3 and T3 continued to enhance cytotoxicity in most patients following 20 µg to 5 µg OKT3 administration. Enhancement by IL-2 was low also and a significant additive effect with OKT3 was not demonstrated in any of the OKT3 treated groups or control group. There was no significant increase in the response to IL-2 *in vitro* following OKT3 administration *in vivo*.

The effectors responsible for cytotoxicity were probably NK cells. Changes in the percentage of circulating NK cells were not closely associated with changes in cytotoxicity. Changes in the number of NK cells were usually associated with changes in cytotoxicity in a similar direction but not necessarily of a similar magnitude. OKT3 did alter the proportion of cells in PBMC preparations which reflected changes in peripheral blood. In particular PBMC preparations were not excessively contaminated by neutrophils and monocytes following therapy. Changes in cytotoxicity were due to changes in the number of NK cells in blood reflected by their proportions in PBMC preparations.

In conclusion, OKT3 was safe to administer and was associated with changes in NK cells numbers that were significantly different to untreated controls. These changes were associated with corresponding changes in cytotoxicity. Depression of cytotoxicity associated with 50 µg OKT3 may be initially detrimental to patients although cytotoxicity was not assessed at 24 hours. In the 5 µg patients, cytotoxicity was significantly enhanced over pre-OKT3 levels at 24 hours. The 20 µg group demonstrated similar enhancement. However, the 10 µg was influenced by patients with widely differing cytotoxicities. The finding that cytotoxicity was sometimes increased without an increase in NK cell numbers, or was increased out of proportion to the increase in NK cell numbers, suggested that increased activation of NK cells either directly by OKT3 or secondary to cytokine release may have occurred in some cases.

Low doses of OKT3 have the effect of enhancing NK activity *in vivo* although the precise dose and proof of benefit at tumour level require further study.

CHAPTER SEVEN

DISCUSSION

7.1   INTRODUCTION.

Current modalities engaged in the treatment of malignant neoplasms are of limited efficacy and are associated with significant toxicity. Given these limitations it would be useful to develop areas of augmenting the hosts own anti-tumour responses. Unfortunately, most non-viral induced human tumours with the exception of melanoma and renal cell carcinoma are not immunogenic (Melief and Kast, 1991a and 1991b). However, the human cellular immune response consists of both a specific element mediated by CTL and a non-specific element mediated principally by NK cells. The role of the immune response and the means by which it may be manipulated in order to eliminate cancer cells has been reviewed in chapters One and Two and had important theoretical bearings on the development of the studies presented in this thesis.

Enhancement of the human immune system has been attempted using both exogenous lymphokine administration and adoptive transfer of *ex vivo* activated cells such as LAK and TIL. It is now established that the major cell population among LAK cells with broad lytic activity against tumour cells consists of activated NK cells with a less important contribution from non-specifically activated T cells (Weil-Hillman et al, 1989 and 1990). All the major biological activity of LAK and TIL preparations against tumours both *in vivo* and *in vitro* can be traced back to activated NK cells or T cells. In LAK cells NK activity predominate. In TIL CD8$^+$ T cells predominate. High-dose systemic IL-2 therapy has the disadvantage of activating large numbers of irrelevant T and NK cells with little or no tumour-reducing capacity. This may theoretically be reduced by stimulating specific populations of effectors for therapy of specific tumours.

Because of the expense and toxicity of these approaches other workers have attempted to boost immune responses by using T-cell receptor specific agents to activate T cells *in vivo*. CTL are known to be an efficient immune mechanism for the destruction of foreign or pathogenic cells. An important molecule in CTL fuction is the CD3 antigen and anti-CD3 mAb have been shown to trigger killing of various human and murine cell lines by CTL lines maintained in continuous culture (Mentzer et al, 1988). Anti-CD3 mAb also have the paradoxicial effect of inhibiting TcR-mediated target cell recognition either by inducing TcR modulation or by physical blockade of the TcR. They may also induce a physiological alteration in mature CTL effectors that inhibits their ability to mediate target cell lysis.

The principle aim of the research presented here was to assess the effect of different mAb on non-MHC-restricted cytotoxicity. The receptors involved in non-MHC-restricted effector cell and target cell interaction and lysis are controversial. The three main receptors that have been identified in triggering this form of lytic activity are CD2, CD3, and the CD16 molecule (Hersey and Bolhuis, 1987). NK are a heterogenous population with respect to surface antigen expression which may be classified by CD56 (NKH-1) and CD16 expression into different populations (Nagler et al, 1989 and 1990). CD56 is expressed on most PBL capable of non-MHC-restricted cytotoxicity. CD16 is

also expressed on the majority of NK cells and also neutrophils. The expression of either of these antigens was used to identify NK cells.

CD16 antigen expression may be related to the maturity of NK cells with CD16^{bright} cells representing the most mature subset. CD56$^+$CD16$^-$ cells are expanded *in vivo* by IL-2 therapy and are responsible for the majority of LAK activity by *in vivo* activated PBL (Weil-Hillman et al, 1989). Although CD3$^+$CD16$^-$ T cells incubated in IL-2 show some LAK activity this is at a lower level and with slower kinetics than IL-2 activated NK cells. These findings demonstrate that different subsets of effector cells mediating non-specific cytotoxicity may be susceptible to selective expansion *in vivo* by specific biological agents. Non-specific mixtures of anti-lymphocyte immunoglobulins stimulate T cells directly but not NK cells *in vitro* (Taniguchi et al, 1990), although NK cells may be activated indirectly. OKT3 has been shown to be an effective addition to adoptive immunotherapy using TIL because it leads to increased proliferation and generation of a large T cell population (Ohta et al, 1991).

Such responses could conceivably provide a powerful antitumour therapy. The induction of widely specific non-MHC-restricted cytolytic activity by anti-CD3 mAb is currently of interest as the therapeutic use of activated killer lymphocytes shows great promise. The activation of numerous effector cell types could also be of benefit since cells within the same polyclonal population can differ in their susceptibility to a specific mechanism of cytolysis.

The type of tumour studied is clearly important with respect to susceptibility to response. Recurrent tumours of gastrointestinal origin are frequently resistant to conventional treatment with radiotherapy or chemotherapy and early surgical resection remains the only effective curative treatment modality. Specific active immunotherapy using BCG and syngeneic tumour cells have been shown to be effective in reducing the number of recurrences following surgical resection of colorectal cancer (Hoover et al, 1985). Although recent reports of the successful use of adoptive cellular immunotherapy in patients with advanced solid organ malignancies have demonstrated objective tumour responses (Rosenberg et al, 1988) most immunotherapy protocols in the treatment of GIT cancer have proved unsuccessful and treatment of colorectal cancer with both IL-2 or IL-2 and LAK cells remains generally disappointing.

The effects of MHC class I expression on tumourogenicity are complex and depend upon the immunogenicity of the tumour in question (Ljunggren and Karre, 1990). In strongly immunogenic tumours which elicit CD8$^+$ CTL responses, down regulation of MHC class I usually increases tumourogenicity. In the case of tumours in which T cell immunity is less important MHC class I down regulation can lead to increased sensitivity to NK cell killing and may enhance *in vivo* tumour resistance (Ljunggren and Karre, 1990). The loss of HLA class I expression is relatively common in colon cancer. Approximately 10% of human colorectal tumours lack expression of any HLA class I antigens (Kaklamanis and Hill, 1992).

The aetiology of MHC loss in colorectal cancer is of considerable interest because if the loss is due to immunoselective pressure, it implies that colorectal tumours may at least initially be powerfully immunogenic. The moderate antitumour activity against many tumours exerted by NK cells, especially those with low levels of MHC class I expression is in line with the concept of NK cells as a first line defence mechanism against neoplastic cells. Blottierre et al, (1992) studied 15 different human colorectal cell lines for HLA class I antigen expression and NK susceptibility and no quantitative correlation was observed between these 2 parameters although cell lines which were defined as lacking HLA class I antigens were more sensitive to killing than class I positive cells. Low or absent HLA class I expression on tumour cells has been interpreted as providing selective advantage to the tumour in avoiding immune mediated destruction.

However, the price of this prompt action and broad lytic specificity is less efficiency and precision. Nevertheless, in view of the lack of expression of MHC class I antigen by gastrointestinal cancers, PBL may prove to be reasonable effector cells against such tumours because they have high LAK activity and are easy to obtain. LAK cells may not be useful against autologous tumours that express MHC class I antigens and in this instance TIL stimulated by IL-2 and OKT3 would be expected to be good effector cells because the of the T cell component.

The work presented in this thesis was a continuation of preliminary studies in our laboratory on the manipulation of K562 cytotoxicity *in vitro* among cancer patients and healthy donors using anti-CD3 mAb (Ubhi et al, 1991). This work represented a preliminary study involving a small number of patients with various solid tumours (including GIT cancers). Results were not conclusive but suggested that fresh PBMC from patients who failed to respond to IL-2 immunotherapy also demonstrated reduced enhancement of cytotoxicity *in vitro* following incubation with anti-CD3 mAb. The implication of these studies was that peripheral blood cells which were responsible for anti-CD3 mAb enhanced cytotoxicity *in vitro* may also be resposible for IL-2-induced tumour regressions *in vivo*. The logical extension of this work was to embark on further studies to investigate the effect of OKT3 administration to cancer patients. This necessitated a more thorough investigation of cellular cytotoxicity and its manipulation using various mAb *in vitro*.

The chief aims of these studies were to establish the cytotoxicity assay as a reliable method for measuring NK activity in our laboratory; to investigate NK activity and its *in vitro* manipulation in healthy donors; to compare NK activity in healthy donors with cancer patients; to investigate the effect of administering an anti-CD3 mAb (OKT3) to cancer patients. These studies were intended to represent a preliminary investigation with a view to developing a protocol for more effective cancer immunotherapy possibly using combined rIL-2 and OKT3.

7.2   CHOICE OF EXPERIMENTAL METHODS.

7.2.1   Selection of subjects.

A   Healthy donors.

Laboratory and hospital staff provided a reliable source of healthy donors. This was important because the assessment of cytotoxicity using the methods outlined below is a lengthy procedure and required the collection of blood samples between 07:00 and 9:00 hours. These donors were initially studied both to assess the parameters which affected cellular cytotoxicity and to explore the mechanisms by which it may be modulated *in vitro*. There are problems associated with using such subjects and a pitfall commonly encountered in studying cancer patients is the use of a poorly controlled 'normal' population. Nevertheless, it was useful to investigate these subjects in the present studies because of the practical constraints of this research (namely funding and manpower restrictions) and also because of the problems associated with matching hospital inpatients suffering with benign conditions to those with cancer.

Subjects were generally healthy although a precise history of drug ingestion, smoking habits, alcohol consumption, diet, and ingestion of the oral contraceptive pill (OCP) was not obtained. These factors may have influenced results and their role cannot therefore be assessed. Nurses who had worked a previous night shift were excluded from study because of the known influence of a circadian rhythm on cytotoxicity. The elderly healthy subjects (≤ 50 years) were recruited principally from nursing staff and volunteer workers at the hospital whereas the younger donors consisted of a greater proportion of laboratory staff. Again a selection bias is difficult to assess with respect to the above variables which are often related to individual lifestyles.

Overall the number of male subjects was similar to that of female subjects. However, of the 28 elderly healthy donors there were 18 females compared with 10 males. The small number of subjects in this group in particular limits the conclusions that can be drawn with respect to influence of gender.

B   Cancer patients.

Gastrointestinal tract cancer patients were selected in all but one case for these studies because of their ready availability on general surgical and medical wards, the often advanced nature of their tumours, and their unresponsiveness to effective medical therapy. Colorectal cancer has also been an area of considerable focus in mAb research and has been the subject of the majority of studies concerned with mAb targeting and therapy (Lange and Martin, 1991). A single patient who had malignant melanoma was selected because this patient also subsequently underwent a course of IL-2 therapy and this patient has been reported elsewhere (Hollingworth et al, 1993). Because there was no formal funding for these studies both the cancer patients from whom a single blood sample was obtained and those subjects receiving OKT3 were investigated during the course of routine clinical management. As for the healthy donors, patients were selected

according to various inclusion and exclusion criteria but these were by necessity limited by their highly variable clinical management, variable pre-existing health, and availability of individuals on the day of study. The cancer patients were significantly older than the elderly healthy donors (median age 70 years, range 38 to 87 years compared with 58.5 years, range 50 to 84 years). This again emphasises the problems of finding a matched control group with which to compare patients in clinical studies.

As for the healthy donors all patients were caucasions. Polymorphism exists with respect to monocyte Fcγ receptors for murine IgG2a (Imai et al, 1981). Defective monocyte accessory function is the cause of T cell unresponsiveness to the mitogenic properties of OKT3 in cultures of PBMC in certain individuals, which is due to a deficiency in Fcγ receptors for IgG2a (Ceuppens et al, 1985). Abo et al, (1984) also demonstrated ethnic differences in the responsiveness of lymphocytes to anti-CD3 mAb. IgG2a subclasses induce proliferation in all individuals whereas IgG1 anti-CD3 mAb fail to do so in a large proportion of Asians including Indian, Japanese, and Chinese subjects. This fact clearly has important consequences in antibody design and patient selection for cancer therapy.

Patients were not included for study if they had undergone a blood transfusion within 28 days. NK cell function has been shown to be significantly impaired (p < 0.001) up to 30 days after surgery in patients transfused with whole blood (Jensen et al, 1992). Whole blood transfusion also causes a decreased T helper : T suppressor lymphocyte ratio (Jensen et al, 1992). It has been suggested that perioperative transfusion increases the recurrence rate and decreases survival rates in patients with colorectal cancer. A few patients had undergone blood transfusions more than 28 days prior to study and this may have had an influence on results.

The use of the Karnofsky classification (Karnofsky et al, 1948) provided only very limited information for defining patient health in this study. Most patients had very advanced disease and only 33 out of the 51 patients studied had tumours that were potentially curable by surgery at the time of study. The median survival of only 2.5 months (range 1 to 12 months) in the 28 patients who died during the course of these studies emphasises the advanced nature of the tumours. Patients were therefore a highly heterogenous group which has a bearing on the interpretation of individual results.

Routine prestudy blood tests were undertaken by the hospital pathology department. This exluded renal failure in all patients and confirmed jaundice in 4 patients. Jaundice did not appear to have a marked influence on cytotoxicity. The facilities of the hospital pathology department were not available to monitor patients routinely during study. CT scanning and ultrasonography was used in some patients to assess the extent of abdominal malignancy but again this service was not routinely available and the size of tumour masses was not routinely measured. Accurate staging was not always possible and patients were crudely separated into those with liver metastases and without liver metastases. Grading of tumours was performed although this information was collected

retrospectively and was subject to variation in interpretation between the different pathologists involved.

Initially it was planned to treat all patients with a single 50 µg dose of OKT3 based on the report by Wiseman et al, (1991) with a one month follow up. Six patients and 4 controls receiving normal saline were treated in this way. However, practical constraints involved with obtaining subsequent samples following hospital discharge meant that this approach was abandoned in favour of administering varying doses of OKT3. This again emphasises the practical problems associated with conducting the clinical studies. Only clinical toxicity was assessed using a highly simplified adaptation of the WHO criteria because of the limitations imposed by the availability of hospital pathology services and therefore routine post-OKT3 haematological and biochemical monitoring was not undertaken.

Cancer patients may have been subjected to changes in NK activity as a consequence of psychological changes associated with hopitalization as well as their disease. It has been shown that stress in the form of noise results in a reduction in NK activity up to 72 hours later although there were significant individual differences between subjects (Siebern et al, 1992). This effect was highly variable and was not associated with changes in either the number of circulating NK or T cell subsets. Even modest life stresses such as academic examinations have been shown to reduce NK activity (Kiecolt-Glaser et al, 1984). Depressed patients have been shown to exhibit lower levels of NK activity than matched controls which is due to both alterations in the availability and killing capacity of NK cells in peripheral blood (Evans et al, 1992). This study demonstrated these changes for male patients only and demonstrated striking depression related gender differences.

Reduced NK activity has been shown to occur in men with depression and is independent of the effects of hospitalisation (Caldwell et al, 1991). Hospitalisation was not shown to be a factor in reduction of NK activity which is an important consideration in the studies presented here where the length of hospitalization varied considerably.

OKT3 was reconstituted within 48 hours of administration and in the limited samples measured total OKT3 content was found to vary by up to 2 fold. This probably had little influence on results in view of the small doses administered and the rapid dilutional effect following injection. No attempt was made to vary the dose of OKT3 administered with respect to body mass or surface area. Because of the complexity of OKT3 kinetics following injection circulating blood volume may have been a more important factor but was not easy to assess.

7.2.2 CHOICE OF EFFECTORS.

The variables that should be considered in interpretation of NK activity include the following; the type of effector cell performing the NK activity ie. typical NK cells, CD3+ NK cells, and T cells; the experimental methods used; the histological type of tumour; the patients age and gender. Discrepancies may arise due to variations in the NK cell assays used among different laboratories which include the choice of target cell, the effector to target cell ratio, and numerous other experimental parameters that can affect the results of the analysis.

Freshly isolated PBMC were obtained for these studies because these were considered to be representative of the *in vivo* situation at least with respect to events in peripheral blood. However, this may not be representative of the situation at tumour level. Assessment of the overall killing capacity of PBMC preparations measures both their levels of activation and numbers of relevant effectors. The method for separation of PBMC described by Boyum (1968) has been shown to be reproducible in numerous studies and also in this study where sufficient numbers of (> 95 % viable) PBMC were routinely obtained.

Analysis of PBMC preparations was essential because levels of cytotoxicity measured in assays depends upon both the relative constituents of effectors present and also the consistency of the separation process particularly important when serial analysis of NK activity is assessed. Both EDTA and heparin have the same effect on yields of PBMC (heparin was routinely used in these studies). Boyum obtained considerably greater yields of lymphocytes (> 98%) with less contamination by monocytes and neutrophils than were obtained in these studies. Although we found contamination to be greater it was consistent from one preparation to the next. The separation technique resulted in modest purification of NK cells with < 25% contamination by neutrophils and monocytes. The method does however, have the disadvantage of being a time consuming technique to perform. The method described by Rees and Platts (1983) using whole blood is rapid and provides a better reflection of the *in vivo* situation and may be worth incorporating in future studies. The proportion of NK cells in PBMC preparations was shown to closely correlate with the proportions of circulating NK cells which was an essential observation in relating *in vitro* results to *in vivo* cytotoxicity.

7.2.3 MEASUREMENT OF CYTOTOXICITY.

The 4-hour ^{51}Cr-release assay incorporating K562 target cells can be reliably performed in the laboratory provided sufficient quality control measures are undertaken to ensure reproducibility and minimise daily variability (Whiteside and Herberman, 1989). The latter becomes essential when serial measurements of NK activity are needed as in monitoring of patients during the course of their disease or during immunotherapy. Criteria for the acceptance of the NK cell assay need to be established for each laboratory with the study of a population of healthy donors. A circadian rhythm has been noted with

maximum activity occuring in the morning or early afternoon (Whiteside and Herberman, 1989). Enumeration of NK cells by flow cytometry alone is not a substitute for assessment of cytotoxic reactivity. A consistent aim of BRM is to increase NK activity in patients with cancer. Evaluation of patients treated with BRM should include assays of NK activity. The usefullness of reliable NK cell measurements in screening patients for therapy using combination chemotherapy or BRM is obvious.

The ^{51}Cr-release assay was chosen for measuring cytotoxicity principally because of our prior experience with this method and also because it can be completed within a single day. There was however, great potential for technical variation and standardization within the laboratory was important. The incorporation of controls within assays has been advocated (Pross and Maroun, 1984). Batch cryopreservation of healthy donor lymphocytes was attempted but proved unsuccessful and was abandoned (results not described). With respect to manipulation of cytotoxicity *in vitro,* unstimulated PBMC acted as internal controls with which PBMC incubated in mAb or IL-2 could be compared.

All assays were performed in foetal calf serum. However, it is crucial to consider the influence of human serum on any possible *in vivo* use of antibodies. IgG in human serum competes with OKT3 for the Fc receptors on human monocytes. The strict dependence of OKT3-mediated PBMC proliferation on monocytes and its inhibition by human serum that were initially described by Van Wauwe et al, (1981) illustrates this point. This was therefore a factor to consider in drawing comparisons between the *in vitro* and *in vivo* situation in the present studies.

Calculation of results in LU was accepted as the most appropriate means of data expression. The lytic unit has become the principle means by which levels of activity are summarised in cytotoxicity assays (Bryant et al, 1992). A correlation coefficient (r) of $\leq$ 0.95 was accepted in the present studies although r was usually > 0.98. Our studies demonstrated that variation in the E:T ratio had an effect on results expressed in LU with a decrease in cytotoxicity noted at lower E:T ratios. Generally this was only a problem in the relatively small number of assays performed following administration of 50 µg of OKT3. A criticism of LU is that the dose response curve is often fitted to assay data using computer software and technical details of the calculation may not be apparent to the user. The same method of calculation of LU was employed throughout these studies so that consistency was obtained. It was decided to express results of cytotoxicity against NK resistant targets as % specific lysis because results in LU were too small to be meaningful.

Considerable time was also devoted to investigating culture conditions of target cells before a standard approach for labelling with ^{51}Cr was adopted. Ultimately a method was chosen whereby target cells were cultured overnight with ^{51}Cr which was found to result in substantially improved incorporation of radioactivity by cells without affecting their susceptibility to lysis. We also demonstrated was the finding that

prolonged (up to one hours) standing of whole heparinised blood did not result in variation of cytotoxicity which was important with regard to the practical limitations of the clinical studies when a time delay was often necessary prior to PBMC separation.

Other reagents such as sodium azide which is present as the preservative in most mAb preparations did not influence cytotoxicity at the final concentration present in assays. Non-specific antibody preparations were also without significant effect on cytotoxicity and these were not routinely included in assays as a control. The necessity for the inclusion of a non-specific antibody preparation which would have substantially added to the length of the procedure was therefore avoided.

7.2.4 FLOW CYTOMETRY.

The use of monoclonal antibodies allows the precise determination of T cell and NK cell subsets and promises the development of therapeutic protocols that can be designed to manipulate selected lymphocyte populations. For example human lymphocytes with NK activity have been reported to display a combination of B cell, macrophage (CD11), and T cell markers (CD2, CD3, CD7, CD8, and CD25) (Reynolds and Ortaldo, 1987). CD3 and CD8 are expressed on subsets of cells with NK activity. Although the major effector cells mediating NK activity in humans are the CD3⁻ NK cells there is a small but significant amount of cytotoxic activity associated with the CD3⁺ LGL population (approximately 5% of the lytic potential of PBL). Leu 19 (CD56, NKH-1) is used as a pan-NK-associated marker which is useful for detecting resting as well as activated NK cells (Ritz et al, 1988). The CD56 adhesion molecule is the major determinant for detecting non-MHC-restricted mononuclear cells and these cells also invariably express CD16 (Van Tol et al, 1992).

Peripheral blood cell subsets were studied using flow cytometry in an attempt to correlate specific effectors with both baseline K562 cytotoxicity and cytotoxicity enhanced with anti-CD3 and anti-CD16 mAb. Although flow cytometry is a standard procedure for assessing cells bearing specific surface antigens the method used in calculating results is important. In these studies this involved "gating" specific populations and expressing these as a proportion of the total population. However, the density of antigen expression was not assessed and cells which express variable levels of antigen were therefore not so clearly defined. This fact probably accounts for the relatively high numbers of CD3⁺NK cells detected which express CD3 in association with NK markers (CD16 and CD56) in comparison to other studies.

7.2.5 ELISA TECHNIQUES.

Measurement of cytokine and sIL-2Rα levels was undertaken using commercially available ELISA techniques. Although extremely reliable the prohibitive cost of the 'kits' limited the number of samples investigated. It was for this reason that the assessment of

only the samples considered most likely to be associated with cytokine release ie. following 50 μg OKT3, were investigated.

7.2.6 STATISTICS.

Statistical advice was sought from the Department of Epidemiology at Leicester University and in view of the small number of subjects studied simple paired and unpaired parametric and non-parametric tests were adopted. Subjects were divided into groups depending on age, gender, tumour grade, stage etc. for analysis wherever possible.

7.3 ASSESSMENT OF HEALTHY DONORS.

7.3.1 Cellular cytotoxicity.

One of the largest studies of NK activity in healthy individuals was undertaken by Pross and Baines (1982) who investigated 539 donors from once to 213 times over a 7 year time span against the K562 tumour cell line. Although a broad range of activity was noted it was impossible to determine without repeated testing whether or not any particular individual was a high or low NK donor. Repeated testing did demonstrate that the level of NK activity is not random within an individual and healthy individuals can be ranked into consistently high or consistently low NK donors. The rank order of cytotoxicity was maintained when healthy donors were tested together. An increase in cytotoxicity from birth to adulthood was noted as was a higher level in males compared with females. Although age and sex differences are slight, their existance should be considered when studies of NK activity are analysed.

The fact that individual NK donors are consistent in their cytotoxic function is extremely important and essential if meaningful statistics are to be undertaken on patient populations. The relative cytotoxic activity due to NK cells is a characteristic of the individual donor and is independent of the target cell which is used for its assesment. This was confirmed in the present studies where an individual with high reactivity against K562 targets also had relatively high reactivity against other tumour cell lines.

Median levels of K562 cytotoxicity in these studies were 3.3 LU/10^7 PBMC ranging between 0.0 to 110.0 LU/10^7 PBMC for all 103 healthy donors. Mean levels were 15.8 LU/10^7 PBMC because of the markedly positively skewed distribution of results. There was no significant association between K562 cytotoxicity and either age or gender. All other cell lines investigated demonstrated low levels of susceptibility to lysis by freshly isolated PBMC. The one individual whom was studied most frequently (JH) demonstrated considerable variation in K562 cytotoxicity between 0.0 and 144.5 LU/10^7 PBMC (median level of 38.0 LU/10^7 PBMC) over an 18 month period. Although similar variation was seen in all the 6 individuals tested repeatedly the rank order of cytotoxicity was generally maintained. Some workers (Heppner et al, 1975) have also found considerable variation in the activity of normal donors tested repeatedly while other

workers (Pross and Baines, 1982; Pross and Maroun, 1984) have found good reproducibility when assays were run several days in a row with the same normal individual but wider divergence when run at more widely spaced intervals. Susceptibility to killing may also vary from day to day depending on the health of the target cells. Incorporation of a standard cryopreserved PBMC preparation as an internal control in the assays would have partly helped to resolve this issue. Because normal donors are relatively consistent in their activity they have been used as a bank of normal controls against which patient data can be normalized (Pross and Maroun, 1984). A panel of healthy donor controls was not used because of the practical restrictions of this study relating to time and availability of donors.

OKT3 and T3 mAb were found to optimally enhance K562 cytotoxicity at a concentration of 1 μg ml^{-1}. This was slightly greater than in previous studies (Ubhi et al, 1991) although a broad plateau was seen over the range of concentrations chosen. High concentrations of these anti-CD3 mAb (100 μg ml^{-1}) did not inhibit cytotoxicity. Duration of exposure of PBMC to OKT3 and T3 in the assay did not increase enhancement when assessed over a 150 minute time period and 30 minutes was chosen as the most convenient preincubation time for PBMC. OKT3 and T3 did not substantially enhance cytotoxicity against the NK resistant cell line daudi nor against the other cell lines studied which probably reflected the short incubation times which were insufficient to generate LAK activity.

Leeuwenberg et al, (1985) demonstrated that although an anti-CD3 mAb (T3) inhibited antigen-specific lysis by CTL generated in mixed lymphocyte cultures, it consistently augmented non-MHC-restricted cytotoxicity against cell lines daudi, raji, and K562. These antibodies had no effect on unstimulated PBL in contrast to the present studies and only Fc receptor positive cell lines were susceptible to this enhancement of non-specific cytotoxicity. Anti-CD3 mediated cytotoxicity could be inhibited by aggregated human IgG which supported the involvement of Fc receptors in the process. The mechanism of enhancement of cytotoxicity was considered most likely to involve the crosslinking by anti-CD3 of lymphocytes to the Fc receptors on target cells.

Murine T cells have been shown to be capable of redirected killing by IgG2a murine Mab provided the target cell expresses receptors for IgG2a. In other words the mAb cross-bridges between the antigen-receptor on the CTL and the FcR on the target cell (Staerz and Bevan, 1985). This is in contrast to ADCC where the FcR resides on the effector cell. It would be advantageous to focus a strong T cell response to a chosen target ie. a cancer cell. Heteroconjugates (hybrid antibodies) in which one of the component binding sites is an anti-T cell receptor and the other is directed against any chosen target antigen can focus T cells to act at the chosen site and may prove usefull in cancer immunotherapy in the future.

The mechanism of killing *in vitro* clearly depends upon many factors which include, the duration of exposure to anti-CD3, and the continued presence of anti-CD3 in

the assay. An additional requirement for anti-CD3 activation of lymphocytes is the presence of accessory cells which produce mediators such as IL-1. In contrast to resting T cells activated T cells do not require IL-1 for either the induction of proliferation or IL-2 production (Manger et al, 1985). Monocytes were present in the PBMC preparations in the present studies but their role in presenting mAb to lymphocytes remains undetermined. Susceptibility of tumour cells to lysis *in vitro* has been shown to correlate with binding of the Fc fragment of anti-CD3 to the tumour cell surface resulting in crosslinking of CTL to tumour cells (Mentzer et al, 1988). K562 cell lines and daudi cells were efficiently lysed in the presence of anti-CD3 at concentrations as low as 0.1 μg ml^{-1} in the above studies. Purified Fc fragments inhibited the cytolysis induced by the intact anti-CD3 mAb. Binding of the T cell clone to the target cell involves binding of the target cell to the Fc part of the mAb as evidenced by the fact that F(ab)'$_2$ fragments thoroughly depleted of all intact antibody fail to induce non-specific cytotoxicity whereas they continue to effectively block antigen-specific cytotoxic activity (Spits et al, 1985). F(ab)'$_2$ fragments were not investigated in the present studies because although they are relatively easy to generate it remains difficult to confirm the purity of the preparations.

Enhancement of K562 cytotoxicity by OKT3 and T3 was a constant feature in all individuals tested in the present study. T3 was substantially greater (2606 %) than for OKT3 (1201 %). A rat anti-CD3 mAb ('humanized' OKT3) has been developed in an attempt to reduce a significant anti-globulin response (Routledge et al, 1991). A small quantity was obtained for these studies as a generous gift (Dr Routledge). This mAb consists of a human IgG1 isotype and exhibits enhanced complement mediated cell lysis. It has been shown to be less powerfull in modulating the CD3 antigen than bivalent anti-CD3. These features make it more likely to destroy T cells rather than activate them and it has been developed essentially with a view to the treatment of CD3$^+$ T cell malignancies. It was incorporated in these studies to investigate the efficacy of a monovalent antibody in enhancing cytotoxicity. Enhancement by hOKT3 was not found to be significant in 11 individuals studied.

CD16 (FcγRIII) is the only IgG receptor expressed on NK cells and is responsible for ADCC (Lanier and Phillips, 1992). Mab directed against CD16 leads to triggering of cytotoxicity and cytokine secretion. CD16 is associated with either the ζ chain of CD3 or the γ subunit of the high affinity IgE receptor. The ζ chain is tyrosine phosphorylated as a consequence of CD16 engagement in an analogous way to ζ subunit phosphorylation during CD3-TcR activation (O'Shea et al, 1991). Anti-CD16 significantly enhanced K562 cytotoxicity to levels intermediate between that of OKT3 and T3 which were significantly greater than OKT3. Addition of anti-CD2 and anti-CD16 mAb, to mixtures of effector cells and target cells has been shown to induce conjugate formation of IgG FcR$^+$ target cells followed by non-specific cytolysis which is dependent upon the IgG subclass specificity of the FcR on the target cell (Van de Griend et al,

1987). This suggests that these mAb in particular may warrant further investigation as they may have a role to play in immunotherapy protocols.

Limited studies using anti-CD2, anti-CD4, anti-CD8, anti-CD7, and an anti-ζ mAb alone demonstrated variable (but not statistically significant) enhancement of cytotoxicity. However, the small numbers of subjects tested limited the conclusions that could be drawn. CD2 is one of the earliest T cell differentiation markers and is present on all blood derived T cells and NK cells (Bolhuis et al, 1986). Anti-CD2 mAb have been shown to be capable of activating both T cells and NK cells to non-specific cytotoxicity (Siliciano et al, 1985). In the case of T cells a synergistic effect is seen between different anti-CD2 Mab. Although the ζ chain is a key subunit of the TcR its direct stimulation does not appear to have a marked influence on cytotoxicity in contrast to anti-CD16 mAb.

CD8 and CD4 act as coreceptors respectively for class I and class II MHC molecules (Springer 1990). The experiments in this study with anti-CD4 and anti-CD8 mAb were performed to assess whether they influenced cytotoxicity. Results of previous studies have been conflicting. Anti-CD8 has been shown to inhibit cytotoxicity induced by anti-CD3 and anti-CD2 in T cell clones (Van Seventer et al, 1988). In this respect CD4 and CD8 do not merely serve as passive adhesion structures but also exert regulatory effects on T cell activity. However, in contrast to T cell activity, non-specific cytotoxicity was reported not to be influenced by anti-CD8 mAb (Van Seventer et al, 1988). Anti-CD4 mAb were also shown to inhibit T cell proliferation induced by anti-CD2. Other studies have demonstrated that anti-CD4 and anti-CD8 do not induce cytolytic activity to allogeneic target cells when T cells are incubated with these mAb (Suthanthiran et al, 1984). Similarly mAb to CD4 and CD8 did not increase NK activity of LGL enriched fractions of PBMC from normal individuals against K562 in contrast to anti-CD3. Anti-CD4 and anti-CD8 did modestly enhance cytotoxicity in the present studies but insufficient subjects were investigated to draw meaningful conclusions.

Studies using mAb against adhesion molecules were undertaken to gain insight into other factors that may affect cytotoxicity. Manipulation of adhesion molecules plays a fundamental part in understanding cellular cytotoxicity *in vitro* and *in vivo*. The adhesive interactions of cells with other cells mediated by adhesion receptors have a central role in the functions of the immune system and regulate the migration of lymphocytes and the interactions of activated cells during the immune response (Springer, 1990). Adhesion molecules are not just important with respect to interaction between effector cells and tumour targets. They are relevant in migration of effector cells between tissues *in vivo*. Lymphocyte migration is an important prerequisite for an adequate and efficient immune defence mechanism (Duijvestijn and Hamann, 1989). Extravasation of effector cells from the circulation involves a lymphocyte adhesion step that is mediated by interaction of particular receptors on lymphocytes with ligands displayed by vascular endothelium. IL-1, TNF-α, and IFN-γ have been shown to be potent inducers of endothelial adhesiveness for lymphocytes (Duijvestijn and Hamann, 1989).

Limited studies using mAb against adhesion molecules (anti-LFA-1, anti-ICAM-1, anti-PECAM, and anti-ELAM) demonstrated significant inhibition of K562 cytotoxicity by anti-LFA-1 and anti-ICAM-1 (CD54) in particular. These findings confirmed what is known from previous studies.

Cell adhesion molecules (CAM) participate in interactions between lymphocytes, accessory cells, and target cells that are critical in the generation of effective immune responses. The lymphocyte function associated molecule (LFA-1) (CD11a/CD18) plays a key part in lymphocyte adhesion. Stimulation of CD2 and CD3 molecules results in cellular activation which strongly promotes LFA-1 dependent adhesion. NK cells express LFA-1 (CD11a/CD18) and LFA-3 (CD58) (Robertson et al, 1990). NK cells also express ICAM-1 (CD54) at greater levels than T cells. The ligand for LFA-1 is CD54 and for CD2 it is CD58.

In vitro incubation of fresh NK cells with IL-2 also demonstrates 4 to 6 fold increases in surface levels of LFA-1, CD2, CD54, and CD56. Increases in CAM are associated with enhanced formation of E:T conjugates and enhanced killing of NK sensitive targets. LAK activity induced by exogenous IL-2 has been shown to be partially inhibited by anti-CD2, anti-CD11a, or anti-CD54 antibodies and is almost completely abrogated by anti-CD2 and anti-CD11a in combination. Anti-CD54 has been shown to inhibit killing of both K562 and daudi targets despite the absence of its ligand (LFA-1) on these target cells. Other presently unidentified ligands are therefore clearly involved. CAM are therefore capable of enhancing both initial conjugate formation and triggering of the lytic process. CAM therefore play a central role in the regulation of NK cytolysis and changes in CAM expression may alter the target cell specificity of activated NK effectors. Further work is needed to elucidate the roles of CAM in mAb enhanced cytotoxicity.

Because a multireceptor recognition process is involved in non-MHC-restricted target cell recognition and lysis activation by one receptor may be enhanced via another using combinations of the relevant mAb. The chimaeric human-mouse anti-T cell mAb (CH2;SDZ 214 - 380) anti-hCD7 was constructed by cloning the variable regions of both the light and heavy chains of a murine hybridoma which has reactivity with the CD7 antigen which is expressed by most NK cells (Heinrich et al, 1989). In the present studies however, combinations of anti-hCD7 and OKT3 demonstrated a partially additive effect only and anti-hCD7 in combination with T3 failed to enhance cytotoxicity over that observed for T3 alone. Further studies are required to investigate synergy between different mAb.

The Fc portion of IgG is the major inhibitor in human serum of OKT3 stimulation of PBMC and inhibition by IgG subclasses correlates to their ability to bind to the monocyte Fc receptor (Looney and Abraham, 1984). Human IgG1 and IgG3 produce greater inhibition than IgG2 and IgG4 in a competitive manner. Anti-CD3 stimulation of PBMC is therefore inhibited by human serum but not by foetal calf serum. At μg

concentrations anti-CD3 is mitogenic even in the presence of 10% human plasma because at these concentrations anti-CD3 can effectively compete for Fc receptors with the IgG present in human plasma.

FcγRII (CD32w) is found on the surface of both K562 and daudi cells (Looney et al, 1986). FcγRII has a higher affinity for mouse IgG1 than IgG2a. Mouse IgG1 binds with relative specificity to FcγRII which is found on K562 cells and on monocytes and this receptor has an important role in the human T cell proliferative response to IgG1 anti-CD3 (T3) (Schreiber et al, 1992). The magnitude of T cell mitogenesis by anti-CD3 is known to correlate with the affinity of the Fc fragment for FcRγI whose relative binding is IgGI = IgG3 > IgG4 for human subclasses of mAb and IgG2a = IgG3 > IgG1 > IgG2b for murine isotypes. Analysis of early T cell activation, proliferation, and lymphokine production indicate that isotype exerts a profound effect on mAb activation potency (IgG2a >> IgG1 > IgG2b) whereas, epitope specificity exerts a minor effect (Woodle et al, 1991). The Fc fragment is therefore an extremely important factor in considering antibody design. The response induced by mIgG2a (OKT3) but not by mIgG1 (T3) anti-CD3 may be blocked by low concentrations of heat aggregated mIgG2a. Conversely the response produced by mIgG1 anti-T3 but not by mIgG2a anti-CD3 may also be blocked by heat-aggregated mIgG1.

An attempt was made to investigate the mechanism of enhancement of cytotoxicity by preincubating K562 cells in antibodies with different isotypes prior to the addition of effectors. Enhancement with OKT3 and T3 was then attempted. None of the antibodies chosen (pooled mIgG, mIgG1, mIgG2a, and hIgG1) had any effect on enhancement observed following addition of anti-CD3. This was surprising in view of previous work suggesting that Fc interactions were important. However, preincubation of K562 with an anti-CD32w (specific for FcγRII) mAb appeared to substantially impair enhancement by T3 in 3 donors. This was in contrast to anti-CD64 (FcγRI) mAb which had no effect. These preliminary experiments suggested that Fc interactions between the IgG1 isotype and FcγRII may be important at least with respect to T3 enhancement. This finding reinforced the theory that the greater enhancement seen with T3 may be related to the greater affinity of IgG1 binding to FcRγII on K562 cells compared with IgG2a (OKT3).

Preliminary experiments were also undertaken to compare rIL-2 enhancement of K562 cytotoxicity with that of OKT3 with a view to exploring synergy. Dose-dependent enhancement was seen with rIL-2 as expected. However when PBMC were incubated with a low-dose of rIL-2 and 1 μg ml^{-1} OKT3 only a partially additive effect was observed rather than a synergistic effect. More work is required to investigate synergy between IL-2 and OKT3 *in vitro* and *in vivo*.

7.3.2   Analysis of peripheral blood cells.

The present study demonstrated an increase in numbers of circulating neutrophils with age which failed to reach statistical significance. Absolute numbers of lymphocytes

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and NK cells were similar in both the < 50 and ≥ 50 years old healthy donors. Mean CD3+NK cells were significantly higher in the elderly donors. The proportion of NK cells were similar to previously reported figures at 12.3% for the young healthy donors compared with 14.6% for the elderly healthy donors. The proportion of CD3+NK cells was significantly greater at 10.9% and 15.3% respectively than levels previously reported.

The significance of CD3+NK cells is uncertain in the present studies. Lanier et al, (1985) defined a functionally unique subset of cytotoxic T lymphocytes in peripheral blood by examining coexpression of CD3 and CD16 antigens. This subset represented only 2% of circulating PBL in most healthy donors. These lymphocytes characteristically expressed LGL morphology but showed little cytolytic activity either before or after IL-2 treatment in contrast to typical CD3⁻ NK cells. However, these CD3⁺ NK cells could be induced to kill NK resistant targets in the presence of anti-CD3 mAb. Radiolabelled IL-2 binding studies have shown that treatment with anti-CD3 mAb results in upregulation of the IL-2Rβ chain on leukemic CD3⁺ LGL (Loughran et al, 1990).

Little information is available regarding the activation of normal CD3⁺ LGL. Anti-CD3 has been shown to induce cytotoxicity in leukemic CD3⁺ LGL but not proliferation and this induction of cytotoxicity occurs through an IL-2-independent pathway. Increased killing of targets was deemed unlikely to be explained by bridging of anti-CD3 coated effector cells to Fc receptor positive target cells since anti-CD3 mediated cytotoxicity also occured in Fc receptor negative target cells such as Molt-4. The explanation may have been that anti-CD3 results in enhanced expression of effector cell adhesion molecules involved in target cell binding. Because non-MHC-restricted cytotoxicity mediated by normal CD3⁻ NK cells can be augmented by IFN-γ and IL-2 anti-CD3 mediated cytotoxicity may be due to production of these lymphokines by activated CD3⁺ LGL and T cells.

There was no gender related difference in any cell subset in the present studies. Balch et al, (1982) demonstrated a correlation between LGL and age and sex where older patients had higher numbers of circulating LGL as did males compared with females. These findings were also confirmed by Ligthart et al, (1986) who also showed a decrease in proportion of T cells with age. These findings stress the importance of using age-related reference values or age and sex matched control groups in such studies.

The existence of a substantial number of CD25 positive T cells in the blood of normal healthy individuals may reflect prior activation of these cells. Since CD4 cells are the major source of IL-2 production , persistence of the CD25 antigen may result from chronic stimulation by this factor. Quantitative measurement of antigen levels rather than enumeration of positive cells is probably required to assess the significance of CD25 expression. CD4 and CD8 expression was assessed with CD25 (IL-2Rα) in a small number of donors and CD25 expression was shown to be greater on CD4 lymphocytes compared with CD8 lymphocytes. Although the range of results obtained was as

expected from previous work no further conclusions were possible. IL-2Rα has been extensively studied as an activation marker in many clinical situations and the normal baseline values have been generally considered to be expressed on less than 10% of lymphocytes. In one previous study using a phycoerythrin-conjugated antibody to the IL-2Rα chain approximately 30% of normal PBL have been found to be positive IL-2Rα and 66% of these cells were in the CD4+ subset and mostly within the CD45RA negative fraction associated with activated or memory T cells (Jackson et al, 1990).

Contradictory results can be explained by the fact that the sensitivity of the detection method varies with respect to both immunofluorescent labelling techniques and instrumentation for fluorescence detection. Flow cytometers which are designed to pass cells through the excitation source while the cells are encased in a quartz cuvette (eg, FACScan) as employed here, rather than using a liquid stream-in-air configuration provide for more efficient optimal collection of fluorescence. More extensive studies were not performed to study CD4 or CD8 expression because of the expense of the fluorescein-conjugated mAb and it was considered unlikely to yield significant results on the small number of patients in this trial.

In order to determine the relative importance of different cell subsets in mediating cytotoxicity circulating cells were correlated with levels of killing. A highly significant correlation was observed between proportions of circulating NK cells and levels of K562 cytotoxicity ($r = 0.37$, $p < 0.005$) for all healthy donors and a significant correlation was not seen for CD3+NK and K562 cytotoxicity. That better correlations were not seen probably reflects the variation in killing capacity between individual NK cells. Variation in the proportion of NK cells in one individual (JH) also corresponded with variation in K562 cytotoxicity. This confirmed that NK cells are responsible for the majority of K562 cytotoxicity in fresh PBMC and CD3+ cells expressing NK markers must play only a minor role if at all. The marked variation in cytotoxicity in one individual with time is may be a consequence of variation in the proportion of circulating NK cells as well as changes in their activity.

Enhancement of K562 cytotoxicity by OKT3 was closely related to baseline levels of cytotoxicity but unrelated to circulating numbers of CD3+NK cells or T cells. This was also the case for the elderly healthy donors in whom greater levels of circulating CD3+NK were seen. Anti-CD16 enhancement of cytotoxicity was also unrelated to proportions of circulating NK cells in 12 healthy donors studied. Therefore it seems reasonable to conclude that the action of anti-CD3 mAb on K562 cytotoxicity is mediated largely through NK cells and is indirect although the role of CD3+ cells cannot be excluded.

7.4   ASSESSMENT OF CANCER PATIENTS.

7.4.1   Cellular cytotoxicity.

Cancer patients were studied to assess the effect of malignancy on non-MHC-restricted cellular cytotoxicity *in vitro* and to investigate its modulation using anti-CD3 mAb. Cancer patients underwent similar investigations to those described for healthy donors with whom they were compared. There is considerable controversy in the literature with regard to NK cell abnormalities in cancer patients. There are probably technical as well as biological explanations for these divergent results which include cell purification techniques, target cells used, and culture conditions. Since there are such a large number of variables to be considered in analysing data about NK cell function it is not therefore suprising that there is ambiguity in the literature about their role in cancer. The variables that should be considered in interpretation of NK activity include the type of effector cell performing the NK activity, the experimental methods used, the histological type of tumour, and the patients age and gender.

It is generally reported that patients with advanced cancer exhibit lower natural cytotoxicity against K562 target cells than patients with localised malignancy or normal individuals (Steinhauer et al, 1982). NK activity has also been shown to be significantly diminished in patients with advanced gastrointestinal tumours compared with patients with local disease and healthy controls (Monson et al, 1987). Surgically treated patients with colorectal cancer who have had potentially curative excision of primary tumours have been demonstrated to have a return in NK activity and cell numbers to nearly normal levels (Whiteside and Herberman, 1989). Other patients despite having very advanced cancers (84% had distant metastases) had levels of NK activity that were similar to age matched controls (Balch et al, 1984). LAK cell activity against NK resistant targets has also been shown to be diminished compared with non-malignant control subjects (Monson et al, 1987).

As for healthy donors great variation in baseline K562 cytotoxicity was observed ranging between 0.0 and 197.2 LU/10^7 PBMC (median 4.4 LU/10^7 PBMC). As observed for the healthy donors Daudi cytotoxicity was substantially lower than K562 cytotoxicity in all cases. No statistically significant difference in levels of K562 cytotoxicity was observed between all 51 cancer patients and all 103 healthy donors. When cancer patients were compared with healthy donors aged $\geq$ 50 years levels of cytotoxicity were also not significantly different (4.4 LU/10^7 PBMC and 2.0 LU/10^7 PBMC respectively). There was no significant difference in levels of K562 cytotoxicity between patients aged < 70 years or $\geq$ 70 years or between males and females. Patients with upper GIT tumours had higher median levels of cytotoxicity (9.3 LU/10^7 PBMC) compared with patients with lower GIT tumours (2.1 LU/10^7 PBMC) although the significance of this finding is uncertain.

Surprisingly patients with liver metastases had higher median levels of cytotoxicity (8.0 LU/10^7 PBMC) compared to those without liver involvement (3.3

LU/10^7 PBMC). This may have been explained by the fact that the patients without liver metastases still had very advanced tumours. That levels of cytotoxicity are maintained in cancer patients is an important observation and suggests optimism for its enhancement in immunotherapy protocols.

A similar pattern of cytotoxicity to that observed among healthy donors was also demonstrated among cancer patients following incubation with mAb. The amount of enhancement with OKT3 and T3 was similar to that observed among healthy donors. T3 resulted in substantially greater enhancement than OKT3. PBMC from 14 cancer patients were incubated with anti-CD16 and median cytotoxicity was enhanced from 0.74 LU/10^7 PBMC to 17.53 LU/10^7 PBMC but because of the large range in cytotoxicity observed post-anti-CD16 (0.1 to 1292.5 LU/10^7 PBMC) this failed to reach statistical significance (p = 0.07). Synergy was not observed between OKT3 and rIL-2 although a partially additive effect was seen. As with the healthy donors enhancement of daudi cytotoxicity was seen with OKT3 and T3 to levels which reflected the low levels of baseline cytotoxicity. OKT3 and IL-2 in combination resulted in a partially additive effect with regard to enhancement of cytotoxicity as was seen in the healthy donors. To conclude it can be stated that K562 cytotoxicity could be manipulated in the same way in cancer patients as in healthy donors.

7.4.2 Analysis of peripheral blood cells.

The white blood cell count was greater in the cancer patients than in the healthy donors due to a neutrophilia (p < 0.05). Patients with liver metastases also had higher levels of circulating neutrophils than patients without liver involvement (p = NS). This may have been a response to necrotic tumour tissue or subclinical infection. The proportion of circulating NK cells was significantly higher (p < 0.05) compared with healthy donors. Mean CD3$^+$NK cells were also significantly higher in the cancer patients compared with healthy donors (15.7% compared with 12.9%). When cancer patients were compared with the elderly healthy donors these trends were maintained but with the exception of a monocytosis were not statistically significant.

Overall the number of circulating LGL in advanced cancer patients has been shown to parallel NK activity (Steinhauer et al, 1982). However, advanced cancer patients with low NK activity showed no decrease in the number of LGL from that of other cancer patients or healthy donors. It was suggested that the defect in NK activity may arise from reduced recycling capacity of NK cells. Balch et al, (1982) demonstrated that levels of NK cells were depressed more significantly in colon cancer patients than among any other cancer type, although they found no difference in levels of NK cells within different stages of disease. LGL determined by HNK-1 (CD57) antigen expression have been shown to correlate with NK function in 66% of healthy donors compared with only 33% of colon cancer patients (Balch et al, 1984). It was concluded that other cell populations such as T cells may contribute more significantly to NK activity

in colon cancer patients compared with healthy donors. CD3+NK cells may also have been responsible for a significant contribution to NK activity in these studies. CTL responses have not been shown to be significantly different between subjects with and without gastrointestinal cancer and patients with advanced gastrointestinal cancer have been shown to possess normal numbers of lymphocytes expressing the CD3 antigen (Monson et al, 1987).

Balch et al, (1982 and 1984) demonstrated a correlation between LGL and age and sex in patients with cancer. Older patients had higher numbers of circulating LGL as did males compared with females. Cancer patients (colon, lung, breast, and head and neck) also had levels of LGL which were significantly lower than healthy age and sex-matched controls. Median levels of LGL were 9% in colon cancer patients compared with 16.5% in age-matched normal individuals (Balch et al, 1984). These findings were not true however, of all tumour types and patients with melanoma and sarcoma did not have deficient levels of LGL compared with age and sex-matched controls. Not all studies have confirmed these findings and circulating NK cell numbers have not been shown to be diminished in patients with advanced malignancies which included colorectal cancer (Monson et al, 1987). Monson et al, (1987) subsequently concluded that because activated NK activity against K562 targets was normal in patients with advanced cancer a defect in the T cell component of LAK precursors may be responsible for the impaired LAK activity in these patients.

In the present studies the proportion of circulating CD3+NK cells were significantly greater (p = 0.04) in the patients with liver metastases (21.9%) compared with patients without liver involvement (11.7%) although there was no difference in the proportion of NK cells. The reason for this is unclear. However, if these cells can be shown to play a role in tumour elimination they may provide a specific target for activation in immunotherapy protocols.

As was observed among the healthy donors, PBMC preparations reflected the proportion of circulating cells present in whole blood with similar levels of contamination by neutrophils and monocytes (< 25%) and relative purification of mean (± SEM) NK cells to $27.0 \pm 2.9\%$ compared with $16.8 \pm 1.3\%$ in whole blood. The % of circulating NK cells significantly correlated with % NK cells in PBMC preparations. NK cells were responsible for the majority of K562 cytotoxicity in patients without liver metastases in whom a significant correlation was seen between % NK cells and K562 cytotoxicity (r = 0.52, p < 0.05) but not CD3+NK cells. However, the relative role of NK and CD3+NK cells in patients with metastases was less clear. In patients with liver involvement NK cells were not correlated with K562 cytotoxicity but were associated with CD3+NK (r = 0.54, p < 0.05). This suggests that these cells may have a more important role in K562 cytotoxicity in patients with liver metastases.

7.5   ADMINISTRATION OF OKT3 TO CANCER PATIENTS.

7.5.1 Introduction.

The situation is likely to be extremely complicated with respect to mAb therapy in cancer patients and the *in vivo* effects are not easily predictable from *in vitro* studies where experimental conditions may limit the multitude of interactions that are observed in the clinical setting. The basis for the administration of anti-CD3 mAb to cancer patients was largely theoretical and it is worthwhile reviewing the limited data available.

Studies into the effects of anti-CD3 mAb (both in humans using OKT3 and in murine studies using the hamster mAb 145-2C11) demonstrated transient T-cell activation *in vitro* as evidenced by proliferation and lymphokine secretion (Newell et al, 1991). T cell activation is also a consequence of *in vivo* administration of anti-CD3 (Hirsch et al, 1989). These workers showed that 40 µg to 400 µg of 145-2C11 administered intraperitoneally to mice resulted in activation of T cells which was followed by T cell depletion, TcR modulation, and ultimately immunosuppression. T cell activation was reflected by increased IL-2Rα expression and enhanced proliferation *in vitro* to both exogenous IL-2 and allogeneic cells. Evidence supporting *in vivo* activation of T cells by OKT3 in humans has recently been presented by Ellenhorn et al, (1990b) in which lymph node T cells from allograft recipients were excised before and after OKT3 and were shown to display increased IL-2Rα expression as well as increased proliferation to exogenous IL-2 and allogeneic cells. Following administration of OKT3 to organ transplant recipients, OKT3 can be demonstrated coating lymph node cells within 2 hours and these cells show increased responsiveness to IL-2 *in vitro* at 6 hours post-OKT3 (Ellenhorn et al, 1990).

In specifically considering cancer therapy Ellenhorn et al, 1988 had previously demonstrated that low doses of 145-2C11 could be exploited to augment the inadequate immune response of mice to a weakly immunogenic malignant UV-induced fibrosarcoma (1591 PRO-4L line). While a relatively high dose of intraperitoneal 145-2C11 (40 µg and 400 µg) was found to be immunosuppressive and was associated with tumour progression, a 4 µg dose was associated with decreased tumour incidence and with the generation of lasting immunity to further tumour challenge. This effect was not mediated through NK cells but through activation of CD8+ cytotoxic T cells which also required the presence of CD4+ helper cells. The immunopotentiating effects resulting in tumour progression may be manifested by increased tumour specific T cells, LAK cells, or the induction of anti-tumour cytokines and in this respect TNF was detected in the serum of anti-CD3 treated mice but not normal controls (Ellenhorn et al, 1988). Studies in humans are limited but metastatic gastrointestinal adenocarcinoma has been previously treated with combinations of polyclonal IgG2a mAb and IFN-γ (Blottierre et al, 1990) although in this study no significant clinical responses were seen.

Following the preliminary results of the *in vitro* studies and theoretical postulates that anti-CD3 mAb are capable of enhancing non-MHC-restricted cytotoxicity *in vitro* and

in vivo in animal models OKT3 was administered to human cancer patients. A 50 μg dose of OKT3 was initially chosen based on the report by Wiseman et al, (1990). Control patients were given 10 mls of normal saline only. In retrospect it may have been more appropriate to administer a non-specific immunoglobulin as a control preparation rather than nomal saline. Previous studies have shown that administration of high doses of intravenous immunoglobulins increase the activity of NK cells and numbers of circulating CD16$^+$ cells in humans (Finberg et al, 1992).

Therapeutic monitoring strategies for OKT3 therapy include measuring the % and absolute numbers of PBL subclass populations (CD2, CD3, CD4, and CD8), serum drug levels, and antimouse antibodies. Anti-mouse antibodies are usually seen 3 to 4 weeks after cessation of therapy although they may occur as early as 8 to 10 days after the onset of therapy. Anti-mouse antibodies were not assessed in these studies because only a single dose of OKT3 was administered and long-term follow up was not possible.

7.5.2 Safety and toxicity.

Significant adverse reactions may be noted during the first days of therapy for transplant rejection and constitute chills, fever, nausea, vomiting, diorrhea, dyspnea wheezing and aseptic meningitis which commence within the first one to two hours of administration (Ortho Multicenter Study Group 1985). Rarely patients develop an aseptic meningitis. Such side effects are termed the first dose reaction and patients have been shown to demonstrate a significant rise in TNF-α levels which correspond with the severity of symptoms as well as elevated levels of IL-2 and IFN-γ which are elevated at 2 hours post-OKT3 (Gaston et al, 1991). In this study patients with more severe reactions also had greater numbers of CD3$^+$ and CD4$^+$ lymphocytes prior to OKT3 administration. It seems likely that TNF-α possibly of T lymphocyte origin is a key mediator of the OKT3 first-dose reaction. This 'cytokine release syndrome' is therefore probably due to T cell activation rather than lysis. T cell clones have been found to be capable of producing TNF-α following stimulation with anti-CD3 and IL-2 (Turner et al, 1987). Cyokines are not released after the second and third injections of OKT3.

Symptoms following low-dose OKT3 administration were qualitatively similar to those seen following 5 mg doses used in transplant therapy. However, they were dose related, transient, and acceptable to patients. Intervention using paracetamol to control rigors was required in only one patient. All side effects had subsided by 8 hours post-therapy. Long-term side effects were not assessed because of problems with patient follow up and short survival times. The long-term consequences of T cell stimulation by OKT3 are also unknown. T cell dysfunction in mice has been shown to persist for a period of time after TcR re-expression when T cells appear to possess impaired CTL and MLR reactivity (Hirsch et al, 1988). The clinical significance of such findings requires further investigation.

7.5.3   OKT3 kinetics.

OKT3 in serum was undetectable even at 15 minutes following injection and was associated with rapid binding to circulating lymphocytes. Even after 5 µg OKT3 approximately 60% of lymphocytes (equivelent to the CD3+ population) demonstrated detectable surface bound OKT3. The 0.5 µg dose was associated with detectable levels of OKT3 on 16.7% and 10.3% of lymphocytes at 60 minutes post-injection in the two individuals studied. OKT3 did not appear to modulate the CD3 antigen when assessed simultaneously with CD2 expression but rather was associated with physical depletion of lymphocytes from the circulation.

Plasma levels of OKT3 are dependent upon the dose and duration of administration and the kinetics of ligand binding and subsequent lymphodepletion (Goldstein et al, 1986). The half-life of OKT3 is about 18 hours (Hooks et al, 1991). The route of metabolism of OKT3 is not clear; it may be removed by opsonization by the reticuloendothelial system when bound to T cells, or by human anti-mouse antibody production which is seen in 85% of patients receiving OKT3. Plasma OKT3 is eliminated predominantly if not exclusively by binding to T cells following initial intravenous injections. There thus appear to be two phases of removal which involve firstly binding to T cells followed in transplant patients by a slower elimination phase after 6 to 10 days of therapy. Following 5 mg doses of OKT3 plasma 24-hour trough levels have been shown to rise over the first 3 days of therapy and reached mean ($\pm$SEM) steady state levels of 902 ± 71 ng ml^{-1}.

In vitro OKT3 blocks the specific killing of tumour targets by cytotoxic T cells at concentrations of 1000 ng ml^{-1}. Whole mAb and F(ab')$_2$ fragments of OKT3 have been shown to effectively coat the TcR complex. However, whole mAb is more effective at modulating the TcR because of the importance of Fc interactions (Woodle et al, 1991). OKT3 induces maximal mitogenesis at a concentration of 10 to 29 ng ml^{-1} (Van Wauwe et al, 1980). Monovalent Fab fragments are 100 times less potent than parent IgG. Quantitative studies using ^{125}I-labelled Fab fragments of OKT3 showed an average of 5.1×10^4 receptor sites per cell. The application of classic pharmacokinetic analysis to the elimination of OKT3 is therefore not appropriate. The optimal dose of OKT3 required to induce activation rather than immunosuppression remains unresolved.

7.5.4   Serum cytokine and sIL-2Rα levels.

Cytokine levels were detected in only one patient (TNF-α at 60 minutes post 50 µg OKT3 injection). *In vivo* injection of the hamster anti-murine CD3 Mab 1452C11 into BALB/c mice has been shown to induce a massive systemic release of cytokines. TNF-α was detected at 90 minutes as well as IL-2 and IFN-γ.(Ferran et al, 1990). Release of cytokines is a transient phenomenon. Elevated levels of TNF-α, IL-2, and IFN-γ are observed in the serum of kidney transplant recipients after the first injection of OKT3

(Abamowicz et al, 1989). TNF-α was noted to peak at 1 hour and IL-2 and IFN-γ at 2 hours post-OKT3.

It is known that NK activity can be strongly enhanced by TNF-α and IL-2 (Ostensen et al, 1987). Limited studies to detect serum factors that may influence cytotoxicity following OKT3 administration using a variation of the ^{51}Cr-release assay and K562 cells and PBMC from a healthy donor were difficult to interpret and did not demonstrate a constant or significant effect by cancer patients serum following 50 μg or 20 μg OKT3. This simple biological assay technique was relatively crude in comparison to the commercial ELISA techniques. There was a depression of cytotoxicity up to 30 minutes following 50 μg OKT3 in 5 of the 6 patients. One patient who developed rigors requiring treatment with paracetamol demonstrated early enhancement of cytotoxicity at 30 minutes but the patient with elevated TNF-α levels did not. TNF-α may not therefore be the factor involved.

Studies were not undertaken to compare the effect on K562 cytotoxicity of serum from cancer patients with that of healthy donors although a soluble factor produced by colon cancer cells has been shown to moderately suppress mitogen-induced T cell proliferation (Ebert et al, 1990). This factor also impaired the development of LAK cells when present during the incubation of PBMC with IL-2. It did not inhibit the lysis of target cells by either NK or LAK however, because these events are not dependent upon lymphocyte proliferation. Such factors may favour tumour survival in the host and further work on serum factors in cancer patients is required.

Levels of sIL-2Rα were not increased over pre-treatment values in the present studies. Quantification of lymphocyte activation by measuring sIL-2Rα levels may give insight into immunological dynamics following immunotherapy (Windsor et al, 1991). Human PBMC have been shown to release sIL-2Rα following OKT3 which is dependent on cellular activation and the release of sIL-2Rα appears to be a characteristic marker of T lymphocyte activation (Rubin et al, 1985). Quantitation of sIL-2Rα may therefore be a useful laboratory measure of *in vivo* immune system activation. Studies have suggested that sIL-2Rα levels offer a rapid, reliable, and non-invasive measure of disease activity and response to therapy (Rubin and Nelson, 1990).

7.5.5   <u>Effect of OKT3 on peripheral blood cells.</u>

During the initial 60 minutes following 50 μg OKT3 a significant decline in circulating neutrophils, monocytes, and lymphocytes was observed. Neutrophil levels had recovered by 240 minutes and remained elevated at 480 minutes although the decline in lymphocytes and monocytes persisted up to 480 minutes. A modest decline in mean circulating CD3+ lymphocytes and NK cells was also observed in the control patients at 60 minutes following normal saline which had partially recovered at 240 minutes and fully recovered at 480 minutes. NK cells in the control patients were more susceptible to

this fall which remained below pre-normal saline values at 480 minutes. OKT3 induced a similar rate of decline in both $CD4^+$ and $CD8^+$ T cells.

The rate of decline of circulating $CD3^+$ lymphocytes following OKT3 was dose-related being initially rapid following 50 μg OKT3 and demonstrating no sign of recovery at 480 minutes. Partial recovery was seen following the 20 μg to 5 μg doses at 8 hours and 24 hours. NK cells demonstrated a similar pattern with a dose related fall in circulating numbers. Although NK cells remained substantially below pre-50 μg levels at 8 hours a mean rebound lymphocytosis was seen at 24 hours in the 5 μg treated patients.

The most important observation was that the decline in lymphocytes following OKT3 was unrelated to CD3 antigen expression. That a systemic factor released from activated lymphocytes may be responsible for changes in circulating blood cells was supported by the studies of Ulich et al, (1987). TNF-α administration to rats resulted a marked neutrophilia and lymphopaenia within 90 minutes which had returned to normal by 24 hours. These findings are identical to the changes in circulating lymphocytes that occur following administration of OKT3. Therefore stimulation of TNF-α secretion is likely to contribute to early haematological changes following OKT3.

7.5.6 Effect of OKT3 on cellular cytotoxicity.

Describing the effect of OKT3 on cellular cytotoxicity was complicated by the small number of patients in each treatment group and the large variability in pre-treatment levels between individuals. A semi-quantitative analysis involving individual patients was performed because meaningful statistical analysis was not valid. The conclusions that could be drawn were therefore limited. Following 50 μg OKT3 a progressive and significant decrease in levels of K562 cytotoxicity was observed up to 240 minutes in comparison to both pre-treatment levels and the control patients. The control patients also demonstrated an initial decline in K562 cytotoxicity which had recovered by 240 minutes and this may have been related to the decline in circulating NK cells. Cytotoxicity was enhanced by OKT3 and T3 *in vitro* which was proportional to baseline values obtained in culture medium. No increase in cytotoxicity was observed against daudi targets because the period of study was too short (240 minutes) to induce LAK activity.

The effect of the lower doses of OKT3 were highly variable. Mean K562 cytotoxicity was significicantly enhanced at 24 hours following 5 μg OKT3 compared to pre-treatment levels. K562 cytotoxicity was also enhanced at 24 hours following 20 μg OKT3 although this failed to reach significance. The patients receiving 10 μg OKT3 demonstrated a mean decline in K562 cytotoxicity at 24 hours compared with pre-treatment levels. Studies at 24 hours would have been useful in the patients receiving 50 μg OKT3. It appears that OKT3 is capable of enhancing cytotoxicity 24 hours following administration however, further studies are required to determine the optimal dose.

7.5.7 Relationship between peripheral blood cells and cytotoxicity.

The influence of OKT3 on K562 cytotoxicity as assessed by the ^{51}Cr-release assay depends upon a combination of its ability to activate lymphocytes and to influence the proportion of circulating cells. Insufficient PBMC were obtained from some of the patients receiving 50 μg OKT3 to perform assays from a maximum E:T ratio of 40:1. As previously demonstrated this reduces cytoxicity as measured in lytic units in its own right. However, although this experimental artefact of the assay may account for some of the decline in cytotoxicity observed it is unlikely to account for it all. The relative proportion of lymphocytes in peripheral blood remained approximately the same following 50 μg OKT3 which was presumably reflected in the PBMC preparations (although this was not specifically analysed in this group). The decline in cytotoxicity may be explained by the selective removal of that part of the NK cell population which is capable of mediating most of the K562 cellular cytotoxicity. It has already been noted that NK cells are highly heterogenous with respect to levels of activation and killing capacity. This is the chief reason why simple measurement of NK cell numbers is not an adequate means of assessing NK activity.

Following a qualitative description of NK cells and K562 cytotoxicity for individual patients, changes in K562 cytotoxicity generally reflected changes in numbers of circulating NK cells. It seems likely that over short periods of time the influence of OKT3 *in vivo* is principally mediated by its ability to influence the availability of active circulating NK effectors possibly by altering their patterns of migration. Cellular activation may have a more important role following more prolonged exposure to OKT3.

7.6   CONCLUSIONS AND PROSPECTS FOR FUTURE RESEARCH.

Techniques for antibody engineering are now overcoming many of the problems that have prevented mAb being used routinely in clinical practice (Hawkins et al, 1992). Effective therapy using unmodified mAb has been elusive . Recently mAb therapy has been revolutionized by advances in genetic engineering (Waldmann, 1991). Engineering of antibodies by even a single amino acid substitution in the Fc receptor binding segment of mAb has a profound effect on modulating the biological properties of the antibody (Alegre et al, 1992). This may be a useful approach in enhancing the clinical effects while reducing unwanted side effects. Most anti-human CD3 mAb are directed at the ε subunit of the CD3 complex which suggests that in genetically engineering mAb the Fc fragment is the appropriate site (Transey et al, 1989).

The development of host antibodies to OKT3 was not assessed in these patients and deserves further consideration in view of the probable need for repeated infusions or injections of OKT3 in immunotherapy protocols. Xenogenic antibodies are highly immunogenic in man. Jaffers et al, (1986) demonstrated that anti-OKT3 antibodies appeared in 50% of transplant patients peaking at 20 to 33 days after the last dose of OKT3. These antibodies interfered with the therapeutic effectiveness of OKT3 in at least

one instance. However, although chimaerization of antibodies can diminish HAMA responses, attention also needs to be payed to the variable region which is also immunogenic (Bruggemann et al, 1989). Host antibodies to both the isotype and idiotype developed 2 to 3 weeks after therapy in 86% of patients receiving OKT3 alone but in significantly fewer (39%) patients receiving concomitant immunosuppression. Antibodies to OKT3 have not been shown to produce significant symptoms of hypersensitivity, anaphylaxis, or serum sickness so that their main significance is their potential for reducing levels of OKT3.

Chimaeric antibodies therefore have at least two advantages over mouse antibodies (Riechmann et al, 1988). Firstly effector functions can be tailored as desired, eg. human IgG1 and IgG3 appear to be more effective for complement and cell-mediated lysis and therefore for killing tumour cells. Secondly human antibodies should minimise the anti-globulin response during therapy by avoiding anti-isotypic antibodies since the most immunogenic part of the antibody is the Fc region. After a single infusion of a non-specific murine mAb the plasma half life generally varies between 20 and 24 hours although great variation is observed (Mellstedt et al, 1991). Chimaeric (mouse/human) mAb have a longer plasma half-life of between 5 and 6 days and a reduced incidence of HAMA.

There is a close association between levels of NK activity and the ability to eliminate circulating tumour cell emboli (Hanna, 1992). NK cells have been shown to reduce the number of metastases in mice following injection of tumours (Gorelik et al, 1982). The ability to activate NK cells may provide a basis for therapy in the control of tumour metastases. Activation of such host effector mechanisms may prove more efficient in destroying circulating tumour cells and preventing heterogenous metastatic spread of malignant neoplasms even though they are inneffective in causing regression of established primary tumours. Single tumour cells or small clumps entering the circulation may be more susceptible to destruction by immune mechanisms and mechanisms that are active in blood may not operate within a primary solid tumour mass. This suggests that even if protocols incorporating anti-CD3 mAb fail to induce regression of advanced tumours they may have a role in the adjuvant therapy of cancer.

There remains a considerable need for assays of cell-mediated immunity to human tumours which can be used reliably to monitor the course of disease in cancer patients. The interest in studying NK activity in cancer bearing humans was generated largely by data in selected experimental systems suggestive of a role of NK cells in surveillance to metastasis and in mode of action of immunomodulatory agents. There are often considerable problems associated with the use of the NK assay to monitor therapeutic interactions which are largely related to the spontaneous fluctuation of the assay, particularly prominent in cancer patients (Introna and Mantovani, 1983). To specifically define the role of NK cells in the prognosis, diagnosis, and pathogenesis of human disease further it will be necessary to monitor human NK cell function and numbers in

both health and disease on a regular basis rather than under the special circumstances dictated by a particular research interest.

Although leucophoresis yields and numbers of LAK cells generated in culture correlates with peak lymphocyte counts achieved with IL-2, neither tumour reduction or clinical toxicity have been shown to correlate with the dose of IL-2, cytolytic activity of LAK cells or with other laboratory parameters which include baseline lymphocyte counts and rebound lymphocytosis (Boldt et al, 1988). There remains a need to identify other laboratory and clinical parameters more predictive of the likely outcome of immunotherapy and its toxicity in general.

Augmentation of NK activity in the blood compartment is not necessarily representative of changes at the tumour site and studies of the eventual destination of NK effectors leaving the blood have obvious bearing on the monitoring of BRM in humans. Interpretation of data is hampered by a lack of information concerning whether and how NK cells leave the bloodstream and enter neoplastic tissues. Moreover freshly isolated tumour cells are important in obtaining *in vitro* data of *in vivo* significance.

Future studies examining the site of a tumour rejection response may give very useful information as to which of the potential effector cell types are actually represented there, although the presence of a particular cell type does not necessarily mean that it is critical to the rejection process. It is unclear how blood NK activity relates to tissue levels of reactivity. The number of mononuclear cells in the peritumoural stroma of colorectal cancers has been shown to be significantly higher in 5-year cancer-free survivors compared to patients who are dead from cancer within 5 years after operation (Svennevig et al, 1984). It is however speculative whether tumour antigenicity or tumour necrosis is responsible for attracting mononuclear cells to the tumour site.

Some idea of the relative effectors can be obtained by the study of constituents of TIL (Itoh et al, 1986 and 1988; Muul et al, 1987). The percentage of TIL from different cancers vary widely before IL-2 therapy and there is considerable diversity with regard to lymphocyte infiltration, phenotypic expression, and functional capacity (Balch et al, 1990). The functional correlation of TIL with properties of different tumour cells is important in studying the host-tumour relationship. Breast cancer, colon cancer, and sarcomas do not appear to elicit as vigorous an immune response in terms of numbers of TIL present or their cytotoxic efficiency in contrast to melanomaand renal cell carcinoma. These observations are consistent with the clinical observation that melanomas and renal cell carcinomas are more likely to undergo spontaneous regression and respond to IL-2 therapy. The low levels of NK activity of tumour-associated lymphoid cells are primarily related to a low frequency in the relevent effector cells at the tumour site although suppression of the *in vitro* maintenance of cytotoxicity by in situ macrophages and lymphocytes has been described in a few patients (Introna and Mantovani, 1983).

Future development of protocols combining IL-2 with mAb may provide effective cancer immunotherapy. T cells obtained from peripheral blood after IL-2 therapy have

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been shown to display decreased responses to mitogens and alloantigens, and do not proliferate *in vitro* in response to further IL-2, or mediate non-MHC-restricted cytotoxicity or even targeted lysis in the presence of bispecific mAb (Weil-Hillman et al, 1992). These T cells are however not irreversibly inactivated and could be activated *in vitro* by anti-CD3 mAb together with IL-2. It can be concluded that T cell lytic function is partially inhibited by IL-2 therapy. Exposure of T cells to anti-CD3 mAb prior to *in vivo* IL-2 treatment generates T cell lytic activity *in vitro* and suggests that a combined *in vivo* protocol of anti-CD3 Mab and IL-2 giving anti-CD3 first may cause activation of T cells in addition to activation of NK cells. Just as the most effective chemotherapy seems to involve combinations of independently acting cytotoxic agents, a multimodal immunologic attack would be more therapeutic than a single immune mechanism. Thus a combined approach involving both NK and T cell activation coupled with infusions of intact mAb may be more effective than NK cell activation with IL-2 alone.

Fianally two properties of anti-CD3 Mab may eventually limit their clinical application for potentiating host immune responses. Firstly there is a very fine balance between the activating and suppressive properties of anti-CD3 mAb and secondly at present the long term effects on T-cell function are unknown.

The following is a summary of the conclusions that can be drawn from this work.

1. The ^{51}Cr-release assay is a reproducible method for assessing cellular cytotoxicity *in vitro* provided it is highly standardised in the laboratory with attention to the many technical variables which may influence results.

2. NK activity shows considerable individual variation within both cancer patients and healthy donors and significant levels are maintained even in the presence of advanced malignancy.

3. NK activity may be manipulated over short periods of time using mAb and rIL-2.

4. Anti-CD3 mAb are capable of enhancing K562 cytotoxicity mediated by fresh PBMC *in vitro* among both cancer patients and healthy donors.

5. The mechanism of enhancement of cytotoxicity by mAb *in vitro* remains unproven but is likely to involve enhanced conjugation between effector cells and target cells possibly mediated via Fc receptor interactions. Indirect activation of NK cells secondary to cytokine release by directly activated T cells cannot be excluded. The role of lymphocytes expressing both the CD3 antigen and NK cell markers (CD3$^+$NK) remains unproven.

6. Combinations of mAb may provide a means of enhancing cytotoxicity further although in the limited experiments presented the effect was only partially additive rather than synergistic and their possible role *in vivo* is highly speculative.

7. The relevance of *in vitro* modulation of NK activity by OKT3 to its effects observed *in vivo* are uncertain. Further studies are required to assess its effect on lymphocyte migration and events at tumour level.

8. OKT3 may be safely administered to cancer patients and rapidly influences NK activity in peripheral blood in a dose-dependent manner probably mediated through retrafficking of circulating lymphocytes.
9. The therapeutic efficacy of OKT3 administration in cancer patients remains unproven.
10. Further studies are required to investigate the optimal dose, mode of administration, and possible synergy with other agents in the development of an effective therapeutic protocol incorporating OKT3.

APPENDIX A
MONONUCLEAR CELL PREPARATION

Materials:
1)   Hanks balanced salt solution. (HBSS, Imperial Laboratories Ltd.).
2)   25 mls universal containers (Media, Ltd.).
3)   Ficoll-Hypaque (Pharmacia Ltd.).
4)   Preservative-free heparin (C.P. Pharmaceuticals Ltd.).
5)   10 mls pipettes (Costar Ltd.).
6)   Automatic pipette (Sarstedt Ltd.).
7)   Digital pipette (Jencons Ltd.).
8)   1 X RPMI 1640 tissue culture medium (Northumbria Biologicals Ltd.(NBL))
        supplemented with 10% heat inactivated foetal calf serum (NBL), L-glutamine
        (2mM) (Imperial Laboratories Ltd.), and HEPES (10mM) (NBL) hereafter
        referred to as complete tissue culture medium.
9)   Invert fluorescence microscope (E. Leitz Ltd.).
10)   HM-LUX light microscope (E. Leitz Ltd.).
11)   Whirlimixer (Fisons Scientific Equipment Ltd.).
12)   Gentian violet (Hopkin and Williams Ltd.).
13)   Acridine Orange and Propidium Iodide (Sigma London Chemical Co.Ltd.).
14)   25 cm^2 tissue culture flask (Nunc Ltd.).
15)   Haemocytometer (Richardsons Ltd.).
16)   30 cm rotor centrifuge (Minifuge GL, Haraeus Equipment Ltd.).

Methods:
A.1 PBMC Separation
All separations were performed at room temperature.

(1) Between 10 and 50 mls of blood was obtained by venepuncture of a forearm vein
using a 21 G needle and a tourniquet, and gently mixed in a 25 cm^2 tissue culture flask
containing 500 units of preservative free heparin. Samples were held at room
temperature prior to processing.

(2) 10 mls of F-H (Pharmacia) was pipetted into 25 ml universal tubes.

(3) The blood was diluted with an equal volume of HBSS and 15 mls was carefully
layered onto the F-H.

(4) The tubes were spun at 500 g in a centrifuge for 20 minutes.

(5) This resulted in a sharp white band of mononuclear cells between the upper plasma layer and the slightly opaque layer of F-H. The red cells and granulocytes formed a pellet at the bottom of the tubes (Plate 2).

(6) Using a 10 mls pipette and automatic dispenser the mononuclear cells were removed from each universal container, taking care to avoid the F-H layer and were transferred to another universal container to which was added HBSS to a total volume of 20 mls. The samples were spun at 650 g for 20 minutes.

(7) The supernatants were decanted and the cell pellets resuspended and combined in a single universal container containing 20 mls of HBSS. Cells were washed twice in HBSS at 400 g for 10 minutes

(8) The supernatant was discarded and the pellet was resuspended in 2 ml tissue culture medium.

A.2 Cell Counting

(1) An "Improved Neubauer" haemocytometer was used and the cells were introduced beneath the coverslips. Cells were counted using a light microscope.

(2) A stock solution of white blood cell counting fluid was made comprising gentian violet in a 2% acetic acid solution and stored at room temperature. Erythrocytes are lysed by acetic acid and the lymphocyte nuclei are stained blue by gentian violet.

(3) For visualization of PBMC 50 μl of the cell suspension was added to 450 μl of counting fluid in a 5 mls plastic tube and was gently mixed in a Whirlimixer.

(5) Cells were counted at a X 100 magnification (10 X ocular and 10 X objective lens) in the 1 mm X 1 mm area formed by the centre of the cross. Cells which touched the upper and left-hand perimeters were included but cells which touched the lower and right hand perimeters were excluded.

(6) The number of PBMC per ml of the original undiluted suspension is given by the formula:

$$\text{No. cells } (10^6)/\text{ml} = n/100 \text{ X y}$$

Where n = cells counted

y = the dilution factor

In the case of PBMC determination y = 10. y = 1 for target cells which did not require staining for visualization and were undiluted prior to counting.

(7) The formula is based on the known dimensions of the haemocytometer. The centre square (comprising 25 squares) has an area of 1 mm². When the coverslip is pressed down over the grid so that interference patterns appear, the depth of the chamber is 0.1mm. The total volume of the large square is therefore

$$1 \times 1 \times 0.1 = 0.1 mm^3$$
$$= 10^{-4} ml$$

The total number of cells counted is n in a volume of 10^{-4}ml therefore the number of cells expressed as millions per ml is n/100 X y. Counting was performed three times for each sample and the mean obtained.

(8) To perform a standard assay in triplicate at 4 dilutions from a highest effector: target ratio of 40:1 required 2.4 million PBMC.

A.3 Assessment of Viability by Fluorescence Microscopy

(1) A stock solution of acridine orange and propidium iodide (AO/PI) was made according to the formula shown below and frozen in 1 ml aliquots.

> 15 mg acridine orange (AO)
> 50 mg propidium iodide (PI)
> 1 ml 95% ethanol
> 45 ml PBS

(2) Stock solution was thawed prior to use, diluted to 1:10 in PBS and stored at 4°C in a dark bottle for up to one month.

(3) 0.2 mls PBMC at 8×10^6 ml^{-1} were added to one well of a 96 well microtitre culture plate to which was added 2µl AO/PI. Target cells were prepared in identical fashion except that they were stained prior to resuspension at 10^5 ml^{-1}.

(4) Following 5 minutes incubation at room temperature, cells were gently mixed by agitating the plate and were introduced under the coverslip of an 'improved Neubauer' haemocytometer following which they were examined at 100 X magnification (using a Leitz Diavert Inverted Microscope with a 450 (blue) /490 (green) nm excitation filter. All cells incorporate acridine orange and appear green when visualised using the blue excitation filter. Non viable cells incorporate only propidium iodide and appear red when visualised using the green excitation filter (Plates 3 and 7). Cells were counted in the four 1 mm X 1 mm corner squares and the mean count calculated. A minimum of 100 cells was counted Percentage cell viability was expressed by the formula

$$\% \text{ non-viable cells} = \frac{\text{Red (non-viable) cells}}{\text{Green (viable and non-viable) cells}} \times 100$$

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APPENDIX B

ASSESSMENT OF CELL-MEDIATED CYTOTOXICITY

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<u>Materials:</u>

1)   All cells used in these studies were cultured under sterile conditions at $37^{\circ}C$ in complete RPMI 1640 tissue culture medium (NBL Ltd.) in a humidified atmosphere of 5% CO_2 and 95% air and were prepared and maintained in the Department of Surgery Transplant laboratories.

        Prophylactic antibiotics were not routinely used.

        The following cell lines were studied.

        K562 - an NK cell sensitive erythroleukemia cell line.

        Daudi - an NK cell resistant, LAK cell sensitive Burkitt lymphoma line.

        A549 - an epithelial cell line.

        HUVECS - Human umbilical vein endothelial cells isolated enzymatically from the vein lining of human umbilical cord.

        LCL - a lymphoblastoid cell line derived from a renal patients' lymphocytes (undergoing transplantation) and transformed by Epstein Barr virus to confer proliferative immortality.

        Prostate tumour cell line (generous gift of Dr. E. Kehinde, Dept of Surgery, LGH).

2)   K562, Daudi, and A549 cell cultures were originally obtained from the European Collection of Animal Cell Cultures (ECACC) (Porton Down, Salisbury, UK). HUVEC and the LCL cell lines were prepared in the Department of Surgery Laboratories

3)   75 cm^2 tissue culture flasks and 175 cm^2 megaflasks (Costar).

4)   Mycoplasma detection kit (enzyme immunoassay) (Boehringer Mannheim Ltd.).

5)   Ca/Mg free HBSS (Imperial Laboratories Ltd.).

6)   0.5% trypsin and 0.2% EDTA in PBS (NBL Laboratories Ltd.).

7)   Sodium ^{51}chromate (Amersham Ltd.).

8)   24 well flat-bottomed tissue culture flask with lid (Nunc Ltd.).

9)   1280-ultragamma gamma-counter (Plate) (LKB Ltd.) Plate 6.

10) CO_2 Gased Incubator (Flow Laboratories Ltd.).

<u>Methods:</u>

<u>B.1 Maintainance of Target Cells</u>

(1) K562, Daudi, and LCL cell lines were maintained at between 0.3 and 0.9 X $10^6 ml^{-1}$ in a 75 cm^2 tissue culture flask (Costar) HUVEC and A549 cells were grown as an adherent monolayer in 175 cm^2 megaflasks (Costar) and required detachment prior to passage and use in assays.

(2) Cells were passaged every 2 to 3 days by resuspending in fresh medium after centrifugation at 600 g for 4 minutes. Cultures were regularly monitored for mycoplasma infection using a commercially available assay kit (enzyme immunoassay) (Boehringer Mannheim Ltd.).

(3) Prior to and following ^{51}Cr-labeling, a suitable aliquot of cells was removed from stock culture counted and viablity assessed using fluorescence microscopy (Appendix A-iii). Viable cells routinely exceeded 95%.

B.2 Preparation of Adherent Cells

(1) The overlying culture medium was discarded and adherent cells were rinsed once with approximately 20 - 30 mls Ca/Mg free HBSS.

(2) A 1 ml solution of trypsin and EDTA made up to 10 mls in Ca/Mg free HBSS was added to the megaflask and incubated for 5 minutes at 37°C in a 5% CO_2 air mixture.

(3) Cells were checked for detachment into individual cells and added to 20 ml complete tissue culture medium and centrifuged at 200 g for 5 minutes at room temperature.

(4) The supernatant was discarded and the cells resuspended in 10 mls complete tissue culture medium.

B.3 ^{51}Chromium Labelling of Target Cells

(1) Between 3×10^6 and 5×10^6 cells were washed in tissue culture medium by centrifugation at 200 g for 10 minutes. Cells were resuspended in approximately 1ml tissue culture medium in a 24 well culture plate (Nunc) to which was added between 5 and 10MBq ^{51}Cr. The plate was then either incubated for a minimum of 2 hours or if possible overnight (approximately 18 hours) at 37°C in a 5% CO_2 air mixture.

(2) The suspension was transferred to a 25 ml universal container with a screw top (Media) and washed three times in tissue culture medium by centrifuging at 200 g for 10 minutes.

(3) After the final wash cells were resuspended in 5 mls complete tissue culture medium, were counted (Appendix A-ii), and were resuspended at a final concentration of 10^5 ml^{-1}.

(4) Total incorporation was measured for 6 samples in a gamma-counter by measuring the radioactivity emitted from a 50μl aliquot (5000 cells) placed in a minicentrifuge tube.

B.4 The Four Hour ^{51}Chromium-Release Assay

Materials:

1)   96 well round-bottomed microtitre culture plate (Plate)with lid (Nunc Ltd.).
2)   Multichannel digital pipette (Flow Laboratories Ltd.).
3)   Microcentrifuge tubes (Elkay Ltd.).
4)   Nonidet P-40 detergent (Sigma Chemical Co. Ltd.).

Methods:

(1) PBMC were separated from heparinised blood as previously
described (Appendix 1.1).

(2) The final cell pellet was suspended in tissue culture medium, counted (Appendix B-
ii) and then resuspended by further dilution with HBSS at a concentration of 8 X 10^6
ml^{-1}. When insufficient yields of PBMC were obtained to obtain a dilution of < 8 X 10^6
ml^{-1} in 2 mls, cells were corrected to 4 X 10^6 ml^{-1} and assays performed at four E:T
ratios from 20:1. All experiments were performed in triplicate.

(3) 50μl of complete tissue culture medium was added to each well in rows B to E of a
96 well round bottomed microtitre culture plate (Plate 5) using a digital multichannel
pipette.

(4) To the first two rows of the culture plate (A and B) was added 50μl of suspended
PBMC in triplicate using a digital multichannel pipette.

(5) PBMC were then serially diluted by transferring 50μl of the contents of row B after
thorough mixing by repeated pipetting, into row C and then repeating this manouvre to
transfer 50μl from row C to row D. The 50 μl from row D was discarded. Row E
contained no PBMC and was used to measure of background release (BG).

(6) 50μl of culture medium was then added to each well in the first five rows (Rows A to
E).

(7) Target cells were prepared to a cell suspension of 10^5 ml^{-1} (Appendix B3), placed in
a petri dish and 100μl was pipetted into each well in rows A to E of the culture plate.
Constant mixing of cells by gentle agitation was necessary to avoid settling during
pipetting.

(8) 100μl of target cells was added to three wells in the last row and to this was pipetted 100μl of NP-40 to induce complete lysis and provide a measure of maximum ^{51}Cr release (MR).

(9) The lid was located on the culture plate which was incubated at 37°C in a humidified 5% CO_2 air mixture for four hours.

(10) The plate was then centrifuged at 200 g without breaking and 100μl of supernatant removed from each well, and transferred to a microcentrifuge tube taking care not to disturb the cell pellet at the bottom.

(11) The amount of radioactivity in the supernatant was then measured over a minute in a gamma counter (Plate 6)

(12) Percentage specific target cell lysis was then calculated from the formula

Lysis (%) = $\dfrac{\text{release in sample (CPM) - spontaneous release (CPM)}}{\text{maximum release (CPM) - spontaneous release (CPM)}}$ X 100

(13) Results are calculated on the basis of lytic curves, established by plotting the percentage of specific lysis for each effector to target cell (E:T) ratio (Pross et al, 1981). Logarithmic conversion of E:T results in a linear correlation and the number of PBMC required to result in lysis of a given percentage of target cells can be calculated. Results are expressed as LU/10^7 PBMC, where 1 LU is arbitarily defined as the number of PBMC required to obtain 30% specific lysis of target cells.
Lytic units are calculated from the equation

No. of lytic units (LU)/10^7 PBMC = $\dfrac{10^7}{T \times X_p}$

where T = No. of targets (10^4)

X_p = the effector:target ratio at which 30% of target cells are lysed.

The calculation depends upon the curve being linear and correlation coefficients (r) were usually > 0.98. Assays were discarded if r was < 0.95. Assays were also discarded if spontaneous release exceeded 10% of maximal release or if maximal release was less than 4000 CPM.

APPENDIX C
IN VITRO MANIPULATION OF CELL-MEDIATED CYTOTOXICITY

Materials:
1)   Recombinant interleukin 2 (Proleukin) was purchased from Eurocetus Ltd.
2)   Details of antibodies studied are shown in Table C1.
3)   Sodium azide (Na3NH) (Sigma Chemical Co. Ltd.).

Methods:
(1) PBMC were separated and serially diluted in a microtitre culture plate as described (Appendix 1 and 2).

(2) MAb (Table C.1) or rIL-2 were freshly reconstituted in tissue culture medium on the day of assay to a known isotype concentration. Serum was thawed completely at room temperature on the day of assay.

(3) Columns 1 to 3 were prepared with tissue culture medium (Appendix B-iv (6). 50 μl of mAb, rIL-2 solution, or undiluted serum was added to the appropriate wells in triplicate in place of tissue culture medium to columns 4 to 6, 7 to 9 etc.

(4) The lid was located on the culture plate which was then incubated at 37°C in a humidified 5% CO_2 air mixture for 0, 30, 60 and 120 minutes in initial experiments and for 30 minutes in subsequent experiments when this time was found to be optimal.

(5) Target cells were added and the assay completed (Appendix B-iv (7) to (14)).

(6) The effect of mAb and rIL2 on cytotoxicity was compared with baseline cytotoxicity obtained from the first three columns in which cells were incubated with tissue culture medium alone.

Table C1 - Antibodies studied in the cytotoxicity assay.

Antibody	Source	Subclass	Initial conc	CD antigen
OKT3	Cilag Biotech	murine IgG2a (κ)	1mg/ml	CD3
T3	Dako	murine IgG1 (κ)	1mg/ml	CD3
mCD7	Sandoz*	murine IgG2	4.4mg/ml	CD7
hCD7	Sandoz*	human IgG1	7.0mg/ml	CD7
mhOKT3	Gift (*EGR)	h monovalent OKT3	1mg/ml	CD3
T11	Dako	murine IgG1 (κ)	137μg/ml	CD2
Leu-19	Dako	murine IgG1 (κ)	50μg/ml	CD56
TCR-zeta	Coulter	murine IgG1 (κ)	1mg/ml	Zeta chain
Anti-CD4	Dako	murine IgG1 (κ)	21μg/ml	CD4
Anti-CD8	Dako	murine IgG1 (κ)	65μg/ml	CD8
Anti-ICAM	British Biotech	murine IgG1	1mg/ml	αICAM-1 (CD54)
Anti-VCAM	" "	murine IgG1	1mg/ml	αVCAM-1
Anti-ELAM	" "	murine IgG1	1mg/ml	ELAM-1
Anti-PECAM	" "	murine IgG1	1mg/ml	αPECAM
mIgG1	Sigma	murine IgG1 (k)	1mg/ml	polyclonal
mIgG2a	Sigma	murine IgG2a (k)	1mg/ml	polyclonal
hIgG1	Sigma	human IgG1	1mg/ml	polyclonal
mIgG	Sigma	Pooled murine IgG	1mg/ml	polyclonal
Anti-CDw32	Serotec	mouse IgG2a	0.2mg/ml	CDw32 (IgGγRII)
Anti-CD64	Serotec	mouse IgG1	50μg/ml	CD64 (IgGγRI)
Anti-CD18	Serotec	rat IgG2b	2mg/ml	CD18 (LFA-1)
Anti-CD16	serotec	rat IgG2b	50μg/ml	CD16 (FcRγIII)

*Gift

*EGR Generous gift of Dr E. G. Routledge

<u>APPENDIX D</u>
<u>FLOW CYTOMETRY</u>

<u>Materials:</u>

1) FACScan flow cytometer with Hewlett packard computer and Simulset software (Becton-Dickinson UK Ltd.) (Plate 8).

2) Fluoresceine-confugated and unconjugated antibodies are shown in Table D1.

3) 5 mls bottles containing EDTA (L.I.P. Equipment and Services Ltd.).

4) Phosphate buffered saline (PBS X 20) (Mercia Diagnostics Ltd.).

5) Sheath Fluid Isoton II (Coulter Electron Ltd.).

6) FACs lyse solution (10 X) (BD).

7) Ultrapure water (0.22 μ filtered water in an Elgastat Spectrum (11091), Elga Ltd.).

8) FACScan Tubes (12mm X 75mm) (Elkay Products Inc.).

9) Sodium azide (Sigma Chemical Co. Ltd).

10) Paraformaldehyde (Sigma Chemical Co. Ltd).

<u>Methods:</u>

(1) PBC in whole blood and PBMC separated on F-H were both analysed following the same procedure. The whole blood lysis method was employed

(2) 5 mls of blood was collected into a glass bottle containing EDTA as the anticoagulant and gently mixed. PBMC were prepared as described (A-i) and resuspended in complete medium to between 8 and 4 X 10^6 ml^{-1}.

(2) A manual WBC was performed by diluting 50μl whole blood (taking care to wipe excess blood from the pipette tip) in 450μl of WBC counting fluid. Cells were counted in 4 large grids on an 'improved neubauer' haemocytometer as described in the Appendix (A-ii) and the mean WBC calculated.

(3) FACScan test tubes were labelled in the following way
    a) No addition
    b) Control-SL
    c) Leucogate
    Subsequent tubes were labelled according to the mAb used.

(4) 50μl of whole blood or PBMC suspension were added to each tube.

(5) 20μl of fluorochrome conjugated monoclonal antibodies were added to tubes as labelled except for the first tube.

(6) Tubes were vortexed on a whirlimixer to mix cells and were incubated for 25 minutes at room temperature in the dark.

(7) 2 mls of FACs lyse (10 X) solution diluted 1 in 10 with ultra pure water was added to each tube which were vortexed again to mix, and incubated for a further 10 minutes at room temperature in the dark.

(8) Tubes were centrifuged at 650 g for 5 minutes at room temperature, the supernatant was decanted and the cell pellet vortexed to resuspend cells.

(9) 3 mls of 0.1% PBS/azide solution (prepared by adding 1g sodium azide to 1 litre of PBS (prepared by diluting 50 mls of 20 X PBS concentrate in 950 mls of ultra pure water) was added to each tube.

(10) Tubes were centrifuged at 650 g, for 5 minutes at room temperature.

(11) A stock solution of 200 mls 1% Paraformaldehyde was prepared. 180 mls of PBS was heated to 70°C to which 2g of paraformaldehyde was added. The solution was allowed to cool, topped up to 200 mls and filtered through a 0.45μ filter.

(12) The cell pellet was resuspended in 0.225 ml of the 1% Paraformaldehyde in PBS solution and tubes vortexed again.

(13) Samples were usually processed immediately or were stored at 4°C in the dark and analysed later the same day.

Calculation of Results

(1) The FACScan was set up using the parameters stored on the computer disk for whole blood (Simulset software).

(2) The tube containing the leukogate antibody was inserted into FACScan and 10000 ungated events were collected. The Leukogate data was then used to manually set the lymphocyte gate.

(3) The remaining tubes were analysed on this lymphocyte gate and 5000 cells counted for each sample.

(4) The absolute lymphocyte count was obtained from the total whole blood white cell count and the three way differential count obtained from the ungated Leucogate sample which allowed separation of lymphocytes, monocytes, and neutrophils. From this differential WCC absolute numbers cells bearing specific markers were calculated.

Table D1 - Monoclonal Antibodies Used For Flow Cytometry.

Antibody	CD	IgG class/subclass	Fluorochrome	Assigned Specificity
H Le-1[a]	CD45	Mouse IgG1,κ	FITC	Leucocytes
Leu-M3	CD14	Mouse IgG2b,κ	PE (Simultest prep)	Monocytes/macrophage
Leu-4[a]	CD3	Mouse IgG1	FITC	T cells
Leu-11c	CD16	Mouse IgG,κ	PE	NK cells
Leu-19	CD56	Mouse IgG,κ	PE (Simultest prep)	NK cells
anti-CD2[b]	CD2	Mouse IgG,κ	PE	Lymphocytes
Leu-4[a]	CD3	Mouse IgG1	PE	T cells
Anti-CD4[b]	CD4	Mouse IgG1,κ	PE	Helper T cell subset
Anti-CD8[b]	CD8	Mouse IgG1,κ	PE	Cytotoxic T cell subset
Anti-IL-2R[a]	CD25	Mouse IgG1,κ	FITC	Activated lymphocytes
Leu-16[b]	CD20	Mouse IgG1,κ	PE	B cells
Anti-mIgG[c]	mIgG	Mouse IgG1,κ	FITC	Mouse IgG coated cells

[a] Becton Dickinson Ltd., Cowley, Oxford.

[b] Dako Ltd. High Wycombe, Bucks.

[c] Caltag Laboratories, San Francisco, California.

MEASUREMENT OF IL-2, IFN-γ, TNF-α, AND sIL-2Rα LEVELS BY ENZYME-LINKED IMMUNOSORBANT ASSAYS (ELISA)

Materials:

1) The following ELISA kits were purchased from Laboratory Impex Diagnostic Ltd.

   Biokine IL-2 Test Kit (T Cell Sciences Inc.) - stated minimum sensitivity = 8.8 pg 0.1 ml^{-1}.

   Human Interferon Gamma Elisa (Janssen Biochemica Ltd.) - stated minimum sensitivity = 5 pg ml^{-1}.

   Tumour Necrosis Factor-α Elisa Kit (Cistron Biotechnology Ltd.) - stated minimum sensitivity = 20 pg ml^{-1}.

   All these kits measured soluble (biologically active cytokines).

   IL-2R Test Kit (T Cell Diagnostics Inc.) - stated minimum sensitivity = 50 Uml^{-1}.

2) Automatic plate reader (Titertek Multiskan, Flow Laboratories Ltd.).

3) Rotating platform (Sterling Instruments Ltd.).

4) Automatic plate washer (Ultrawash, Dynatech Ltd.).

5) 2N H_2SO_4 (Sigma Chemical Co. Ltd.).

6) 10 mls glass blood bottles (LPS Ltd.).

Methods:

Serum samples

(1) A 10 mls blood sample was collected into a glass tube and allowed to clot by standing at room temperature for approximately 30 minutes.

(2) The tube was centrifuged at 1750 g for 10 minutes and serum removed by pipetting.

(3) Serum was stored at -70°C in 1 ml aliquots and completely thawed at room temperature prior to use.

The procedure followed was as described in the manufacturer's instructions. In each assay a standard curve was constructed using either recombinant cytokine or IL-2R standards and each sample was assayed in duplicate in the 96 well plate provided.

<u>APPENDIX F</u>
<u>MEASUREMENT OF OKT3 LEVELS</u>

<u>Materials:</u>

1)   FACScan flow cytometer with Hewlett packard computer and Simulset software
(Becton-Dickinson UK Ltd.).

2)   Phosphate buffered saline (PBS X 20) (Mercia Diagnostics Ltd.) and 0.1%
sodium azide (Sigma London Chemical Co. Ltd.) (PBS/Azide).

3)   Sheath Fluid Isoton II (Coulter Electronics Ltd.).

4)   Propidium Iodide (Sigma London Chemical Co.Ltd.).

5)   AB pooled human serum (Blood Transfusion Service, Sheffield), and normal
goat serum (Vector Laboratories, Peterborough).

6)   Goat anti-mouse IgGγ FITC-conjugate (Sigma immunochemicals).

7)   Assorted tubes and pipettes as listed in appendices A to D.

8)   OKT3 1 mg ml^{-1} (Cilag Biotech Ltd.).

9)   HUT 78 cell line (ECACC).

<u>Methods:</u>

(1) HUT 78 cells were maintained in continuous culture and an aliquot of cells removed
on the day of assay and incubated for 5 minutes at room temperature with propidium
iodide at a concentration of 1 μg /10^6 cells.

(2) Cells were then washed in tissue culture medium by centrifugation at 400g for 10
minutes, resuspended in PBS/Azide to a volume of 20 mls and washed again at 400 g for
10 minutes.

(3) Cells were then resuspended in a mixture of PBS/Azide and 5% AB serum.

(4) Serum samples or OKT3 samples were thawed to room temperature on the day of
assay and diluted five times by a series of halving dilutions in PBS/Azide and 5% AB
serum.

(5) A stock solution of OKT3 was reconstituted to 1 μg ml^{-1} in AB serum and this was
then diluted by a series of halving dilutions to generate samples of known concentration
(standards).

(6) To 100 μl of each standard or unknown sample was added 100 μl of the HUT 78 cell
suspension in Facscan tubes.

(7) Tubes were then vortexed to ensure mixing of cells with OKT3 solution and
incubated for 30 minutes at 4°C.

(8) Cells were then washed in 3 mls of PBS/Azide by centrifugation at 650 g for 5 minutes. The supernatant was decanted and the cells resuspended by gentle agitation.

(9) Goat anti-mouse IgGγ FITC-conjugate was diluted 1 in 20 in PBS/Azide and 5% AB serum and 1% normal goat serum and 100 µl added to the HUT 78 cells which were incubated for 30 minutes in the dark at 4ºC.

(10) Cells were then washed (twice) with PBS/Azide by centrifugation at 650 g for 10 minutes and resuspended in 150 µl of Isoton II.

(11) Cells were processed through the FACScan using an aquisition gate to exclude cells stained with propidium iodide and 2000 events collected.

(12) The median fluorescence value of each population of cells including the experimental sample was calculated by the flow cytometer and used to calculate a standard curve over a concentration range of 20 to 500 ng ml^{-1}.

(13) The OKT3 concentration for each dilution of the unknown sample was then calculated using the standard curve and the mean value determined.

APPENDIX G

RECONSTITUTION AND ADMINISTRATION OF OKT3 FOR PATIENT STUDIES

Introduction

OKT3 was purchased from Cilag Biotechnology Ltd. in the form of a glass vial containing 5mg OKT3 in 5 mls of 5% glucose. A stock solution of 50 μg OKT3 was reconstituted under sterile conditions to a 10 mls solution of 5% glucose and 0.2% human albumin. The Leicestershire Health Authority District Pharmacy Sterile Products Unit and the Pharmaceutical Service at Leicester General Hospital kindly supplied this preparation using the following protocol. OKT3 was was stored at 4°C and always administered to patients within 72 hours of reconstitution.

Materials:

1)   OKT3 (5000 μg in 5 mls) (Cilag Biotechnology Ltd.).

2)   4.5% Human Albumin (Immuno Ltd.).

3)   5% glucose solution (Baxter Health Care Ltd.).

4)   10 mls 3 piece Luer lock syringes with blind hubs (B. Braun Medical Supplies Ltd.).

5)   0.2 μ hydrophobic (airvent) filter (Sartorius Ltd.).

Methods:

(1) 1 ml OKT3 was removed from the glass vial containing the original solution using a 1 ml syringe and transferred to a 10 mls sterile glass vial to which was then added 4 mls of 5% glucose. The remaining OKT3 was stored at 4°C for susequent use.

(2) 0.5 mls of this solution was then added to 0.45 mls of 4.5% human albumin solution (drawn up using the airvent filter) then using a 10 mls syringe fitted with a syringe connector this was made up to 10 mls with 5% glucose and the syringe sealed with a blind hub.

(4) For the 20μg, 10μg, and 5μg doses, 4 mls, 2 mls, and 1 ml of this stock solution respectively, was reconstituted to 10 mls using 0.9% saline. For reconstitution of 0.5μg OKT3 a 5μg preparation was reconstituted and 1 ml of this solution was again reconstituted to 10 mls. This was performed immediately prior to administration.

(5) OKT3 was given by rapid (2 minutes) bolus intravenous injection into an antecubital fossa vein through a 19 gauge intravenous cannula.

RESULTS OF CYTOTOXICITY ASSAYS AND FLOW CYTOMETRY

Table 3.7 - General characteristics of cancer patients.

Patient	Sex	Age	OKT3 Trial (μg)	Karnofsky Performance status (%)	Date studied	Symptoms prior to diagnosis (months)	Survival following diagnosis (months)
BL	F	71	Cont	70	17.11.92	6	2
HW	M	84	Cont	100	17.11.92	4	2
IA	F	79	Cont	60	3.2.93	2	1
GW	M	67	Cont	60	3.2.93	4	2
AG	F	69	Cont	90	15.2.93	3	3
NS	M	64	Cont	60	15.2.93	8	3
DH	M	74	50	100	24.3.92	12	6
AP	F	56	50	60	24.3.92	3	Unknown
IB	F	70	50	60	13.4.92	4	2
WD	M	70	50	60	14.4.92	6	2
ME	F	69	50	70	14.4.92	3	2
WW	M	72	50	90	13.4.92	2	Alive
MD	F	75	Cont	90	23.4.92	4	Unknown
PW	M	83	Cont	60	23.4.92	6	2
GG	M	51	Cont	80	6.4.92	4	Alive
HC	M	61	Cont	80	10.3.92	3	12
DC	M	60	20	60	28.9.92	4	2
BJ	F	58	20	90	28.9.92	2	2
GR	M	48	20	90	28.9.92	4	Alive
DD	M	38	20	60	24.2.92	2	3
AE	M	58	20	100	9.3.93	12	Alive
NM	M	62	20	90	25.3.93	4	7
IT	F	81	10	60	23.9.92	3	1
WG	M	82	10	90	23.9.92	4	Alive
WL	F	53	10	90	23.9.92	1	3
HB	M	76	10	90	24.2.92	6	Alive
KC	M	71	10	60	9.3.93	8	3
RW	F	71	10	60	25.3.93	4	Alive
FB	M	65	5	90	26.10.92	4	6
DB	M	76	5	80	26.10.92	5	Alive
RD	M	69	5	90	26.10.92	3	3
AB	M	83	5	60	7.7.92	6	4
RH	M	38	5	100	7.7.92	None	Alive
DS	M	55	5	70	7.7.92	2	5
TH	M	52	0.5	80	8.7.92	4	5
DV	M	66	0.5	60	8.7.92	3	Alive
AA	M	86	No	80	11.3.93	4	Alive
MB	M	69	No	70	5.3.93	3	Alive
FD	M	75	No	60	5.3.93	6	Alive
AS	M	60	No	80	10.3.93	12	Alive
AH	M	61	No	90	10.3.93	5	Alive
HO	M	61	No	90	1.3.93	10	4
LH	M	87	No	60	1.3.93	6	Alive
ABT	M	72	No	80	18.3.93	3	2
JMR	F	72	No	70	18.3.93	5	Alive
AJR	M	82	No	90	18.3.93	3	Unknown
BWL	F	75	No	70	18.3.93	6	2
TJ	M	65	No	90	10.3.93	4	Alive
MM	F	74	No	90	23.3.93	3	Alive
EY	M	86	No	80	23.3.93	6	2
KB	F	67	No	70	23.3.93	6	Alive

Table 3.8 - Previous treatment and management following study.

Patient	Previous surgery (months (m))	Tumour at time of study	Treatment following study
BL	No	Primary	Laparotomy and medical palliation
HW	No	Primary	Medical palliation
IA	Yes (resection, 12m)	Recurrence	Medical palliation
GW	No	Primary	Laparotomy and medical palliation
AG	Yes (bypass, 1m)	Primary	Medical palliation
NS	No	Primary	Palliative intubation
DH	No	Primary	Palliative intubation
AP	No	Primary	Palliative intubation
IB	No	Primary	Laparotomy and medical palliation
WD	No	Primary	Hartmann's resection and DXT
ME	No	Primary	Palliative intubation
WW	No	Primary	Biliary stent and medical palliation
MD	No	Primary	Resection
PW	No	Primary	Palliative intubation
GG	No	Primary	Resection
HC	No	Primary	Resection
DC	No	Primary	Medical palliation
BJ	No	Primary	Laparotomy and medical palliation
GR	No	Primary	Resection
DD	Yes (resection, 18m)	Recurrence	Palliative intubation
AE	No	Primary	Resection
NM	Yes (resection, 24m)	Recurrence	Resection
TT	No	Primary	Palliative intubation
WG	No	Primary	Resection
WL	No	Primary	Laparotomy and medical palliation
HB	No	Primary	Resection
KC	No	Primary	Palliative intubation
RW	No	Primary	Palliative intubation
FB	Yes (laparoscopy, 2m)	Primary	Interleukin 2
DB	No	Primary	Resection
RD	Yes (resection, 1m)	Residual	Medical palliation
AB	Yes (resection, 2m)	Residual	Medical palliation
RH	Yes (resection, 18m)	Recurrence	Resection
DS	Yes (resection, 1m)	Residual	Medical palliation
TH	Yes (resection, 1m)	Residual	Medical palliation
DV	Yes (resection, 18m)	Recurrence	Medical palliation
AA	No	Primary	Resection
MB	Yes (resection, 18m)	Recurrence	Medical palliation
FD	No	Primary	Resection
AS	No	Primary	Palliative intubation
AH	No	Primary	Resection
HO	No	Primary	Palliative intubation
LH	No	Primary	Resection
ABT	Yes (Denver shunt 2m)	Primary	Medical palliation
JMR	No	Primary	Medical palliation
AJR	Yes (resection, 1m)	Residual	Medical palliation
BWL	No	Primary	Laparotomy and medical palliation
TJ	No	Primary	Resection
MM	No	Primary	Resection
EY	No	Primary	Resection
KB	No	Primary	Resection

Table 3.9 - Characteristics of tumours in individual patients.

Patient	Primary site	Histology	Stage	Grade (Differentiation)	Liver mets	Date diagnosed
BL	pancreas	adeno	Fixed	Moderate	No	16.11.92
HW	unknown	adeno	Liv mets	Poor	Yes	16.11.92
IA	rectum	adeno	Liv mets	Moderate	Yes	9.1.92
GW**	pancreas	adeno	Fixed	Poor	No	4.2.93
AG*	pancreas	adeno	Fixed	Poor	Yes	15.2.93
NS	oesophagus	adeno	No op.	Moderate	No	5.11.92
DH	oesophagus	adeno	No op	N.S.	No	4.3.92
AP	oesophagus	squamous	No op	Moderate	No	25.2.92
IB	stomach	adeno	Fixed	Moderate	Yes	13.4.92
WD	rectum	adeno	Dukes C	N.S.	No	13.4.92
ME	oesophagus	adeno	No op	N.S.	Mets	18.3.92
WW*	biliary	adeno	No op	Well	No	19.2.92
MD	colon	adeno	Dukes B	Moderate	No	16.4.92
PW	oesophagus	squamous	No op	Poor	No	22.4.92
GG	oesophagus	adeno	$T_2N_1M_0$	Poor	No	3.4.92
HC	oesophagus	adeno	$T_3N_1M_0$	Poor	No	20.2.92
DC	stomach	adeno	No op	Poor	Mets	17.7.92
BJ	stomach	adeno	Fixed	N.S.	Mets	25.9.92
GR	oesophagus	adeno	$T_2N_0M_0$	Moderate	No	25.9.92
DD	oesophagus	adeno	No op	Poor	No	15.2.93
AE	oesophagus	adeno	$T_2N_0M_0$	N.S.	No	27.2.93
NM	rectum	adeno	Dukes C	Moderate	No	19.3.93
TT	oesophagus	adeno	No op	Poor	Mets	30.7.92
WG	rectum	adeno	Dukes B	Well	No	17.9.92
WL	biliary	adeno	Fixed	Poor	Mets	14.9.92
HB	rectum	adeno	Dukes A	Moderate	No	21.2.92
KC	oesophagus	adeno	No op	Well	No	12.2.93
RW	oesophagus	squamous	Fixed	Moderate	No	8.3.93
FB	unknown	melanoma	Liv mets	N.S.	Yes	29.9.92
DB	stomach	adeno	$T_3N_0M_0$	Moderate	No	22.10.92
RD	colon	adeno	Liv mets	Moderate	Yes	2.10.92
AB	rectum	adeno	Liv mets	Moderate	Yes	24.6.92
RH	rectum	adeno	Liv mets	Moderate	Yes	7.7.92
DS	colon	adeno	Liv mets	Moderate	Yes	22.6.92
TH	rectum	adeno	Liv mets	N.S.	Yes	17.6.92
DV	rectum	adeno	Dukes A	N.S.	No	15.5.92
AA	colon	adeno	Dukes B	Moderate	No	5.2.93
MB	rectum	adeno	Liv mets	Poor	Yes	22.1.93
FD	stomach	adeno	$T_2N_0M_0$	N.S.	No	26.2.93
AS	oesophagus	adeno	No op	Poor	No	10.3.93
AH	colon	adeno	Dukes B	Poor	No	9.3.93
HO	oesophagus	adeno	No op	Poor	Yes	5.2.93
LH	rectum	adeno	Dukes C	Moderate	No	8.1.93
ABT*	pancreas	adeno	No op	Poor	No	2.3.93
JMR	unknown	adeno	No op	N.S.	Yes	18.3.93
AJR	stomach	adeno	$T_4N_0M_1$	N.S.	Yes	5.3.93
BWL	stomach	adeno	$T_2N_0M_1$	N.S.	Yes	2.2.93
TJ	rectum	adeno	Dukes C	Well	No	10.3.93
MM	rectum	adeno	Dukes B	N.S.	No	23.3.93
EY	oesophagus	adeno	$T_3N_0M_0$	Well	No	2.3.93
KB	oesophagus	adeno	$T_2N_0M_0$	Moderate	No	23.3.93

RH underwent resection of 2 liver metastases, having previously had an abdomino-perineal resection. N.S. not stated.
** Jaundiced at time of study.
* Previously jaundiced.

Table 3.15 - Influence of non-specific mAb at 1 µg ml^{-1} on K562 cytotoxicity in healthy
donors.

	Medium	PmIgG	mIgG1	mIgG2a	hIgG1
JH	100.7	109.8	-	-	70.3
AH	0.3	0.2	-	-	-
PP	0.0	-	0.1	1.0	0.1
SV	0.0	-	0.0	0.0	0.0
WN	0.0	-	0.0	0.0	0.0
NP	72.6	99.6	-	-	-
KS	14.8	29.6	-	-	-
TH	90.3	90.5	-	-	95.3
RH	2.9	4.9	-	-	2.5
NK	8.6	7.5	-	8.7	-
WV	0.5	0.5	-	0.5	-
GW	25.1	17.6	-	17.1	-
SB	0.0	0.0	0.0	-	-
PF	0.6	0.5	0.5	-	-
MD	16.2	15.2	13.6	-	-
JEH	25.7	15.4	-	-	-
GP	0.3	0.0	-	-	-
n	17	14	6	6	6
mean	21.1	28.0 (25.6)	2.4 (2.9)	4.6 (5.8)	28.0 (32.4)
med.	2.9	11.4 (11.7)	0.1 (0.2)	0.8 (0.4)	1.3 (1.6)
SEM	8.1	10.7 (9.5)	2.7 (2.3)	2.9 (4.1)	17.6 (20.0)

Figures in parentheses are results of respective samples incubated in tissue culture
medium only.

Table 3.16 - Variation in total OKT3 content (µg) of 10 syringes.

Patient	DH	ME	WD	IB	WW	FB	JR	DC	MD	RW
OKT3	63.6	66.7	65.0	62.0	58.8	62.24	103.8	96.3	62.2	105.6

Table 4.1 - Individual K562 cytotoxicity with OKT3 and T3 (LU/10^7 PBMC) among 103 healthy male and female donors (donors underlined are assays performed by RC).

MALES					FEMALES				
Subject	Age	medium	OKT3	T3	Subject	Age	medium	OKT3	T3
AI	19	0.1	-	38.4	LP	20	3.8	3.8	4.0
JE	22	4.8	37.4	-	AN	21	0.0	2.7	42.1
IS	22	35.9	67.9	56.4	AW	22	0.0	0.2	-
SF	22	25.6	231.4	-	NK	22	8.6	42.7	-
AP	23	0.0	11.4	58.7	DOW	23	10.4	18.0	18.7
SWH	23	1.2	2.7	-	SB	23	0.01	2.4	-
DW	24	15.2	18.9	-	SEH	23	1.0	21.6	72.8
JL	24	85.2	85.2	152.4	LU	24	0.0	0.2	-
DU	25	11.2	12.4	19.1	AL	25	0.0	0.1	-
MRA	25	2.4	66.8	95.3	NG	25	0.5	6.0	-
SW	26	5.5	38.3	69.0	NN	25	0.5	2.8	-
CAM	27	19.7	33.3	-	SH	25	37.5	31.7	-
CE	27	54.3	-	106.7	CEW	26	1.1	1.5	-
AR	28	2.6	-	29.3	LA	26	16.8	-	51.0
GRB	28	50.3	69.9	-	DA	27	82.0	106.4	-
IS	28	0.3	35.5	70.4	AH	28	0.3	-	9.9
IU	28	0.6	4.5	-	PB	28	8.2	-	57.8
PA	28	6.2	46.0	-	SLC	28	35.0	100.6	180.1
WM	29	13.2	35.3	85.6	DH	31	0.4	1.7	3.2
JH	30	12.1		97.2	BP	32	4.8	18.4	-
JM	30	0.0		38.4	EB	33	0.0	-	1.5
MR	30	1.9	4.1	-	NOP	33	72.6	147.9	-
SV	30	0.0	0.4	3.6	WN	33	0.0	3.4	7.2
GM	33	0.4	4.5	-	HH	34	1.5	-	-
GW	33	25.1	42.9	92.6	VS	34	102.4	135.9	-
TD	33	73.1	120.5	-	CW	40	6.5	-	42.3
TB	34	3.4	-	-	SM	40	6.6	-	26.3
BR	35	0.3	-	56.9	NP	43	72.6	125.4	
EN	36	13.3	32.7	-	CS	44	0.6	3.8	18.6
RH	37	2.9	-	-	CB	45	22.6	-	358.4
PU	38	11.2	12.4	-	JAH	45	47.4	53.5	-
NM	42	40.3	-	331.0	MD	45	0.5	2.8	-
PKD	42	0.0	-	84.8	LF	46	33.6	70.4	148.2
RJ	43	0.1	-	0.7	PP	48	0.2	0.7	-
WS	43	4.7	-	-	WV	48	0.5	0.3	-
PSV	44	2.3	-	-	TC	49	7.5	15.4	33.4
FJ	45	0.7	14.0	-	WW	49	0.49	3.6	-
TH	45	25.8	-	168.1	CAH	50	35.4	47.5	83.3
AT	50	3.9	26.6	36.8	PF	53	0.6	17.3	61.3
APN	56	0.0	0.3	2.0	RDK	53	0.0	2.2	31.5
SMC	57	1.8	25.3	-	DS	55	80.1	121.7	-
MH	58	1.3	3.7	35.7	PS	55	38.8	91.8	104.7
RI	62	0.4	7.5	12.7	DN	57	8.7	17.3	-
TOM	62	0.8	44.1	44.5	EW	57	2.2	37.8	37.8
LDA	66	0.0	2.9	35.5	GB	57	0.9	57.8	105.3
JB	82	12.5	22.7	49.1	JP	57	27.5	53.3	841.0
KJ	83	0.7	14.0	74.0	MA	57	84.3	161.8	210.1
ARP	84	0.0	1.4	4.0	GC	59	110.0	179.8	286.1
					KS	59	14.9	36.8	46.7
					YB	59	3.3	2.9	-
					DC	60	0.9	2.8	35.0
					AC	61	1.0	20.4	42.8
					KLB	61	12.6	16.1	34.4
					AST	63	18.4	60.0	127.7
					EO	66	0.0	25.3	34.3

Table 4.4 - K562 cytotoxicity (LU/10^7 PBMC) with varying concentrations of OKT3.

		OKT3 concentration (μg ml^{-1})			
Subject	Medium	0.01	0.1	1.0	10
IU	0.6	0.3	4.5	5.7	1.1
AL	0.1	0.0	0.0	0.3	0.0
AH	4.5	22.1	32.7	31.7	32.3
NM	8.8	46.5	44.8	52.7	47.6
NG	0.5	4.4	6.0	6.0	2.3
GM	0.4	3.0	4.5	4.5	0.9
DW	15.2	14.8	22.2	18.9	24.3
CW	1.1	6.2	6.4	1.5	2.1
TH	32.3	98.0	81.3	79.4	75.3
JH	67.1	66.2	68.0	71.9	75.9
JL	119.1	93.5	153.9	151.1	160.3
TB	25.0	48.5	53.2	61.0	64.8
SW	45.1	65.8	83.3	77.5	75.3
SM	51.8	62.9	67.6	59.9	64.9
LA	19.7	29.8	33.6	33.3	34.4
Mean	26.1	37.43	44.1	43.7	44.1
Median	15.2	29.76	33.	33.3	34.4
SEM	8.6	8.77	10.9	10.8	11.4

Table 4.5 - K562 cytotoxicity (LU/10^7 PBMC) with varying concentrations of T3.

		T3 concentration (μg ml^{-1})			
Name	Medium	0.01	0.1	1.0	10
AH	0.0	0.0	0.2	0.3	0.3
JH	5.6	21.0	44.7	59.5	46.2
LA	7.8	23.4	41.8	58.1	41.8
SC	0.9	0.4	0.9	6.1	3.2
CS	4.7	3.0	15.2	48.2	59.5
Mean	3.8	9.6	20.5	34.4	30.0
Median	4.7	3.0	15.2	48.2	41.8
SEM	1.5	5.2	9.7	12.9	12.0

Table 4.6 - K562 cytotoxicity (LU/10^7 PBMC) with varying concentrations of mCD7.

		mCD7 concentration (μg ml^{-1})			
Name	Medium	0.01	0.1	1.0	10
AH	11.4	9.2	30.9	40.3	49.0
JH	15.5	21.1	25.8	29.0	3.1
LA	19.7	19.2	28.0	23.3	29.5
Mean	15.6	16.5	28.2	30.9	37.9
Median	15.5	19.2	28.0	29.0	35.1
SEM	2.4	3.7	1.5	5.0	5.8

Table 4.7 - K562 cytotoxicity (LU/10^7 PBMC) with varying concentrations of hCD7.

		hCD7 concentration (μg ml^{-1})			
Subject	Medium	0.01	0.1	1.0	10
SW	0.0	0.5	1.1	0.4	0.4
TH	0.0	0.4	2.4	1.7	1.3
PSV	2.3	3.4	12.6	6.9	6.2
AL	0.1	0.6	2.3	1.8	1.6
AH	0.0	0.1	0.6	0.3	0.4
JH	5.6	19.7	24.8	26.5	26.4
LA	7.8	18.3	21.1	22.6	19.9
Mean	2.3	6.1	9.2	8.6	8.0
Median	0.1	0.6	2.4	1.8	1.6
SEM	1.2	3.4	3.9	4.2	4.0

Table 4.8 - Effect of variation in total assay time (hours) on K562 cytotoxicity (LU/10^7 PBMC) with T3.

	TIME	1	2	3	4	5	6
AH	medium	0.0	0.0	0.0	0.5	0.6	0.4
	T3	0.01	0.5	1.5	9.9	29.8	30.6
JH	medium	8.7	11.3	23.9	71.0	80.0	71.9
	T3	11.1	204.5	322.6	781.3	909.1	680.4
TH	medium	2.1	17.0	36.2	89.3	105.7	94.2
	T3	4.7	29.0	69.9	160.3	209.2	234.8
Medium	Mean	3.6	9.5	20.1	53.6	62.1	55.5
	Median	2.1	11.4	23.9	71.0	80.0	71.9
	SEM	2.6	5.0	10.6	27.1	31.6	28.3
T3	Mean	5.3	78.0	131.4	317.2	382.4	315.2
	Median	4.7	29.0	69.9	160.3	209.2	234.8
	SEM	3.2	63.8	97.7	236.1	268.5	191.9

Table 4.9 - Effect of variation in total assay time (hours) on K562 cytotoxicity (LU/10^7 PBMC) with hCD7.

	TIME	1	2	3	4	5	6
BP	medium	0.0	0.2	1.2	4.8	5.0	8.3
	hCD7	0.0	1.4	13.0	22.2	29.4	37.6
VS	medium	9.0	21.8	45.1	102.4	154.1	114.8
	hCD7	13.4	24.1	73.9	141.3	214.6	173.6
MR	medium	0.3	2.1	8.9	20.4	28.6	34.0
	hCD7	0.2	3.0	16.3	22.1	27.3	35.9
Medium	Mean	3.1	8.0	18.4	42.5	62.6	52.4
	Median	0.3	2.1	8.9	20.4	28.6	34.0
	SEM	3.0	6.9	13.5	30.3	46.3	32.1
hCD7	Mean	4.5	9.2	34.4	61.9	90.4	82.4
	Median	0.2	2.1	16.3	22.2	29.4	37.6
	SEM	4.4	7.5	19.8	39.7	62.1	45.6

Table 4.10 - Influence of OKT3 and T3 on K562 cytotoxicity ($LU/10^7$ PBMC).

CYTOTOXICITY	Medium	OKT3		T3	
Number of subjects	103	81		59	
Mean	15.8	37.7	(17.1)	83.4	(13.6)
Median	3.3	18.9	(3.3)	46.7	(2.4)
SEM	2.5	5.3	(3.0)	16.2	(3.0)
Range Min	0.00	0.1		0.7	
Max	110.0	231.4		841.0	
CHARACTERISTICS	Medium	OKT3		T3	
Males (n)	48	34		29	
Females (n)	55	47		30	
Age - Median	34	34		43	
Range Min	19	20		19	
Max	84	84		84	

Figures in parentheses are corresponding values for PBMC incubated in
tissue culture medium.

Table 4.11 - Influence of OKT3 and T3 on K562 cytotoxicity ($LU/10^7$ PBMC) in 42
healthy donors analysed simultaneously.

CYTOTOXICITY	Medium	OKT3	T3
Mean	13.6	34.0	81.5
Median	1.8	21.0	45.6
SEM	3.9	6.1	20.5
Range Min	0	0.3	2.0
Max	110.0	179.8	841.0

Table 4.12 - Comparison of hOKT3, OKT3, and T3 on K562 cytotoxicity ($LU/10^7$
PBMC).

Subject	medium	hOKT3	OKT3	T3
YB	3.3	5.1	2.9	8.3
SM	4.4	5.3	6.8	24.2
JH	35.9	82.6	74.8	166.9
AH	1.2	2.0	2.5	5.1
MD	16.2	13.7	28.0	84.3
RH	2.9	2.7	13.8	25.4
NK	8.6	44.1	42.7	114.3
WV	0.5	0.7	0.3	43.3
GW	25.1	45.9	42.9	92.6
SB	0.0	0.2	2.4	1.6
PF	0.6	0.4	3.5	30.6
Mean	9.0	18.4	20.1	54.2
Median	3.4	5.1	6.8	30.6
SEM	3.6	8.2	7.3	16.1

Table 4.13 - Enhancement of K562 cytotoxicity (LU/10^7 PBMC) by OKT3, T3, and anti-CD16 mAb.

Subject	Medium	OKT3	T3	Anti-CD16
CAH	35.4	47.5	83.3	29.2
PF	0.6	17.3	61.3	13.2
RDK	0.0	2.2	31.5	83.9
PS	38.8	91.8	104.7	106.0
AW	0.0	0.3	2.0	3.9
GB	0.9	57.8	105.3	118.8
JP	27.5	53.3	841.0	54.3
GC	110.0	179.8	286.1	222.5
KLB	12.6	16.1	34.4	20.4
AC	1.0	20.4	42.8	50.4
TOM	0.8	44.1	44.5	30.3
RI	0.4	7.5	12.7	85.4
JH	144.5	256.4	591.7	432.9
CS	0.2	2.8	8.4	10.5
PP	6.9	16.5	28.7	40.9
Mean	25.3	54.3	151.9	86.8
Median	1.0	20.4	44.5	50.4
SEM	11.4	18.8	63.0	28.7

Table 4.14 - Modulation of K562 cytotoxicity (LU/10^7 PBMC) by anti-CD2, anti-CD4, and anti-CD8 mAb.

Subject n = 10	medium	CD2	Subject n = 11	medium	CD4	Subject n =11	medium	CD8
YB	3.3	1.9	YB	3.3	2.7	YB	3.3	6.7
SM	4.4	3.4	SM	4.4	16.5	SM	4.4	13.5
PP	0.2	12.8	PP	0.2	48.8	PP	0.2	27.2
JH	25.7	28.3	JH	25.7	64.9	JH	25.7	48.8
AH	0.3	1.0	AH	0.3	1.1	AH	0.3	0.4
MD	0.5	1.0	MD	0.5	5.0	MD	0.5	3.8
CE	58.5	69.7	MR	1.9	4.0	MR	1.9	6.1
JAH	25.7	28.3	CE	58.5	103.2	CE	58.5	53.8
JP	0.3	0.1	JAH	25.7	69.9	JAH	25.7	48.8
WM	0.5	1.0	JP	0.3	1.1	JP	0.3	0.4
			WM	0.5	5.0	WM	0.5	3.8
Mean	11.9	14.6		11.0	29.3		11.0	19.4
Median	1.9	2.6		1.9	5.0		1.9	6.1
SEM	6.1	7.1		5.6	10.9		5.6	6.4

Table 4.15 - Modulation of K562 cytotoxicity (LU/10^7 PBMC) by anti-mCD7, anti-hCD7, and anti-ζ mAb.

Subject N = 11	medium	mCD7	Subject N = 15	medium	hCD7	Subject N = 3	medium	anti-ζ
LU	0.0	4.4	SW	0.0	0.4	LF	33.57	49.4
PA	6.2	76.9	TH	0.0	1.7	JAH	47.35	32.5
SWH	1.2	34.1	PSV	2.3	6.9	CS	0.58	1.3
CE	74.5	164.5	AL	0.1	1.8			
AH	11.4	40.3	AH	0.0	0.3			
LA	19.7	23.3	JH	5.6	26.5			
PB	38.2	57.8	LA	7.8	22.6			
GB	50.3	69.9	BP	4.8	22.2			
DA	82.0	106.4	VS	102.4	141.3			
SM	6.3	7.8	MR	20.4	22.1			
JH	15.5	9.0	CE	5.7	31.5			
			LU	0.0	1.5			
			PA	6.2	118.5			
			SWH	1.2	48.1			
			JB	12.5	34.1			
Mean	27.8	55.9		11.3	32.0		27.2	27.8
Median	15.5	40.3		4.8	22.2		33.6	32.5
SEM	8.9	14.3		6.7	11.0		13.9	14.1

Table 4.17 - Effect on K562 cytotoxicity (LU/10^7 PBMC) of combining OKT3 and hCD7.

Subject	Medium	OKT3	hCD7	OKT3 + hCD7
EN	13.3	32.7	20.7	37.9
JH	22.8	34.6	56.8	83.0
PB	10.3	24.2	13.2	30.3
AST	33.8	73.7	76.9	100.9
NOP	72.6	147.5	122.6	150.8
KS	14.9	36.8	34.0	38.6
mean	27.9	58.3	54.0	73.6
median	18.8	35.7	45.4	61.0
SEM	9.6	19.2	16.7	19.3

Table 4.20 - K562 cytotoxicity (LU/10^7 PBMC) with OKT3 and T3 for K562 target cells
preincubated in tissue culture medium or polyclonal antibody preparations

Target preparation	Subject	Medium	Blocking mAb	OKT3	T3
K562 + medium	AH	1.7	2.0	4.5	30.7
K562 + pmIgG		1.8	2.1	4.7	31.5
K562 + medium	JH	20.0	12.4	44.6	161.3
K562 + pmIgG		24.7	15.7	42.2	153.1
K562 + medium	MD	2.4	2.9	9.2	34.7
K562 + pmIgG		4.4	1.5	11.3	31.3
K562 + medium	NK	8.6	8.7	42.7	114.0
K562 + mIgG2a		6.9	12.6	44.8	131.4
K562 + medium	WV	0.5	0.5	0.3	43.3
K562 + mIgG2a		0.3	0.5	0.2	38.6
K562 + medium	GW	25.1	17.1	42.9	92.6
K562 + mIgG2a		31.2	21.8	41.6	102.9
K562 + medium	SB	0.0	0.0	2.4	1.6
K562 + mIgG1		0.0	0.0	0.4	1.1
K562 + medium	PF	0.6	0.5	3.5	30.6
K562 + mIgG1		0.6	0.7	6.1	40.0
K562 + medium	MD	16.2	13.6	28.0	84.3
K562 + mIgG1		14.4	15.9	23.4	58.2
K562 + medium	AH	1.2	0.3	2.5	5.1
K562 + hIgG1		3.0	1.1	6.5	4.9
K562 + medium	JH	35.9	22.0	74.8	166.9
K562 + hIgG1		38.6	31.4	120.3	168.4
K562 + medium	RH	2.9	2.5	13.8	25.4
K562 + hIgG1		4.0	2.4	13.2	34.9

Table 4.21 - K562 cytotoxicity (LU/10^7 PBMC) from 12 experiments using polyclonal
(non-specific antibody - NSA) antibody preparations.

	Medium	NSA	OKT3	OKT3 K562+NSA	T3	T3 K562+NSA
Mean	9.6	15.4	22.4	26.2	65.9	65.5
Median	2.7	5.6	11.5	12.2	39.0	37.5
SEM	3.5	8.7	6.9	9.8	16.5	16.8

Table 4.25 - WBC, differential count, and cells expressing NK markers (CD3 NK)
$(10^9 L^{-1})$ for healthy donors aged < 50 years.

Donor	WBC	Neut	Lymph	Mono	CD3+L	NK cells	CD3 NK
AJ	6.0	4.80	0.86	0.35	0.62	0.07	0.09
LP	5.3	3.98	1.05	0.31	0.73	0.06	0.09
AN	6.6	4.34	1.95	0.39	1.65	0.15	0.31
SF	6.0	2.82	2.72	0.46	2.08	0.35	0.34
AW	5.4	3.13	1.63	0.65	1.03	0.18	0.08
JS	8.4	4.85	3.02	0.52	1.92	0.56	0.28
SWH	5.3	3.51	1.42	0.38	1.10	0.08	0.07
AP	4.8	3.23	1.26	0.31	0.99	0.14	0.28
SEH	3.4	2.09	0.46	0.14	0.35	0.03	0.04
JL	6.9	4.32	2.03	0.55	1.37	0.44	0.04
DW	6.1	3.16	2.57	0.35	1.84	0.36	0.21
NG	6.4	4.31	1.54	0.56	1.09	0.20	0.12
AL	4.2	2.85	1.07	0.27	0.81	0.09	0.14
MRA	3.8	2.28	1.14	0.38	0.42	0.08	0.07
SW	3.8	2.46	1.04	0.30	0.63	0.18	0.09
CEW	6.0	3.92	1.68	0.40	1.29	0.14	0.11
CE	5.0	2.71	1.91	0.38	1.18	0.38	0.17
IU	7.4	5.66	1.70	0.57	0.86	0.09	0.08
SLC	5.2	3.97	0.88	0.35	0.63	0.19	0.02
IS	6.5	4.66	1.42	0.45	1.06	0.17	0.34
AR	3.9	2.68	0.86	0.36	0.73	0.05	0.18
JH	3.0	2.32	0.51	0.25	0.36	0.07	0.08
JM	5.2	3.69	1.27	0.23	0.93	0.08	0.17
TD	5.5	3.48	1.61	0.41	1.26	0.12	0.32
GM	6.4	4.53	1.47	0.40	1.17	0.15	0.10
EB	5.0	3.36	1.27	0.36	0.99	0.06	0.35
BR	6.0	4.39	1.19	0.48	0.94	0.16	0.19
SM	3.1	2.19	0.72	0.19	0.46	0.12	0.05
CW	5.8	4.44	1.04	0.06	0.72	0.12	0.15
NM	5.8	4.23	1.27	0.30	0.85	0.17	0.13
PKD	5.1	3.90	0.93	0.27	0.76	0.10	0.09
RJ	5.7	4.06	1.30	0.33	1.05	0.06	0.16
PSV	4.2	2.40	1.52	0.28	1.04	0.25	0.15
TH	4.6	3.05	1.29	0.26	0.76	0.26	0.05
CB	4.0	2.67	1.09	0.25	0.59	0.21	0.11
n	35	35	35	35	35	35	35
Mean	5.31	3.55	1.38	0.36	0.98	0.17	0.15
Median	5.33	3.51	1.27	0.35	0.94	0.14	0.12
SEM	0.21	0.15	0.10	0.02	0.07	0.02	0.02
%		67	26	7	71	12	11

Table 4.26 - WBC, differential count, and cells expressing NK markers (CD3 NK)
(10⁹ L⁻¹) for healthy donors aged ≥ 50 years.

Donor	WBC	Neut	Lymph	Mono	CD3⁺ L	NK cells	CD3 NK
AT	5.1	3.78	1.02	0.30	0.80	0.12	0.17
CAH	6.1	4.31	1.42	0.39	1.08	0.12	0.22
PF	9.6	6.16	2.79	0.61	1.87	0.74	0.26
RDK	6.9	4.42	2.09	0.40	1.49	0.35	0.34
PS	5.9	3.92	1.46	0.52	1.16	0.13	0.23
APN	5.6	4.14	0.94	0.48	0.67	0.15	0.07
DN	5.0	2.68	1.94	0.39	1.49	0.12	0.22
SMC	4.2	2.78	1.01	0.37	0.47	0.17	0.14
MA	7.7	5.14	2.10	0.45	1.50	0.35	0.53
EW	4.9	3.20	1.35	0.36	0.95	0.12	0.29
GB	5.5	3.59	1.53	0.38	1.02	0.27	0.23
JP	3.2	1.92	0.97	0.31	0.63	0.18	0.14
MH	8.0	4.94	2.25	0.81	1.90	0.14	0.43
GC	6.1	4.43	1.27	0.40	0.81	0.28	0.10
KLB	7.2	6.26	0.56	0.38	0.41	0.17	0.10
AC	6.4	4.85	1.13	0.41	0.78	0.13	0.07
TOM	3.7	2.73	0.64	0.32	0.36	0.19	0.07
RI	5.5	3.36	1.74	0.40	1.28	0.19	0.31
LDA	5.2	3.61	1.11	0.49	0.92	0.13	0.25
EO	5.4	3.65	1.37	0.38	1.07	0.13	0.17
n	20	20	20	20	20	20	20
Mean	5.86	3.99	1.44	0.43	1.03	0.21	0.22
Median	5.53	3.99	1.36	0.39	0.98	0.16	0.22
SEM	0.34	0.25	0.13	0.03	0.10	0.03	0.03
%		68	25	7	72	14	15

% neut, lymph, mono = mean % of WBC, NK and CD3NK = mean % lymphocytes.

Table 4.27 - Expression of CD3, CD20, CD4, CD8 , and CD25 (IL-2Rα) among young healthy donors (10^9 L^{-1}).

Donor	CD3 L	(CD20)	CD4	CD4/CD25	CD8	CD8/CD25
ARJ	0.62	-	-	-	-	-
LP	0.73	-	-	-	-	-
AN	1.65	-	-	-	-	-
SF	2.08	0.48	1.06	0.29	1.17	0.69
AW	1.03	0.25	0.79	0.24	0.58	0.03
JS	1.92	-	-	-	-	-
SWh	1.10	0.26	0.80	0.22	0.34	0.16
AIP	0.99	-	-	-	-	-
SEH	0.35	-	-	-	-	-
JL	1.37	0.34	-	-	-	-
DW	1.84	0.47	1.25	0.21	0.84	0.18
NG	1.09	0.30	0.49	0.07	0.65	0.01
AL	0.81	0.16	0.46	0.17	0.36	0.04
MA	0.42	-	-	-	-	-
SW	0.63	0.25	0.43	0.14	0.37	0.02
CW	1.29	0.33	0.85	0.09	0.47	0.01
CE	1.18	0.35	-	-	-	-
IU	0.86	0.30	0.55	0.19	0.39	0.03
SLC	0.63	-	-	-	-	-
IS	1.06	-	-	-	-	-
AR	0.73	-	-	-	-	-
JH	0.36	0.47	0.80	0.33	0.34	0.01
JM	0.93	0.30	0.43	-	0.37	-
TD	1.26	0.48	-	-	-	-
GM	1.17	-	0.85	0.18	0.47	0.02
EB	0.99	-	-	-	-	-
BR	0.94	-	-	-	-	-
SM	0.46	0.33	-	-	-	-
CIW	0.72	-	-	-	-	-
NM	0.85	0.35	0.46	0.19	0.36	0.18
PKD	0.76	-	-	-	-	-
RJ	1.05	-	-	-	-	-
PSV	1.04	0.46	0.61	0.13	0.66	0.16
TH	0.76	-	0.53	0.16	0.56	0.14
CB	0.59	-	-	-	-	-
n	35	17	15	14	15	14
Mean	0.98	0.35	0.69	0.19	0.53	0.06
SEM	0.07	0.02	0.06	0.02	0.06	0.02
%	71	25	45	12	35	4

% is mean of lymphocytes for respective donor.

Table 5.1 - K562 cytotoxicity (LU/10^7 PBMC) among 51 cancer patients (with enhancement by OKT3 and T3. (Patients underlined are assays performed by RC).

MALES					FEMALES				
Patient	Age	medium	OKT3	T3	Patient	Age	medium	OKT3	T3
RH	38	0.8	0.9	53.0	WL	53	1.1	1.3	6.6
DD	38	1.2	9.8	11.8	AP	56	34.4	57.6	125.5
GR	48	50.3	60.2	67.6	BJ	58	39.4	74.9	117.1
GG	51	197.2	507.6	519.5	KB	67	0.0	3.8	10.6
TH	52	0.0	0.1	2.9	ME	69	42.8	54.5	40.0
DS	55	0.1	0.4	0.9	AG*	69	0.0	2.3	17.6
AE	58	0.3	21.5	227.5	BL	71	24.7	42.5	93.2
DC	60	4.7	15.8	25.6	RW	71	3.1	6.9	12.3
AS	60	0.0	1.8	2.1	JMR	72	0.7	0.0	-
HC	61	97.0	141.4	233.1	MM	74	3.3	27.6	6.5
AH	61	0.6	30.4	71.3	MD	75	0.8	7.7	10.5
NM	62	4.4	24.8	44.9	BWL	75	7.3	17.5	-
NS	64	0.0	6.9	17.7	IB	76	9.3	27.7	45.2
FB	65	52.4	63.5	-	IA	79	23.0	75.8	177.3
TJ	65	57.2	97.7	302.7	IT	81	46.3	276.2	869.6
DV	66	0.9	1.4	1.2					
GW**	67	21.9	71.0	144.5					
RD	69	8.6	17.9	-					
MB	69	63.4	108.1	153.8					
WD	70	13.1	14.7	20.9					
KC	71	3.3	16.5	106.2					
WW*	72	0.0	0.0	8.7					
ABT**	72	53.9	118.0	-					
DH	74	24.9	44.0	123.2					
FD	75	0.3	4.0	22.1					
DB	76	21.2	33.1	-					
HB	76	70.4	124.8	326.8					
WG	82	0.2	19.0	363.6					
AJR	82	33.1	182.0	-					
PW	83	24.7	49.3	61.3					
AB	83	20.5	21.0	29.7					
HW	84	0.0	0.0	4.1					
HO	85	0.0	4.7	267.7					
AA	86	0.0	0.5	9.3					
EY	86	3.8	-	-					
LH	87	0.0	0.0	0.6					

** Bilirubin elevated at time of study.

* Bilirubin elevated within the previous month but within normal range at time of study.

Table 5.3 - K562 cytotoxicity (LU/10^7 PBMC) and *in vitro* modulation by OKT3 in 50 cancer patients compared with 50 healthy donors including 28 elderly healthy donors.

Group	BASELINE			OKT3			Q of enhancement		
	mean	sem	median	mean	sem	median	mean	sem	median
All	21.2	4.9	4.5	49.8	12.1	20.0	28.5	8.0	11.1
Males	23.6	6.7	4.4	51.8	15.6	19.0	28.2	9.6	11.1
Females	15.7	4.5	7.3	45.1	17.9	27.6	29.4	14.8	11.7
< 70 yrs	27.1	8.9	4.4	55.0	20.4	21.5	27.9	12.2	11.1
≥ 70 yrs	15.4	3.8	7.3	44.6	13.3	19.0	29.2	10.6	11.9
U. GIT	27.9	9.2	9.3	70.3	24.2	27.7	42.5	16.4	11.9
L GIT	14.8	5.6	2.1	31.8	9.6	18.5	17.0	4.7	8.1
Bil/panc	16.9	8.7	11.5	39.2	19.6	22.4	22.3	11.4	10.1
Unknown	17.7	17.3	0.7	21.2	21.2	0.4	3.5	3.8	0.0
Jaundice	19.0	12.7	11.0	47.9	28.6	36.7	28.9	16.3	25.7
Mets	17.7	4.7	8.0	47.2	16.0	17.7	29.6	13.0	9.8
No mets	23.6	7.5	3.3	51.5	17.3	23.1	27.9	10.3	15.5
All healthy	17.1	3.0	3.3	37.7	5.3	18.9	20.6	3.2	11.4
≥ 50 years	16.5	5.5	2.0	39.3	8.9	24.0	22.9	4.1	18.1

Table 5.4 - K562 cytotoxicity (LU/10^7 PBMC) and *in vitro* modulation by T3 in 43 cancer patients compared with 59 healthy donors and including 24 elderly healthy donors.

Group	BASELINE			T3		
	mean	sem	median	mean	sem	median
All	20.6	5.5	3.3	111.9	25.4	45.2
Males	21.9	7.7	1.0	107.5	24.5	48.9
Females	17.5	5.0	9.3	122.1	64.1	45.2
< 70 yrs	26.8	9.6	1.2	95.5	26.3	44.9
≥ 70 yrs	13.4	4.2	3.3	130.7	46.0	53.3
U. GIT	29.0	10.5	7.0	145.3	64.4	47.3
L GIT	15.2	5.9	0.9	95.9	30.0	44.9
Mets	16.8	5.5	4.7	120.7	57.2	40.0
No mets	22.6	8.0	3.2	107.1	25.1	61.4
All healthy	13.6	3.0	2.4	83.4	16.2	46.7
≥ 50 years	15.3	5.7	1.2	107.14	25.1	61.4

Table 5.5 - Individual results of the effect of anti-CD16 on K562 cytotoxicity (LU/10^7 PBMC) in 14 cancer patients compared with OKT3.

Patient	Medium	OKT3	Anti-CD16
MB	63.4	108.1	63.5
FD	0.3	4.0	0.2
AS	0.0	1.8	0.9
AH	0.1	30.4	0.1
HO	0.0	4.7	0.1
LH	0.0	0.0	0.1
ABR	53.9	118.0	97.2
JMR	0.7	0.0	1.0
AJR	33.1	182.6	1292.5
BWL	7.3	17.5	5.5
TJ	57.2	97.7	202.8
MM	3.3	27.6	23.7
EY	3.8	-	29.8
KB	0.0	3.8	12.4
Mean	16.9	45.8 (15.9)	123.56
Median	0.7	17.53 (2.0)	9.0
SEM	7.0	16.6 (6.6)	91.2

Figures in parentheses are respective values in medium for OKT3.

Table 5.7 - WBC, differential, and cells expressing NK markers (CD3 NK) (X 10^9/L) for cancer patients.

Patient	WBC	Neut	Lymph	Mono	NK cells	CD3 NK
BL	3.1	2.07	0.77	0.27	0.12	0.07
HW	9.4	5.94	2.86	0.60	0.32	0.42
IA	6.0	3.22	2.08	0.70	0.44	0.48
GW	8.9	6.17	2.03	0.71	0.38	0.33
AG	4.7	2.99	1.30	0.40	0.13	0.09
NS	5.9	3.84	1.39	0.67	0.08	0.03
DH	5.3	2.40	2.35	0.51	0.37	0.49
AP	3.5	1.83	1.30	0.37	0.15	0.14
IB	7.6	5.20	1.68	0.71	0.23	0.54
WD	4.6	3.57	0.71	0.31	0.15	0.11
ME	9.5	7.15	1.57	0.8	0.30	0.48
WW	7.0	4.70	1.87	0.43	0.22	0.44
MD	7.0	5.30	1.19	0.50	0.11	0.12
PW	9.1	7.84	0.89	0.67	0.26	0.10
GG	4.8	3.13	1.38	0.30	0.51	0.09
HC	6.7	4.17	1.81	0.72	0.47	0.20
DC	12.4	10.22	1.71	0.48	0.35	0.21
BJ	7.2	5.03	1.56	0.59	0.21	0.50
GR	7.8	5.78	1.68	0.57	0.30	0.09
DD	4.9	3.62	0.96	0.33	0.15	0.07
AE	5.9	3.72	1.85	0.33	0.32	0.27
NM	5.3	4.00	0.95	0.35	0.18	0.03
TT	8.9	6.46	1.92	0.51	0.07	0.53
WG	7.0	5.21	1.24	0.53	0.04	0.37
WL	15.5	11.66	2.20	1.64	0.18	0.32
HB	3.9	2.38	1.21	0.32	0.27	0.13
KC	6.6	5.10	0.91	0.59	0.24	0.05
RW	7.7	6.18	0.97	0.55	0.15	0.03
FB	9.5	6.38	2.41	0.70	0.39	0.49
DB	4.6	3.08	1.11	0.42	0.20	0.12
RD	7.8	5.80	1.42	0.53	0.28	0.96
AB	7.5	5.48	1.02	1.00	0.15	0.11
RH	3.6	2.30	0.97	0.31	0.11	0.28
DS	6.4	3.66	1.50	1.24	0.21	0.12
TH	6.0	4.78	0.78	0.46	0.06	0.03
DV	11.9	9.40	1.51	0.99	0.26	0.18
AA	5.0	3.59	0.81	0.59	0.09	0.08
MB	8.07	6.71	0.87	0.48	0.32	0.14
FD	11.4	10.10	0.66	0.64	0.03	0.13
AS	5.2	4.05	0.77	0.38	0.03	0.03
AH	9.1	6.99	1.16	0.53	0.26	0.09
HO	5.6	4.32	0.93	0.32	0.20	0.11
LH	10.0	7.59	1.68	0.73	0.39	0.18
ABT	10.9	7.61	1.38	1.04	0.45	0.23
JMR	6.3	4.25	1.63	0.44	0.14	0.14
AJR	6.8	5.13	1.36	0.27	0.37	0.35
BWL	4.4	2.95	1.13	0.35	0.10	0.48
TJ	5.4	3.35	1.27	0.79	0.14	0.34
MM	4.1	2.75	1.04	0.34	1.18	0.08
EY	6.9	4.97	1.39	0.51	0.21	0.11
KB	10.1	8.23	1.35	0.48	0.20	0.14
n	51	51	51	51	51	51
Mean	7.11	5.09	1.39	0.57	0.23	0.24
SEM	0.36	0.31	0.07	0.04	0.02	0.03
%		72	20	8	16	17

Table 5.8 - Expression of CD4, CD8, and coexpression of CD25 (IL-2Rα) among 10 cancer patients (10^9 L^{-1}).

Donor	CD4	CD4/CD25	CD8	CD8/CD25
DH	1.25	0.21	0.62	0.01
AP	0.57	0.08	0.42	0.02
IB	0.97	0.10	0.49	0.06
WD	0.40	0.07	0.25	0.05
ME	0.77	0.18	0.42	0.02
WW	0.83	0.12	0.74	0.02
MD	0.66	0.10	0.33	0.04
PW	0.32	0.03	0.40	0.06
GG	0.66	0.12	0.33	0.04
HC	0.91	0.21	0.68	0.02
Mean	0.73	0.12	0.47	0.03
SEM	0.09	0.09	0.05	0.01
%		16		6

Table 5.9 - Cellular constituents of whole blood and PBMC from healthy donors (n = 8) and cancer patients (n = 15).

HEALTHY DONORS

Donor	WBC	BLOOD % (WBC X10^9/L) Neut	Mono	Lymph	NK	PBMC% Neut	Mono	Lymph	NK
MD	5.6	65.9	7.2	27.0	16.6	15.6	8.3	76.1	30.8
JH	7.8	77.5	5.9	16.6	18.0	5.3	22.3	72.4	29.4
SW	7.1	69.3	6.2	24.5	21.9	15.8	13.9	70.3	39.1
AL	4.7	67.8	6.8	25.4	19.4	8.9	11.6	79.1	30.1
TH	4.4	68.4	6.3	25.4	14.6	7.2	14.5	78.3	24.1
CE	5.9	71.3	8.6	20.1	17.7	3.0	27.8	68.3	25.0
CS	5.7	65.9	5.1	29.1	5.2	11.4	14.4	74.2	8.4
PP	9.8	53.9	8.6	37.5	5.4	9.5	13.4	77.1	16.3
Mean	6.3	67.5	6.8	25.7	14.9	9.6	15.8	74.5	25.4
SEM	0.5	2.4	0.4	2.2	2.2	1.6	2.2	1.4	3.4

CANCER PATIENTS

Donor	WBC	Neut	Mono	Lymph	NK	Neut	Mono	Lymph	NK
BL	3.1	66.8	8.7	24.8	15.6	10.9	23.9	65.3	20.0
HW	9.4	63.2	6.4	30.4	11.2	7.3	19.0	73.7	18.2
IA	6.0	53.7	11.7	34.7	21.2	11.6	13.6	74.7	33.9
GW	8.9	69.3	8.0	22.8	18.7	17.4	18.5	64.1	34.6
AG	4.7	63.6	8.5	27.7	10.0	10.2	14.7	75.2	10.0
NS	5.9	65.1	11.4	23.6	5.8	11.6	22.1	66.4	8.6
DD	4.9	73.9	6.7	19.6	15.6	11.8	7.0	81.1	22.1
AE	5.9	63.1	5.6	31.4	17.3	7.9	10.5	81.6	25.8
NM	5.3	75.5	6.6	17.9	18.9	1.5	14.8	69.8	22.6
HB	3.9	61.0	8.2	31.0	22.3	5.9	11.0	83.1	30.4
KC	6.6	77.3	8.9	13.8	26.4	9.1	28.5	62.4	40.3
RW	7.7	80.3	7.1	12,6	15.5	10.9	29.7	59.5	19.6
FB	9.5	67.2	7.4	25.4	16.2	21.0	22.5	56.5	47.5
DB	4.6	67.0	9.1	24.1	18.0	10.6	13.7	75.7	35.3
RD	7.8	74.4	6.8	18.2	19.7	12.9	11.9	75.2	35.6
Mean	6.4	68.1	8.1	23.9	16.8	11.6	17.4	71.0	27.0
SEM	0.6	1.8	0.5	1.7	1.3	1.0	1.7	2.1	2.9

Table 6.2 - Percentage of lymphocytes with detectable membrane bound OKT3 with time (minutes) following administration of varying doses of OKT3.

Patient	Dose (μg)	Pre-OKT3	15	30	60	240	480	1440
DH	50	3.0	66.8	62.5	59.5	56.8	58.9	-
AP	50	1.5	62.5	59.2	54.7	37.5	22.7	-
IB	50	1.5	67.4	60.6	59.9	21.6	23.7	-
WD	50	1.4	58.6	56.1	46.6	33.6	44.6	-
ME	50	0.4	53.9	59.5	53.1	18.4	0.1	-
WW	50	2.8	74.6	71.3	70.0	50.1	39.6	-
DC	20	2.1	-	-	62.7	52.5	25.9	19.5
BJ	20	2.4	-	-	68.1	58.3	56.2	39.3
GR	20	1.2	-	-	68.7	58.9	60.6	47.0
DD	20	0.8	-	-	67.1	57.2	56.0	28.9
AE	20	0.5	-	-	68.3	60.4	64.4	28.7
NM	20	2.6	-	-	57.1	47.9	43.3	4.3
IT	10	4.0	-	-	63.1	28.2	8.5	2.4
WG	10	7.0	-	-	67.9	55.0	54.0	20.8
WL	10	1.9	-	-	53.2	47.6	40.5	11.4
HB	10	0.6	-	-	63.5	48.9	47.7	29.1
KC	10	2.1	-	-	53.7	36.0	35.9	14.3
RW	10	4.6	-	-	65.5	21.0	6.1	3.3
FB	5	4.0	-	-	52.6	35.8	24.5	14.5
DB	5	2.1	-	-	56.5	51.4	28.8	36.6
RD	5	5.9	-	-	66.7	52.2	52.1	30.6
AB	5	5.4	-	-	57.9	40.4	4.6	7.6
RH	5	4.1	-	-	65.7	48.3	14.7	14.8
DS	5	1.7	-	-	37.2	18.6	2.4	4.3
TH	0.5	2.5	-	-	16.7	9.9	6.8	9.6
DV	0.5	0.6	-	-	10.3	11.6	5.0	10.3

Table 6.3 - sIL-2R levels (U ml^{-1}) following 50 μg OKT3 compared with control patients.

Patient	OKT3 (μg)	PreOKT3/ normal saline	30 minutes	60 minutes	480 minutes
DH	50	248.67	220.70	210.80	212.69
AP	50	466.48	443.73	430.49	542.40
IB	50	337.69	318.75	343.71	417.24
WD	50	1135.05	1097.16	1186.74	1144.51
ME	50	845.27	780.87	765.72	814.96
WW	50	682.39	646.40	640.72	729.74
MD	0	525.19	527.08	536.55	502.46
PW	0	981.63	909.66	850.95	904.00
GG	0	409.66	375.57	343.37	354.74
HC	0	343.71	-	301.71	301.71

Table 6.4 - Percentage K562 cytotoxicity and effect of patients serum following OKT3/normal saline (minutes) (figures in parentheses are serum and K562 without PBMC).

Patient	OKT3 (μg)	Pre-OKT3/ normal saline	15	30	60	240	480	1440
DH	50	51.5	48.2	46.9	52.4	53.6	55.4	-
		(0.0)	(-0.3)	(-0.2)	(0.1)	(0.0)	(0.4)	-
AP	50	57.0	54.3	52.5	57.8	54.3	50.9	-
		(0.5)	(0.8)	(0.4)	(0.7)	(0.5)	(0.5)	-
IB	50	45.0	48.4	50.2	54.2	46.8	49.4	-
		(0.4)	(0.7)	(0.7)	(0.6)	(0.8)	(0.6)	-
WD	50	49.6	46.2	45.0	47.1	44.9	42.1	-
		(0.3)	(1.1)	(0.6)	(0.8)	(0.7)	(0.9)	-
ME	50	39.3	37.1	34.7	44.0	43.1	40.7	-
		(0.5)	(0.1)	(0.0)	(0.2)	(-0.1)	(0.3)	-
WW	50	40.3	39.3	41.1	44.1	43.4	40.8	-
		(1.2)	(0.7)	(0.8)	(1.1)	(0.9)	(0.6)	-
DC	20	19.1	-	-	19.8	18.6	23.4	24.4
		(-0.3)	-	-	(-0.1)	(0.1)	(0.1)	(0.1)
BJ	20	25.1	-	-	28.8	28.6	27.3	30.1
		(0.2)	-	-	(0.5)	(0.0)	(0.0)	(0.7)
GR	20	28.7	-	-	28.8	31.7	31.2	28.1
		(-0.2)	-	-	(0.3)	(0.2)	(0.2)	(-0.1)
DD	20	25.6	-	-	24.5	24.2	28.6	25.5
		(0.1)	-	-	(0.0)	(0.5)	(-0.3)	(0.5)
AE	20	26.2	-	-	22.3	22.3	25.7	21.5
		(-0.6)	-	-	(0.6)	(0.0)	(0.2)	(0.1)
NM	20	24.1	-	-	27.6	27.6	27.3	27.1
		(0.2)	-	-	(0.3)	(0.3)	(0.4)	(-0.2)
BL	0	44.6	-	-	42.4	43.0	40.3	40.6
		(1.4)	-	-	(0.5)	(-0.3)	(0.8)	(1.1)
HW	0	26.0	-	-	28.0	30.0	30.8	30.5
		(0.5)	-	-	(0.2)	(0.1)	(0.3)	(1.4)
IA	0	25.4	-	-	29.9	29.3	30.3	25.6
		(0.8)	-	-	(0.8)	(0.9)	(1.4)	(0.8)
GW	0	18.4	-	-	18.7	21.6	19.8	15.5
		(0.6)	-	-	(0.6)	(0.5)	(0.5)	(0.6)
AG	0	18.8	-	-	22.4	27.4	29.1	28.8
		(0.6)	-	-	(0.4)	(0.0)	(0.5)	(0.6)
NS	0	30.5	-	-	29.9	30.4	28.4	35.4
		(1.2)	-	-	(0.7)	(1.1)	(0.5)	(0.1)

Table 6.5 - WBC with time 50 μg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	5.3	4.6	3.8	2.4	6.8	3.5
AP	50	3.5	2.9	2.5	1.5	6.4	5.0
IB	50	7.6	8.6	5.2	4.0	13.0	11.4
WD	50	4.6	4.6	4.5	4.2	5.9	5.5
ME	50	9.5	7.0	8.4	6.8	8.7	5.8
WW	50	7.0	6.7	5.7	5.1	9.9	7.9
MD	0	7.0	7.7	8.8	8.9	8.6	7.5
PW	0	9.1	10.1	9.4	9.4	9.2	9.6
GG	0	4.8	5.9	5.0	4.7	8.4	7.6
HC	0	6.7	6.4	5.5	5.4	7.9	6.3

Table 6.6 - Neutrophils with time 50 µg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (µg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	2.40	2.38	2.29	1.62	5.83	2.83
AP	50	1.83	1.55	1.55	1.08	6.01	4.75
IB	50	5.20	6.47	4.19	3.21	12.53	10.84
WD	50	3.57	3.82	3.78	3.68	5.50	5.02
ME	50	7.15	5.33	6.51	5.47	7.73	4.90
WW	50	4.70	5.14	4.49	4.47	9.37	7.46
MD	0	5.30	5.91	6.86	7.15	6.67	5.85
PW	0	7.84	8.46	7.92	8.08	8.04	8.46
GG	0	3.13	3.65	3.31	3.06	6.92	5.18
HC	0	4.17	4.10	3.43	3.32	5.58	4.03

Table 6.7 - Lymphocytes with time 50 µg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (µg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	2.35	1.69	1.17	0.66	0.56	0.40
AP	50	1.30	1.02	0.70	0.39	0.23	0.11
IB	50	1.68	1.30	0.66	0.74	0.14	0.25
WD	50	0.71	0.49	0.44	0.34	0.20	0.23
ME	50	1.57	0.91	1.06	0.84	0.70	0.60
WW	50	1.87	1.11	0.88	0.42	0.23	0.29
MD	0	1.19	1.20	1.32	1.18	1.39	1.28
PW	0	0.89	0.81	0.65	0.55	0.47	0.45
GG	0	1.38	1.87	1.41	1.31	1.14	1.90
HC	0	1.81	1.67	1.52	1.55	1.76	1.71

Table 6.8 - Monocytes with time 50 µg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (µg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	0.51	0.53	0.37	0.12	0.42	0.28
AP	50	0.37	0.36	0.26	0.30	0.16	0.15
IB	50	0.71	0.83	0.36	0.40	0.34	0.30
WD	50	0.31	0.29	0.28	0.17	0.20	0.26
ME	50	0.80	0.76	0.84	0.43	0.28	0.37
WW	50	0.43	0.45	0.34	0.20	0.31	0.15
MD	0	0.50	0.60	0.80	0.58	0.55	0.38
PW	0	0.67	0.83	0.83	0.78	0.69	0.69
GG	0	0.30	0.47	0.32	0.32	0.34	0.54
HC	0	0.72	0.63	0.50	0.54	0.47	0.55

Table 6.9 - NK cells with time 50 µg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (µg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	0.37	0.33	0.22	0.11	0.07	0.03
AP	50	0.15	0.14	0.10	0.04	0.04	0.01
IB	50	0.23	0.17	0.07	0.10	0.02	0.03
WD	50	0.15	0.11	0.11	0.10	0.05	0.07
ME	50	0.30	0.15	0.17	0.12	0.07	0.06
WW	50	0.22	0.12	0.11	0.04	0.03	0.03
MD	0	0.11	0.12	0.16	0.15	0.19	0.12
PW	0	0.26	0.20	0.15	0.13	0.08	0.09
GG	0	0.51	0.73	0.43	0.30	0.45	0.43
HC	0	0.47	0.52	0.48	0.44	0.49	0.37

Table 6.10 - CD3$^+$ cells with time 50 μg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	1.61	1.09	0.75	0.41	0.28	0.20
AP	50	0.86	0.63	0.41	0.22	0.10	0.03
IB	50	1.22	0.91	0.43	0.46	0.04	0.09
WD	50	0.51	0.33	0.28	0.20	0.11	0.12
ME	50	0.98	0.56	0.64	0.50	0.38	0.35
WW	50	1.45	0.83	0.62	0.29	0.10	0.17
MD	0	0.85	0.87	0.95	0.82	0.99	0.95
PW	0	0.53	0.52	0.41	0.33	0.26	0.28
GG	0	0.73	0.92	0.79	0.79	0.53	1.18
HC	0	1.14	0.97	0.91	0.91	1.07	1.16

Table 6.11 - CD4 cells with time 50 μg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	1.25	0.93	0.63	0.38	0.25	0.19
AP	50	0.57	0.44	0.28	0.18	0.07	0.02
IB	50	0.97	0.73	0.36	0.39	0.03	0.09
WD	50	0.40	0.29	0.25	0.19	0.12	0.13
ME	50	0.77	0.44	0.52	0.40	0.32	0.29
WW	50	0.83	0.60	0.46	0.23	0.09	0.14
MD	0	0.66	0.65	0.66	0.57	0.65	0.72
PW	0	0.32	0.29	0.23	0.21	0.16	0.19
GG	0	0.66	0.87	0.72	0.70	0.49	1.12
HC	0	0.91	0.79	0.69	0.74	0.86	0.88

Table 6.12 - CD8 cells with time 50 μg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	0.62	0.41	0.26	0.12	0.07	0.04
AP	50	0.42	0.34	0.20	0.09	0.05	0.02
IB	50	0.49	0.30	0.14	0.17	0.02	0.03
WD	50	0.25	0.15	0.13	0.10	0.05	0.06
ME	50	0.42	0.21	0.24	0.19	0.12	0.11
WW	50	0.74	0.31	0.22	0.08	0.04	0.03
MD	0	0.33	0.34	0.41	0.37	0.50	0.34
PW	0	0.40	0.35	0.25	0.19	0.14	0.14
GG	0	0.33	0.49	0.30	0.28	0.29	0.33
HC	0	0.68	0.66	0.60	0.61	0.71	0.57

Table 6.13 - % CD3 cells coexpressing IL-2R (CD25) with time 50 μg and controls (4) (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	15.3	15.7	15.6	16.1	22.3	19.8
AP	50	15.1	16.2	15.8	17.9	12.5	9.2
IB	50	10.7	10.8	13.6	13.6	6.6	9.5
WD	50	16.1	19.2	20.0	17.8	19.5	17.4
ME	50	19.8	22.9	22.9	25.3	25.4	24.9
WW	50	13.5	13.5	11.8	14.1	14.2	11.7
MD	0	12.3	11.5	11.1	7.7	9.6	13.3
PW	0	10.1	9.0	12.2	11.4	14.9	15.4
GG	0	16.8	17.0	20.4	18.4	14.8	23.7
HC	0	21.1	19.8	21.8	24.0	25.8	26.6

Table 6.14 - % CD4 cells coexpressing IL-2R (CD25) with time 50 µg and controls (4) $(X\ 10^9\ L^{-1})$.

Patient	OKT3 (µg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	17.1	18.1	16.5	16.5	24.1	20.8
AP	50	13.9	12.3	12.5	15.0	9.9	7.9
IB	50	10.7	11.7	13.4	12.9	4.9	10.8
WD	50	17.4	20.6	18.9	16.7	21.3	18.9
ME	50	23.6	24.7	25.9	25.6	24.6	28.3
WW	50	14.3	15.7	12.5	16.3	12.5	13.8
MD	0	14.8	12.6	11.7	9.5	12.3	14.7
PW	0	10.5	10.0	11.0	11.3	14.8	15.9
GG	0	17.7	17.0	21.1	20.5	17.9	24.7
HC	0	23.0	21.6	22.2	22.8	23.8	24.1

Table 6.15 - % CD8 cells coexpressing IL-2R (CD25) with time 50 µg and controls (4) $(X\ 10^9\ L^{-1})$.

Patient	OKT3 (µg)	0 mins	15 mins	30 mins	60 mins	240 mins	480 mins
DH	50	2.4	2.0	1.6	1.1	2.9	1.6
AP	50	4.3	4.5	6.0	6.4	3.2	4.3
IB	50	1.2	1.3	2.2	2.0	0.9	3.6
WD	50	2.1	2.9	3.0	2.3	3.1	2.8
ME	50	3.7	5.0	4.3	4.8	4.7	5.6
WW	50	2.3	2.2	2.0	2.0	3.4	2.2
MD	0	1.2	0.9	0.8	0.5	0.7	1.3
PW	0	1.5	1.9	2.9	1.7	2.3	2.6
GG	0	1.2	1.5	2.1	1.4	1.8	2.2
HC	0	3.5	3.3	3.2	4.0	4.5	4.8

Table 6.16 - WBC following OKT3 (minutes) 20 μg to 0.5 μg and controls (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0	60	240	480	1440
BL	0	3.1	3.5	5.1	3.4	4.8
HW	0	9.4	9.6	8.4	7.9	8.3
IA	0	6.0	4.6	5.5	6.6	6.6
GW	0	8.9	7.7	7.5	7.1	7.3
AG	0	4.7	4.7	7.0	5.9	4.8
NS	0	5.9	6.3	8.4	8.0	5.5
DC	20	12.4	10.2	11.0	10.9	10.3
BJ	20	7.2	5.1	7.4	4.8	5.8
GR	20	7.8	6.6	7.6	8.0	9.2
DD	20	4.9	4.8	5.2	5.2	5.4
AE	20	5.9	4.8	9.9	6.6	9.3
NM	20	5.3	5.3	5.8	5.6	6.4
TT	10	8.9	10.6	11.6	7.6	13.6
WG	10	7.0	7.4	7.3	7.5	6.9
WL	10	15.5	13.3	12.0	12.0	16.9
HB	10	3.9	3.9	4.9	4.7	4.7
KC	10	6.6	8.1	9.5	9.2	9.1
RW	10	7.7	8.0	10.2	8.6	7.2
FB	5	9.5	9.8	8.6	8.5	9.8
DB	5	4.6	4.5	5.3	6.3	4.4
RD	5	7.8	7.8	10.1	7.1	8.6
AB	5	7.5	7.7	7.7	7.8	8.9
RH	5	3.6	3.6	3.9	5.4	5.3
DS	5	6.4	6.2	6.5	7.3	7.1
TH	0.5	6.0	6.4	6.3	5.9	5.8
DV	0.5	11.9	10.4	9.8	9.3	6.6

Table 6.17 - Neutrophils following OKT3 (minutes) 20 μg to 0.5 μg and controls (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0	60	240	480	1440
BL	0	2.07	2.51	3.76	2.39	3.37
HW	0	5.94	5.86	5.71	4.91	4.73
IA	0	3.22	2.57	2.51	3.30	4.03
GW	0	6.17	6.29	5.98	5.51	5.21
AG	0	2.99	3.15	5.22	4.17	3.26
NS	0	3.84	4.38	6.19	7.22	3.78
DC	20	10.22	8.99	10.24	9.36	8.56
BJ	20	5.03	3.96	6.51	3.79	4.18
GR	20	5.78	4.83	5.30	5.58	7.02
DD	20	3.62	3.81	4.27	4.13	3.94
AE	20	3.72	3.47	9.31	6.18	7.72
NM	20	4.0	4.14	4.73	4.40	4.92
TT	10	6.46	8.73	9.20	6.30	11.27
WG	10	5.21	5.75	5.15	5.62	5.15
WL	10	11.66	10.40	9.26	9.08	14.52
HB	10	2.38	2.47	3.54	3.53	3.08
KC	10	5.10	6.60	8.04	7.74	7.45
RW	10	6.18	6.39	8.35	6.71	5.70
FB	5	6.38	5.69	6.58	6.44	7.22
DB	5	3.08	3.16	4.01	4.37	3.05
RD	5	5.80	5.79	7.92	5.17	4.46
AB	5	5.48	5.82	6.10	6.05	6.26
RH	5	2.30	2.53	2.75	3.93	3.74
DS	5	3.66	3.76	3.92	4.63	4.22
TH	0.5	4.78	5.33	5.04	4.41	4.39
DV	0.5	9.40	8.69	8.21	7.81	4.99

Table 6.18 - Lymphocytes following OKT3 (minutes) 20 µg to 0.5 µg and controls (X 10^9 L^{-1}).

Patient	OKT3 (µg)	0	60	240	480	1440
BL	0	0.77	0.71	0.98	0.77	1.03
HW	0	2.86	3.14	2.32	2.46	2.97
IA	0	2.08	1.42	2.28	2.48	1.76
GW	0	2.03	0.99	0.94	1.08	1.43
AG	0	1.30	1.23	1.32	1.31	1.15
NS	0	1.39	1.29	1.37	0.44	1.07
DC	20	1.71	0.93	0.52	1.18	1.34
BJ	20	1.56	0.73	0.63	0.61	1.15
GR	20	1.68	1.26	1.57	1.80	1.44
DD	20	0.96	0.66	0.66	0.75	1.05
AE	20	1.85	1.06	0.52	0.32	1.06
NM	20	0.95	0.73	0.66	0.78	1.06
TT	10	1.92	1.23	1.80	1.34	1.55
WG	10	1.24	1.16	1.57	1.28	1.27
WL	10	2.20	1.50	1.60	1.76	1.15
HB	10	1.21	1.05	0.97	0.85	1.18
KC	10	0.91	0.77	0.76	0.71	0.81
RW	10	0.97	0.95	1.08	1.21	1.05
FB	5	2.41	1.31	1.30	1.41	1.80
DB	5	1.11	0.96	0.92	1.38	0.99
RD	5	1.42	1.37	1.53	1.33	1.57
AB	5	1.02	0.93	0.92	0.73	1.47
RH	5	0.97	0.69	0.70	1.11	1.23
DS	5	1.50	1.37	1.51	1.59	1.59
TH	0.5	0.78	0.54	0.71	0.95	0.94
DV	0.5	1.51	0.87	0.69	0.67	0.81

Table 6.19 - Monocytes following OKT3 (minutes) 20 μg to 0.5 μg and controls (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0	60	240	480	1440
BL	0	0.27	0.27	0.36	0.25	0.37
HW	0	0.60	0.61	0.38	0.53	0.62
IA	0	0.70	0.61	0.72	0.83	0.80
GW	0	0.71	0.42	0.59	0.51	0.65
AG	0	0.40	0.33	0.47	0.42	0.38
NS	0	0.67	0.66	0.84	0.34	0.66
DC	20	0.48	0.29	0.23	0.36	0.41
BJ	20	0.59	0.41	0.26	0.40	0.47
GR	20	0.57	0.51	0.73	0.62	0.75
DD	20	0.33	0.33	0.27	0.31	0.35
AE	20	0.33	0.27	0.09	0.10	0.53
NM	20	0.35	0.44	0.41	0.43	0.42
IT	10	0.51	0.64	0.60	0.31	0.78
WG	10	0.53	0.49	0.58	0.61	0.48
WL	10	1.64	1.40	1.15	1.08	1.25
HB	10	0.32	0.39	0.39	0.32	0.43
KC	10	0.59	0.71	0.70	0.76	0.84
RW	10	0.55	0.65	0.77	0.68	0.45
FB	5	0.70	0.80	0.73	0.65	0.78
DB	5	0.42	0.38	0.37	0.55	0.37
RD	5	0.53	0.64	0.66	0.60	0.58
AB	5	1.00	0.95	0.97	1.02	1.17
RH	5	0.31	0.38	0.37	0.36	0.36
DS	5	1.24	1.07	1.06	1.08	1.29
TH	0.5	0.46	0.53	0.54	0.54	0.47
DV	0.5	0.99	0.84	0.83	0.82	0.80

Table 6.20 - NK cells following OKT3 (minutes) 20 μg to 0.5 μg and controls (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0	60	240	480	1440
BL	0	0.12	0.11	0.13	0.11	0.14
HW	0	0.32	0.56	0.36	0.41	0.40
IA	0	0.44	0.25	0.46	0.49	0.30
GW	0	0.38	0.13	0.10	0.16	0.29
AG	0	0.13	0.12	0.13	0.19	0.11
NS	0	0.08	0.06	0.07	0.08	0.03
DC	20	0.35	0.23	0.05	0.22	0.25
BJ	20	0.21	0.10	0.05	0.05	0.16
GR	20	0.30	0.16	0.26	0.25	0.18
DD	20	0.15	0.10	0.15	0.15	0.23
AE	20	0.32	0.10	0.08	0.02	0.19
NM	20	0.18	0.08	0.10	0.08	0.23
IT	10	0.07	0.06	0.07	0.07	0.05
WG	10	0.04	0.04	0.06	0.04	0.02
WL	10	0.18	0.12	0.12	0.13	0.04
HB	10	0.27	0.23	0.26	0.17	0.24
KC	10	0.24	0.22	0.23	0.22	0.24
RW	10	0.15	0.16	0.16	0.15	0.15
FB	5	0.39	0.13	0.16	0.18	0.32
DB	5	0.20	0.16	0.25	0.28	0.19
RD	5	0.28	0.31	0.22	0.21	0.38
AB	5	0.15	0.13	0.14	0.20	0.32
RH	5	0.11	0.06	0.06	0.10	0.16
DS	5	0.21	0.26	0.27	0.40	0.39
TH	0.5	0.06	0.03	0.03	0.07	0.06
DV	0.5	0.26	0.10	0.12	0.08	0.14

Table 6.21 - CD3$^+$NK cells following OKT3 (minutes) 20 μg to 0.5 μg and controls (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0	60	240	480	1440
BL	0	0.07	0.06	0.09	0.07	0.07
HW	0	0.42	0.42	0.39	0.39	0.41
IA	0	0.48	0.37	0.63	0.62	0.49
GW	0	0.33	0.12	0.10	0.16	0.22
AG	0	0.09	0.08	0.09	0.09	0.07
NS	0	0.03	0.05	0.04	0.02	0.21
DC	20	0.21	0.09	0.04	0.17	0.16
BJ	20	0.50	0.21	0.13	0.11	0.39
GR	20	0.09	0.07	0.08	0.12	0.13
DD	20	0.07	0.04	0.07	0.10	0.08
AE	20	0.27	0.09	0.03	0.01	0.14
NM	20	0.03	0.02	0.01	0.03	0.05
IT	10	0.53	0.37	0.53	0.38	0.40
WG	10	0.37	0.32	0.48	0.39	0.41
WL	10	0.32	0.17	0.16	0.23	0.09
HB	10	0.13	0.09	0.08	0.10	0.14
KC	10	0.05	0.04	0.04	0.04	0.05
RW	10	0.03	0.03	0.02	0.05	0.03
FB	5	0.49	0.18	0.19	0.23	0.31
DB	5	0.12	0.09	0.10	0.19	0.07
RD	5	0.96	0.85	0.10	0.92	0.92
AB	5	0.11	0.07	0.06	0.06	0.14
RH	5	0.28	0.16	0.21	0.25	0.41
DS	5	0.12	0.13	0.14	0.14	0.20
TH	0.5	0.03	0.03	0.03	0.03	0.07
DV	0.5	0.18	0.10	0.08	0.07	0.13

Table 6.22 - CD2 cells following OKT3 (minutes) 20 µg to 0.5 µg and controls (X 10^9 L^{-1}).

Patient	OKT3 (µg)	0	60	240	480	1440
BL	0	0.60	0.54	0.77	0.62	0.82
HW	0	2.56	2.85	2.11	2.26	2.75
IA	0	1.41	0.89	1.59	1.58	1.13
GW	0	1.40	0.63	0.60	0.73	1.09
AG	0	1.15	1.09	1.17	1.15	1.02
NS	0	1.21	1.10	1.19	0.33	0.93
DC	20	1.40	0.70	0.33	0.94	1.09
BJ	20	1.14	0.50	0.39	0.38	0.80
GR	20	1.36	0.98	1.17	1.43	1.18
DD	20	0.83	0.55	0.53	0.66	0.90
AE	20	1.41	0.78	0.31	0.20	0.74
NM	20	0.67	0.48	0.42	0.55	0.74
IT	10	1.72	1.03	1.56	1.18	1.37
WG	10	1.10	1.03	0.40	1.13	1.15
WL	10	1.86	1.23	1.34	1.53	0.87
HB	10	1.06	0.90	0.82	0.71	1.04
KC	10	0.73	0.57	0.56	0.51	0.62
RW	10	0.76	0.67	0.77	0.93	0.80
FB	5	2.10	1.15	1.08	1.21	1.54
DB	5	0.96	0.81	0.78	1.19	0.82
RD	5	1.19	1.08	1.21	1.08	1.28
AB	5	-	-	-	-	-
RH	5	-	-	-	-	-
DS	5	-	-	-	-	-
TH	0.5	-	-	-	-	-
DV	0.5	-	-	-	-	-

Table 6.23 - CD3 cells following OKT3 (minutes) 20 µg to 0.5 µg and controls (X 10^9 L^{-1}).

Patient	OKT3 (µg)	0	60	240	480	1440
BL	0	0.50	0.46	0.68	0.53	0.68
HW	0	2.31	2.38	1.75	1.98	2.39
IA	0	1.09	0.74	1.22	1.33	1.04
GW	0	1.37	0.59	0.60	0.69	0.95
AG	0	1.10	0.99	1.05	1.01	0.93
NS	0	1.16	1.07	1.11	0.27	0.93
DC	20	1.40	0.53	0.30	0.23	0.94
BJ	20	1.15	0.50	0.37	0.35	0.45
GR	20	1.08	0.82	0.95	1.21	1.02
DD	20	0.69	0.46	0.43	0.52	0.71
AE	20	1.28	1.71	0.29	0.19	0.66
NM	20	0.55	0.42	0.34	0.50	0.62
IT	10	1.69	0.97	1.51	1.14	1.32
WG	10	1.09	0.93	1.39	1.12	1.13
WL	10	1.76	1.18	1.27	1.45	0.92
HB	10	0.81	0.66	0.58	0.55	0.80
KC	10	0.56	0.42	0.42	0.36	0.48
RW	10	0.63	0.60	0.65	0.81	0.71
FB	5	1.79	1.07	0.96	1.06	1.26
DB	5	0.78	0.67	0.58	0.97	0.66
RD	5	0.99	0.88	1.04	0.89	0.97
AB	5	0.72	0.67	0.69	0.73	0.95
RH	5	0.73	0.50	0.63	0.79	0.95
DS	5	1.09	0.88	0.95	1.03	1.03
TH	0.5	0.54	0.37	0.45	0.64	0.67
DV	0.5	0.86	0.51	0.48	0.36	0.70

Table 6.24 - % CD3 cells coexpressing IL-2Rα (CD25) following OKT3 (minutes) 20 μg, to 0.5 μg and controls (X 10^9 L^{-1}).

Patient	OKT3 (μg)	0	60	240	480	1440
BL	0	8.2	8.5	8.5	9.6	7.8
HW	0	5.4	4.7	5.6	5.2	3.6
IA	0	3.9	5.5	5.0	5.2	6.4
GW	0	5.7	5.1	3.0	6.0	4.7
AG	0	20.9	18.8	18.7	17.5	21.1
NS	0	33.6	33.7	28.4	20.4	35.0
DC	20	-	-	-	-	-
BJ	20	-	-	-	-	-
GR	20	-	-	-	-	-
DD	20	21.2	19.9	15.7	-	-
AE	20	-	-	-	-	-
NM	20	19.3	20.3	-	-	-
IT	10	3.5	3.4	6.6	3.2	4.3
WG	10	6.6	7.8	8.6	8.6	4.7
WL	10	10.5	12.1	12.3	12.9	11.9
HB	10	10.6	11.1	10.6	-	-
KC	10	-	-	-	-	-
RW	10	11.2	12.8	-	-	6.0
FB	5	12.9	16.6	-	-	-
DB	5	11.8	17.7	-	-	-
RD	5	20.8	17.6	-	-	-
AB	5	-	-	-	-	-
RH	5	-	-	-	-	-
DS	5	-	-	-	-	-
TH	0.5	-	-	-	-	-
DV	0.5	-	-	-	-	-

Table 6.25 - K562 cytotoxicity (LU/10^7PBMC) and *in vitro* manipulation in control group undergoing 24 hour investigations.
* = performed at E:T of 20:1, ** ≤ 15:1

Patient	mAb/IL-2	Minutes			
		0	60	240	1440
BL	Medium	24.7	14.5	28.5	6.7
	OKT3	42.5	31.1	42.0	73.3
	T3	93.2	68.4	91.2	103.5
	IL-2	65.1	54.1	88.4	128.2
	IL-2 + OKT3	55.4	41.4	52.5	102.3
HW	Medium	0.0	0.0	0.0	0.0
	OKT3	0.0	0.1	0.1	0.0
	T3	4.1	7.3	4.4	3.6
	IL-2	0.0	0.0	0.0	0.0
	IL-2 + OKT3	0.0	0.2	0.3	0.1
IA	Medium	23.0	13.3	24.1	8.6
	OKT3	75.8	56.6	34.2	10.9
	T3	177.3	169.5	112.4	44.0
	IL-2	59.5	38.7	41.5	10.8
GW	Medium	21.9	15.7	5.2	18.7
	OKT3	71.0	38.6	10.0	35.9
	T3	144.5	58.4	24.6	58.8
	IL-2	55.1	28.7	12.1	23.6
AG	Medium	0.0*	0.0*	0.1*	0.0*
	OKT3	2.3*	1.8*	11.1*	0.0*
	T3	17.6*	39.7*	40.2*	10.2*
	IL-2	0.2*	0.1*	1.5*	0.3*
NS	Medium	0.0	0.0	0.0	0.0
	OKT3	6.9	6.3	9.4	4.3
	T3	17.7	12.2	18.0	8.9
	IL-2	0.1	0.0	0.4	0.1

Table 6.26 - K562 cytotoxicity (LU/10^7PBMC) and *in vitro* manipulation in 50 μg group undergoing 8 hour investigations.

Patient	mAb	Minutes			
		0	30	60	240
DH	Medium	24.9	17.6	5.9	0.0*
	OKT3	44.0	14.4	4.2	0.0*
	T3	123.2	29.1	13.8*	0.0*
AP	Medium	34.4	43.5	4.2*	0.0
	OKT3	57.6	53.0	19.1*	6.8
	T3	125.5	98.5	41.0*	13.5
IB	Medium	9.3	4.0	0.0*	0.2*
	OKT3	27.7	5.0	0.9*	0.4*
	T3	45.2	7.1	6.5*	0.3*
WD	Medium	13.1*	26.8*	26.5*	0.1**
	OKT3	14.7*	18.6*	26.7*	0.0**
	T3	20.9*	26.1*	26.6*	0.0**
ME	Medium	42.8	45.1	34.3	8.8
	OKT3	54.5	53.8	33.8	14.6
	T3	40.0	36.5	27.6	12.8
WW	Medium	0.0	0.0	0.0*	0.0**
	OKT3	0.0	0.0	0.0*	0.0**
	T3	8.7	0.5	0.0*	0.0**

Table 6.27 - Daudi cytotoxicity (LU/10^{7}PBMC) and *in vitro* manipulation in 50 μg group undergoing 8 hour investigations.

Patient	mAb	Minutes			
		0	30	60	240
DH	Medium	0.0	0.0*	0.0*	0.0*
	OKT3	0.0	0.0*	0.0*	0.0*
	T3	0.0	0.0*	0.0*	0.0*
AP	Medium	0.0	0.0	0.0*	0.0*
	OKT3	0.0	0.0	0.0*	0.0*
	T3	27.0	3.9	0.0*	6.8*
IB	Medium	0.7	0.0	0.0*	0.0*
	OKT3	1.8	0.0	0.0*	0.0*
	T3	1.6	0.0	0.0*	0.0*
WD	Medium	0.0*	0.0*	0.0*	0.0**
	OKT3	0.0*	0.0*	0.0*	0.0**
	T3	0.0*	0.0*	0.0*	0.0**
ME	Medium	10.4	5.7	2.3	0.1
	OKT3	5.0	4.0	1.4	0.2
	T3	3.4	2.3	1.4	0.0
WW	Medium	0.0	0.0	0.0*	0.0**
	OKT3	0.0	0.0	0.0*	0.0**
	T3	0.0	0.0	0.0*	0.0**

Table 6.28 - K562 cytotoxicity (LU/10^{7}PBMC) and *in vitro* manipulation in control group undergoing 8 hour investigations.

Patient	mAb	Minutes			
		0	30	60	240
MD	Medium	0.8	6.2	1.1	1.0
	OKT3	7.7	23.1	16.1	18.0
	T3	10.5	35.2	23.2	19.5
PW	Medium	24.7	7.7	9.8	0.2
	OKT3	49.3	12.7	13.2	0.7
	T3	61.3	21.5	12.7	0.3
GG	Medium	197.2	79.4	47.2	115.7
	OKT3	507.6	142.3	65.0	114.7
	T3	519.5	171.2	68.5	135.5
HC	Medium	97.0	81.8	48.0	226.8*
	OKT3	141.4	150.6	268.8	323.6*
	T3	233.1	217.9	242.1	467.3*

Table 6.29 - Daudi cytotoxicity (LU/10^{7}PBMC) and *in vitro* manipulation in control group undergoing 8 hour investigations.

Patient	mAb	Minutes			
		0	30	60	240
MD	Medium	0.1	0.0	0.2	0.0
	OKT3	0.8	0.0	0.7	0.1
	T3	1.3	1.0	1.3	0.4
PW	Medium	0.0	0.0	0.0	0.0
	OKT3	0.0	0.0	0.0	0.0
	T3	0.0	0.1	0.1	0.2
GG	Medium	38.4	11.1	9.9	8.7
	OKT3	50.1	23.8	17.7	11.6
	T3	88.5	18.4	15.8	9.2
HC	Medium	0.0	0.0	0.0	0.0*
	OKT3	0.0	0.4	1.1	0.6*
	T3	0.0	0.3	0.4	2.7*

Table 6.30 -   K562 cytotoxicity (LU/10^{7}PBMC) and *in vitro* manipulation in
20 µg group undergoing 24 hour investigations.

Patient	mAb/IL-2	0	Minutes 60	240	1440
DC	Medium	4.7	3.6	0.0	25.7
	OKT3	15.8	6.1	0.0	41.3
	T3	25.6	13.9	0.0	76.6
BJ	Medium	39.4*	13.9*	2.6*	56.4*
	OKT3	74.9*	17.7*	8.4*	65.9*
	T3	117.1*	32.4*	5.0*	91.2*
GR	Medium	50.3*	34.4*	54.8*	44.6*
	OKT3	60.2*	36.5*	49.6*	52.3*
	T3	67.6*	39.0*	58.7*	63.3*
DD	Medium	1.2*	1.7*	2.2*	86.4*
	OKT3	9.8*	14.1*	2.4*	113.9*
	T3	11.8*	8.5*	7.4*	107.4*
	IL-2	20.6*	22.4*	2.7*	91.0*
	IL-2 + OKT3	22.4*	23.6*	5.4*	121.4*
AE	Medium	0.3	0.4	0.0*	4.6*
	OKT3	21.5	0.3	0.2*	8.8*
	T3	227.5	33.5	6.0*	155.8*
	IL-2	17.3	2.9	0.1*	12.0*
	IL-2 + OKT3	28.7	3.1	0.5*	13.6*
NM	Medium	4.4*	2.0*	0.6*	32.4*
	OKT3	24.8*	2.8*	3.3*	110.5*
	T3	44.9*	4.4*	10.6*	208.5*
	IL-2	8.5*	5.3*	3.3*	52.8*
	IL-2 + OKT3	47.4*	24.5*	34.8*	101.6*

Table 6.31 -   K562 cytotoxicity (LU/10^{7}PBMC) and *in vitro* manipulation in
10 µg group undergoing 24 hour investigations.

Patient	mAb/IL-2	0	Minutes 60	240	1440
IT	Medium	46.3	51.7	62.4	0.2
	OKT3	276.2	104.9	342.5	0.9
	T3	869.6	271.7	943.5	6.5
WG	Medium	0.2	0.3	4.6	0.0
	OKT3	19.0	14.6	10.5	0.1
	T3	363.6	283.3	729.1	26.5
WL	Medium	1.1	0.0	1.9	0.0
	OKT3	1.3	0.0	2.0	0.0
	T3	6.6	3.0	16.4	0.0
HB	Medium	70.7*	88.3*	146.4*	238.1*
	OKT3	124.8*	126.7*	212.8*	442.5*
	T3	326.8*	284.7*	420.2*	980.4*
	IL-2	171.2*	180.0*	222.7*	260.8*
	IL-2 + OKT3	179.2*	143.1*	234.5*	453.3*
KC	Medium	3.3	3.8	4.8	19.5*
	OKT3	16.5	8.7	5.7	35.4*
	T3	106.2	68.1	56.0	153.1*
	IL-2	22.9	17.8	13.8	45.4*
	IL-2 + OKT3	18.4	10.3	7.5	52.3*
RW	Medium	3.1*	6.7*	0.1*	0.0*
	OKT3	6.9*	5.4*	0.1*	0.2*
	T3	12.3*	3.6*	0.8*	5.9*
	IL-2	0.3*	1.7*	0.3*	0.1*
	IL-2 + OKT3	12.6*	8.6*	0.8*	0.3*

Table 6.32 -   K562 cytotoxicity (LU/10^{7}PBMC) and *in vitro* manipulation in
5 µg group undergoing 24 hour investigations.

Patient	mAb/IL-2	0	Minutes 60	240	1440
FB	Medium	52.4	19.1	27.3	138.7
	OKT3	63.5	21.7	34.7	146.2
	IL-2	72.2	32.0	49.2	278.6
	IL-2 + OKT3	67.3	34.8	4.1	235.9
DB	Medium	21.2	37.7	39.4	81.9*
	OKT3	33.1	44.4	42.8	100.5*
	IL-2	41.4	50.4	58.9	124.4*
	IL-2 + OKT3	45.4	59.7	63.7	136.4*
RD	Medium	8.6	7.9	4.2	21.5
	OKT3	17.9	11.7	10.2	36.1
	IL-2	14.6	14.2	9.6	37.8
	IL-2 + OKT3	26.8	20.3	17.0	52.6
AB	Medium	20.5	27.6	27.8	65.5
	OKT3	21.0	25.1	28.4	91.2
	T3	29.7	42.8	41.6	127.7
RH	Medium	0.8	1.3	0.8	1.6
	OKT3	0.9	1.0	0.7	4.5
	T3	53.0	52.9	32.4	28.0
DS	Medium	0.1	0.2	0.34	14.3
	OKT3	0.4	0.5	0.9	10.8
	T3	0.9	0.9	0.9	17.1

Table 6.33 -   K562 cytotoxicity (LU/10^{7}PBMC) and *in vitro* manipulation in
0.5 µg group undergoing 24 hour investigations.

Patient	mAb	0	Minutes 60	240	1440
TH	Medium	0.0	0.0	0.0	20.1
	OKT3	0.1	0.2	0.1	32.7
	T3	2.9	1.3	0.6	33.6
DV	Medium	0.9	1.8	1.7	0.4
	OKT3	1.4	1.9	1.5	1.3
	T3	1.2	1.3	1.6	2.0

Table 6.34 - Comparison of % cytotoxicity against K562 and Daudi cells at an E:T ratio of 20:1 in 10 cancer patients.

Patient	Time (mins)	K562			DAUDI		
		medium	OKT3	T3	medium	OKT3	T3
DH	0	17.6	28.6	39.4	1.1	3.4	7.8
	30	22.2	21.8	27.2	6.7	4.7	7.1
	60	16.9	17.5	15.8	4.1	2.9	6.8
	240	5.9	5.5	9.2	3.5	2.5	3.5
AP	0	26.2	32.0	45.7	6.6	10.3	30.1
	30	27.9	31.8	38.9	10.9	8.0	20.6
	60	9.2	15.7	20.1	3.1	3.0	4.1
	240	10.4	13.6	15.7	7.2	6.5	18.7
IB	0	15.3	23.2	28.5	8.1	2.3	16.1
	30	11.2	13.3	15.7	5.5	10.0	7.8
	60	7.6	11.7	17.7	3.9	3.5	6.7
	240	10.1	10.0	10.6	2.5	3.8	3.0
WD	0	14.9	16.2	19.6	1.9	1.8	3.8
	30	23.8	20.9	24.5	4.6	2.5	3.3
	60	24.9	24.0	24.3	3.7	2.8	4.7
	240	2.9	1.9	1.9	0.0	0.3	0.0
ME	0	27.6	31.1	29.7	20.8	18.1	20.0
	30	28.0	30.2	25.4	16.2	16.5	16.2
	60	22.9	25.1	23.2	12.8	11.4	12.5
	240	13.6	14.8	15.9	6.7	8.1	9.5
WW	0	4.3	7.2	19.3	0.4	2.7	12.3
	30	5.9	5.4	10.2	2.2	0.5	5.4
	60	2.6	2.9	7.8	0.9	1.5	3.8
	240	3.6	2.5	5.5	2.3	1.5	3.0
MD	0	10.1	17.6	20.5	3.4	4.1	6.2
	30	9.6	20.2	25.8	3.2	4.3	5.6
	60	5.0	16.1	21.9	3.8	6.9	8.5
	240	10.5	19.8	20.0	3.4	4.6	5.4
PW	0	20.1	32.8	32.7	4.3	5.1	6.9
	30	13.9	18.6	23.1	3.4	5.7	7.3
	60	15.2	16.9	18.5	2.9	5.7	6.8
	240	9.8	8.9	8.4	2.9	1.8	4.6
GG	0	56.0	61.6	62.4	28.7	31.9	35.5
	30	40.5	47.1	50.2	21.0	28.4	21.1
	60	29.6	34.8	31.9	15.4	14.9	17.7
	240	44.2	41.4	47.0	13.3	13.4	12.1
HC	0	42.2	47.5	49.9	3.3	8.4	11.6
	30	37.8	49.9	51.9	4.9	10.4	11.6
	60	29.2	50.4	43.1	4.0	12.1	13.9
	240	53.5	59.7	65.9	3.9	11.3	16.9

Table 6.35 - % cells in blood and PBMC preparations from 6 cancer patients undergoing serial monitoring of NK function.

TIME	(hours)	BLOOD				PBMC			
		0	1	4	24	0	1	4	24
BL	mono	8.6	7.8	7.0	7.6	23.9	24.3	14.2	18.5
	neut	66.6	71.8	73.8	70.1	10.9	11.1	6.7	8.3
	lymphs	24.8	20.2	19.2	21.4	65.3	64.7	79.1	73.1
	NK	15.6	14.9	13.3	13.6	20.0	20.3	24.8	20.0
HW	mono	6.4	6.3	4.5	6.7	19.0	16.5	9.2	7.9
	neut	63.2	61.0	68.0	62.2	7.3	9.6	6.9	6.9
	lymphs	30.4	32.7	27.6	31.1	73.7	73.9	83.9	85.2
	NK	11.0	17.8	15.5	13.5	18.2	16.2	22.5	18.6
IA	mono	11.7	13.2	13.0	12.1	13.6	7.2	12.2	22.5
	neut	53.6	55.9	45.6	61.1	11.6	5.8	9.7	21.9
	lymphs	34.7	30.9	41.4	26.6	74.7	87.0	78.1	55.6
	NK	21.3	17.7	20.1	17.1	33.9	27.5	27.9	21.4
GW	mono	8.0	5.4	7.8	9.0	18.5	19.9	18.7	19.8
	neut	69.3	81.7	79.7	71.4	17.4	23.7	14.7	30.3
	lymphs	22.8	12.9	12.5	19.6	64.1	56.4	66.6	49.4
	NK	18.6	13.4	10.7	20.1	34.6	28.3	19.6	27.3
AG	mono	8.6	7.0	6.7	8.0	14.7	12.2	13.7	7.0
	neut	63.7	67.0	74.6	68.0	10.2	5.7	5.1	9.5
	lymphs	27.7	26.1	18.8	24.0	75.2	82.1	81.3	83.5
	NK	9.9	9.9	10.0	9.3	10.0	11.6	12.1	15.3
NS	mono	11.4	10.5	10.0	11.9	22.1	25.1	26.8	20.9
	neut	65.1	69.5	73.7	68.7	11.6	6.8	5.5	7.8
	lymphs	23.5	20.5	16.3	19.4	66.4	68.2	67.7	71.3
	NK	5.4	4.8	5.3	3.2	8.6	6.3	7.1	4.5
Mean	mono	9.1	8.4	8.2	9.2	18.6	17.5	15.8	16.1
SEM		0.8	1.2	1.2	0.9	1.6	2.9	2.5	2.8
Mean	neut	63.6	67.8	69.2	66.9	11.5	10.5	8.1	14.1
SEM		2.2	3.7	5.0	1.7	1.4	2.8	1.5	4.0
Mean	lymphs	27.3	23.9	22.6	23.7	69.9	72.1	76.1	69.7
SEM		1.9	3.0	4.3	1.9	2.1	4.6	3.0	5.9
Mean	NK	13.6	13.1	12.5	12.8	20.9	18.4	19.0	17.9
SEM		2.4	2.1	2.1	2.4	4.6	3.6	3.2	3.1

Table 6.36 - Variation in lymphoctes and NK cells in PBMC fractions compared with blood following OKT3.

Patient	Hours	% lymphs blood	%NK blood	% lymphs PBMC	%NK PBMC	NK in PBMC $(X\ 10^5\ ml^{-1})$
FB	0	25.4	16.0	56.5	47.5	3.80
(5 µg)	1	16.8	10.0	60.4	39.4	3.15
	4	15.1	12.5	54.7	44.4	3.55
	24	18.3	17.8	64.5	42.9	3.43
DB	0	18.1	18.1	75.7	35.3	2.82
(5 µg)	1	16.6	16.6	72.2	30.6	2.45
	4	27.6	27.6	77.4	39.7	3.18
	24	19.3	19.3	71.6	31.6	2.53
RD	0	18.9	18.7	75.2	35.6	2.85
(5 µg)	1	17.6	22.2	73.0	37.5	3.00
	4	15.1	14.5	64.0	36.4	2.91
	24	18.2	24.1	74.7	42.1	3.37
HB	0	30.9	22.1	83.1	30.4	2.43
(10 µg)	1	26.8	21.9	73.9	30.8	2.46
	4	19.8	27.2	84.8	36.0	2.88
	24	25.2	20.5	90.0	29.7	2.38
KC	0	13.8	26.4	62.4	40.3	3.22
(10 µg)	1	9.5	28.9	46.5	36.4	2.91
	4	8.0	29.7	48.3	35.9	2.87
	24	8.9	29.3	58.1	38.6	3.09
RW	0	12.6	15.6	59.5	19.6	1.57
(10 µg)	1	11.9	16.9	60.6	19.4	1.55
	4	10.6	14.3	57.3	14.0	1.12
	24	14.6	14.5	62.6	20.7	1.66
DD	0	19.5	15.7	81.1	22.1	1.77
(20 µg)	1	13.8	15.7	78.7	21.1	1.69
	4	12.6	29.5	86.9	30.6	2.45
	24	19.5	22.2	82.8	30.8	2.46
AE	0	31.4	17.5	81.6	25.8	2.06
(20 µg)	1	22.1	9.5	77.1	14.0	1.12
	4	5.2	16.2	81.3	21.7	1.74
	24	11.4	18.3	73.4	23.8	1.90
NM	0	18.0	19.0	69.8	22.6	1.81
(20 µg)	1	13.8	11.5	63.8	10.5	0.84
	4	11.3	14.6	64.7	14.0	1.12
	24	16.6	21.9	71.5	31.1	2.49

Table 6.37 - Variation in NK % cells (means ($\pm$ SEM)) in blood following OKT3 compared with changes in K562 cytotoxicity (medians) (LU/10^7 PBMC).

OKT3		Minutes				
		0	30	60	240	1440
50 µg	NK	15.5	15.4	15.6	15.4	-
(n = 6)		1.6	2.4	3.0	2.2	-
	cyto	19.0	22.2	5.1	0.1	-
Controls	NK	24.4	24.3	23.1	24.5	-
(n = 4)		5.9	4.5	3.6	5.8	-
	cyto	60.9	43.5	28.5	58.4	-
20 µg	NK	17.3	-	14.4	14.6	17.8
(n = 6)		1.0	-	2.2	2.2	1.6
	cyto	4.5	-	2.8	1.4	38.5
10 µg	NK	13.2	-	13.9	14.5	12.1
(n = 6)		4.0	-	4.1	4.7	4.6
	cyto	3.2	-	5.2	4.7	0.1
5 µg	NK	15.7	-	15.2	15.9	20.1
(n = 6)		1.2	-	2.2	2.6	1.8
	cyto	14.6	-	13.5	15.8	43.5
Controls	NK	13.7	-	13.1	12.4	12.8
(n = 6)		2.4	-	2.1	2.1	2.5
	cyto	11.0	-	6.6	2.6	4.3

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