AN INVESTIGATION OF THE INTERACTIONS BETWEEN LYMPHOKINE ACTIVATED KILLER CELLS AND T-LYMPHOCYTES IN VITRO.

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This thesis is dedicated to
my parents for their encouragement over the years
and to the memory of
Mr. Robert Chambers and Mrs. Elizabeth Cameron.

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ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
BCG	Bacille Calmette Guérin
CD	Cluster of differentiation antigen
CEA	Carcinoembryonic antigen
cpm	Counts per minute
CTL	Cytotoxic T-lymphocyte
CTLL	Cytotoxic T-lymphocyte line
DNA	Deoxyribonucleic acid
EBNA	Epstein Barr Virus nuclear antigens
EBV	Epstein Barr Virus
ELISA	Enzyme-linked immunoabsorbent assay
F(ab)	Fragment antigen-binding
Fc	Fragment crystalline
FCS	Foetal calf serum
GM-CSF	Granulocyte-macrophage colony stimulating factor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLA	Human leucocyte antigen
ICAM	Intracellular adhesion molecule
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IU	International units
kD	Kilodalton
LAK	Lymphokine-activated killer cell
LCL	Lymphoblastoid cell line
LFA	Lymphocyte function-associated antigen
LGL	Large granular lymphocyte
LMP	Latent membrane protein

MBq	Mega Becquerel
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MNC	Mononuclear cells
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCMC	Natural cell-mediated cytotoxicity
NIBSC	National Institute of Biological Standards and Controls
NK	Natural Killer cells
NKCF	Natural Killer cell cytolytic factor
OD	Optical density
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rh	Recombinant human
RPMI	Roswell Park Memorial Institute
TAA	Tumour-associated antigen
TBS	Tris buffered saline
T-cell	T-lymphocyte
TCGF	T-cell growth factor
TCR	T-cell receptor
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TIL	Tumour infiltrating lymphocytes
TNFα	Tumour necrosis factor alpha
TNFβ	Tumour necrosis factor beta
TSA	Tumour specific antigen
U	Units
UV	Ultraviolet

SUMMARY

Lymphokine activated killer (LAK) cells are efficient mediators of tumour destruction in experimental models of metastatic cancer, but their mode of action in vivo is poorly understood. The general efficacy of LAK cell therapy in humans has been disappointing, although notable individual successes were observed in clinical trials, particularly in patients with melanoma or renal cell carcinoma. However, the mechanism by which LAK cells mediate tumour regression in these patients remains undefined. Although LAK cells are capable of directly killing tumour cells in vitro, evidence that they localise at the site of the tumour in vivo is sparse: it has been shown that following administration, LAK cells do not accumulate at or infiltrate into metastatic lesions. Intravenous infusion of LAK cells into the peripheral circulation results in LAK cell trafficking via the lungs to the liver and spleen for clearance. Since LAK cells in vivo appear to infiltrate the spleen, which contains a lymphoid reservoir of T-cells and monocytes, the opportunity for LAK cells to interact with the hosts' immunocytes may exist. Consequently, any effects which LAK cells exert on tumour regression may be indirect, via the local secretion of secondary cytokines and/or their interactions with other cells of the immune system. This project therefore investigates the hypothesis that regulatory cytokines, known to be produced by LAK cells, may augment a cytotoxic T-lymphocyte mediated anti-tumour response, assuming that T-cells primed to the tumour are present in the spleen.

The premise for this hypothesis is based on previous observations in a murine model, which demonstrated that the systemic administration of interleukin-2 (IL-2) to mice enhanced the delayed hypersensitivity reaction to a contact allergen. It was subsequently shown that the effects of IL-2 observed on this T-cell mediated response could be modulated by LAK cells, suggesting that the presence of LAK cells altered the effects of IL-2 in the spleen. It therefore appeared that LAK cells could interact with activated T-cells within the immune system and thus, modulate their function. This is particularly interesting since LAK cells can secrete secondary cytokines which may be capable of regulating T-cell activity in vitro.

To address the proposal that LAK cells could modulate T-cell function in vitro, through the release of secondary cytokines, LAK cells were induced with either recombinant human IL-2 or soluble monoclonal anti-CD3. By varying both time in culture and dose of stimulus, the production of cytokines (tumour necrosis factor alpha, interferon gamma and interleukin-1) and the cytotoxic capacity of various LAK cell populations were compared. Both stimulants induced LAK activity although the LAK activity of these effector cells showed poor correlation with the cytokines produced.

To determine whether LAK cell-derived supernatants had the capability of regulating T-cell cytotoxicity in vitro, modulatory effects on a cytotoxic T-cell line were studied. The ability of these cells to kill an EBV transformed target cell line, of appropriate HLA haplotype, was not enhanced by any of the supernatants generated over a five hour period. Increasing exposure of both the T-cell and target cell lines to the supernatants to twenty-four hours produced complications: exposure of target cells to supernatants alone for a prolonged period induced lysis. A few, random LAK supernatants augmented cytotoxicity when the T-cell line was preactivated with supernatant for nineteen hours prior to incorporating target cells into the assay. However the enhanced killing observed was not considerably different to the lytic activities obtained in the initial five hour cytotoxicity assay. Furthermore, for those supernatants involved, no predictive pattern could be determined when considering cytokine levels within the supernatant itself or the parameters under which the supernatant was generated. Thus, in this system, secondary cytokines produced by LAK cells were unable to enhance T-cell cytotoxicity in vitro, with only a few exceptions.

The ability to regulate T-cell function by increasing T-cell proliferation was also addressed. Peripheral T-cells proliferated in response to the IL-2 induced supernatants over seventy-two hours. However, stimulation with these supernatants plus exogenous, soluble anti-CD3 enhanced the response considerably. The proliferation stimulated by the anti-CD3 induced supernatants was more pronounced, although the addition of exogenous anti-CD3 had marginal effect on the proliferative response observed.

In conclusion, recombinant IL-2 and soluble monoclonal anti-CD3 were both capable of inducing LAK activity and cytokine secretion in normal peripheral blood mononuclear cells. However, the secondary cytokines produced by these LAK populations had only marginal effects on T-cell cytotoxicity and/or proliferation in vitro within this system. These results imply that secondary cytokine production by LAK cells would fail to modulate T-cell function in vivo, but further study would be required to confirm whether this is the case.

INTRODUCTION

The foundations of tumour immunology were derived from the work of Paul Ehrlich in the early part of this century. His concept of immunological mechanisms being occasionally directed against autologous cancer cells expressing antigenic structures which the host could recognise as foreign and the idea that this immune surveillance was responsible for the relatively low frequency of tumours still remains largely unproven. Early transplantation studies in the 1950s led to Thomas and Burnet proposing the "immune surveillance theory". They postulated that one function of the immune system was to recognise and destroy nascent neoplastic cells, therefore serving as a mechanism for the control of carcinogenesis (**Grossman and Herberman, 1986; Kripke, 1988; Johnson et al ,1989**). Although subject to much debate, the concept of immunosurveillance has kindled much research into tumour immunology over the past few decades.

1.1 : THE IMMUNOSURVEILLANCE THEORY

The hypothesis of immune surveillance against cancer was based on the following assumptions:

(1) Cancer cells are antigenic, as transformed and normal cells differ antigenically.
(2) Cancer cells can be recognised as "foreign" and destroyed by the immune system.
(3) A systemic, antigen-specific T-cell mediated immune response is involved.
(4) Neoplasia is associated with increased immunosuppression.

It was proposed that cancer arose when the immune system of the host was depressed or when the transformed cells lacked antigenicity. The aetiology of the cancer was thought to determine tumour antigenicity and the identification of three broad tumour categories followed:

(1) VIRALLY-INDUCED TUMOURS - generally antigenic, inducing protective immunity to all tumour associated with the virus.
(2) CHEMICALLY-INDUCED TUMOURS - generally antigenic with varying ability to induce protective immunity (usually specific for immunising tumour).
(3) SPONTANEOUS TUMOURS - lacked ability to confer protective immunity due to poor immunogenicity.

Immune surveillance, as originally defined, would only operate against highly immunogenic tumours. Studies in experimental animals suggests that immune surveillance operates effectively against tumours induced by oncogenic viruses or U.V. radiation, which would be highly immunogenic. Thus, our present knowledge of

experimental tumour systems might suggest that only highly-antigenic human cancers would be subject to immune surveillance. However differing opinions exist. **Hewitt et al (1976)** argue that highly antigenic experimental tumours in animals induced by chemical carcinogens, are not representative of human cancers. These are more likely to resemble spontaneous murine tumours, which are uniformly non-immunogenic in nature (**Kripke, 1988**). The validity of this argument is dependent on the premise that the majority of human tumours are spontaneous rather than virally, chemically or radiologically induced. Therefore, perhaps the important distinction between human and experimental tumours is their aetiology and consequently the immunogenicity conferred.

Grossman and Herberman (1986) propose a further school of thought, postulating "immune surveillance" without immunogenicity, where tumour escape may be a direct result of escape from the regulation of differentiation. They discuss the potential role of lymphoid cells in preventing the accumulation of small, irregular phenotypic and karyotypic changes in normal tissue. Normal developing cells should differentiate and mature towards a specialised phenotype and function, under the influence of growth and differentiation factors and their microenvironment. In the absence of such control, perhaps as a result of genetic changes, these cells may stay in a state of cell division instead of reaching maturity. An event of this nature may also render these cells less responsive to differentiation. Such a "transformation" towards a state of cellular immortality would develop as the cell adapts to express those genes associated with division at the expense of those genes responsible for the karyotype and function of the cell. Thus, changes in the responsiveness to haematopoeitic growth factors/cytokines, produced by both lymphoid cells and the cells within the microenvironment, may alter the regulatory pressure on cell maturation. This could lead to a population imbalance in either haematopoeitic tissue or tissue with a rapid cell turnover, and possibly to malignancy in these areas.

Unstimulated peripheral blood mononuclear cells (PBMC) from non-tumour bearing individuals have been shown to lyse some tumour targets in vitro. A restricted range of transformed cells, usually of lymphoid origin, were susceptible to lysis whilst cells originating from solid tumours were resistant. The phenomenon was called natural cell-mediated cytotoxicity (NCMC). NCMC was innate, occurring without prior exposure to tumour targets, and was non-MHC restricted. Large granular lymphocytes (LGL) were found to mediate NCMC and termed "natural killer (NK)" cells. NK cells lack the ability to lyse solid tumour cells and therefore probably have little relevance in immune lysis of solid tumours. However NK cell recognition of "abnormal" haematopoeitic

cells suggests a role for NK cells in the immunosurveillance of lymphoproliferative malignancies (**Herberman and Ortaldo, 1981; Trinchieri, 1989**).

1.2 : EVIDENCE FOR IMMUNE RECOGNITION OF TUMOURS, ANTI-TUMOUR IMMUNE RESPONSES AND TUMOUR ESCAPE MECHANISMS

Cell-mediated immunity to a tumour may be either specific or non-specific in nature. A specific anti-tumour response is dependent on the tumour being recognised in the context of major histocompatibility complex (MHC) antigens. The majority of MHC-restricted anti-tumour responses are mediated by $CD8^+$ cytotoxic T-lymphocytes (CTL) and $CD8^+$ tumour infiltrating lymphocytes (TIL) via T-cell receptor (TCR) interactions with MHC class I molecules and antigen, although MHC class-II restricted responses mediated by $CD4^+$ T-lymphocytes have also been reported (**Topalian et al, 1994**). Non-specific responses are non-MHC restricted and include direct anti-tumour cytotoxicity mediated by NK cells, lymphokine-activated killer (LAK) cells or monocytes.

Recognition and immune response to tumours may depend on several factors:

(1) The MHC expression on the tumour.

(2) The existence of tumour-specific antigens (TSA) and tumour-associated antigens (TAA).

(3) Infiltration of the tumour by lymphoid cells.

(4) Cellular interactions: communication between lymphoid cells and other cells of the body via surface antigens, MHC, adhesion molecules and cytokines.

(5) The existence of tumour immunosuppressive effects.

(1) MHC EXPRESSION

Variation in the expression of both MHC-class I and class II molecules has been observed on human tumours. The loss of MHC class I antigens due to the lack of β 2-microglobulin messenger ribonucleic acid (mRNA) expression has been observed in colorectal carcinoma (**Momburg and Koch, 1989**). In contrast, the expression of MHC class II molecules in malignant melanoma is associated with a poor prognosis and the early development of metastases (**Bröcker et al, 1985**). The failure of tumours to express classical MHC antigens may have important implications for the success of a specific CTL anti-tumour response. Induction of MHC antigens on tumour targets by cytokines has been shown to render targets more susceptible to specific CTL-mediated lysis. Conversely however, tumour cell lysis by non-specific effector cells is reduced when MHC expression is augmented (**Guillou, 1990**). Thus, aberrant MHC expression

on human tumours may be a pivotal factor in determining whether immune recognition or tumour escape occurs.

(2) TUMOUR ANTIGENS

In order to produce an anti-tumour response, the immune system must be capable of recognising and discriminating any structural and antigenic differences between tumour and normal cells. In addition to the identification of oncofetal antigens such as carcinoembryonic antigen (CEA), monoclonal antibody technology has aided in the search for TSA or TAA. Many new tumour associated antigens have been identified on human tumours including: gastrointestinal tumours, bladder carcinoma, renal cell carcinoma, bronchial carcinoma, lymphoma and melanoma (reviewed by **Johnson et al, 1989**) The development of the monoclonal antibody "14 C1" (**Al-Azzawi et al, 1987**) has led to the discovery of a 32 kilodalton (kD) molecule associated with ovarian cancer (**Gallagher et al, 1991**).

Perhaps the most extensively researched tumour antigens are those associated with melanoma, via analysis at the molecular level and the assessment of cytotoxic T-cell (CTL) responses. **Lotze and Tomita et al (1989)** demonstrated that anti-human melanoma CTL clones ($CD8^+$ TIL) can be generated directly from tumour-infiltrating lymphocytes. They developed CTL clones specific for autologous fresh tumour but non-reactive with either allogeneic tumour or autologous targets, such as fibroblasts or Epstein Barr Virus (EBV) transformed B-cells. Lysis of autologous tumour could be blocked by the W6/32 antibody against MHC class I, implicating an obligatory role for class I molecules in antigenic processing and presentation. This was substantiated by the observation that pre-treatment of autologous tumour with interferon gamma (IFNγ) increased lysis by a cloned T-cell line, probably via increased MHC expression in response to IFNγ stimulation **(Farrar and Schreiber, 1993)**.

A more extensive study was conducted by **Hersey et al (1988)** in order to elucidate the nature of the antigens recognised by CTL derived from melanoma patients. They assessed the degree of proliferation of melanoma-derived CTL clones in response to antigen(s), as cellular extracts from melanoma patients, following immobilisation on nitrocellulose. Proliferation was observed and the antigen(s) responsible for stimulation in all instances were around 48 kD in molecular weight, consisting of protein and glycolipid components. CTL clones specific for melanoma were not stimulated by those extracts derived from non-melanoma cell preparations. These proliferative responses were again shown to be dependent on the presence of autologous or histocompatible antigen presenting cells (APC). In contrast to **Lotze and**

Tomita et al (1989), antigen presentation appeared to be via MHC class II molecules since only antibodies against class II antigens inhibited the proliferative response. Pre-treatment of CTL with anti-CD3 antibody likewise inhibited the process, suggesting the CTL response was mediated via interactions with the T-cell receptor (TCR).
At a molecular level the identification of the human gene MAGE-1 (**Brasseur et al, 1992**) has been an interesting discovery. It is believed that this gene directs the expression of the MZ2-E antigen, a molecule potentially associated with tumour rejection. The study reports that five of the fourteen melanoma cell lines examined expressed the MAGE-1 gene. Likewise several primary melanoma tumour samples expressed MAGE-1 whereas a panel of normal tissues proved negative for expression of the gene. The MZ2-E antigen has been shown to be recognised on the MZ2-MEL melanoma cell-line by autologous $CD8^+$ CTL, with recent evidence indicating MZ2-E presentation to be HLA-A1 restricted. This paper also reports MAGE-1 expression in a significant number of breast carcinomas (20% of tumours examined were positive) whilst no expression was detected in normal breast tissue samples, including one control sample from a carcinoma patient.

In addition to studies with potentially immunogenic human tumours, such as melanoma, tumour antigen studies have been conducted on those cancers associated with viruses, particularly papillomavirus (cervical carcinoma) and Epstein-Barr Virus (immunoblastic lymphomas of the immunosuppressed, endemic Burkitt's lymphoma, Hodgkins disease). **Rickinson et al (1992)**, have researched T-cell recognition of EBV associated lymphomas extensively, showing that reactive CTL with operational specificity for EBV can be cloned. Such CTL were found to be largely MHC class-I restricted. They suggest that there are at least eight independent virally-coded proteins with the potential to provide target epitopes for the EBV specific
T-cell response and that, in each case, epitope choice will be critically dependent on the HLA restricting molecule. The eight viral proteins of interest consist of six nuclear antigens (EBNA 1, 2, 3A, 3B, 3C and LP) and two latent membrane proteins (LMP1 and 2). Of the known EBV latent proteins EBNA 3A, 3B and 3C are frequently the dominant CTL targets. Responses have also been observed against the other proteins with the exception of EBNA1. Immunoblastic lymphomas express the complete spectrum of EBV latent proteins and therefore remain sensitive to EBV specific CTL recognition. In contrast, no such CTL responses are observed in Burkitt's lymphoma since the following factors may contribute to immune escape: expression of EBV latent protein is restricted to EBNA 1 only; allele specific downregulation of MHC class I expression; absent or low expression of relevant cellular adhesion molecules.

Such studies emphasise that tumour antigens and MHC expression have obligatory roles in the immune recognition and response to tumours and this survey implies that tumour immunity and immunosurveillance exists.

(3) CELLULAR INFILTRATION AND INTERACTIONS IN TUMOURS

In addition to CTL anti-tumour responses against TSA/TAA in the context of MHC as discussed, the presence of infiltrating cells within tumours is proposed as further evidence of immunity against tumours. Tumour infiltrates appear to be heterogeneous populations of cells consisting of CTL, non-MHC restricted T-cells, natural killer (NK) cells, macrophages and granulocytes (**Herberman et al, 1990**).The inflammatory component of tumour stroma is thought to play an important role in the modulation of tumour expansion. Local release of chemotactic factors within the tumour microenvironment probably recruits infiltrating monocytes with the potential to elicit anti-tumour cytotoxicity (**Martinet et al, 1992**). The cellular infiltrate also includes NK cells which may contribute a number of cytokines into the cytokine network within the tumour, regulating the activity of resident neoplastic cells and lymphoid cells infiltrating the tissue. NK cells secrete interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα), the latter of which is capable of direct lysis of neoplastic cells. Furthermore NK-derived interleukin-4 (IL-4) and interleukin-6 (IL-6) can alter the proliferation and metastatic potential of malignant cells in vitro (**Lorenzen et al, 1991**).

In a recent review on TIL (**Whiteside et al, 1992**), the mononuclear cell (MNC) infiltrates of human, solid tumours are discussed in considerable detail. Many human, solid tumours contain numerous MNC situated around the tumour stroma. The intensity of MNC infiltration is variable between tumours and it is still subject to debate whether the degree of MNC infiltration indicates a vigorous local inflammatory response, an anti-tumour immune response or both within the tumour. Crudely, for those tumours where a correlation exists between intensity of MNC infiltration and prognosis/ survival, the MNC could be viewed as effectors of local anti-tumour immunity. Where such correlations are not possible, infiltrating MNC are viewed as inflammatory cells mediating non-specific interactions, rather than tumour-specific interactions.

Immunohistochemical studies suggest that $CD3^+$ T-lymphocytes are the predominant component of tumour MNC, although monocytes are also prominent in many tumours. With the exception of melanoma, B-lymphocytes are rarely found. Although often present, NK cell numbers are relatively small. The CD4/CD8 T-lymphocyte ratio is reported to be higher in the stroma than in the parenchyma. This suggests that $CD4^+$ T-lymphocytes are mainly in the stroma whilst $CD8^+$ T-lymphocytes are more likely to be in contact with tumour cells. Phenotypic analysis of fresh TIL suspensions by flow

cytometry produced similar phenotypic data to the immunohistochemical observations: $CD3^+$ TIL were the major component although some TIL suspensions contained variable proportions of NK cells ($CD3^+CD56^+$) and non-MHC restricted T-lymphocytes ($CD3^+CD56^+$). Also the CD4/CD8 ratios were often greater than one indicating that $CD4^+$ T-lymphocytes, not $CD8^+$ T-lymphocytes, predominate in many tumours.

From both immunohistochemistry and flow cytometry analysis, the $CD3^+$ T-lymphocyte population of the MNC appear to be activated as they express activation markers: both HLA-DR (up to 65% of $CD3^+$ cells by flow cytometry) and CD25/Tac antigen (up to 25% of $CD3^+$ cells by flow cytometry) are detectable. Although substantial numbers of T-lymphocytes express HLA-DR antigens, CD25 expression is less frequent. However, the proportions of $CD25^+$ T-lymphocytes is generally higher in TIL when compared to autologous peripheral blood lymphocytes (PBL). Polymerase chain reaction (PCR) analysis of TIL from a variety of solid tumours suggests that TIL are $CD3^+$ lymphocytes which express TCR $\alpha\beta$ and utilise a restricted repertoire of TCR Vβ genes.

Further evidence for local activation of tumour MNC was obtained by in situ hybridisation studies for cytokine mRNA expression. Tumours found to be profusely infiltrated by MNC contained considerable proportions of TIL in the stroma which expressed mRNA for IL-2, TNFα, IFNγ and IL-2 receptor. Since human solid tumours seem to differ in their ability to modulate the cytokine gene expression of TIL, both the immunogenicity of the tumour and its immunoinhibitory potential may determine the degree of TIL activation in situ.

In summary, the presence of this mononuclear cell infiltrate in solid tumours might indicate a type of anti-tumour immune response and thus suggests that the immune system may recognise and respond to tumours. Since tumour-specific cytolytic T-lymphocytes (CTL) have been detected in several studies, TIL have thus became an area of research with potential therapeutic value (**Whiteside et al, 1992; Rosenberg, 1991**) as discussed in a later section on cellular immunotherapy.

(4) TUMOUR IMMUNOSUPPRESSIVE EFFECTS

Immunosuppression of an anti-tumour response may be mediated by the presence of suppressor cells and/or the release of immunosuppressive factors. In murine and rat models, with chemically or virally induced tumours, $CD4^+$ T-lymphocytes were found to suppress adoptive therapy with $CD8^+$ T-lymphocytes (**Melief and Kast, 1991**).

Non-specific suppressor cells of monocytic origin, capable of suppressing NK and LAK cell activation in response to IL-2 or interferons in vitro, have also been described in human tumour systems. It has been demonstrated that a limited number of human tumour types contain T-lymphocytes with the capacity to suppress both non-specific and tumour-specific T-lymphocyte responses in vitro. These suppressor cells are of the $CD3^+CD8^+$ phenotype and may undergo expansion in vivo following the administration of IL-2 therapy, since they express IL-2 receptors (**Guillou, 1990**).

Factors which have been postulated to be tumour-derived immunosuppressive agents fall into three broad groups: prostaglandins; putative tumour antigens and tumour-derived products (eg. cytokines) which impair lymphocyte proliferation and occasionally cytokine production (**Guillou, 1990**):

(1) Prostagladins: A number of tumour cells can synthesise and release a variety of prostaglandins which possess suppressive properties in some, but not all, cases.

(2) Putative tumour antigens: In experimental animals of melanoma, soluble tumour antigens capable of binding T-cell receptors can inhibit CTL during the effector phase. It has also been suggested that soluble antigen can induce suppressor T-cells capable of blocking CTL development.

(3) Tumour-derived products: Factors which can impair lymphocyte proliferation and in some instances cytokine production, have been demonstrated from tumour cell-lines and fresh tumours of both human and murine origin. Thus, tumour cells themselves may exert local effects which prevent T-lymphocytes from reaching full anti-tumour efficacy. Malignantly transformed cells secrete the cytokines transforming growth factor alpha and beta (TGFα and TGFβ). The latter, is known to inhibit the IL-2 dependent proliferation of T-cells and the induction of alloreactive CTL in the mixed lymphocyte reaction. Furthermore, transfection of the TGFβ gene into a highly immunogenic tumour cell-line abrogated the development of a CTL anti-tumour response (**Melief and Kast, 1991**). Thus TGFβ is a likely candidate as a tumour-derived immunosuppressive factor. However in a series of experiments conducted by **Guillou (1990)** TGFβ was not found to be the immunosuppressive factor isolated. They showed that serum-free supernatants derived from "suppressor" and "non-suppressor" tumour cell-lines (**Guillou, Ramsden et al, 1989**) showed a similar capacity to the tumour cell-lines themselves to suppress LAK cell generation (**Guillou, Sedman et al, 1989**). Further comparison between dose, time of administration and onset of LAK suppression implied that the ability of tumour cells to mediate suppression was via the release of soluble immunosuppressive factors. Physiochemical

analysis of these supernatants suggests that a protein of around 56kD in molecular weight was responsible: neutralising antibodies to TGFβ did not inhibit the immunosuppressive effects observed whilst the chemical characteristics of TGFβ differed to those observed in this case **(Guillou, 1990)**.

In conclusion, the immune system may be capable of detecting tumours although several potential mechanisms for tumour escape exist. These are:

(1) Absence, loss or regulation of tumour-specific antigens.
(2) Absence of, or aberrant expression of, MHC on tumour cells.
(3) Cytokine-induced protection of tumour cells from cytolysis by effector cells (via up-regulation or down-regulation of MHC).
(4) Induction of suppressor cells which interfere with the cytotoxic function of anti-tumour effector cells.
(5) Synthesis and release of immunosuppressive moieties from tumour cells.
(6) Impaired ability to generate cytolytic effector cells or favourable cytokines/ cytokine receptors for an anti-tumour response.

1.3 ; CANCER THERAPY

Conventional treatment of malignancy comprises of surgery, chemotherapy and radiotherapy alone or in combination. Immunotherapy has become an area of intense investigation, however, particularly as a means of treating those cancers which respond poorly to conventional methods. Initial approaches involved the development of "passive" immunotherapy which attempted to non-specifically stimulate the host's immune system towards the growing neoplasm via the administration of immunoglobulins or immunisation with antigens appropriate to the disease (ie. "active" immunotherapy). The use of mitogenic agents such as Bacillus Calmette-Guerin (BCG) and Corynebacterium parvum are examples of non-specific, active immunotherapy. Although largely unsuccessful, treatment with these agents is still used today in some instances (eg. bladder and ovarian cancer). The interest in "adoptive" immunotherapy followed, involving the systemic transfer of immunologic reagents (eg. specific antibody or syngeneic lymphocytes) into the tumour-bearing host.

1.3a ; ADOPTIVE IMMUNOTHERAPY

Adoptive immunotherapy can be defined as cancer treatment that acts primarily through natural host defence mechanisms via the administration of natural mammalian substances capable of mediating anti-tumour effects directly or indirectly (**Rosenberg, 1991**). Several therapeutic approaches have been extensively researched including the

administration of cytokines, antibodies and immunoreactive cells in order to potentiate the host's anti-tumour response.

(1) ANTIBODY THERAPY: "THE MAGIC BULLET"

Monoclonal antibodies have been used to target cytotoxic reagents to the tumour, including radionuclides, drugs, toxins and cytotoxic cells. Pharmacological delivery of the toxic plant lectins abrin and ricin via antibodies has been associated with toxicity mediated by the B chains of these molecules which can attach to mammalian cells following cleavage in vivo. Bispecific F(ab'γ)2 antibody has been used to deliver saporin, a ribosome-inactivating protein (RIP), in the treatment of lymphoma (**Glennie et al, 1988**). Saporin is a single-chain RIP with similarities to the A chain of ricin but it displays a lower level of toxicity than ricin owing to the absence of a B chain molecule in its molecular structure. Conjugation to a bispecific antibody of appropriate specificity allowed saporin to bind and enter target cells, permitting efficient immunotherapy via the shutdown of protein synthesis. The use of F(ab'γ)2 fragments diminished problems with steric hindrance and undesired antibody dependent cell-mediated cytotoxicity (ADCC).

Tutt et al (1991) have developed trispecific F(ab')3 antibody derivatives to activate and redirect resting CTL by co-operative signalling via the TCR/CD3 complex and the accessory molecule CD2, thereby directing the cytotoxicity of unprimed effectors towards the target, regardless of MHC status.

Gillies et al (1992) have applied the antibody-conjugate delivery system to cytokine therapy. They observed that antibody-targeted IL-2 stimulated T-cell killing of autologous tumour cells and they hope to apply this concept to the treatment of melanoma and neuroblastoma. Whilst IL-2 should aid the expansion of CTL, having been targeted to the tumour site via antibodies with anti-tumour specificity, the antibody itself should be able to mediate ADCC by recruiting Fc receptor-bearing cells. The therapeutic value of antibody-cytokine conjugates remains an area of active investigation.

(2) CYTOKINE THERAPY

Several cytokines have been considered as anti-tumour agents either as single modalities or in combination with each other or with standard chemotherapy regimes.

The three families of interferons (alpha, beta and gamma) have been subject to clinical evaluation over the past decade (**Balkwill and Smyth, 1987; Gutterman, 1992**). The alpha interferons (IFNα) have been extensively tested in phase I, II and III trials,

producing anti-tumour activity in several blood-related malignancies: hairy cell leukaemia, non-Hodgkin lymphoma, chronic myelogenous leukaemia and some B-cell malignancies. Local therapy of basal cell, ovarian and bladder carcinoma also showed promise. However, treatment of the more common solid tumours such as breast, bowel and lung showed no convincing response rates whilst results with renal cell carcinoma and melanoma were unclear. Studies in vitro and in laboratory animals have shown IFNα to be synergistic with some cytotoxic drugs, particularly 5F0, alkylating agents and cisplatin. The mechanisms of IFNα anti-tumour activity remain unknown although its primary characteristic is probably an anti-proliferative effect on malignant cells, possibly by direct interference with the cell cycle. By acting in concert with cytotoxic drugs, IFNα may interfere with drug metabolism rendering the tumour more susceptible to drug action. In contrast, there is no evidence for the clinical efficacy of IFNγ. Although some clinical responses were observed with interferon beta (IFNβ), higher doses of the cytokine were required. However, trials with interferons alone have proved of little benefit overall.

The application of interleukin-4 (IL-4) as a potential anti-tumour agent is of great interest at present, owing to its ability to expand T-cell growth in vitro. Phase 1A studies using IL-4 alone or in combination with IL-2, with which IL-4 synergises in vivo, were initiated in 1988. Additionally, several other cytokines, namely IFNα, TNFα, GM-CSF and IL-7, have been or are currently being evaluated for their therapeutic efficacy when administered alone or in concert with IL-2 **(Lotze, Jablons et al, 1989; Lotze, 1990; Lotze, Custer et al, 1990).**

Of all the cytokines that have been evaluated for their potential to mediate an anti-tumour response, the one which has been most extensively studied is IL-2, either alone or in combination with other agents including other cytokines, chemotherapy drugs, monoclonal antibodies or adoptively transferred cells **(Rosenberg and Lotze, 1986; Lotze 1989/1990; Lotze, Jablons et al, 1989)**. Results to date are reasonably consistent. The use of IL-2 alone or with adoptively transferred cells (LAK cells) is discussed more expansively in a later section.

(3) CELLULAR THERAPY

Cellular immunotherapy consists of two major strategies, treatment with lymphokine-activated killer (LAK) cells or tumour infiltrating lymphocytes (TIL), both of which are often given in concert with IL-2. A third approach to adoptive tumour immunotherapy is the use of human $CD4^+$/killer T-cells **(Nakamura et al, 1992).**

LAK cells were defined by **Grimm et al (1982)** and are discussed in detail in a subsequent section. Just as LAK cells have been subject to a considerable degree of research, the use of TIL for adoptive immunotherapy has been extensively investigated. Immunohistology studies of TIL suggest that although composed of several types of mononuclear cells, T-cells appear to be the predominant phenotype. The rationale behind TIL therapy involves the possibility of obtaining a cell fraction which consists of T-cells sensitised to tumour-associated antigens on the assumption that these cell selectively accumulate within the tumour. Thus, TIL therapy generally comprises of obtaining tumour-specific cytolytic T-cells (CTL) and administering them to the patient, having potentiated their anti-tumour activity in vitro or by co-administration with agents capable of enhancing TIL function in vivo **(Whiteside et al, 1992; Melief and Kast, 1991)**.

At the National Cancer Institute in the USA, Rosenberg and his colleagues undertook the task of identifying T-lymphocytes that could recognise specific tumour antigens in a MHC-restricted manner **(Rosenberg, 1991)**. They obtained resected tumours and isolated TIL as single-cell suspensions in IL-2, assuming that tumour infiltrating lymphocytes that were expressing IL-2 receptors were reactive to tumour for two reasons: the cells grew in the presence of IL-2 in culture and destroyed any tumour cells growing simultaneously in culture. Murine TIL were exclusively CD8+ whereas human TIL were of both CD4+ and CD8+ phenotypes.

From in vitro experiments with murine TIL coincubated with autologous tumour cells, several observations were made: TIL specifically recognised tumour antigens since they mediated the specific lysis of tumour targets; specific cytokines were secreted; TIL reactivates were MHC class I restricted since anti-class I antibodies blocked the lysis of tumour cells. TIL were therefore thought to be ineffective against tumours with low levels of MHC class I, particularly since class I negative tumour cells were not recognised whereas tumour cells transfected with MHC class I genes or preincubated with IFNγ regressed. Furthermore the specific secretion of certain cytokines such as IFNγ was found to be more critical to TIL anti-tumour reactivity than specific lysis: non-lytic TIL which secrete IFNγ could effectively treat established lung micro-metastases in mice. TIL therapy in mice with lung and liver metastases indicated a potency which was 50 to 100 times greater than that of LAK cells in reducing lung metastases. Likewise TIL in combination with IL-2 and cyclophosphamide, unlike LAK cells administered with these agents, eliminated metastases from the lung and liver in an advanced tumour model.

Based on these animal studies, human pilot studies were begun by treating patients with advanced melanoma using TIL isolated from autologous tumour which had been expanded with IL-2 in vitro. A preliminary report published in 1988 (**Rosenberg, Packard et al, 1988**) indicated that 38% of patients responded to treatment. Response rates were similar, irrespective of whether patients had previously received high dose IL-2 therapy. TIL have been particularly valuable in the treatment of melanoma: TIL with specific cytolytic activity for autologous tumour and not autologous normal tissues can be isolated from approximately one third of melanoma patients. Melanomas from different individuals appear to share common tumour antigens and recognition of these antigens appears to be largely HLA-A2 restricted. Finally, these studies demonstrated that once administered in vivo, TIL recirculate and localise in cancer deposits, unlike LAK cells: up to 0.015% of all injected TIL localised per gram of tumour according to trials in humans using ^{111}Indium labelled TIL in vivo (**Rosenberg, 1991**).

With this knowledge TIL, which have been genetically modified, have been used to deliver cytokines genes to the tumour. The rationale behind this was first tested by inserting marker genes, namely the bacterial gene coding for neomycin phosphotransferase (NeoR) which could induce resistance to the antibiotic neomycin (**Rosenberg, 1991; Morgan and Anderson, 1993**). This allowed the assessment of: the feasibility and safety of using retroviral mediated gene transfer to introduce genes into humans; the long term distribution and survival of autologous TIL; a means of distinguishing between adoptively transferred TIL and endogenous host lymphocytes. Initial studies in 10 human patients showed no indications of NeoR gene-modified TIL resulting in any alterations to the general properties of TIL, TIL phenotype, TIL cytotoxicity, cytokine gene expression by TIL or TCR status of TIL. Since TIL accumulate in tumour deposits, this system is now being used to modify TIL with genes that can improve the anti-tumour effectiveness of these cells (eg. genes for TNFα, IFNγ, IL-2, IL-6 and chimeric TCR).

Other experimental leads for improving immunotherapy with TIL include:

(1) Improvement of the mechanisms for generating TIL with greater efficacy (eg. selection of lymphocyte subpopulations or clones with greater anti-tumour reactivity, repeated in vitro stimulation, in vitro expansion with IL-2 and other cytokines such as IL-4).

(2) Enhancing the in vivo effectiveness of TIL by administering TIL in combination with other cytokines or radiotherapy.

1.4 : THE LYMPHOKINE ACTIVATED KILLER (LAK) CELL PHENOMENON

1.4a : DISCOVERY AND DEFINITION OF LAK ACTIVITY

Lotze et al (1981) and **Hersey et al (1981)** demonstrated that human lymphocytes derived from the peripheral blood of cancer patients, when cultured in T-cell Growth Factor (TCGF), could kill both fresh and cultivated autologous or allogeneic tumour cells. Upon further investigation, the phenomenon was named lymphokine activated killer activity (**Grimm et al, 1982; Grimm, Ramsey et al, 1983; Grimm, Robb et al, 1983**) and the concept that LAK cells represent a unique cytotoxic effector system that may play an important role in immune surveillance against NK resistant tumour cells, was a potential breakthrough for the adoptive immunotherapy of tumours. The induction of PBMC from normal healthy donors, with either purified IL-2 (**Grimm et al, 1982**) or recombinant human IL-2 (**Rosenberg et al, 1984**) generated a population of effector cells defined as LAK cells as characterised by their ability to lyse fresh tumour cells and tumour cell lines resistant to NK cell killing. LAK cytotoxicity was shown to be a non-specific, MHC-unrestricted phenomenon dependent on the proliferation of the IL-2 activated PBMC, as pre-treatment with either mitomycin C or gamma irradiation inhibited LAK induction (**Grimm and Wilson, 1985**). Furthermore, it was demonstrated that lysis appeared to be an intrinsic property of IL-2 activated cells and not merely an artefact due to the presence of lectins or xenogeneic serum which could also generate promiscuous killing.

1.4b : MURINE STUDIES WITH IL-2 ACTIVATED CELLS (LAK CELLS)

In **1982, Eberlein et al** demonstrated that intravenously injected lymphoid cells expanded in IL-2 were capable of mediating tumour regression of both local and disseminated syngeneic lymphoma in C57BL/6 mice. He observed a 93% success rate with cells expanded short-term in IL-2 as compared to 56% of mice cured with lymphoid cells which were multiply expanded in IL-2 in order to generate bulk numbers of cells for infusion.

Further to this study, **Mazumder and Rosenberg (1984)** developed the C57BL/6 B16 melanoma murine model, providing the first report of the in vivo therapeutic efficacy of systemically administered LAK cells against an established metastatic tumour. They showed that intravenous infusion of 1×10^8 LAK cells into mice with B16 pulmonary metastases resulted in a decreased number of lung nodules whilst improving overall survival. This was also true for metastases generated spontaneously from a primary tumour which was excised prior to LAK cell transfer, a crucial observation of clinical significance. LAK cells derived from the splenocytes of tumour bearing mice were

found to be equally active in vivo. Moreover, activities of LAK cells, irrespective of whether they were induced from the spleens of normal or tumour-bearing mice, were similar when examined in vivo or in vitro. This was therapeutically relevant since it suggested that the potential presence of suppressor cells in hosts with neoplasia was not important. Furthermore, the antimetastic effect of these LAK cells was not dependent on the preceding administration of cyclophosphamide or other immunosuppressants. However, the mechanism of LAK therapy was unknown and the ability of LAK cells to diminish metastases at other sites was not studied in this experiment. Also the mice could not be completely cured of the metastases for several , potential reasons: poor migration of LAK cells to the tumour site suggesting that local delivery was necessary; normal clearance mechanisms were too rapid and thus did not permit the cells to persist; LAK cell activity in vivo was probably short-lived and therefore advocated the need for concomitant in vivo administration of IL-2.

Subsequently, **Mulé et al (1984)** conducted a study to evaluate the anti-tumour effectiveness of IL-2 in vivo alone or in concert with LAK cells. They demonstrated the induction of murine LAK cells with anti-metastatic activity in vivo using recombinant human IL-2 (rhIL-2, **Rosenberg et al, 1984).** In contrast to the non-recombinant IL-2 B16 melanoma model, where LAK cells alone substantially inhibited pulmonary metastases, combined treatment with LAK cells and rhIL-2 was necessary for the successful reduction of established pulmonary and hepatic sarcoma metastases. This immunotherapeutic approach appeared effective against multiple sarcomas (MCA-103, MCA-105 and MCA-106) including one of no detectable immunogenicity (MCA-102). In order to have therapeutic value, these LAK cells seemed to require exogenous IL-2 to prolong their survival and maintain their activity. Thus, it was proposed that human tumours that are poorly immunogenic could potentially be treated with systemic infusions of rhIL-2 activated LAK cells and/or rhIL-2.

1.4c : CLINICAL TRIALS WITH LAK CELLS PLUS OR MINUS IL-2

In order to treat human cancer by adoptive immunotherapy, large numbers of cells are required for adoptive transfer. Since human LAK cells can readily be generated from peripheral blood lymphocytes of tumour-bearing individuals (obtained from leukapheresis or thoracic duct drainage) by incubation for 3-5 days with IL-2, the opportunity exists for treatment of human tumours with large numbers of autologous cells.

In a **1985** review, **Rosenberg** reported his findings from the first phase-I clinical trials, conducted between 1982 and 1984, which were established to address the feasibility and toxicity of obtaining and infusing large numbers of LAK cells into tumour-bearing patients whilst determining the toxicity of systemically administered recombinant IL-2. Although no anti-tumour effects were observed, these studies demonstrated that large numbers of activated cells ($> 10^{11}$) could be infused safely into patients without significant toxicity: transient fever and chills observed following infusion were easily controlled. The maximum tolerated dose of IL-2 was also determined in a study comparing source of IL-2, dose schedule and route of administration (infusion by i.v. bolus, i.p. bolus and continuous i.v.). The major toxicity's associated with IL-2 infusion were fever, chills, malaise, mild hepatic dysfunction and fluid retention; the latter being more difficult to control with conventional drugs. The traffic of these activated cells was also monitored, via ^{111}Indium labelling, demonstrating a prompt clearance by the liver and spleen. However subsequent, repeat infusions of activated cells persisted: excretion was significantly delayed and increased numbers of infused cells were detected in the lung.

The first clinical trials using LAK cells plus IL-2 for the treatment of patients with a variety of advanced malignancies were initiated in December 1984 **(Rosenberg and Lotze, 1986)**. Of the 22 evaluable patients with advanced cancer treated, 9 patients showed an objective regression of metastatic cancer. These responses occurred in four different histological types of tumour; colon and renal cell cancer, melanoma and a primary lung adenocarcinoma. During these therapies metastases in the lung, liver and subcutaneous tissue regressed. One patient experienced complete regression of all metastatic melanoma for twelve months following the completion of therapy. In this case, subcutaneous melanoma metastases in the arm, thigh, back and buttock began to regress on completion of therapy and three months later, all lesions had disappeared.

This phase II trial was expanded to include a total of 157 patients with advanced cancer, receiving treatment with LAK cells plus IL-2 or high-dose IL-2 alone between 1984 and 1986 **(Rosenberg, Lotze et al, 1987)**. The progress report indicated that of 106 patients on therapy of LAK cells plus IL-2, 8 patients had complete responses whilst 15 showed partial responses: an overall success rate of 22%. In addition, 10 patients had minor responses. The median duration of response was variable, lasting for 6 or 10 months depending on whether response was partial or complete. The longest complete response noted was for a patient still in remission 22 months after treatment. Tumour regression was observed at a variety of sites, including lungs, liver, bone, subcutaneous tissue, lymph nodes and circulating lymphoma cells. In comparison, of 46 patients on high-dose IL-2 therapy without LAK cells, 1 patient with

renal cell carcinoma showed complete regression and 5 patient with melanoma had partial responses, where remission lasted from 2 to 11 months. Only 1 patient showed a minor response indicating a 13% success rate for IL-2 therapy. Toxic side effects were common but resolved quickly when IL-2 was discontinued. Four patients died in the trial due to complications possibly associated with IL-2 induced "capillary leak syndrome".

Similar clinical studies are still underway at the National Cancer Institute, Bethesda in the USA. Some of the most recent data (**Lotze 1989/1990**), summarised as at 1988 in table 1 overleaf, suggests that the most significant results are observed in patients with renal cell carcinoma, melanoma, colorectal carcinoma and some patients with lymphoma. Responses to LAK/IL-2 therapy in these three major tumour groups ranges from around 35% down to 10%, although around 50% of the renal cell carcinoma patients showed minor responses (i.e. 25% - 50% tumour shrinkage). The duration of response is significantly durable and long-lasting in some cases: one woman with disseminated cutaneous melanoma treated in 1984 (45 months prior) still showing no signs of recurrence.

Collectively, LAK/IL-2 or IL-2 alone produced marked tumour regression in some patients for whom no other effective therapy was available. Response was variable and success rates were poor when compared to the murine data. **Lotze** suggests that, as in animal models, different tumour types will vary in immunogenicity and possibly in their response to LAK and/or IL-2 therapy as a consequence. He believes that in devising therapeutic strategies for humans that tumour immonogenicity must be an important consideration : non immunogenic tumours requiring treatment with enhanced LAK activity in contrast to tumours that are weakly immunogenic perhaps being best treated by exploiting T-cell activity that may pre-exist in the tumour-bearing host. Another influencing factor is the patient's own immune response which may determine the outcome of therapy.

1.4d : STUDIES WITH ANTI-CD3 INDUCED LAK CELLS

The use of anti-CD3 antibodies in the generation of LAK activity was investigated because this mitogen was thought to have two major advantages over the conventional use of rIL-2. Firstly, anti-CD3 was considered as a means of producing selective, potent cytotoxicity against tumour targets. Secondly, anti-CD3 allowed the generation of sufficient numbers of effector cells in a short period of time.

Table Results of Immunotherapy in Patients with Advanced Cancer

Diagnosis	Number of patients receiving each therapy					
	LAK/IL-2			IL-2		
	Total Evaluable[a]	CR	PR	Total evaluable[b]	CR	PR
Renal	72	8	17	52	4	7
Melanoma	48	4	6	37	0	9
Colorectal	30	1	4	12	0	0
Non-Hodgkin's lymphoma	5	1	2	6	0	0
Sarcoma	6	0	0	1	0	0
Lung adenocarcinoma	5	0	0	1	0	0
Breast	1	0	0	2	0	0
Brain	1	0	0	2	0	0
Esophageal	1	0	0	0	0	0
Hodgkin's lymphoma	1	0	0	0	0	0
Ovarian	1	0	0	1	0	0
Testicular	1	0	0	0	0	0
Hepatoma	0	0	0	1	0	0
Gastrinoma	1	0	0	0	0	0
Thyroid	1	0	0	0	0	0
Unknown primary	1	0	0	0	0	0
	175	14	29	115	4	16

[a]Two treated patients not included [one died of therapy (melanoma) and one lost to followup (breast)].

[b]Four treated patients not included [died of therapy (renal)].

Initial studies characterised the in vitro effects of anti-CD3 (anti-T3 from the hybridoma line 145-2C11 which is directed against the ε-chain of the T3 complex) on murine splenocytes isolated from C57BL/6 or DBA/25 mice (**Yun et al, 1988**).Two days after the onset of anti-T3 stimulation the number of viable lymphocytes and the degree of lymphocyte proliferation increased. This activity peaked on day 3 and then quickly declined. The addition of rhIL-2 (20U/ml) augmented both these effects. The cytolytic activity of the anti-T3 induced LAK cells was determined against known murine, LAK sensitive target cell-lines. The killing observed peaked on day 2 of culture and declined thereafter, whereas the cytolytic activity obtained with rhIL-2 induced murine LAK cells appeared on day 3 and increased progressively up to day 5 of culture. The presence of exogenous rhIL-2 in the anti-T3 stimulated cultures failed to affect the maximal cytolytic activity or the duration of response observed. Interestingly, anti-T3 induced LAK cells gave 10 to 50 fold higher cytotoxic activity than the conventional rhIL-2 induced LAK cells. Stimulation of splenocytes via anti-T3 produced peak amounts of IL-2 following 1 day in culture. The antibody was synergistic with rhIL-2 in this respect, producing greater levels of IL-2 than either of these agents alone. The IL-2 levels in anti-T3 activated cultures were found to decrease more rapidly than those of the rhIL-2 activated cultures, perhaps reflecting on a more rapid assimilation of IL-2 by the cells activated with antibody. Antibodies to IL-2 and IL-4 could inhibit the proliferative and cytotoxic response induced by anti-T3, although the antibody concentrations used were a critical factor. Thus, the effects of anti-T3 may be mediated via the endogenous production of cytokines such as IL-2 or IL-4. Finally, the survival of anti-T3 induced LAK cells was variable: the cells could be maintained in culture without actively proliferating for 3 weeks if grown in the presence of both anti-T3 and rhIL-2 subsequent to activation or, if maintained in rhIL-2 alone, the effector cells remained viable and proliferated for 4 weeks. This was a significant observation since rhIL-2 induced LAK cells can only survive for 7 to 10 days in culture.

Using the same anti-T3 antibody and mouse strains, **Yun et al (1989)** then assessed the ability of anti-T3 induced LAK cells to mediate tumour regression in vivo. They reported that 80-100% of the mice challenged with 1 x 10^2 P815 tumour cells remained free of tumour following the administration of 5 x 10^6 anti-T3 induced LAK cells which were expanded with anti-T3 in vitro. However of the mice challenged with either 1 x 10^3 or 1x 10^4 tumour cells prior to therapy, only 20% remained free of tumour although the growth rate of the tumour was slower. In contrast, treatment with either rhIL-2 induced LAK cells or anti-T3 induced LAK cells that were not expanded in culture failed to infer protection against the tumour. However, activation of these effector cells with anti-T3 antibody for 3 hours in vitro prior to infusion protected 30%

of challenged mice. The effector phenotype was $CD4^{-}CD8^{+}$. Flow cytometry analysis suggested that higher levels of LAK activity obtained in the effector populations which had been activated and then expanded with anti-T3 antibody could be attributed to a preponderance of $CD8^{+}$ lymphocytes. Overall, their observations suggest that the in vivo and in vitro anti-tumour activity of anti-T3 activated killer cells is similar.

The efficacy of immunotherapy with anti-CD3 activated killer cells was also examined in a murine model by **Yoshizawa et al (1991)**. They studied the specific adoptive immunotherapy mediated by tumour-draining lymph node cells sequentially activated with anti-CD3 and IL-2 against MCA106 and MCA205 murine sarcomas in C57BL/6 mice (using the same anti-CD3 antibody as the two pervious studies). They made the following conclusions:

(1) Sequential activation with anti-CD3 and IL-2 in vitro was essential, as simultaneous activation did not enhance efficacy.

(2) Effector cell generation is critically dependent on optimal concentrations for generating cytolytic activity (but not optimal proliferation). These concentrations were 1.0μg/ml anti-T3 for 2 days and 2-10U/ml rhIL-2 for the next 3 days.

(3) Adoptive transfer of anti-CD3/IL-2 activated cells alone could mediate regression of established metastases but concomitant administration of IL-2 enhanced the in vivo activity of these cells.

(4) Tumour regression by these effector cells was immunologically specific.

Human PBMC have also been shown to be activated to cytolytic status by anti-CD3 antibodies, as discussed in a subsequent section on the modulation of LAK induction by antibodies. Monoclonal anti-CD3 can induce proliferation and cytolytic activity in resting PBL populations providing the antibody is immobilised (**Jung et al, 1987; Scott et al, 1988**) and additional signals are supplied (eg. IL-1). Monocytes can fulfil both these requirements by binding anti-CD3 to their Fc receptors and secreting IL-1. Several studies have reported that IL-2 and anti-CD3 act synergistically to give greater proliferation, cytotoxicity and IFNγ production. Interestingly, the synergistic effects of IL-2 and anti-CD3 are monocyte independent. In contrast, the ability of soluble anti-CD3 alone to induce non-MHC restricted killing is monocyte dependent, emphasising that immobilisation of the antibody is a prerequisite of activation (**Tovar et al, 1988**).

1.4e: PHENOTYPIC ANALYSIS OF LAK CELLS

Although notable individual successes were observed in clinical trials with LAK cells and/or rhIL-2, the general efficacy of LAK cell therapy in humans was disappointing. Consequently, LAK cells have been and still remain an area of intense research : scientists and clinicians have attempted to optimise the LAK therapy protocol in conjunction with other cytokines/conventional chemotherapy whilst attempting to understand LAK cells phenotypically and mechanistically both in vivo and in vitro.

The phenotypes of LAK progenitor and effector cells have been investigated in both murine models (**Rosenstein et al, 1984; Kalland et al, 1987**) and in man (**Lanier et al, 1987; Sawada et al, 1988; Geller et al, 1991; Ortaldo et al, 1991**). Early studies (**Grimm et al, 1982**) demonstrated that LAK precursors could be found in NK-void thoracic duct lymphocyte populations in addition to the peripheral blood lymphocytes (PBL) of normal individuals and cancer patients, suggesting the precursors to be of T-cell origin. Depletion experiments with monoclonal antibodies implied that LAK effectors expressed serologically detectable T-cell markers (OKT-3, Leu-1, 4F2) but not the monocyte/NK cell marker OKM-1. Further research (**Grimm, Ramsey et al, 1983**) illustrated that the LAK effector cell was recognised by OKT-3 and OKT-8 antibodies whilst the precursor was unsusceptible to OKT-3, Leu-1 depletion. Also, NK-cell enriched fractions obtained from percoll gradients contained LAK precursors which were inducible for LAK activity, even following the elimination of NK activity via depletion with OKM-1, Leu-7 or OKT-11. They concluded that whilst the cell(s) involved in the induction and display of LAK activity had some common features with the classic CTL and NK cell systems, LAK precursor cells were distinct from these cell types.

Phenotypic analysis of the LAK precursor and effector populations has proven a complex undertaking, attempting to characterise a heterogeneous population of cells. A distinct phenotype unique to LAK cells has not been found. The expanse of phenotypic data and the current school of thought is nicely summarised in reviews by **Hersey and Bolhius (1987)** and **Herberman et al (1987)**. From their review of the literature, it can be concluded that LAK cells can be derived from both T-cells and NK cells, although NK cells are generally considered to be the major source. The generation of LAK effector phenotype appears to be governed by the source of precursor cells and the type of stimulation applied. Generally, $CD3^-CD16^+$, $CD3^+CD16^+$ and $CD3^+CD16^-$ cells are potential progenitors of LAK effectors, each giving rise to a predominant effector phenotype (table 2).

TABLE 2: LAK PROGENITOR AND EFFECTOR PHENOTYPES.

PROGENITORS	STIMULUS	PREDOMINANT EFFECTORS
PBL	IL-2	$CD3^-CD8^+CD16^+$
$CD3^+CD16^-$ PBL	IL-2	$CD3^+CD16^-$
$CD3^+CD4^-CD8^-WT31^-$ PBL	IL-2	$CD3^+CD4^-CD8^-CD16^+$

All cell types express CD2 and HNK1 (CD57).

Hersey and Bolhuis address the following issues in their review:
(1) Are LAK cells derived from peripheral NK cells or are T-cells acquiring broad lytic activity once activated?
(2) If T-cells are involved, activity may be mediated via antigen specific TCR-CD3 complex or via different receptors.
(3) T-cells or NK cells involved may express similar or different receptors.

They suggest that the four main receptors involved in triggering MHC-unrestricted cytotoxicity, taking progenitor phenotype and type of mitogenic stimulus into account, are the CD2(LFA-2) molecule, the CD3-associated $\alpha\beta$ chain TCR, the $\gamma\delta$ chain TCR-CD3 complex and the CD16 (IgG-Fc receptor) molecule. The apparent non-specific nature of the killing observed may reflect the widespread expression of the natural ligands for these receptors on susceptible target cells. Thus, assessment of the in vivo distribution of cytotoxic cells which express these receptors within the host, together with the distribution of their corresponding natural ligands, may enable a more favourable rationale for adoptive immunotherapy to be engaged.

1.4f : LAK INDUCTION: MODULATION WITH CYTOKINES AND ANTIBODIES

In addition to studying the phenotypic aspect of the LAK phenomenon, many researchers became interested in improving the efficiency of LAK induction and survival for therapeutic gain. Therefore the modulatory effects of antibodies, particularly anti-CD3, and various cytokines on the induction phase of LAK activity has been the focus of much investigation.

The ability of several cytokines to regulate LAK induction, alone or in concert with IL-2, has been assessed in human studies. **Grimm and Robb et al (1983)** proposed that factors other than IL-2, such as interleukin-1 (IL-1) or IFNγ, could be responsible for inducing LAK activity. The induction of human LAK cells in vitro with IL-2 was shown to be inhibited by the addition of IL-4 (**Brooks and Rees, 1988; Gallagher et**

al, 1988; Spits et al, 1988) although this is contrary to the effects of IL-4 observed in the mouse (**Mulé et al, 1987**). Furthermore, stimulation with IL-4 alone was unable to induce human LAK activity. Likewise it has been demonstrated that transforming growth factor beta-1 (TGFβ-1) can suppress the generation of LAK cells via IL-2 (**Brooks et al, 1990; Smyth et al, 1991**). The suppressive action of both these cytokines was accompanied by various events: inhibition of deoxyribonucleic acid (DNA) synthesis; inhibition of Tac antigen expression; reduction in tumour necrosis factor alpha (TNFα) and IFNγ production (**Brooks et al, 1990**). This inhibitory effect could be abrogated by IFNγ except that Tac antigen expression could not be restored. Contrastingly, TNFα could not abolish the inhibition. IFNγ was also reported to generate LAK cytotoxicity to a lesser extent than IL-2, although this activity was also subject to the inhibitory effects of IL-4 and TGF-β1. **Gallagher et al (1988)** also demonstrated interleukin-3 (IL-3) mediated suppression of IL-2 induced LAK activity and the inability of IL-3 to induce LAK effectors on its own.

Although several of the cytokines investigated had an inhibitory influence on LAK induction, both interleukin-1 (IL-1) and interleukin-6 (IL-6) have been shown to augment the process, acting in synergy with IL-2 (**Gallagher, Al-Azzawi et al, 1989**).

Whilst the regulation of LAK-cell induction by cytokines has been investigated, the potential of such cytokines to alter LAK effector function in vitro was also addressed (**Gallagher, Al-Azzawi et al, 1989**). IL-1, IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF) did not effect target lysis by LAK effectors. On the other hand, IL-3 and IL-4 decreased the cytotoxicity observed whilst IL-2 itself enhanced the ability of LAK cells to kill tumour targets.

These studies demand significant attention when considering the use of cytokines in the induction of LAK cells for therapeutic benefit since cytokines which they may encounter within the body may reduce the efficiency of therapy. Likewise, care must be given when developing multi-cytokine treatment protocols, in combination with LAK cells, since pro-inhibitory cytokines would be counter-productive and detrimental to therapeutic outcome.

The use of antibodies to develop LAK effectors has also been considered. Anti-CD2 has the ability to trigger LAK cytotoxicity since most LAK precursors are $CD2^+$, although the CD2 pathway predominantly stimulates NK-like progenitors. In contrast to the IL-2 data, anti-CD2 triggered LAK activity was not regulated by IL-4. In fact, IL-4 was found to enhance anti-CD2 induced LAK activity and NK cell proliferation possibly as a result of increased TNFα production. Similarly, immobilised monoclonal

anti-CD16 induced LAK activity in the NK-like precursor cells which could not be inhibited by IL-4 (**Robinet et al, 1992**).

Resting human PBL can also be activated to cytolytic activity by anti-CD3 antibodies in the absence of exogenous IL-2 (**Scott et al, 1988**), although linkage to a solid phase may be required, depending on which anti-CD3 antibody is used. At least two different types of cytolytic cells were activated via anti-CD3 antibodies, an NK-like cell ($CD2^+CD3^-CD16^+$) and a CTL-like cell ($CD2^+CD3^+CD8^+CD16^-$). The NK-like cell type was probably activated indirectly via endogenously produced IL-2 subsequent to $CD3^+$ cells becoming activated in response to anti-CD3 binding. This and other studies found that exogenous IL-2 synergised with anti-CD3 (**Tovar et al, 1988; Ochoa et al, 1989; Moutschen et al, 1990**). Furthermore, the lytic ability of cell populations expanded by anti-CD3 plus IL-2 was shown to be enhanced by the exogenous addition of IL-1 beta (IL-1β), IFNβ or IFNγ. **Yang et al (1990)** also demonstrated that the induction of LAK with anti-CD3 (OKT3), followed by culture in low concentrations of IL-2 and TNFα may be advantageous for the long-term generation of LAK effector cells. The combination of these three agents produced significant increases in both LAK activity and proliferation.

Thus, research to date has demonstrated that LAK cells are a phenotypically heterogeneous population of cells whose induction and effector functions are subject to regulation by both antibodies and cytokines. To expand our understanding of this regulation, attempts to elucidate the mechanisms of LAK activity have been made.

1.4g : MECHANISMS OF LAK CYTOTOXICITY

The lytic mechanisms of LAK cells are thought to resemble those or their precursors in some ways, involving the recognition and binding of target cells followed by the delivery of a "lethal hit" from the effector cell which then mediates target lysis. Being of either NK cell or T-cell origin, LAK cells share structural similarities with both large granular lymphocytes (LGL) and CTL, in particular containing similar cytotoxic granules which probably participate in LAK-mediated cytolysis, just as these granules contribute to the mechanistics of both CTL and NK cytotoxicity (**Goldfarb, 1986; Young and Liu, 1988; Tschoop and Jongeneel, 1988; Berke, 1989; Young, 1989; Krähenbühl and Tschoop, 1990; Peters et al, 1991**). This suggests that the cytotoxic mediators NK cytolytic factor (NKCF) and perforin (cytolysin), together with a variety of proteolytic enzymes, have an important role in LAK-mediated lysis.

Although cytotoxic mediators are common to LAK, NK and CTL, the mechanisms by which they recognise and bind their respective targets differ. However, each type of effector cell requires accessory adhesion molecules to assist their anchorage to target cells prior to the lytic event. Several studies have investigated the relative contributions of adhesion molecule expression on both target cells and LAK effectors to the process of LAK binding and target cell susceptibility to LAK lysis.

Naganuma et al (1991) suggest that intracellular adhesion molecule-1 (ICAM-1) expression on target cells contributes to susceptibility to LAK lysis. They used neuroblastoma target cells which expressed ICAM-1 following IFNγ treatment and was accompanied by an increased level of LAK killing. Pre-treatment of target cells with anti-ICAM-1 monoclonals partially decreased LAK killing whilst totally blocking LAK binding, suggesting that adhesion of LAK cells to the targets via ICAM-1 was important. Similar antibody blocking experiments by **Mentzer et al (1988)** implied a pivotal role for the lymphocyte function-associated antigen-1 (LFA-1) in the LAK-target cell interaction. Monoclonal antibody to the CD11a molecule, a functional domain of LFA-1, effectively inhibited LAK killing when added to LAK effectors. This was of particular interest since ICAM-1 is a ligand for LFA-1 **(Marlin and Springer, 1987; Springer, 1990)**. Since **Naganuma et al (1991)** did not observe complete inhibition of killing following anti-ICAM-1 treatment, it would appear that alternative ligands for LFA-1 may exist on the target cells. This possibility was examined by **Jackson et al (1992)** who studied the role of adhesion molecules in LAK-mediated killing of bladder cancer cells. They discovered that all bladder cancer cell-lines examined were susceptible to LAK lysis, irrespective of whether they were low grade cells (ICAM-2^+) or high grade cells (ICAM-1^+). As also observed by **Naganuma et al (1991)** most of these bladder cancer cell-lines increased their expression of ICAM-1 and were more sensitive to LAK killing once treated with IFNγ. Likewise, treatment of target cells with anti-ICAM-1 partially inhibited these effects. However, this inhibition was still incomplete. Greater inhibition of LAK lysis was observed when effectors were treated with anti-LFA-1 antibodies, as previously reported by **Mentzer et al (1988)**. This data supports the idea that the alternative ligands for LFA-1, namely intracellular adhesion molecules-2 and -3 (ICAM-2 and ICAM-3), may also be important in the processes of LAK binding and lysis. In contrast, **Quillet-Mary et al (1991)** found that pre-treatment of target cells with monoclonal antibodies to LFA-1, ICAM-1 or lymphocyte function-associated antigen-3 (LFA-3) had no inhibitory effects on LAK lysis, although the nature of target cell used differed to those of the previous studies. These same antibodies did however, react with LAK effectors to inhibit lysis, as observed for anti-LFA-1 previously,

irrespective of the fact that these LAK effectors were $CD2^+$ $CD3^-$ as opposed to the $CD8^+CD3^+$ effectors used by **Mentzer et al (1988)**.

Adhesion of LAK cells to their target cells therefore seems to be an important event for the successful delivery of a "lethal hit" and the subsequent lytic process. In addition to their cytotoxic activity, the fact that LAK cells themselves produce cytokines must be of considerable relevance to their functional abilities.

1.4h : CYTOKINE SECRETION BY LAK CELLS

The administration of IL-2 to cancer patients has been shown to induce the expression of endogenous cytokine mRNA and various cytokines in vivo **(Kasid et al, 1989; List et al 1992)**. Following high dose IL-2 therapy to patients, **Kasid et al (1989)** found that the circulating PBMC of these individuals expressed significant mRNA levels for TNFα, IL-6 and Tac antigen. IL-1β mRNA was expressed at low levels but mRNA for GM-CSF and TNFβ was not detected. Prior to IL-2 therapy, little or no evidence of mRNA for any of these proteins was observed. However when cultured in vitro for 1 to 2 days with 1000U/ml IL-2, these PBMC were capable of expressing all of the mRNAs mentioned, including GM-CSF and TNFβ mRNA. In no instances was IFNγ mRNA detected. **List et al (1992)** reported that intraventricular injection of IL-2 into patients with brain tumours (leptomeningeal carcinomatosis) resulted in the rapid induction of TNFα, IL-1β, IL-6, IFNγ and soluble IL-2 receptor in the cerebrospinal fluid.

Similarly in studies with human PBMC, IL-2 has been shown to regulate the induction of IFNγ **(Vilcek et al, 1985; Young and Ortaldo, 1987; Heslop et al., 1988)** TNFα **(Nedwin et al 1985; Heslop et al, 1988)** and TNFβ **(Nedwin et al, 1985)**. More detailed studies of cytokine gene expression and cytokine release during the generation of LAK cells by IL-2 have been conducted **(Kovacs et al, 1989; Limb et al, 1989; Schoof et al, 1992; Saraya and Balkwill, 1993)**. All of these studies detect IFNγ and TNFα mRNAs **(Kovacs et al, 1989; Saraya and Balkwill, 1993)** and proteins **(Limb et al, 1989; Schoof et al, 1992; Saraya and Balkwill, 1993)**. IL-1α was detected in LAK supernatants by **Limb et al (1989)** but neither IL-1α itself nor its mRNA was observed by **Saraya and Balkwill (1993)**. In contrast, the detection of IL-1α mRNA was described by **Kovacs et al (1989).**

The mRNAs for both IL-1β and TNFβ were also found **(Kovacs et al, 1989; Saraya and Balkwill, 1993)** with the TNFβ protein **(Nedwin et al, 1985 ; Saraya and Balkwill, 1993)** and the IL-1β protein **(Kovacs et al, 1989; Saraya and Balkwill, 1993)** also being present in the LAK supernatants. The study by **Saraya and Balkwill**

(**1993**) is probably one of the most extensive investigations of cytokine production in response to IL-2 to date, assessing the gene expression and protein synthesis of 13 cytokines. In addition to those cytokines already discussed (IFNγ, TNFα, IL-1α, IL-1β and TNFβ), gro and IL-6 mRNAs were detected early in culture whilst mRNAs for IL-5, GM-CSF and M-CSF were detectable at a later stage. TGF-β mRNA was always detectable although this transcript was also present in cells cultured in the absence of IL-2. The mRNA transcripts for IFNα and IL-4 (and IL-1α as discussed previously) was not detected at any point.

1.5 : LAK CELLS - A ROLE IN IMMUNOMODULATION?

Apart from the afore-mentioned effects of cytokines on LAK activity, the generation of LAK cells by IL-2 has been shown to be inhibited in-vitro by co-culture with either cultured tumour cell-lines (**Guillou, Sedman et al, 1989**) or serum-free supernatants derived from in vitro maintained tumour cell lines (**Guillou, Ramsden et al, 1989**). Furthermore, LAK activity can be modulated by LAK cells interacting with each other (**Gallagher, Findlay et al, 1989**). The regulation of LAK cells by themselves, tumour-derived materials and cytokines may compromise the ability of LAK cells to function once returned to the tumour-bearing host.

The systemic administration of IL-2 to mice has been shown to enhance the delayed hypersensitivity reaction to a contact allergen (**Zaloom et al, 1991**). The effects of IL-2 observed on this T-cell mediated response was subsequently shown to be modulated by LAK cells (**McCulloch et al, 1991**). The presence of LAK cells altered the effects of IL-2 in the spleen, suggesting that LAK cells can interact with activated T-cells within the immune system and thus, modulate their function. This is of particular interest since LAK cells have the ability to secrete secondary cytokines which may be capable of regulating T-cell activity. Although LAK cells can mediate direct cytotoxicity of tumour cells in vitro, evidence that they actually localise at the site of the tumour in vivo is sparse. **Rubin et al (1989)** showed that the LAK cells did not accumulate at or infiltrate into metastatic lesions. This supports reports that following infusion of LAK cells into the peripheral circulation, LAK cells traffic through the lungs to the liver and spleen for clearance (**Rosenberg, 1985**) although **Basse et al (1992)** suggest that controversies about the tissue distribution of infused LAK cells may have arisen as a result of differences in cellular labelling for such studies.

However, since LAK cells in vivo appear to infiltrate the spleen, which is rich in both T-cells and monocytes, the opportunity for LAK cells to interact with the host's immunocytes may exist. Consequently, any effects they exert on tumour regression may be indirect, via the local secretion of secondary cytokines and/or their interactions with other cells of the immune system. This project therefore addresses whether LAK cells, induced with either rhIL-2 or anti-CD3, have the ability to regulate T-cell cytotoxicity and/or proliferation in vitro, through the release of secondary cytokines which can enhance T-cell function.

MATERIALS AND METHODS

2.1: ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) FROM WHOLE BLOOD

Samples of heparinised venous blood (120ml) were obtained from 4 healthy volunteers. Following a 1:1 dilution in sterile 0.01M phosphate buffered saline, pH 7.4 (PBS), the blood was separated by density gradient centrifugation over lymphoprep 1.077 (NYCOMED) at 400g for 30 mins at 20°C (no brake). The mononuclear cell layer was then harvested from the interface by aspiration. After an initial wash in cold PBS at 600g for 15 mins at 4°C, the PBMC were washed twice at 400g for 6 mins in cold PBS (adapted from method of **Boyum et al, 1968**). Viable cells, as determined by trypan blue exclusion, were then diluted to a cell density of 10^6/ml in medium.

2.2 : INDUCTION OF LAK ACTIVITY

PBMC were cultured in 25cm^2 flasks (GREINER) in AIM-V (**Jadus et al , 1988; Finkelstein and Miller, 1991**) serum-free medium (GIBCO), supplemented with 200mM L-glutamine, at a cell density of 10^6/ml. LAK activity was induced by incubation with either recombinant human (rh)IL-2 (GLAXO) at 200, 1000 or 2000 IU/ml or monoclonal anti-CD3 (SHL45.6, Cambridge University*) at 1, 10 or 100ng/ml. Control flasks containing no stimulus were also set up. Measurements of LAK cytotoxicity and cytokine secretion were made on days 1, 4 and 8 after the start of incubation.

Activation of the PBMC with anti-CD3 was carried out at separate times from the IL-2 induction, using repeat blood donations from the same 4 donors. Control cultures, in medium alone, were set up for both the IL-2 and anti-CD3 stimulations.

* SHL45.6 is a bivalent monoclonal anti-CD3 and was a generous gift from Dr M. Clark, Department of Pathology, University of Cambridge.

2.3 : 4-HOUR CHROMIUM-RELEASE ASSAY OF LAK CYTOTOXICITY

LAK cytotoxicity was measured in a standard 4-hour chromium-release assay against the colon adenocarcinoma cell-line SW742 (**Leibovitz et al, 1976**). The target cell pellet was labelled with 3-4 MBq of aqueous sodium chromate (AMERSHAM) for 1 hour at 37°C. The cells were then washed in serum-free RPMI 1640 (GIBCO) at 400g for 6 mins. Target cells were further incubated for 1 hour at 37°C in 10mls of culture medium, RPMI 1640 + 10% foetal calf serum (FCS), in order to absorb any free, residual sodium chromate. After a further wash at 400g for 6 mins, cell viability was determined by trypan blue exclusion and the cells were diluted in serum-free RPMI 1640 to a density of 10^5/ml.

The LAK cells generated from each donor were harvested on days 1, 4 or 8 of culture by centrifugation at 400g for 6 mins. The resulting cell supernatants were collected, centrifuged at 400g for 6 mins and filter sterilised through 0.2µm filters (ICN FLOW) in order to remove any remaining cells or cell debris. These supernatants were then stored at -20°C in 2-3ml aliquots. Meanwhile, each cell pellet was washed in medium at 400g for 6 mins prior to a cellular viability count. The cell density of those effector cells obtained from each stimulation was then adjusted to 25 x 10^5/ml in serum-free RPMI 1640.

In order to determine the degree of LAK cytotoxicities induced, effector (E) and target (T) cells were plated out in triplicate, at an E:T ratio of 25:1, in 96-well round-bottomed microtitre plates (GREINER). Control wells containing targets alone in medium (spontaneous release) and targets lysed with 10% (v/v) Triton-X 100 (maximum release) were also included in the assay. Following a 4 hour incubation at 37°C in a humidified atmosphere, 100µl of supernatant was removed from each well into scintillation vials. The degree of chromium (^{51}Cr) release was then measured on a gamma counter.

Using the mean cpm values for each effector population, the % LAK cytotoxicities were calculated according to the following formula:

$$\% \text{ CYTOTOXICITY} = \frac{\text{Experimental CPM - Spontaneous release CPM}}{\text{Maximum release CPM - Spontaneous release CPM}} \times 100$$

2.4 : BIOASSAY FOR INTERLEUKIN-1

D1O(N4)M cells (**Hopkins and Humphries, 1989 and 1990**) were cultured in RPMI 1640 medium buffered with 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and sodium bicarbonate (BIOLOGICAL INDUSTRIES) supplemented with 10% FCS (GIBCO), 200mM L-glutamine (BIOLOGICAL INDUSTRIES), 3 µg/ml concanavlin A (SIGMA) and 50mM 2-mercaptoethanol (SIGMA). The cells were maintained in $25cm^2$ flasks (GREINER), suspended in 10mls of medium supplemented with 20-50 IU/ml rhIL-2 (GLAXO) and 20-40 pg/ml crude IL-1 (prepared from silica-stimulated monocytes). Cells were subcultured every 3-4 days by diluting the suspension 1:10 in fresh medium.

Using this medium (without the addition of the above cytokines) as diluent, eight serial 3-fold dilutions of each supernatant were made in flat-bottomed microtitre plates (GREINER), leaving a final volume of 50µl per well. The dilution curve series for each sample was prepared in quadruplicate. Likewise, standard curves of two IL-1 standards, recombinant human (rh)IL-1 international reference standard (NIBSC) at 7.5 ng/ml and calibrated conditioned medium from silica-stimulated monocytes ("in-house" standard) were prepared in triplicate by serial 3-fold dilutions. On the third day of culture the D1O(N4)M cells were harvested and washed twice in medium. The cells were resuspended at a density of 10^5/ml in fresh medium supplemented with 60 IU/ml rhIL-2*. On addition of 50ml of cells to each well, the bioassay plates were incubated at 37°C, 5% CO_2 for 3 days.

The cell proliferation in response to the IL-1 present was determined by cellular viability. An index of viable cell numbers was obtained by a colourimetic method (**Mossman 1983, Hasen et al 1989**) based on the reduction of the tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT (ALDRICH) was dissolved at 5mg/ml in PBS and filtered through a 0.2µm filter. 10µl of MTT solution was then added to each well, containing cells in 100µl of culture medium. The cells were incubated with the MTT at 37°C for 2 hours until a purple precipitate (soluble tetrazolium salt) formed. In order to dissolve the formazan precipitate, 100µl of solvent was added to each well.[The solvent used consisted of sodium dodecyl sulphate (20% w/v) dissolved in 50% (v/v) aqueous N,N-dimethyl-formamide at 37°C, both ALDRICH. The pH of the solution was adjusted to 4.7 with hydrochloric acid. The solvent was stored at 37°C to retain the sodium dodecyl sulphate in solution]. The plates were left to stand overnight at room temperature until the precipitate had fully dissolved. The optical densities were then read on a ELISA plate reader (DYNATECH) at 540nm with a reference wavelength at 630nm.

I am most grateful to Dr Peter Winstanley (Department of Biochemistry, Glasgow Royal Infirmary) for his expert assistance in the analysis of this data using the computer programme flexifit (**Guardabasso et al, 1987**). All techniques used are according to the methodology outlined in Tumour Immunobiology - A Practical Approach (edited by Gallagher, Rees and Reynolds, Oxford University Press, 1993).

* The dose-response curve of the D10(N4)M cells to IL-1 is made much steeper by the incorporation of IL-2 into the assay. The addition of a saturating dose of IL-2 to this assay increases the specificity of the assay for IL-1 by neutralising any effect of IL-2 in the test sample.

2.5 : ELISA FOR TNFα AND IFNγ

Each well of a 96-well microelisa plate (GIBCO) was coated with 50µl of either sheep anti-IFNγ polyclonal antibody or goat anti-TNFα polyclonal antibody diluted 1:1000 or 1:500 respectively, in 0.05M carbonate buffer pH 9.6. Following a 2 hour incubation at 37°C in a humidified atmosphere, any non-specific binding sites were blocked overnight at 4°C with 150µl/well of a 2.5% (w/v) solution of bovine serum albumin (BSA) in tris buffered saline (TBS), both BDH.

Calibration curves of each cytokine standard were prepared: standard IFNγ diluted to 1000, 750, 500, 250, 100, 50 or 10 U/ml and standard TNFα diluted to 100, 75, 50, 25, 10, 5 and 1 U/ml. Test samples and standards were added in 50µl volumes to triplicate wells and incubated for 1 hour at 37°C. The plates were then incubated consecutively for 1 hour intervals with the monoclonal antibodies anti-IFNγ (AS3B) diluted 1:500 or anti-TNFα (101/4) diluted 1:1000 and biotinylated anti-mouse immunoglobulins (AMERSHAM RPN 1001) diluted 1:1000, all at 50µl/well. The assay was developed by the addition of 1:500 streptavidin alkaline phosphatase conjugate (AMERSHAM RPN 1234) to each well (50µl/well) for 30 mins and subsequently by incubation with 50µl of alkaline buffer and 50µl of freshly-prepared phosphatase substrate (SIGMA 104) at 4mg/ml in distilled water, respectively. The plates were incubated with substrate for approximately 30 mins to allow sufficient colour to develop [ie. until top standards reached an OD (414nm) of 1.5-2.0].

All incubations were carried out at 37°C. Reagents (except substrate) were diluted in 1% (v/v) FCS/TBS and the plates were washed in 0.1% (v/v) Tween 20/TBS three times between each step in the procedure. The optical densities were read on an ELISA plate reader at a wavelength of 414nm. Concentrations of IFNγ or TNFα were

obtained by reading the values on the standard curves corresponding with the mean optical density of each test sample.

Many thanks to Dr Catherine McIntyre (Department of Cancer Studies, University of Sheffield) for providing reagents and her technical advice for these assays. ELISA protocol adopted is taken from **Meager et al, 1987 and 1989**.

2.6 : MAINTENANCE OF T-CELL AND LYMPHOBLASTOID CELL LINES

The EBV-transformed lymphoblastoid cell line SW-LCL was generated from the B-cells of SW (by the methodology outlined in **Wallace et al, 1985**), and has an MHC-class I haplotype of HLA-A11, A24, B7, B35. SW-LCL cells were grown in RPMI 1640 supplemented with 10% (v/v) FCS and 200mM L-glutamine and maintained at a cell density of 1-2 x 10^6/ml by passaging 1:2 twice a week.

ST-CTLL is a polyclonal, allospecific cytotoxic T-cell line (CTLL) which is HLA-A11, B35 specific since ST is HLA -A3, A24, B7 and the CTLL line was generated against the line SW-LCL according to the method in **Wallace et al, 1985**. The CTLL was obtained from Dr. L. Wallace and was grown in RPMI 1640 supplemented with 10% FCS, 200mM L-glutamine and 20% (v/v) MLA-144 culture supernatant (also courtesy of Dr. L. Wallace) in 24 well plates (GREINER) at a density of 10^6 cells per well. [MLA-144 supernatant was added as a source of IL-2, an essential requirement for the maintenance of the CTLL which are IL-2 dependent]. Fresh medium was added twice a week whilst the cells were passaged 1:2 as required. The specificity of the line was maintained by antigenically stimulating the T-cells with mitomycin-C-inactivated SW-LCL once every 5 days. These SW-LCL "feeder cells" were treated at a density of 10^6/ml with mitomycin C (SIGMA) at a final concentration of 50µg/ml for 2 hours at 37°C. After several washes in medium, the inactivated cells were seeded at 1-2 x 10^5 cells per 10^6 T-cells (ie. per well). The CTLL was only used in cytotoxicity assays 24 hours after antigenic stimulation.

2.7 : 5 -HOUR CHROMIUM-RELEASE ASSAY OF CTL CYTOTOXICITY

The effects of the LAK supernatants on the killing capacity of the ST-CTLL was determined in a 5-hour chromium-release assay. An effector-to-target (E:T) ratio of 1.5 :1 was used, as predetermined in a titration assay.

Each LAK supernatant was added in triplicate to the wells of a 96-well V-shaped microtitre plate at a final concentration of 25% (v/v). The CTLL were harvested and washed once in IL-2 free medium (i.e. culture medium without MLA-144 supernatant). The effector cells were then seeded at a density of 1.5 x 10^4 cells/well. SW-LCL target cells were pelleted by centrifugation at 300-500 g for 5 minutes on high brake. The cell pellet was then incubated with 1.85 MBq of sodium chromate (AMERSHAM) for 1 hour at 37°C with occasional mixing. The labelled targets were washed twice at 500 g for 1 min with RPMI 1640 plus 10% FCS and subsequently added to the assay plate at 10^4 cells/well to give a total volume of 200ml/well. All assay plates were centrifuged at 80 g for 5 minutes with no brake prior to incubation at 37°C for 5 hours. 100µl of supernatant was then removed from each well into glass tubes and the chromium release counted on a gamma counter. The mean cpm values were then used to calculate the % CTL cytotoxicity according to the following equation:

$$\% \text{ LYSIS} = \frac{\text{Test release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} \times 100$$

The spontaneous and maximum release values were obtained from target cells incubated with medium or 10% (v/v) Triton-X 100 respectively, as previously described. The cytotoxicity values observed in the presence of the LAK supernatants were compared to those obtained for CTLL incubated with either medium or rhIL-2 at final concentrations of 200, 1000 or 2000 IU/ml (representative of the IL-2 dose used to induce the LAK activity).

2.8 : 24 HOUR ^{3}H-URIDINE RELEASE ASSAY OF CTL CYTOTOXICITY

Using the same E:T ratio of 1.5:1, ^{3}H-uridine release assays were used to determine the affects of the LAK supernatants over 24 hours on ST-CTLL as adapted from the method of **Fidler et al (1986)**. [^{3}H-uridine was substituted for ^{51}chromium in the 24 hour assays because chromium becomes toxic to the labelled cells for durations exceeding 18 hours]. The SW-LCL target cells (10^6/ml) were labelled with 0.037 MBq/ml ^{3}H-uridine (AMERSHAM) overnight at 37°C. The targets were washed twice in RPMI 1640 + 10% FCS by centrifugation at 400g for 6 minutes prior to use. The assay plates were set up as before with triplicate wells each containing 25% (v/v) LAK supernatant, 1.5 x 10^4 CTL and 10^4 target cells. Likewise, similar controls were included in the assay: maximum and spontaneous release, CTLL in medium alone and CTLL incubated with 200, 1000 or 2000 IU/ml of rhIL-2. Following a 24 hour incubation at 37°C/5% CO_2, 100µl of supernatant from each well was transferred into scintillation vials. On addition of scintillation fluid (PACKARD), the ^{3}H-Uridine

release was measured on a beta-counter for 3 minutes. The mean % CTL cytotoxicity was calculated as before, as outlined in the previous equation.
Similar 5-hour ^{3}H-uridine release assays were conducted where the CTLL were pre-activated at 37^{o}C with the LAK supernatants (25% v/v), medium or rhIL-2 for 19 hours prior to the addition of labelled target cells for 5 hours. The same E:T ratio and methodology was used throughout.

I am very grateful to Professor Alan Rickinson and his colleagues within the "EBV group" in the CRC unit at the University of Birmingham, especially Dr Lesley Wallace, for the opportunity to work within their labs with the allospecific T-cell lines. My thanks for their technical assistance with the 5-hour cytotoxicity assays and for providing me with the cell lines and MLA-144 supernatant for later experiments.

2.9 ; THYMIDINE INCORPORATION PROLIFERATION ASSAY TO DETERMINE MITOGENICITY

PBMC, isolated as described previously, were plated out in duplicate (10^{5} cells/well) into 96-well flat-bottomed plates and incubated with 25% (v/v) LAK supernatant in the presence or absence of exogenous anti-CD3 (SHL45.6) at a final concentration of 5ng/ml for 72 hours. The proliferation induced was determined by ^{3}H-thymidine (AMERSHAM) incorporation. Following a 72 hour incubation at 37^{o}C, 0.037MBq of ^{3}H-thymidine was added to each well of the assay for 4 hours at 37^{o}C. The pulsed cells were then harvested onto filter paper using an automated cell harvester. Filters were dried overnight and then counted for 5 minutes using a beta-counter to determine the ^{3}H-thymidine incorporation as a measure of cellular proliferation. Controls were also included in the proliferation assay of cells plus medium alone (RPMI + 10%FCS) or cells plus concanavlin A (final concentration of 5ng/ml per well) in order to test cellular viability.

RESULTS

3.1 : INDUCTION OF LAK ACTIVITY AND CYTOKINE SECRETION IN PBMC STIMULATED WITH rhIL-2

PBMC isolated from 4 healthy donors (A-D) were activated with rhIL-2 (at 200, 1000 or 2000 IU/ml) for 1, 4 or 8 days in culture. The LAK cytotoxicities and cytokine profiles (TNFα, IFNγ and IL-1) were compared in order to investigate the effects of individual variation, level of stimulus and duration of culture, on LAK induction. As controls, cells were cultured in the absence of rhIL-2 (0 IU/ml).

3.1a : LAK CYTOTOXICITY

Irrespective of donor, the maximal LAK cytotoxicity was always observed on the fourth day of culture (Figures 1a-4a). Maximal killing against SW742 at a 25:1 E:T ratio was generally obtained in the LAK populations activated with 1000 IU/ml of rhIL-2 : donors A, B and C (Figures 1a-3a). However, in the case of donor D (Figure 4a) maximal LAK cytotoxicity was generated with either 200 or 2000 IU/ml rhIL-2, although 1000 IU/ml rhIL-2 induced increasing LAK cytotoxicity with time.

In general, the LAK activity induced by stimulation with rhIL-2 peaked on day 4 and then declined with prolonged time in culture, irrespective of which dose of rhIL-2 was applied. A few exceptions were observed : donor B at 2000 IU/ml, donor C at 200 IU/ml and donor D at 1000 IU/ml all demonstrated peak cytotoxicity on day 8 (Figures 2a, 3a and 4a respectively).

IL-2 induced LAK activity generally appears to be a dose-responsive phenomenon, showing increased cytotoxicity at higher doses of rhIL-2. For three of the donors, PBMC cultured without rhIL-2 demonstrated no significant LAK cytotoxicity (donors A, B and D : Figures 1a, 2a and 4a). Killing was unusually high for donor C (Figure 3a), perhaps as a result of cellular activation during venupuncture and/or the potential to produce high initial levels of TNFα (Figure 3b).

3.1b : PRODUCTION OF TNFα

The concentrations of TNFα produced ranged from 6.2 U/ml to 144.8 U/ml. A similar trend in TNFα secretion was observed in donors A, B and D (Figures 1b, 2b and 4b). The production of TNFα was favoured by high-dose rhIL-2 (1000 or 2000 IU/ml) and appeared to be dose and time-dependent. As the dose of rhIL-2 increased to 1000 or 2000 IU/ml, the production of TNFα usually increased with the time in culture, illustrated in Figures 1b, 2b and 4b (at 2000 IU/ml and 1000 IU/ml respectively). However, some exceptions were observed, namely donor A at 1000 IU/ml (Figure 1b) and donor D at 2000 IU/ml (Figure 4b).

Although the absence of rhIL-2 (0 IU/ml) resulted in the production of little TNFα (6.2 U/ml to 31.7 U/ml) in the cases of donors A, B and D, donor C produced high levels of TNFα (117.9 U/ml to 144.8 U/ml), even at the onset of culture. This may be partially a consequence of cellular activation during venupuncture but may also demonstrate that donor C is a high TNFα producer.

3.1c : PRODUCTION OF IFNγ

Stimulation with low dose rhIL-2 (200 IU/ml) favoured the production of IFNγ in all donors (Figures 1c, 2c and 4c) except for donor C (Figure 3c), where a dose-response effect was evident, showing higher concentrations of IFNγ present as both rhIL-2 dose and duration in culture increased. Both donor A and donor B produced maximal IFNγ (160 U/ml and 60 U/ml respectively) when activated with 200 IU/ml of rhIL-2 for 4 days (Figures 1c and 2c). However, donor D showed a peak in IFNγ on day 8, (53 U/ml) although the same dose of rhIL-2, 200 IU/ml, was responsible for its production (Figure 4c). With the exception of donor C, incubation with no rhIL-2 or high dose rhIL-2 (2000 IU/ml) usually produced little (10-20 U/ml) or no IFNγ (Figures 1c, 2c and 4c). The concentrations of IFNγ observed in the culture supernatants ranged from 0 U/ml to 160 U/ml.

3.1d : PRODUCTION OF IL-1

The production of IL-1 by the donors in response to rhIL-2 showed a similar trend to the IFNγ profiles. Like IFNγ, IL-1 secretion was favoured by low dose rhIL-2 (200 IU/ml) in donors A and D (Figures 1d and 4d). In this case donor B produced negligible amounts of IL-1 (0.04 ng/ml to 1.69 ng/ml), when compared to donors A and D (1.41 to 20.48 ng/ml), irrespective of the stimulating dose of rhIL-2 (Figure 2d). Donor C again showed a dose-response relationship : increasing both dose of rhIL-2 and time in culture elevated the IFNγ concentrations (Figure 3d). However, the higher levels of IL-1 (8.42 ng/ml to 16.8 ng/ml) were also observed for donor C in the absence of rhIL-2 (Figure 3d), whereas the IL-1 concentrations for donors A and D were low under the same conditions (1.77 ng/ml to 8.66 ng/ml, Figures 1d and 4d). Overall, the secretion of IL-1 ranged from 0.04 ng/ml to 20.48 ng/ml.

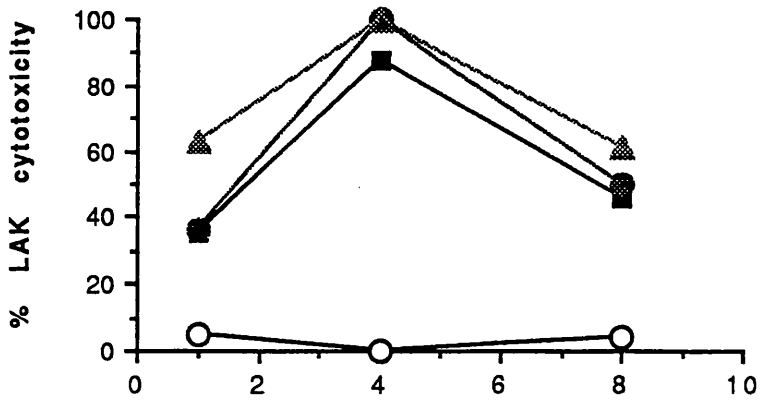
FIGURES 1a - 4a : LAK cytotoxicity induced in the PBMC from four normal volunteers, with rhIL-2 for 1, 4 or 8 days, as determined by 4-hour chromium release assays against SW742 at an E:T ratio of 25:1. Stimulating levels of rhIL-2 are represented by the following symbols: no rhIL-2 (open circles), 200 IU/ml rhIL-2 (squares), 1000 IU/ml rhIL-2 (closed circles) or 2000 IU/ml rhIL-2 (triangles). Donors A to D are represented in Figures 1a to 4a respectively.

FIGURES 1b - 4b : Production of TNFα from the PBMC of four normal donors in response to rhIL-2 stimulation for 1, 4 or 8 days. Cells were cultured without rhIL-2 (open circles) or with 200 IU/ml (squares), 1000 IU/ml (closed circles) or 2000 IU/ml (triangles) of rhIL-2. TNFα concentrations were determined by ELISA and are reported in U/ml. Donors A to D are represented in Figures 1b to 4b respectively. [*The data for donor B, 4 days with no rhIL-2 and at all time points with 2000 IU/ml rhIL-2 was not determined].

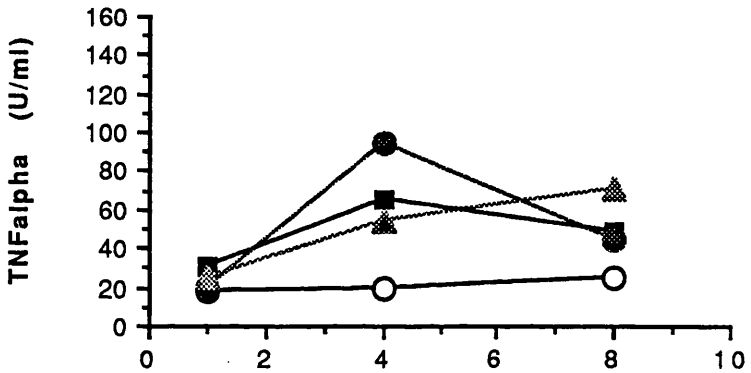
FIGURES 1c - 4c : Production of IFNγ from the PBMC of four normal donors in response to rhIL-2 stimulation for 1, 4 or 8 days. Cells were cultured without rhIL-2 (open circles) or with 200 IU/ml (squares), 1000 IU/ml (closed circles) or 2000 IU/ml (triangles) of rhIL-2. IFNγ concentrations were determined by ELISA and are reported in U/ml. Donors A to D are represented in Figures 1c to 4c respectively.

FIGURES 1d - 4d : Production of IL-1 from the PBMC of four normal donors in response to rhIL-2 stimulation for 1, 4 or 8 days. Cells were cultured without rhIL-2 (open circles) or with 200 IU/ml (squares), 1000 IU/ml (closed circles) or 2000 IU/ml (triangles) of rhIL-2. IL-1 concentrations were determined by bioassay and are reported in ng/ml. Donors A to D are represented by Figures 1d to 4d respectively.

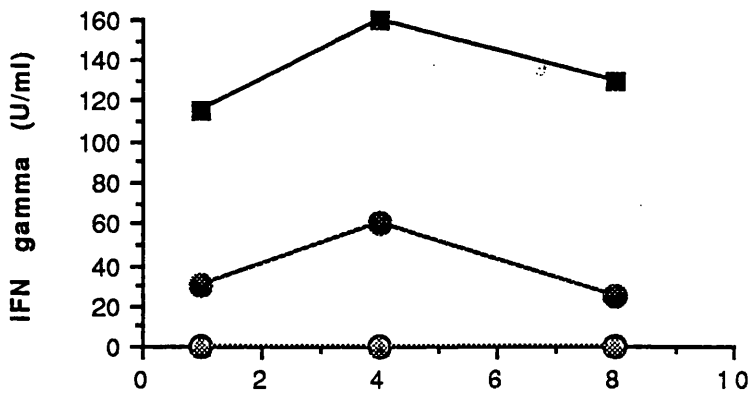
DONOR A : FIGURE 1a



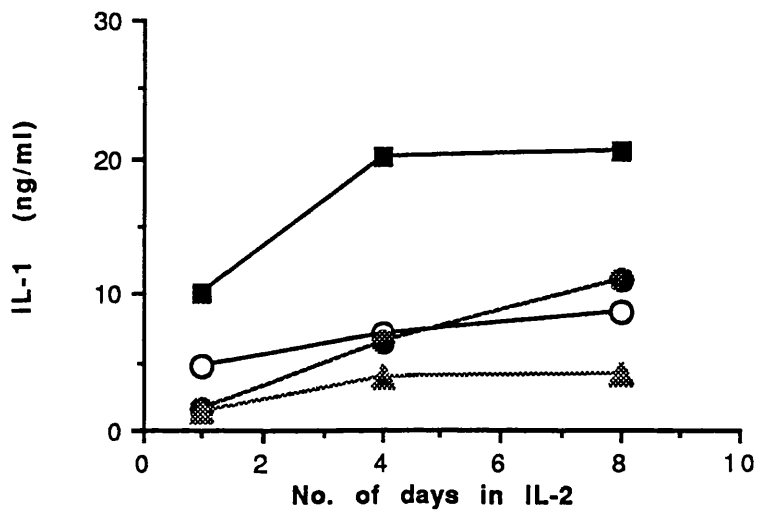
DONOR A : FIGURE 1b



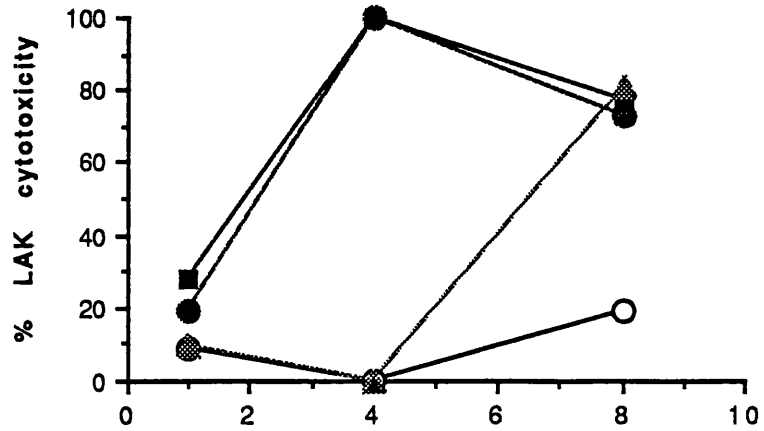
DONOR A : FIGURE 1c



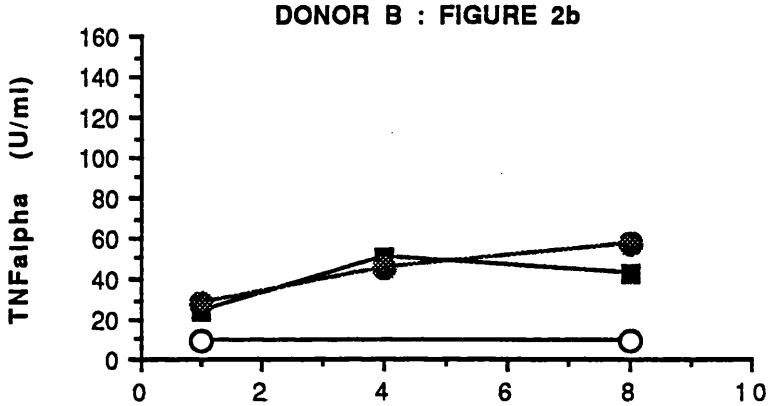
DONOR A : FIGURE 1d



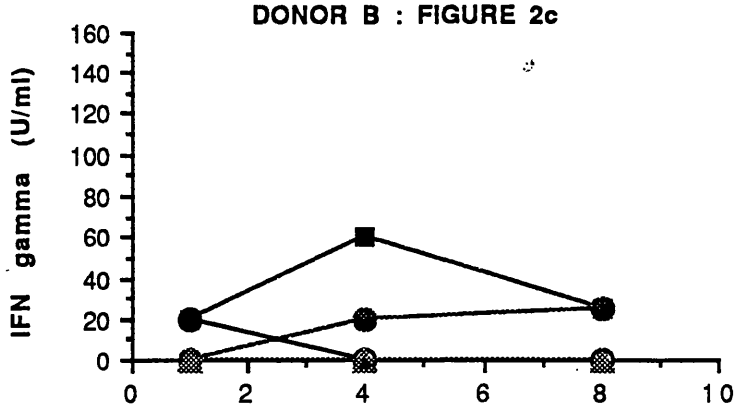
DONOR B : FIGURE 2a



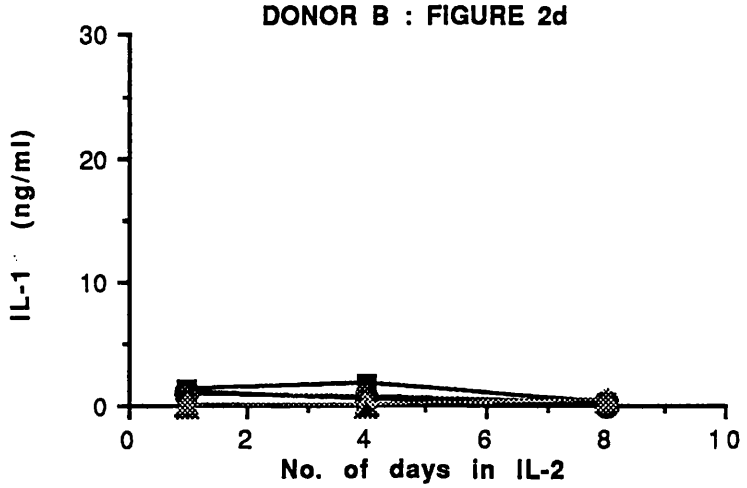
DONOR B : FIGURE 2b



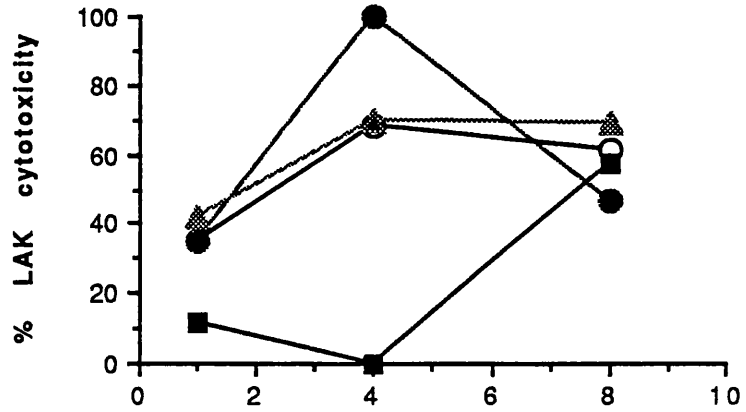
DONOR B : FIGURE 2c



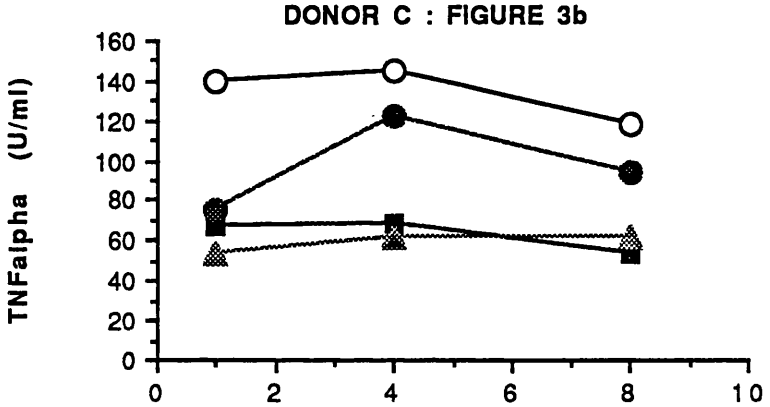
DONOR B : FIGURE 2d



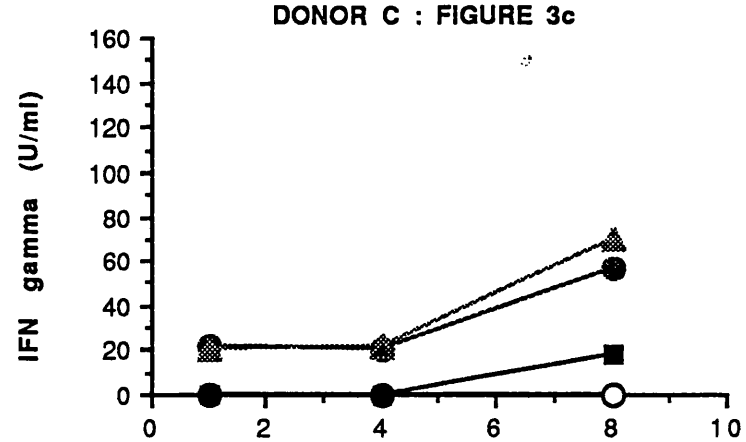
DONOR C : FIGURE 3a



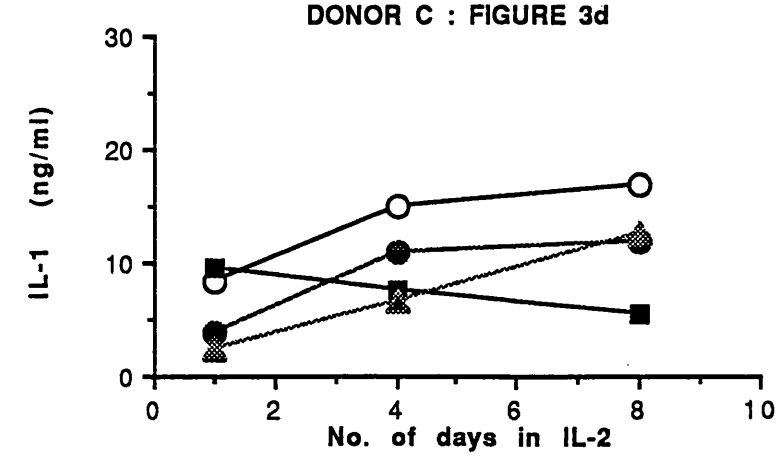
DONOR C : FIGURE 3b

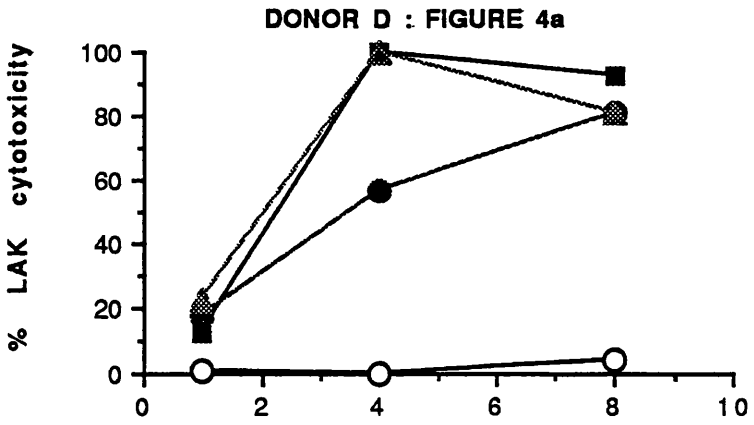


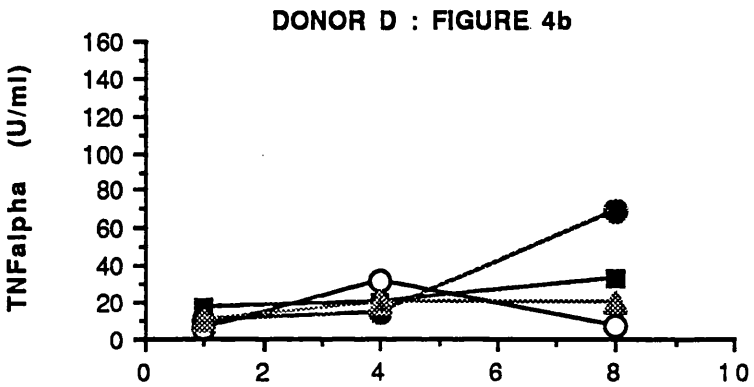
DONOR C : FIGURE 3c

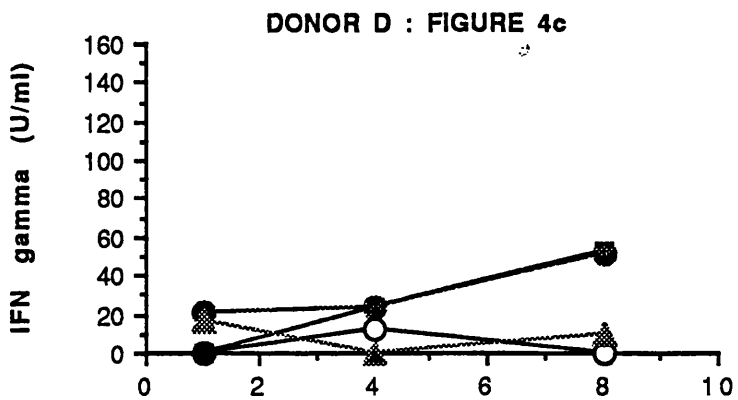


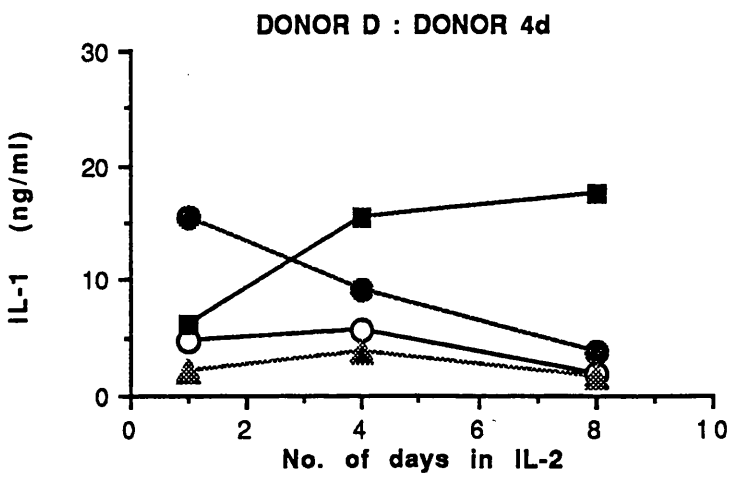
DONOR C : FIGURE 3d




DONOR D : FIGURE 4a
% LAK cytotoxicity
100
80
60
40
20
0
0
2
4
6
8
10


DONOR D : FIGURE 4b
TNFalpha (U/ml)
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10


DONOR D : FIGURE 4c
IFN gamma (U/ml)
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10


DONOR D : DONOR 4d
IL-1 (ng/ml)
30
20
10
0
0
2
4
6
8
10
No. of days in IL-2

3.2 : INDUCTION OF LAK ACTIVITY AND CYTOKINE SECRETION IN PBMC STIMULATED WITH ANTI-CD3

Venupuncture was repeated on donors A-D two weeks after the blood for the rhIL-2 experiments was donated. However, these PBMC were cultured for 1, 4 or 8 days with anti-CD3 antibody (1, 10 or 100 ng/ml). As with IL-2, the resultant LAK cytotoxicities and cytokine levels were compared. Likewise, controls were again established where PBMC were cultured in the absence of antibody (0 ng/ml).

3.2a : % LAK CYTOTOXICITY

Maximal killing was always observed on day 1 of culture at either 10 ng/ml of anti-CD3 antibody (donors A and D, figures 5a and 8a) or 1 ng/ml of anti-CD3 antibody (donors B and C in Figures 6a and 7a). With the exception of donor B with 10 ng/ml anti-CD3 antibody, the % LAK cytotoxicity always peaked on day 1 of culture and then declined with time (Figures 5a - 8a) in response to antibody. By comparison, cytotoxicity peaked on day 4 and then decreased for donor B at 10 ng/ml (Figure 6a). In the absence of anti-CD3 antibody, the LAK cytotoxicities measured were low. However, each donor showed the same general trend of killing, except for donor D where cytotoxicity peaked on day 4 as opposed to day 1 (Figure 8a).

3.2b : PRODUCTION OF TNFα

TNFα production was favoured by high dose anti-CD3 (10 ng/ml or 100 ng/ml) and the levels of TNFα produced in response ranged from 20.3 U/ml to 133.0 U/ml. Maximal TNFα production in donors A, C and D was in response to stimulation with 100 ng/ml of anti-CD3 whereas donor B produced maximal TNFα with 10 ng/ml of anti-CD3. In donor B, TNFα production (Figure 6b) in response to no or low dose anti-CD3 antibody (1ng/ml) peaked on day 1. Higher doses of anti-CD3 (10 ng/ml or 100 ng/ml) produced peak TNFα concentrations on day 8. TNFα secretion at high doses of antibody appeared to increase with time, although the data for the TNFα value on day 1 with 10 ng/ml of anti-CD3 was not available to confirm this trend.

Contrastingly, donor C behaved differently (Figure 7b) : TNFα levels peaked on day 1 and subsequently declined with time, with the exception of the response to 1ng/ml, where TNFα production peaked on day 4 before falling. The response of donor A (Figure 5b) to anti-CD3 antibody, irrespective of dose, resulted in the steady production of TNFα with time, even in the absence of antibody (0 ng/ml). Likewise donor D appeared to follow this pattern of TNFα secretion although this was not conclusive owing to insufficient data for TNFα levels acquired in response to no or

1ng/ml of anti-CD3 antibody. In the absence of antibody, the TNFα concentrations produced ranged from 20.3 to 95.0 U/ml.

3.2c : PRODUCTION OF IFNγ

IFNγ production in donors C and D (Figures 7c and 8c) was very low overall, (ranging between 0 and 30 U/ml). Both these individuals produced their maximal IFNγ concentrations (30 and 23 U/ml respectively) when activated with 100 ng/ml of anti-CD3 antibody. However, the IFNγ level peaked on day 4 with donor C whereas donor D peaked on day 8. In contrast, donors A and B (Figures 5c and 6c) produced maximal IFNγ (120 and 125 U/ml respectively) with 10 ng/ml of antibody, but with different durations in culture : 8 days for donor A (Figure 5c) compared to 4 days for donor B (Figure 6c).

In all other respects, the production of IFNγ by these donors, as a result of anti-CD3 stimulation was unpredictable. The concentrations of IFNγ ranged from 0 to 125 U/ml in these supernatants. In the absence of antibody, little (16 to 36 U/ml) or no IFNγ was detected.

3.2d : PRODUCTION OF IL-1

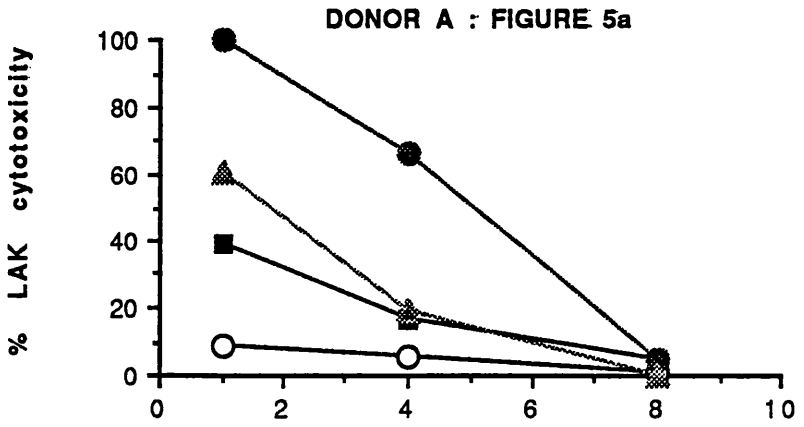
The production of IL-1 (Figures 5d - 8d) in response to monoclonal anti-CD3 was greatest with high doses of antibody (10 or 100 ng/ml), but particularly with 10 ng/ml (donors A, B and D as shown in Figures 5d, 6d and 8d). Although the response to dose is fairly predictable, the IL-1 production with time is more variable. The lack of data for donor D stimulated with any dose of antibody for 8 days made a comparison of IL-1 secretion over time between donors inconclusive. In the absence of anti-CD3 stimulation, IL-1 production was usually negligible (0.24 to 5.21ng/ml). Activation with anti-CD3 produced a range of IL-1 concentrations between 0.24 and 26.09ng/ml.

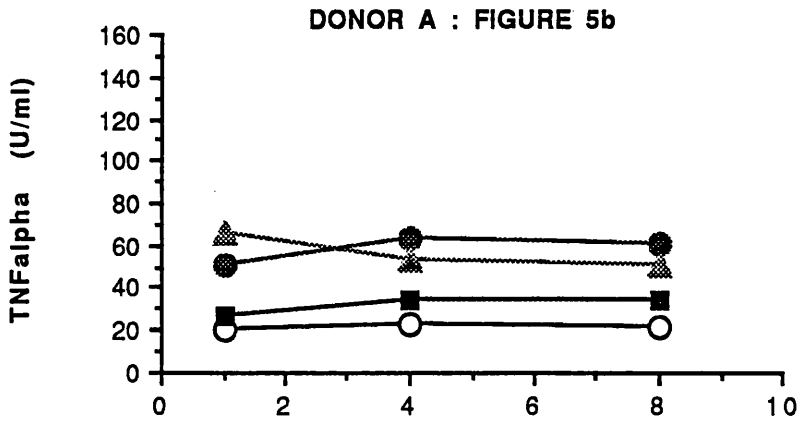
FIGURES 5a - 8a : LAK cytotoxicity induced in the PBMC from four normal volunteers, with monoclonal anti-CD3 for 1, 4 or 8 days, as determined by 4-hour chromium release assay against SW742 at an E:T ratio of 25:1. Stimulating levels of anti-CD3 are represented by the following symbols: no anti-CD3 (open circles), 1ng/ml anti-CD3 (squares), 10ng/ml anti-CD3 (closed circles) or 100ng/ml anti-CD3 (triangles). Donors A to D are represented in Figures 5a to 8a respectively.

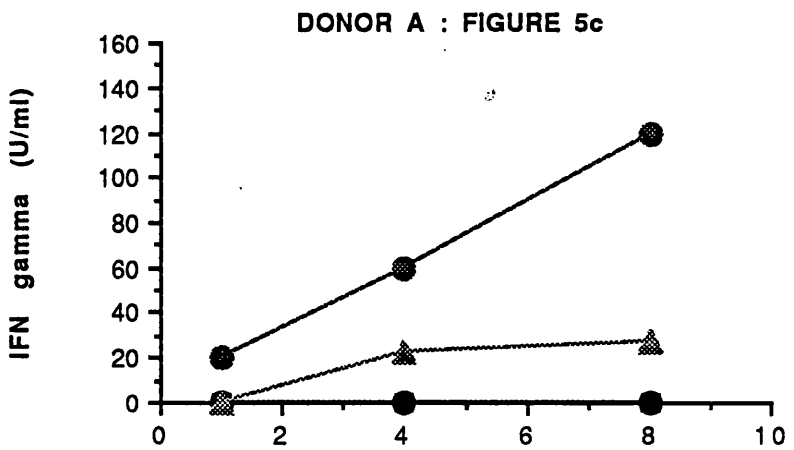
FIGURES 5b - 8b : Production of TNFα from the PBMC of four normal donors in response to anti-CD3 stimulation for 1, 4 or 8 days. Cells were cultured without antibody (open circles) or with 1ng/ml (squares), 10ng/ml (closed circles) or 100ng/ml (triangles) of anti-CD3. TNFα concentrations were determined by ELISA and are reported in U/ml. Donors A to D are represented in Figures 5b to 8b respectively. [*The data for donor B, 1 day with 10ng/ml of anti-CD3 and donor D, at all time points with no or 1ng/ml of antibody was not determined].

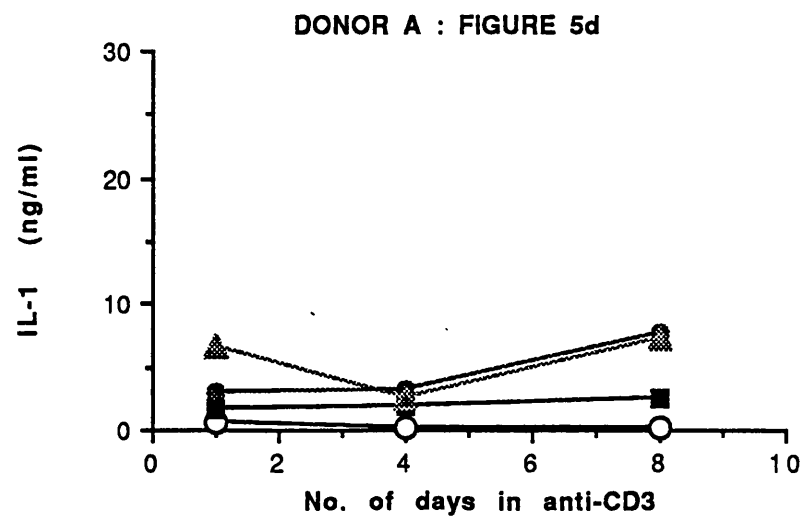
FIGURES 5c - 8c : Production of IFNγ from the PBMC of four normal donors in response to anti-CD3 stimulation for 1, 4 or 8 days. Cells were cultured without antibody (open circles) or with 1ng/ml (squares), 10ng/ml (closed circles) or 100ng/ml (triangles) of anti-CD3. IFNγ concentrations were determined by ELISA and are reported in U/ml. Donors A to D are represented in Figures 5c to 8c respectively.

FIGURES 5d - 8d : Production of IL-1 from the PBMC of four normal donors in response to anti-CD3 stimulation for 1, 4 or 8 days. Cells were cultured without antibody (open circles) or with 1ng/ml (squares), 10ng/ml (closed circles) or 100ng/ml (triangles) of anti-CD3. IL-1 concentrations were determined by bioassay and are reported in ng/ml. Donors A to D are represented in Figures 5d to 8d respectively. [*The data for donor D on day 8 was not determined at any dose of anti-CD3].

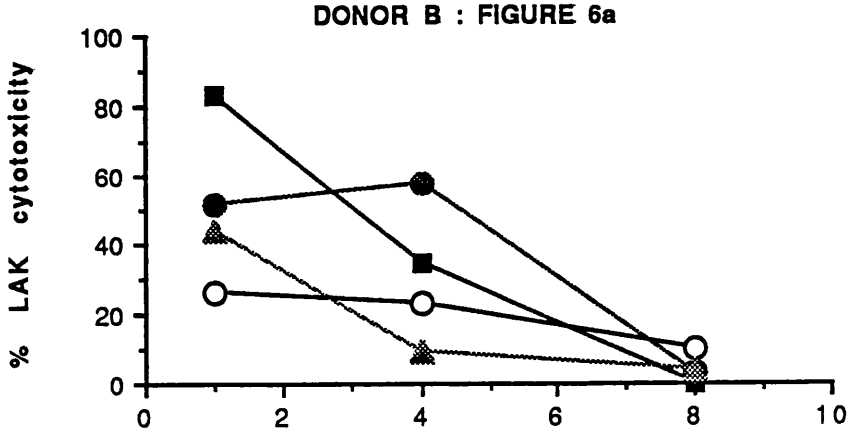

DONOR A : FIGURE 5a
% LAK cytotoxicity
100
80
60
40
20
0
0
2
4
6
8
10


DONOR A : FIGURE 5b
TNFalpha (U/ml)
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10

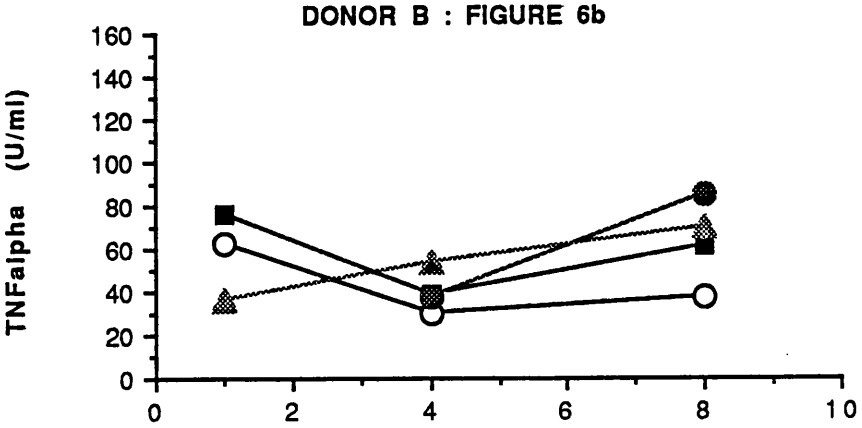

DONOR A : FIGURE 5c
IFN gamma (U/ml)
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10


DONOR A : FIGURE 5d
IL-1 (ng/ml)
30
20
10
0
0
2
4
6
8
10
No. of days in anti-CD3

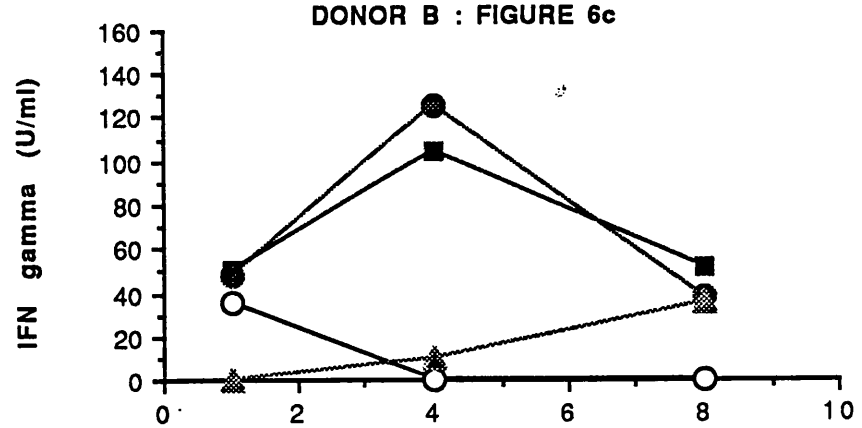
DONOR B : FIGURE 6a



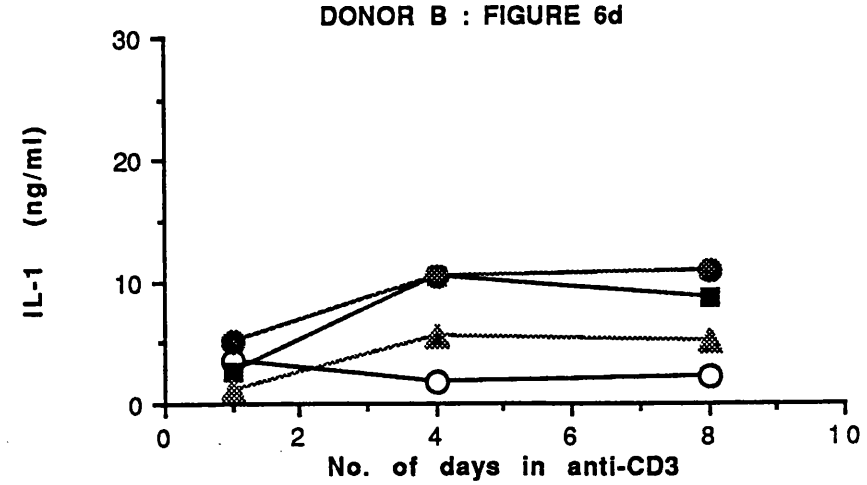
DONOR B : FIGURE 6b



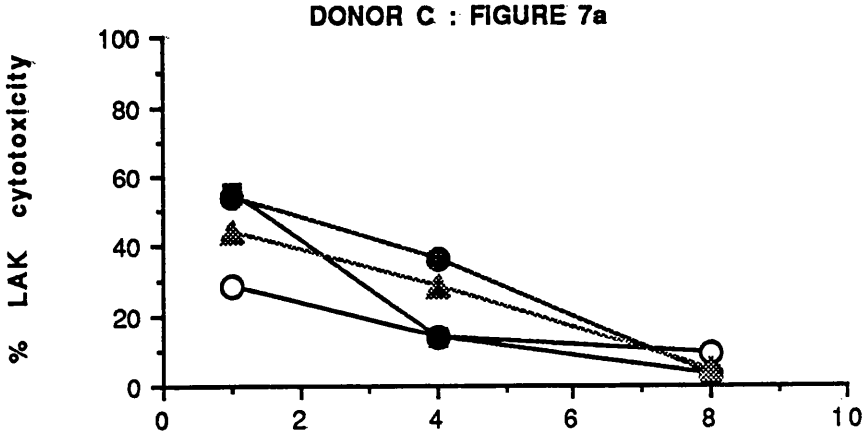
DONOR B : FIGURE 6c



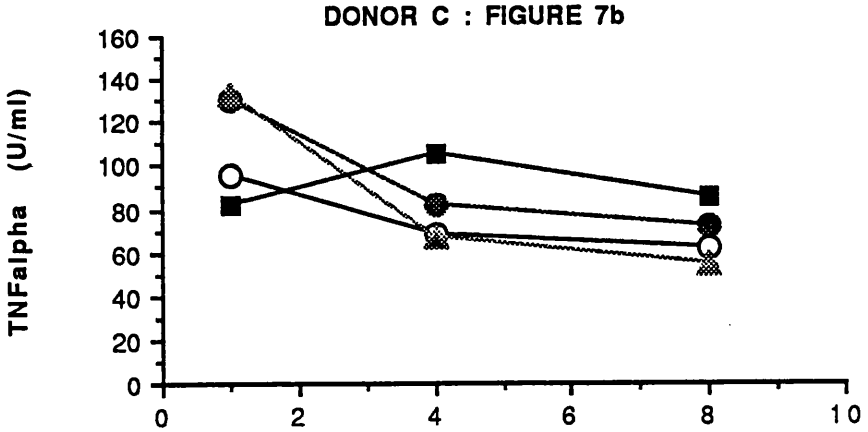
DONOR B : FIGURE 6d



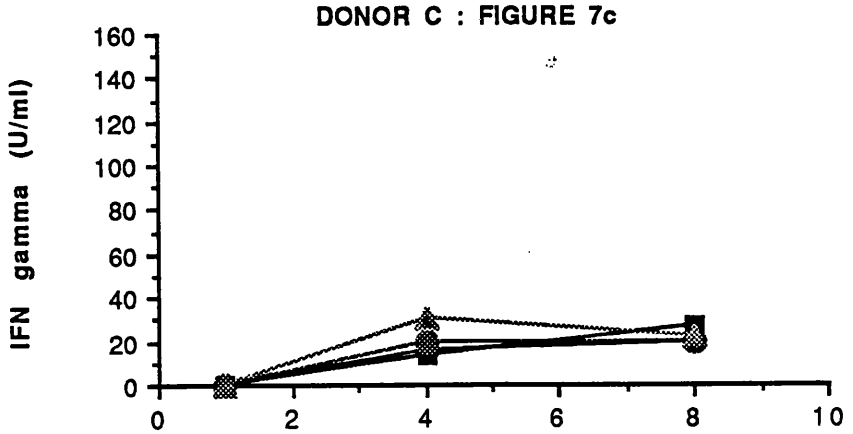
DONOR C : FIGURE 7a



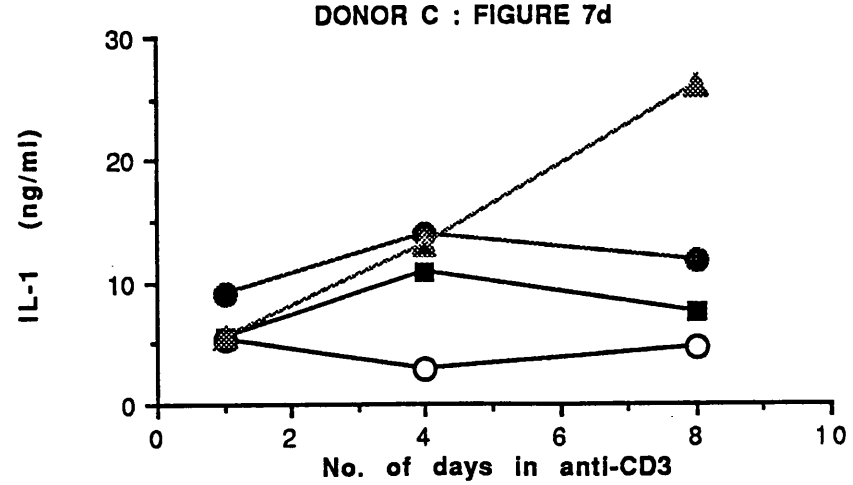
DONOR C : FIGURE 7b



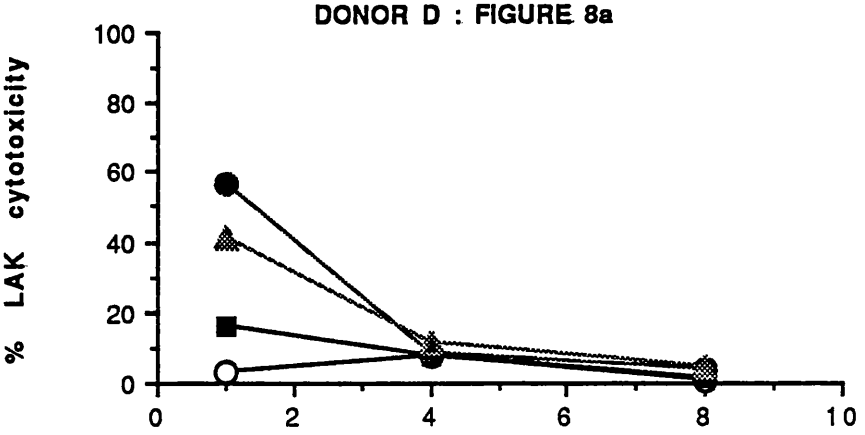
DONOR C : FIGURE 7c



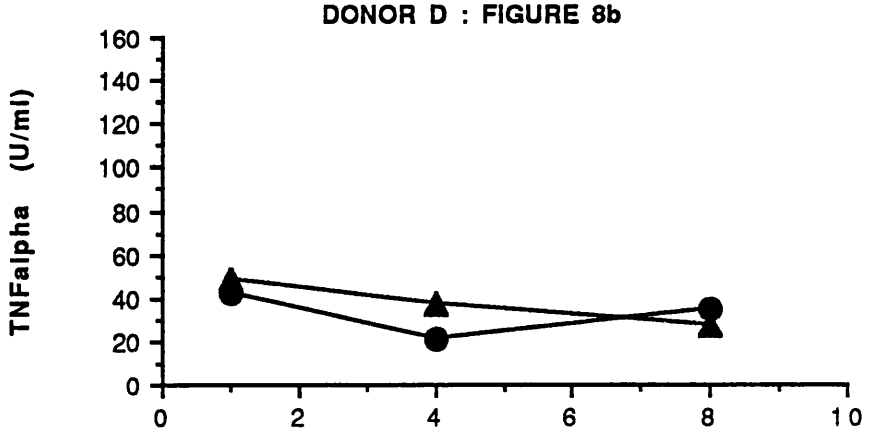
DONOR C : FIGURE 7d



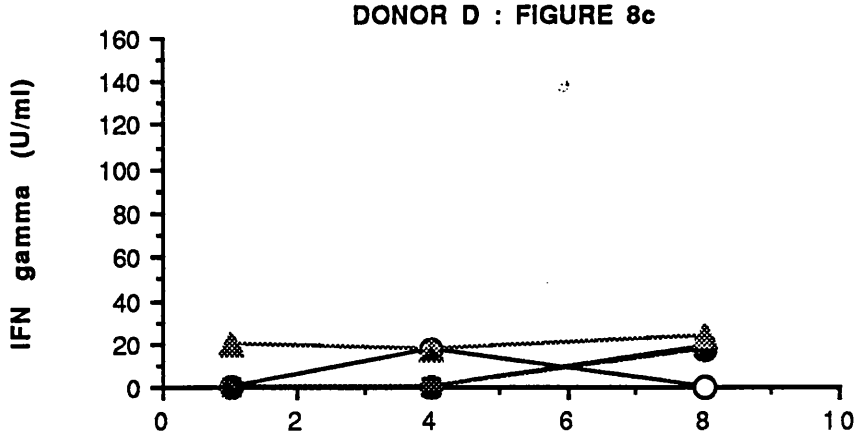
DONOR D : FIGURE 8a



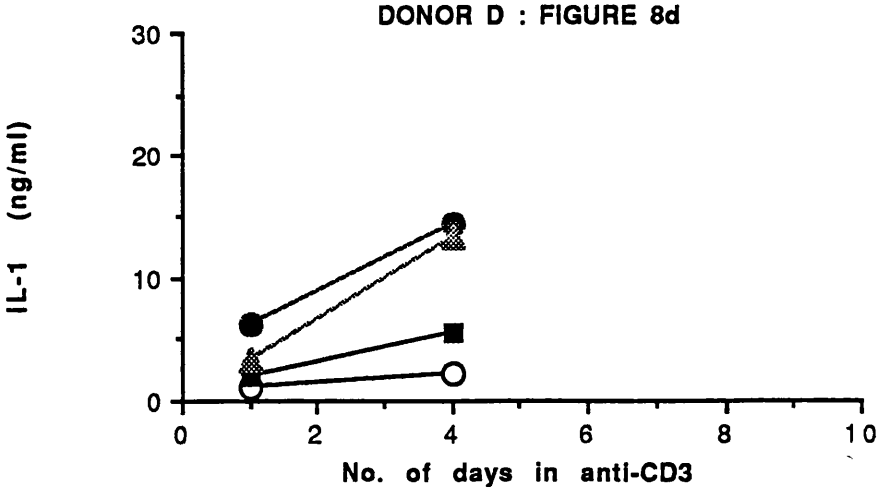
DONOR D : FIGURE 8b



DONOR D : FIGURE 8c



DONOR D : FIGURE 8d



3.3 : EFFECTS OF IL-2 AND ANTI-CD3 INDUCED LAK SUPERNATANTS ON CTL CYTOTOXICITY OVER 5 HOURS

Each LAK supernatant was assessed, at a final concentration of 25%, in 5-hour chromium release assays for the ability to enhance the cytotoxicity of the ST-CTLL against its specific target SW-LCL. The killing observed was compared to the cytotoxicity values obtained for controls (i.e. CTLL with rhIL-2 at final concentrations of 200, 1000 or 2000 IU/ml).

Although fluctuations in killing were observed for both the IL-2 and anti-CD3 induced LAK supernatants (Figures 9a and 9b respectively), there was no significant increase in cytotoxicity over controls (Figure 9a). The % lysis for the controls ranged from 46% to 50%. The % CTL cytotoxicities ranged from a minimum of 32% (supernatant C.I.4.2000) to a maximum of 58% (supernatant C.I.1.1000) for the IL-2 induced supernatants (Figure 9a). The level of killing for the anti-CD3 induced supernatants was similar, ranging from 34% (supernatants B.C.1.0 and B.C.1.1) to 50% (supernatant D.C.1.100), with the exception of the value 77% for supernatant C.C.1.100 (Figure 9b). However, this value was thought to be inaccurate, owing to a experimental error. In the absence of CTLL, none of the LAK supernatants produced any target lysis, confirming that the supernatants had no direct cytotoxic effect on the target cells during the 5-hour assay. In the titration cytotoxicity assay used to determine the E:T ratio (1.5:1) for these experiments, the CTLL produced > 40% lysis. It therefore appears that none of the LAK supernatants significantly enhances the cytotoxic activity of the CTLL.

FIGURES 9a AND 9b : ST-CTLL cytotoxicity generated against SW-LCL following activation with IL-2 induced LAK supernatants (Figure 9a), anti-CD3 induced LAK supernatants (Figure 9b) or rhIL-2 (Figure 9a) for 5 hours. The % lysis was determined by a 5-hour chromium release assay at an E:T ratio of 1.5:1. The LAK supernatants are coded by the following symbols: A, B, C or D represents the donors; I or C represents IL-2 or anti-CD3 induction respectively; 1, 4 or 8 represents the number of days in culture; 0, 200, 1000 or 2000 represents stimulating dose of rhIL-2 (IU/ml); 0, 1, 10 or 100 represents stimulating dose of anti-CD3 (ng/ml).

FIGURE 9a

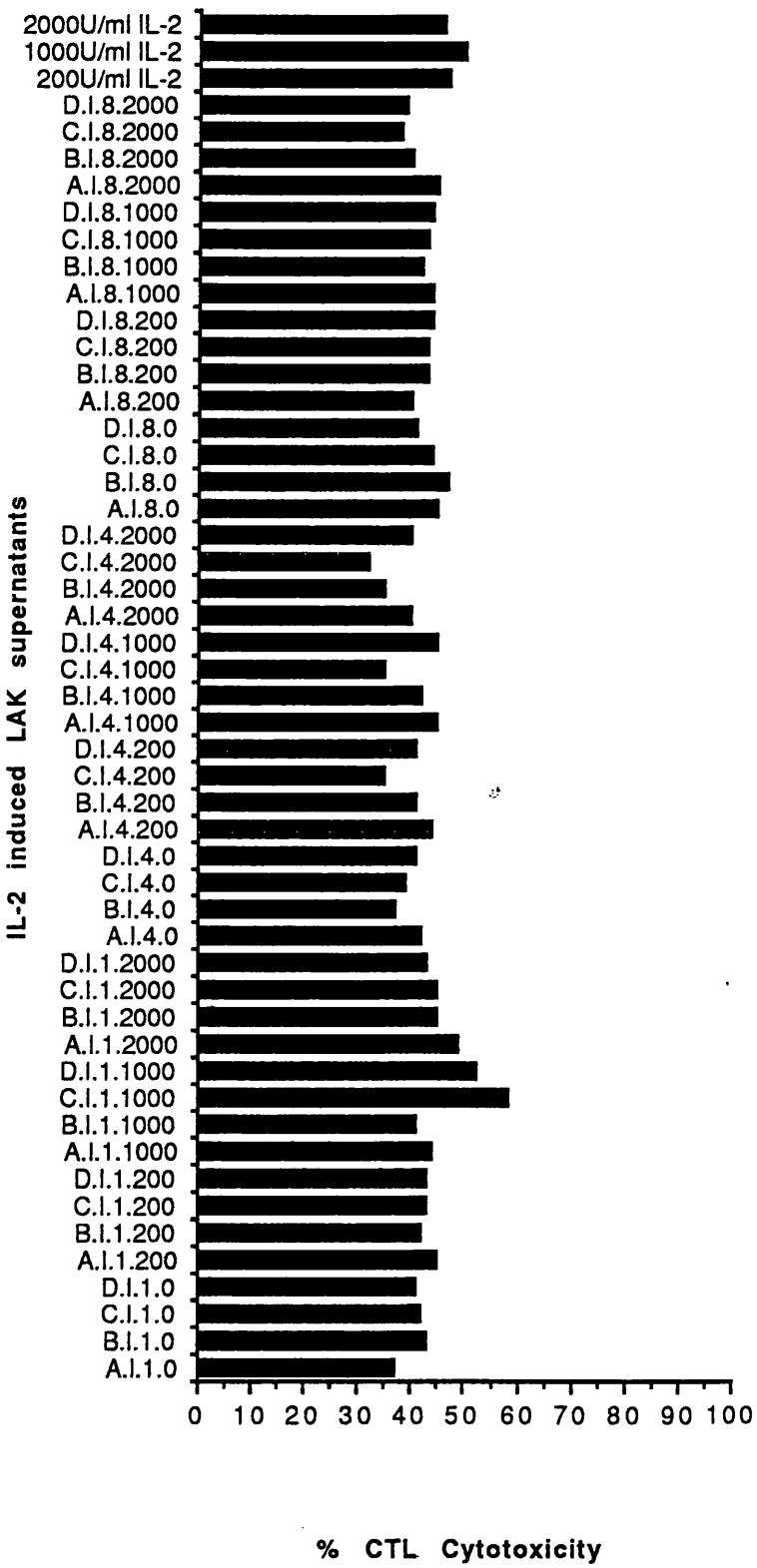
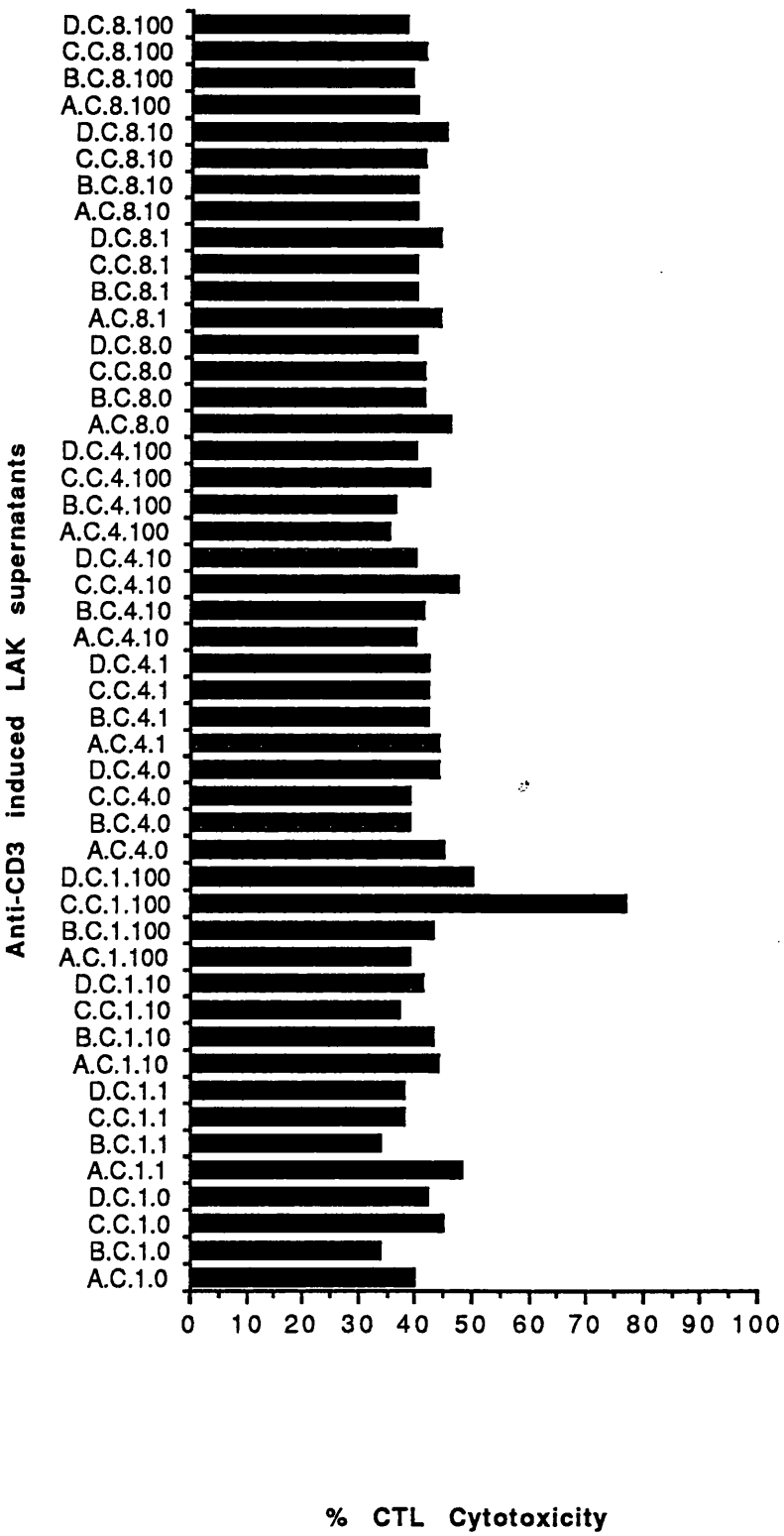

2000U/ml IL-2
1000U/ml IL-2
200U/ml IL-2
D.I.8.2000
C.I.8.2000
B.I.8.2000
A.I.8.2000
D.I.8.1000
C.I.8.1000
B.I.8.1000
A.I.8.1000
D.I.8.200
C.I.8.200
B.I.8.200
A.I.8.200
D.I.8.0
C.I.8.0
B.I.8.0
A.I.8.0
D.I.4.2000
C.I.4.2000
B.I.4.2000
A.I.4.2000
D.I.4.1000
C.I.4.1000
B.I.4.1000
A.I.4.1000
D.I.4.200
C.I.4.200
B.I.4.200
A.I.4.200
D.I.4.0
C.I.4.0
B.I.4.0
A.I.4.0
D.I.1.2000
C.I.1.2000
B.I.1.2000
A.I.1.2000
D.I.1.1000
C.I.1.1000
B.I.1.1000
A.I.1.1000
D.I.1.200
C.I.1.200
B.I.1.200
A.I.1.200
D.I.1.0
C.I.1.0
B.I.1.0
A.I.1.0
IL-2 induced LAK supernatants
0 10 20 30 40 50 60 70 80 90 100
% CTL Cytotoxicity

FIGURE 9b


Anti-CD3 induced LAK supernatants
D.C.8.100
C.C.8.100
B.C.8.100
A.C.8.100
D.C.8.10
C.C.8.10
B.C.8.10
A.C.8.10
D.C.8.1
C.C.8.1
B.C.8.1
A.C.8.1
D.C.8.0
C.C.8.0
B.C.8.0
A.C.8.0
D.C.4.100
C.C.4.100
B.C.4.100
A.C.4.100
D.C.4.10
C.C.4.10
B.C.4.10
A.C.4.10
D.C.4.1
C.C.4.1
B.C.4.1
A.C.4.1
D.C.4.0
C.C.4.0
B.C.4.0
A.C.4.0
D.C.1.100
C.C.1.100
B.C.1.100
A.C.1.100
D.C.1.10
C.C.1.10
B.C.1.10
A.C.1.10
D.C.1.1
C.C.1.1
B.C.1.1
A.C.1.1
D.C.1.0
C.C.1.0
B.C.1.0
A.C.1.0
0 10 20 30 40 50 60 70 80 90 100
% CTL Cytotoxicity

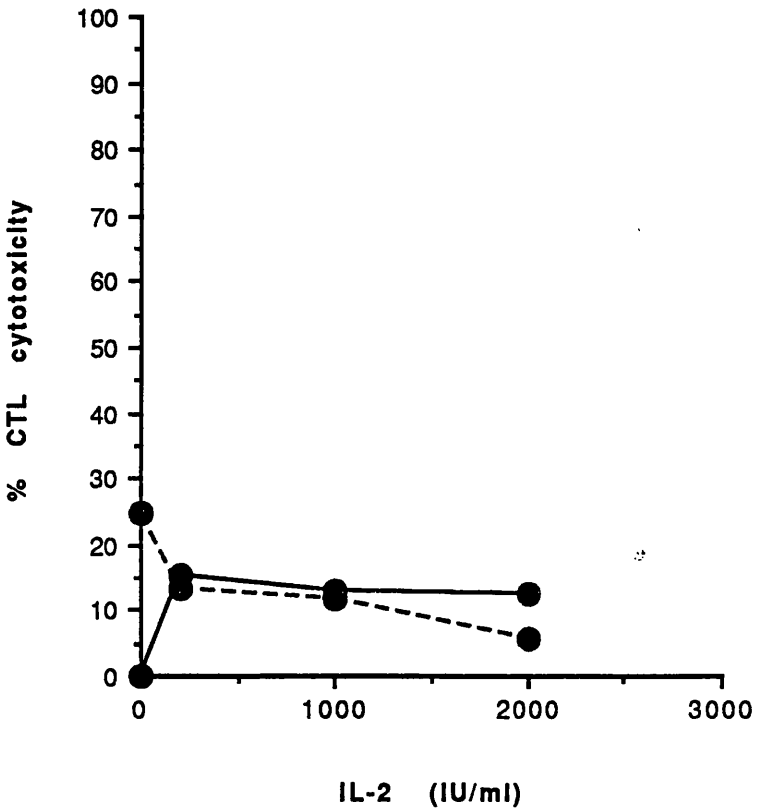
3.4 : EFFECTS OF IL-2 INDUCED LAK SUPERNATANTS ON CTL CYTOTOXICITY OVER 24 HOURS

The majority of IL-2 induced LAK supernatants produced greater CTLL cytotoxicity (Figures 11a to 14d) than controls : CTLL and RPM1 1640 alone resulting in 24.8% lysis whilst CTLL and rhIL-2 at final concentrations of 200, 1000 and 2000 IU/ml produced 15.3%, 13.0% and 12.8% lysis respectively. The cytotoxicity ranged from 11.2% to 71.1% overall.

However, these supernatants appear to have a direct cytotoxic effect on SW-LCL targets cells over a 24 hour period, since the % lysis observed was usually greater in the absence of CTLL (25.1% to 71.1%) when compared to the values obtained when CTLL were present (11.2% to 63.5%) [with the exception of donor D stimulated with 1000 IU/ml rhIL-2, as shown in Figure 14c]. A similar phenomenon was detected with the rhIL-2 controls : the addition of CTLL with 200, 1000 or 2000 IU/ml rhIL-2 resulted in 13.3%, 11.7% and 5.7% lysis respectively, showing an overall reduction in killing (Figure 10).

FIGURE 10 : Cytotoxicity generated against SW-LCL following activation with 0, 200, 1000 or 2000 IU/ml of rhIL-2 for 24 hours in the presence (broken line) or absence (solid line) of ST-CTLL at an E:T ratio of 1.5:1.

FIGURE 10


100
90
80
70
60
50
40
30
20
10
0
% CTL cytotoxicity
0
1000
2000
3000
IL-2 (IU/ml)

FIGURES 11a - 14d : Cytotoxicity generated against SW-LCL following activation over 24 hours with LAK supernatants (25% v/v) induced with no rhIL-2 (open circles), 200 IU/ml rhIL-2 (squares), 1000 IU/ml rhIL-2 (closed circles) or 2000 IU/ml rhIL-2 for 1, 4 or 8 days. The killing observed in the presence (broken line) or absence (solid line) of ST-CTLL at an E:T ratio of 1.5:1 is shown. Donors A to D are represented by Figures 11 to 14 respectively.

FIGURE 11a : DONOR A (0 IU/ml IL-2)

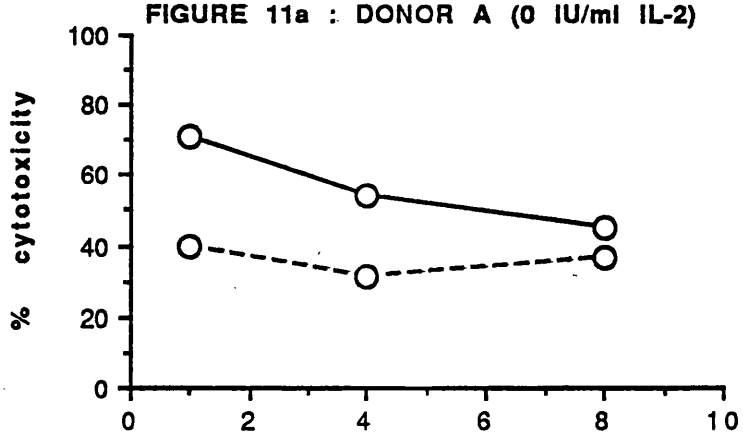


FIGURE 11b : DONOR A (200 IU/ml IL-2)

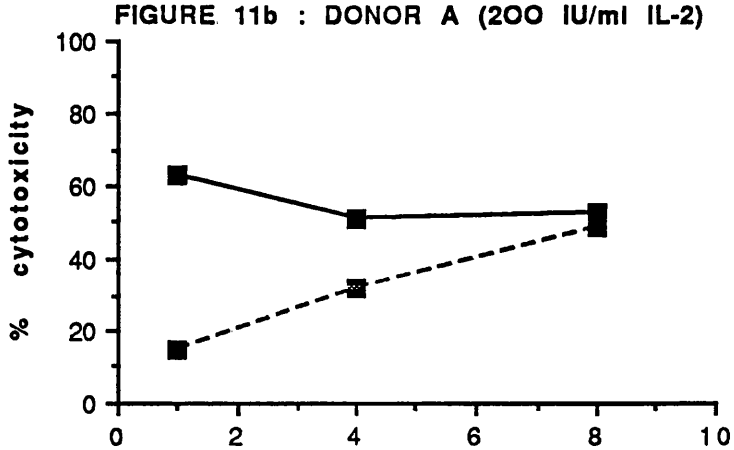


FIGURE 11c : DONOR A (1000 IU/ml IL-2)

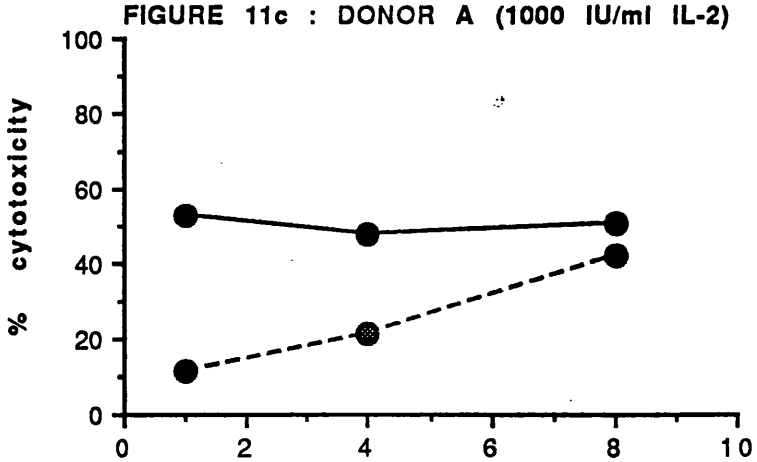


FIGURE 11d : DONOR A (2000 IU/ml IL-2)

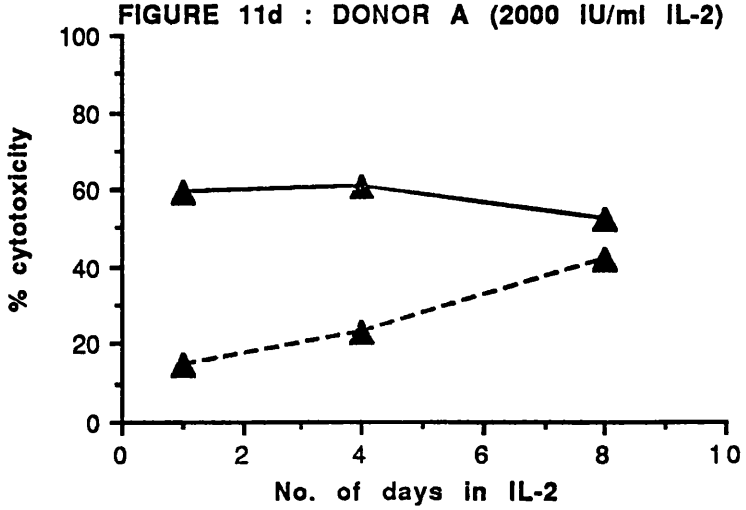


FIGURE 12a : DONOR B (0 IU/ml IL-2)

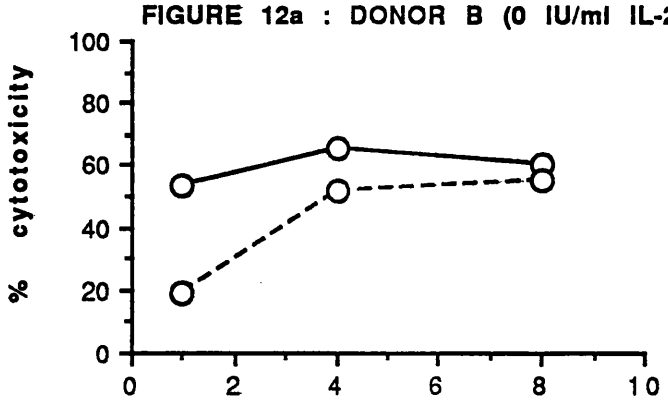


FIGURE 12b : DONOR B (200 IU/ml IL-2)

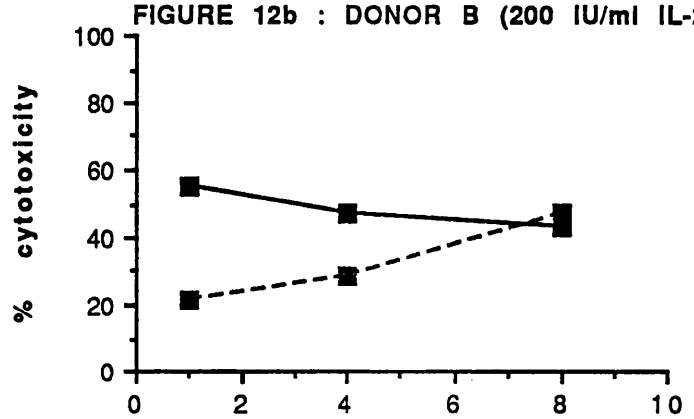


FIGURE 12c : DONOR B (1000 IU/ml IL-2)

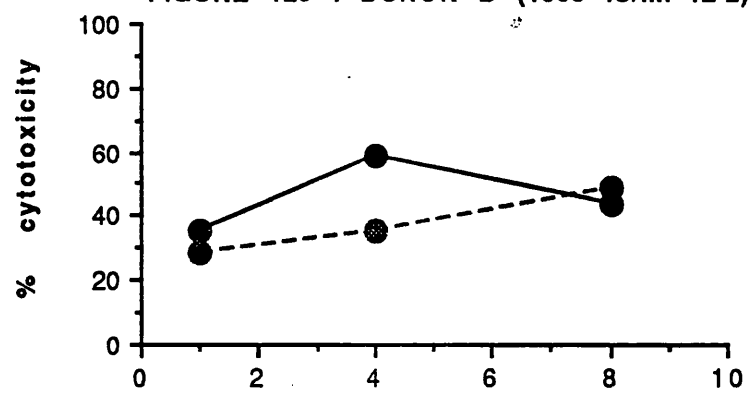


FIGURE 12d : DONOR B (2000 IU/ml IL-2)

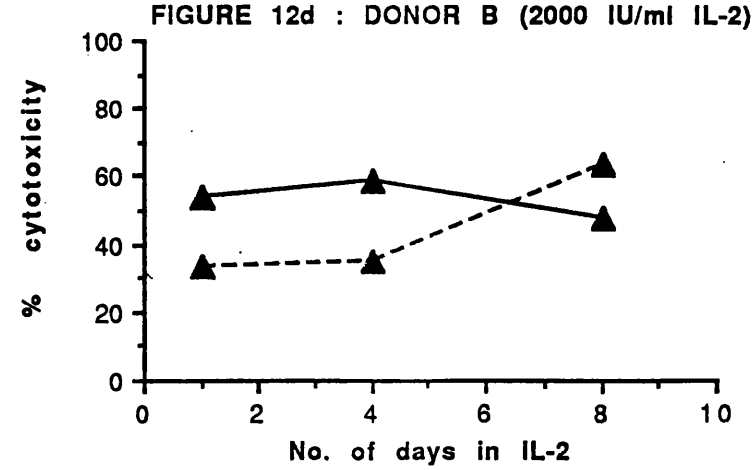


FIGURE 13a : DONOR C (0 IU/ml IL-2)

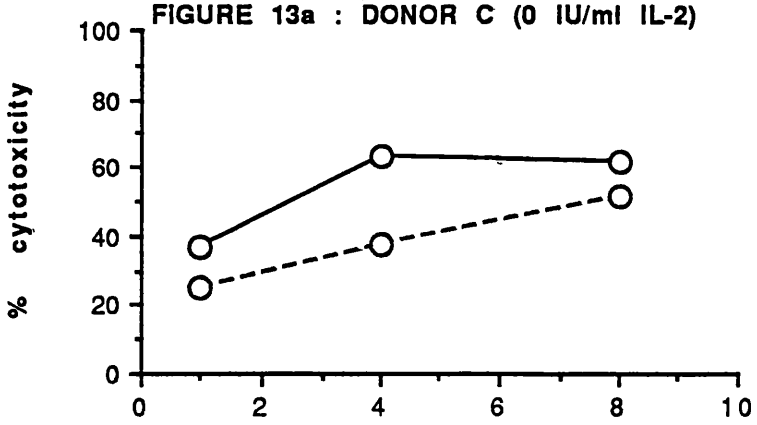


FIGURE 13b : DONOR C (200 IU/ml IL-2)

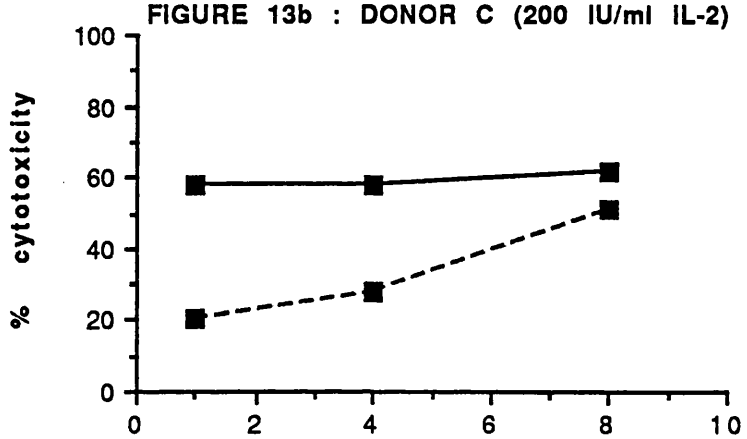


FIGURE 13c : DONOR C (1000 IU/ml IL-2)

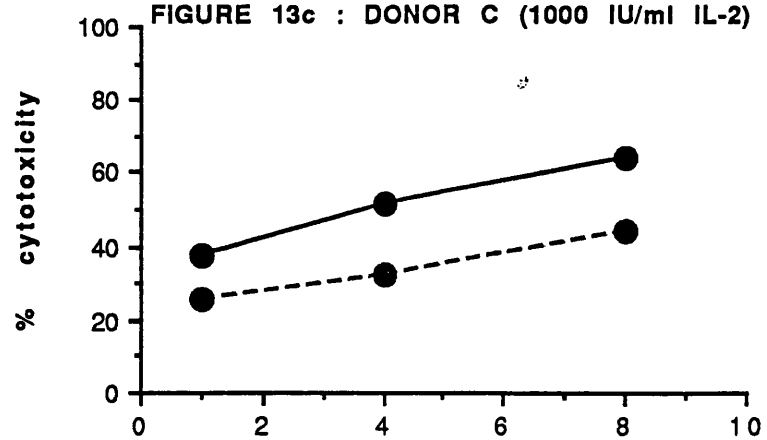


FIGURE 13d : DONOR C (2000 IU/ml IL-2)

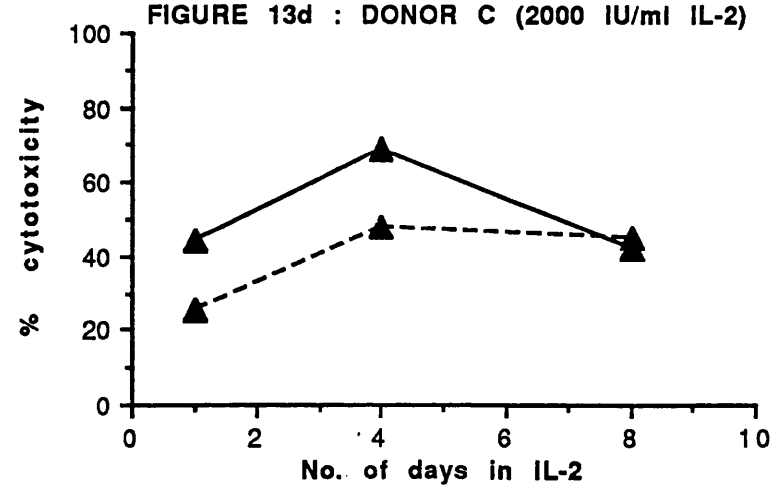


FIGURE 14a : DONOR D (0 IU/ml IL-2)

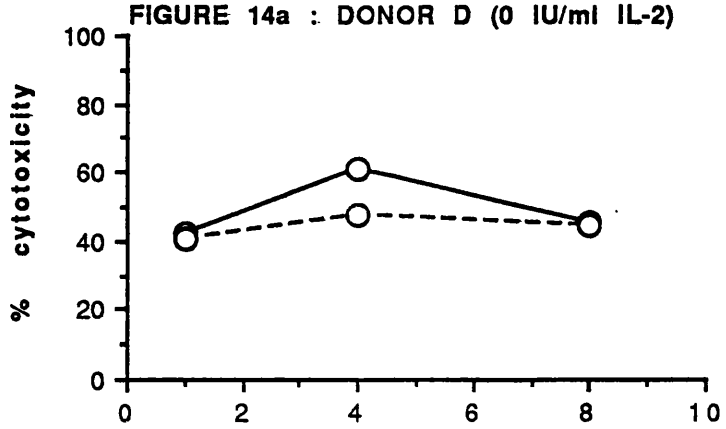


FIGURE 14b : DONOR D (200 IU/ml IL-2)

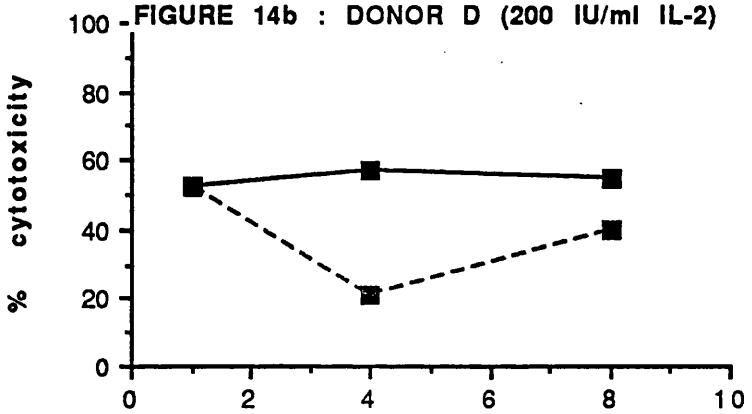


FIGURE 14c : DONOR D (1000 IU/ml IL-2)

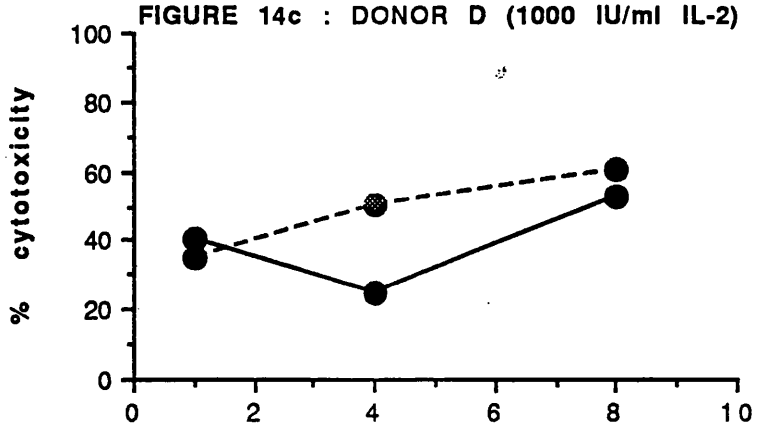
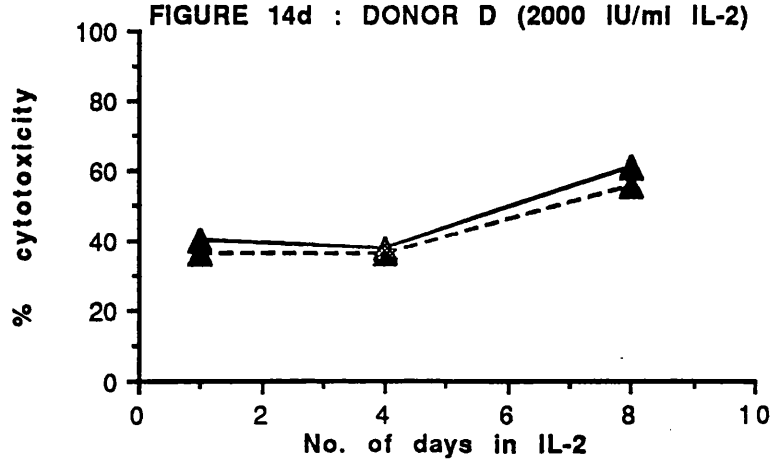


FIGURE 14d : DONOR D (2000 IU/ml IL-2)



3.5 : EFFECTS OF ANTI-CD3 INDUCED LAK SUPERNATANTS ON CTL CYTOTOXICITY OVER 24 HOURS

The anti-CD3 induced LAK supernatants demonstrated a similar pattern of results to the IL-2 induced LAK supernatants (Figures 15a to 18d). These supernatants likewise produced greater cytotoxicity (28.1 to 69.0%) than unstimulated CTLL (24.8%) or the rhIL-2 controls (12.8 to 15.3%). Irrespective of whether CTLL were present or not, the killing observed for the anti-CD3 induced supernatants was always higher than that of the controls, ranging from 28.1% to 76.5% overall. Although the % CTL cytotoxicity for the IL-2 induced LAK supernatants fell as low as 11.2%, the minimum % lysis for the anti-CD3 induced supernatants was 28.1%. Again, a direct cytotoxic action on SW-LCL target cells was apparent following 24 hour exposure to these supernatants since lysis in the absence of CTLL ranged from 37.3 to 76.5%.

FIGURES 15a - 18d : Cytotoxicity generated against SW-LCL following activation over 24 hours with LAK supernatants (25% v/v) induced with no anti-CD3 (open circles), 1ng/ml anti-CD3 (squares), 10ng/ml anti-CD3 (closed circles) or 100ng/ml anti-CD3 for 1, 4 or 8 days. The killing observed in the presence (broken line) or absence (solid line) of ST-CTLL at an E:T ratio of 1.5:1 is shown. Donors A to D are represented by Figures 15 to 18 respectively.[*The effects of the following supernatants on CTLL cytotoxicity over 24 hours was not determined: C.C.4.0, C.C.1.100, C.C.4.100 or C.C.8.100].

FIGURE 15a : DONOR A (0 ng/ml anti-CD3)

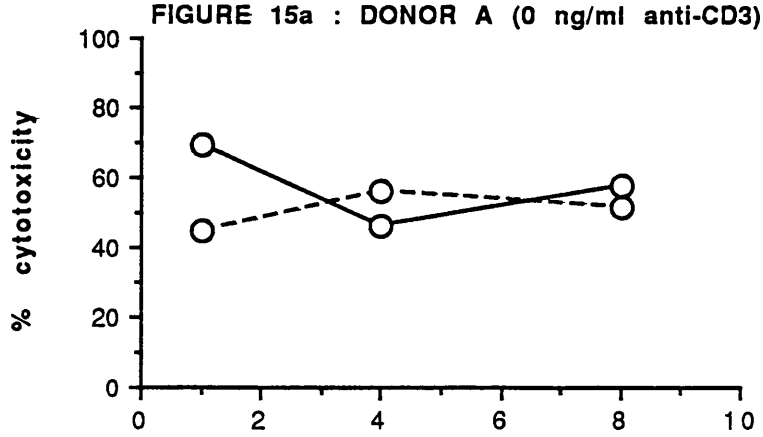


FIGURE 15b : DONOR A (1 ng/ml anti-CD3)

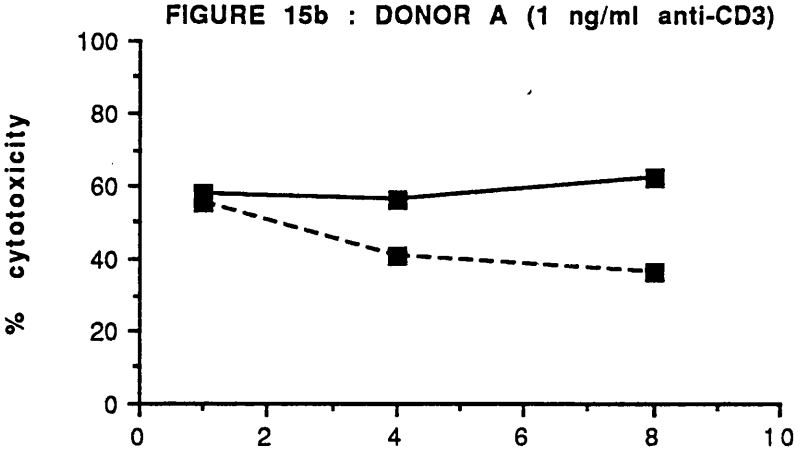


FIGURE 15c : DONOR A (10 ng/ml anti-CD3)

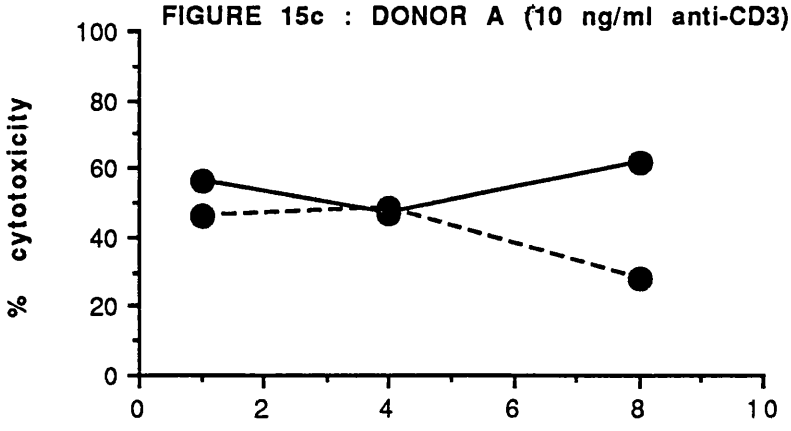


FIGURE 15d : DONOR A (100 ng/ml anti-CD3)

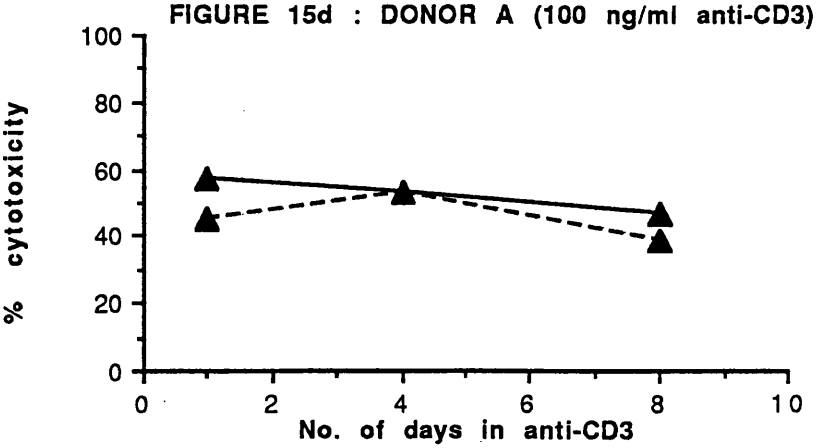


FIGURE 16a : DONOR B (0 ng/ml anti-CD3)

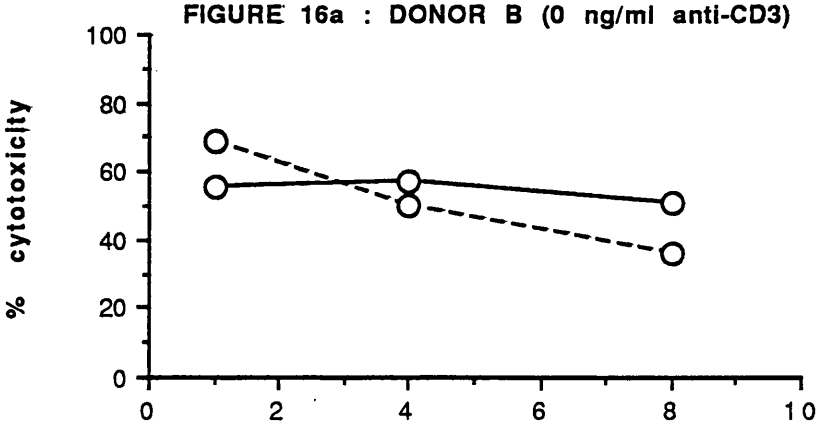


FIGURE 16b : DONOR B (1 ng/ml anti-CD3)

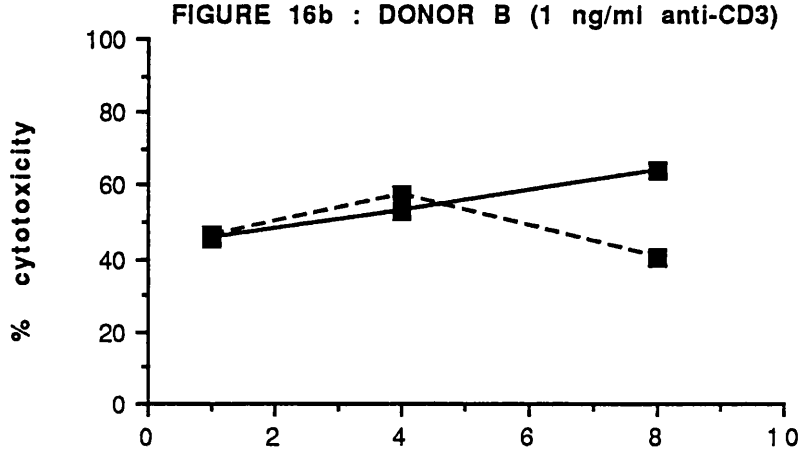


FIGURE 16c : DONOR B (10 ng/ml anti-CD3)

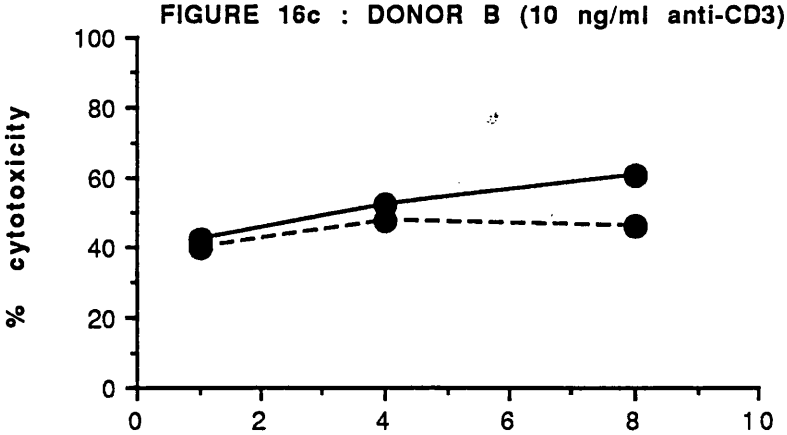


FIGURE 16d : DONOR B (100 ng/ml anti-CD3)

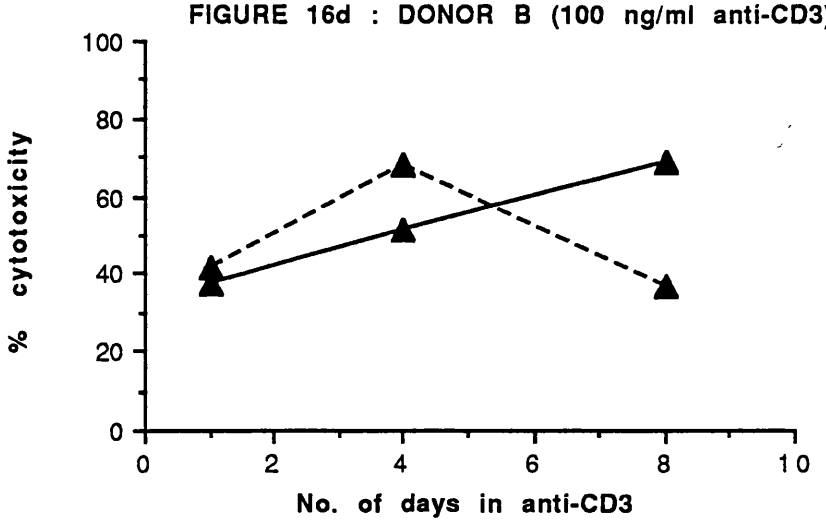


FIGURE 17a : DONOR C (0 ng/ml anti-CD3)

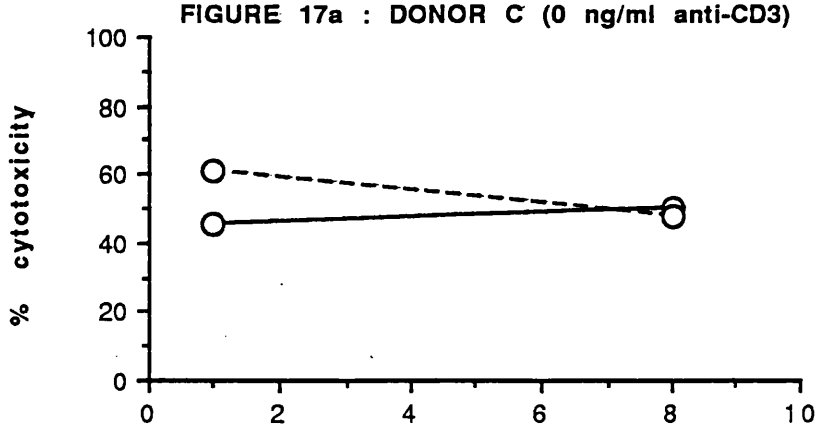


FIGURE 17b : DONOR C (1 ng/ml anti-CD3)

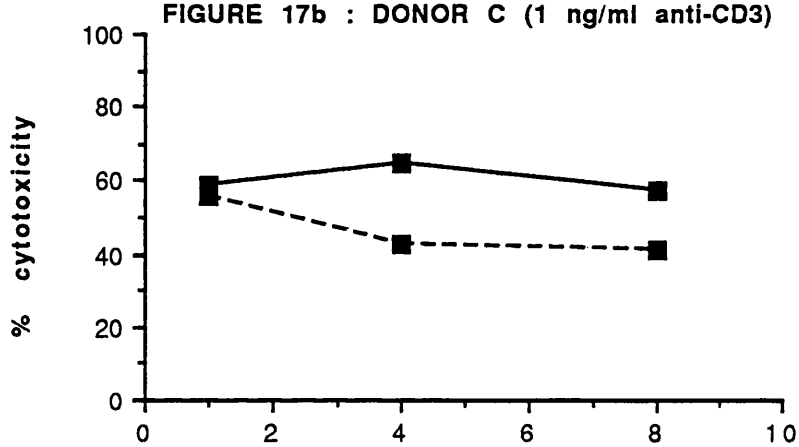


FIGURE 17c : DONOR C (10 ng/ml anti-CD3)

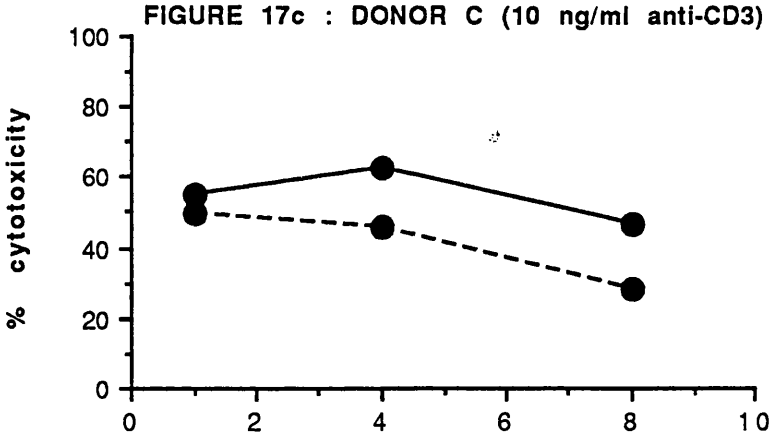


FIGURE 18a : DONOR D (0 ng/ml anti-CD3)

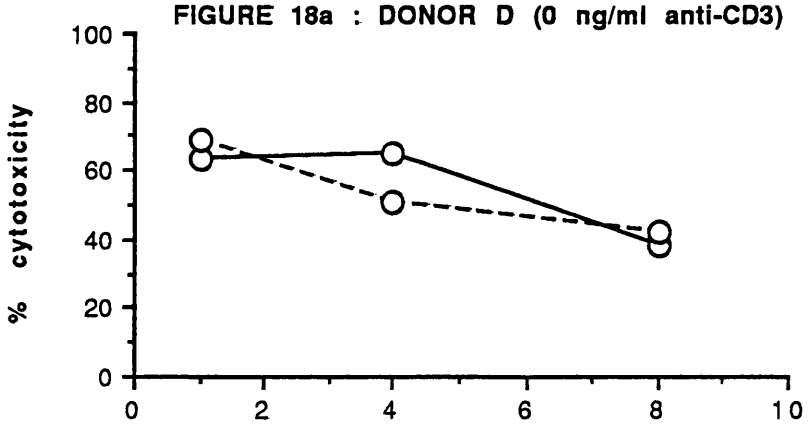


FIGURE 18b : DONOR D (1 ng/ml anti-CD3)

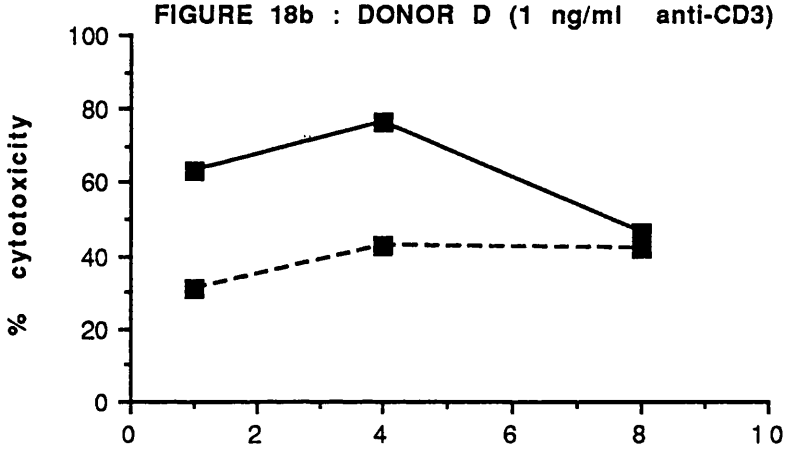


FIGURE 18c : DONOR D (10 ng/ml anti-CD3)

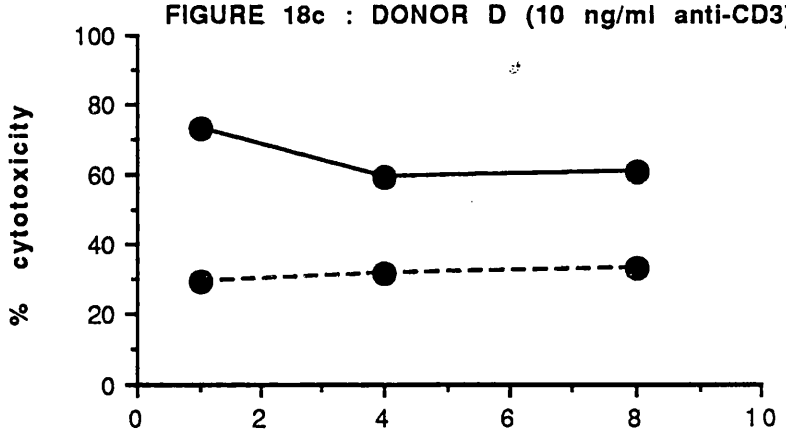
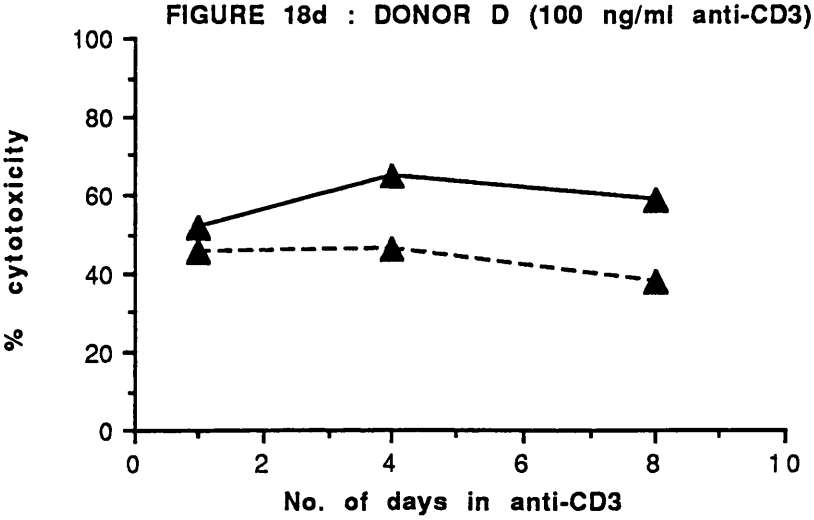


FIGURE 18d : DONOR D (100 ng/ml anti-CD3)



3.6 : EFFECTS OF PREACTIVATION WITH IL-2 OR ANTI-CD3 INDUCED LAK SUPERNATANTS FOR 19 HOURS ON CTL CYTOTOXICITY

Five hour cytotoxicity assays were conducted to investigate changes in SW-LCL lysis when CTLL effector cells had been preactivated with IL-2 or anti-CD3 induced LAK supernatants for 19 hours prior to the addition of target cells. The results were compared to the cytotoxicity values obtained in response to 200, 1000 or 2000 IU/ml rhIL-2 or in the absence of any mitogen (ie. RPMI 1640 alone). The % target lysis for each supernatant or control was also determined in the absence of effector cells, in order to discover whether the supernatants were directly cytotoxic to the target cells in this assay system.

Only 15 of the 96 supernatants generated produced killing (Table 3) in the presence of CTLL, ranging from 8.0% (C.I.8.0) to 55.9% (B.C.1.100). The remaining supernatants resulted in no or negligible killing (0.13 to 5.30%). All 15 samples produced greater lysis in the presence of CTLL than the medium (0%), 200 IU/ml rhIL-2 (0%) or 1000 IU/ml rhIL-2 (0.63%) controls. However, some of these supernatants (10 of the 15) resulted in lysis in the absence of CTLL (3.3 to 40.71%), as did the control 2000 IU/ml rhIL-2 (32.6%). Of these supernatants, 5 of the samples produced greater cytotoxicity when CTLL were present (samples C.I.8.0, B.C.1.10, B.C.1.100, C.C.1.0 and D.C.1.100) whereas the other 5 supernatants produced greater lysis in the absence of CTLL (samples B.C.4.100, D.C.4.100. B.C.8.100, C.C.8.0 and D.C.8.100). Only one of these later samples (D.C.8.100) produced greater killing (40.71%) than either of the 2000 IU/ml rhIL-2 controls (26.8 or 32.6%). Thus this enhanced CTLL cytotoxicity was only observed for a few, random samples [D.I.1.2000, B.C.1.10, B.C.1.100, C.C.1.0, D.C.1.100], producing similar or greater lysis (32.4 to 55.9%) than the supernatants alone or the IL-2 controls.

The fact that no lysis was observed with CTLL in medium alone was probably due to the absence of IL-2 over 24 hours since the CTLL is IL-2 dependent and the addition of 2000 IU/ml rhIL-2 produced lysis.

TABLE 3 : Cytotoxicity generated against SW-LCL in a 5-hour assay, following preactivation of ST-CTLL with either rhIL-2 or IL-2/anti-CD3 induced LAK supernatants for 19 hours prior to the addition of target cells.

TABLE 3

SUPERNATANT	% CYTOTOXICITY CTLL PRESENT	% CYTOTOXICITY CTLL ABSENT
medium only	0	0
200 IU/ml rhIL-2	0	0
1000 IU/ml rhIL-2	0.63	0
2000 IU/ml rhIL-2	26.80	32.60
B.I.1.1000	11.80	0
B.I.8.2000	12.00	0
C.I.8.0	8.00	3.30
D.I.1.1000	18.50	0
D.I.1.2000	53.20	0
B.C.1.10	35.30	18.60
B.C.1.100	55.90	22.00
B.C.4.100	8.47	19.85
B.C.8.100	13.27	18.84
C.C.1.0	32.40	7.70
C.C.1.100	9.40	0
C.C.8.0	15.30	16.18
D.C.1.100	42.50	18.10
D.C.4.100	10.62	12.01
D.C.8.100	9.48	40.71

3.7 : MITOGENIC EFFECTS OF IL-2 INDUCED LAK SUPERNATANTS IN THE PRESENCE OR ABSENCE OF EXOGENOUS ANTI-CD3 ON T-CELLS

Generally, proliferation in response to IL-2 induced LAK supernatants was low (Figures 20a - 23a) in all four donors, ranging from 28 cpm to a maximum of 8,967 cpm.

Minimal proliferation was observed for the supernatants, from three of the four donors (donors A, C and D), obtained from a 1 day LAK induction. Similarly, supernatants from three of the donors (donors A, B and D), when cultured in the absence of rhIL-2, produced minimal proliferation.

Maximal proliferation was observed following activation with supernatants derived from 8 day LAK inductions with 1000 IU/ml rhIL-2 for donors A and B. However donors C and D induced maximal proliferation with supernatants from LAK populations induced with 2000 IU/ml rhIL-2 for 4 days. Overall, the maximal proliferation observed ranged from 7,386 to 8,967 cpm.

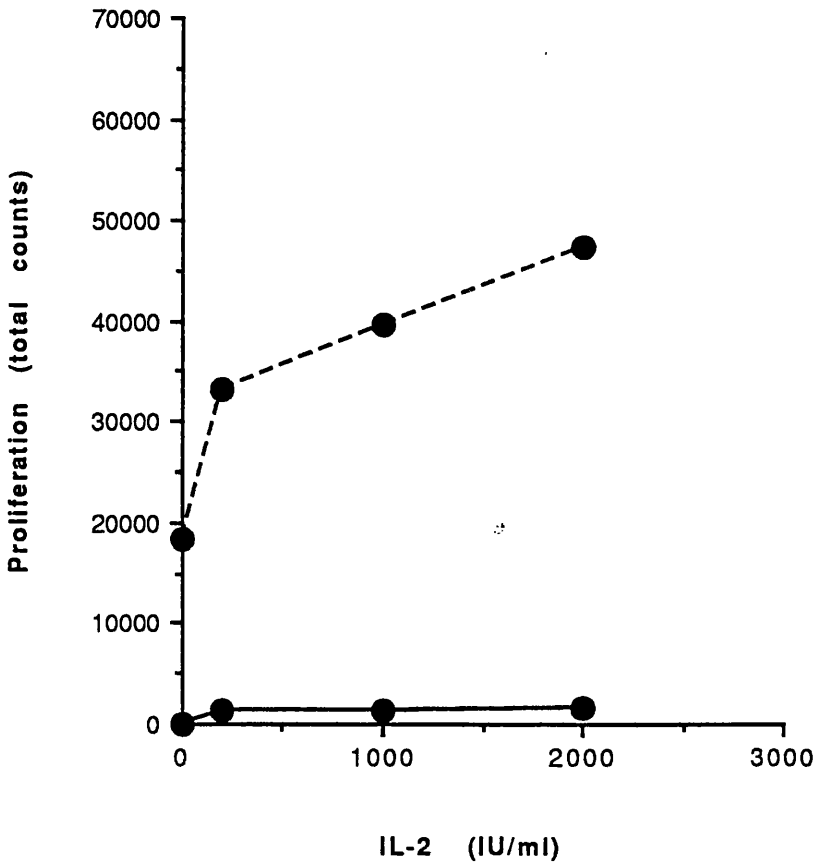
Apart from these observations, no trends were observed between proliferation of peripheral T-cells and either the duration of LAK induction or dose of rhIL-2 used to induce LAK activity. Much variation occurred between individuals in these experiments.

The addition of exogenous anti-CD3 (5 ng/ml) greatly enhanced the proliferation observed for all four donors (Figures 20b - 23b) producing counts ranging from 8,254 cpm to 69,155cpm. However any increase in proliferation obtained, did not appear to be associated with the IL-2 induced LAK supernatants themselves or the culture parameters under which they were generated.

IL-2 itself (200, 1000 or 2000 IU/ml) produced negligible proliferation (1,238 to 1,553 cpm). The addition of exogenous anti-CD3 enhanced proliferation (33,315 to 47,397 cpm), with proliferative response increasing as IL-2 concentration increased (Figure 19).

FIGURE 19 : Proliferation of peripheral T-cells in response to stimulation with 0, 200, 1000 or 2000 IU/ml rhIL-2 for 72 hours in the presence (broken line) or absence (solid line) of exogenous anti-CD3 (5ng/ml).

FIGURE 19


70000
60000
50000
40000
30000
20000
10000
0
Proliferation (total counts)
0
1000
2000
3000
IL-2 (IU/ml)

FIGURES 20a - 23a : Proliferation of peripheral T-cells in response to stimulation over 72 hours with LAK supernatants (25% v/v) induced with no rhIL-2 (open circles), 200 IU/ml rhIL-2 (squares), 1000 IU/ml rhIL-2 (closed circles) or 2000 IU/ml rhIL-2 (triangles) for 1, 4 or 8 days.

FIGURES 20b - 23b : Proliferation of peripheral T-cells in response to stimulation over 72 hours with both LAK supernatants (25% v/v), induced with no rhIL-2 (open circles), 200 IU/ml rhIL-2 (squares), 1000 IU/ml rhIL-2 (closed circles) or 2000 IU/ml rhIL-2 (triangles) for 1, 4 or 8 days, and exogenous anti-CD3 (5ng/ml).

FIGURE 20a : DONOR A

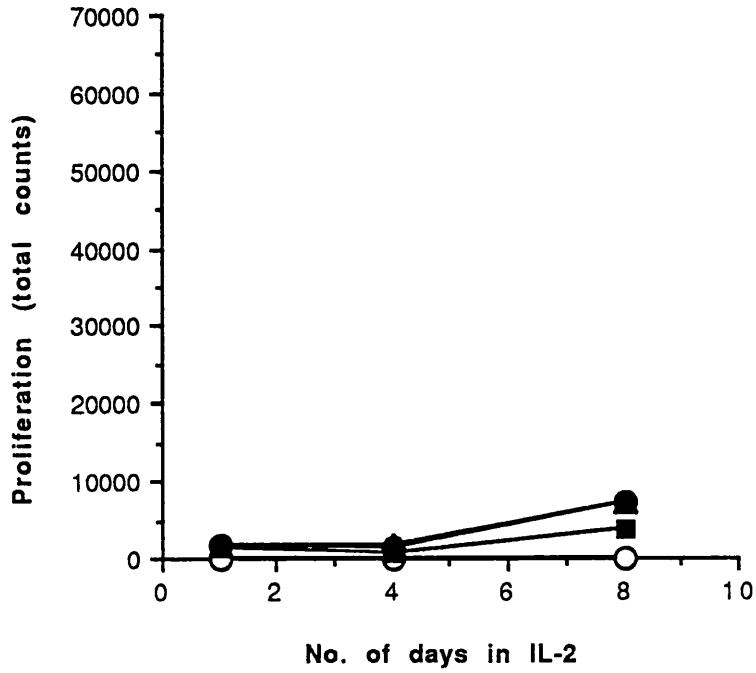


FIGURE 20b : DONOR A

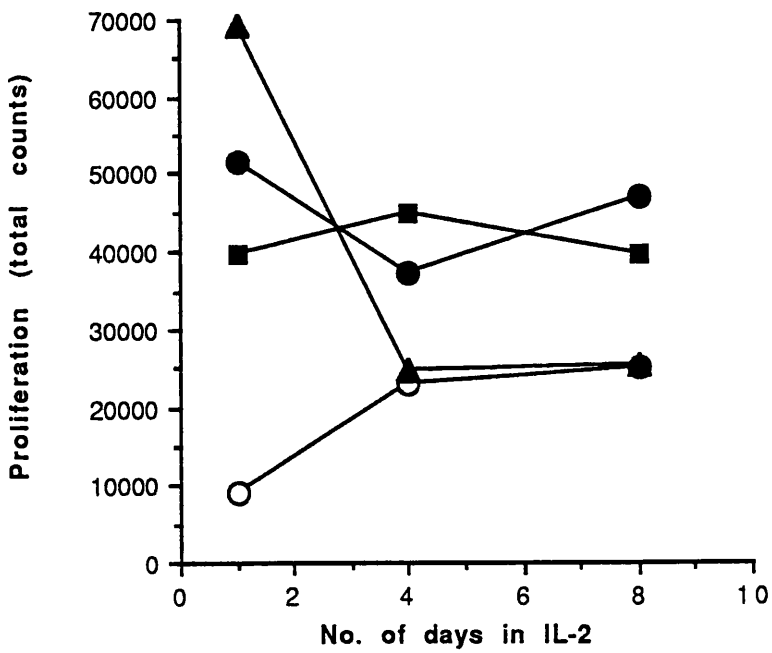


FIGURE 21a : DONOR B

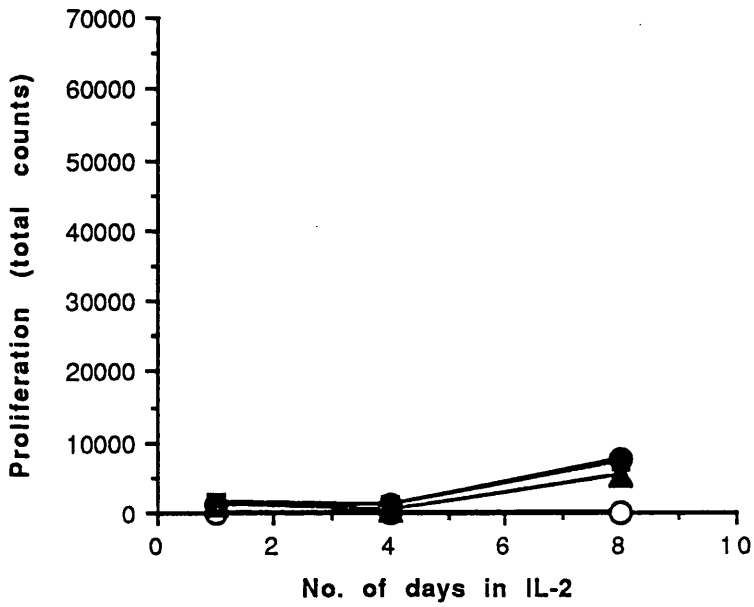


FIGURE 21b : DONOR B

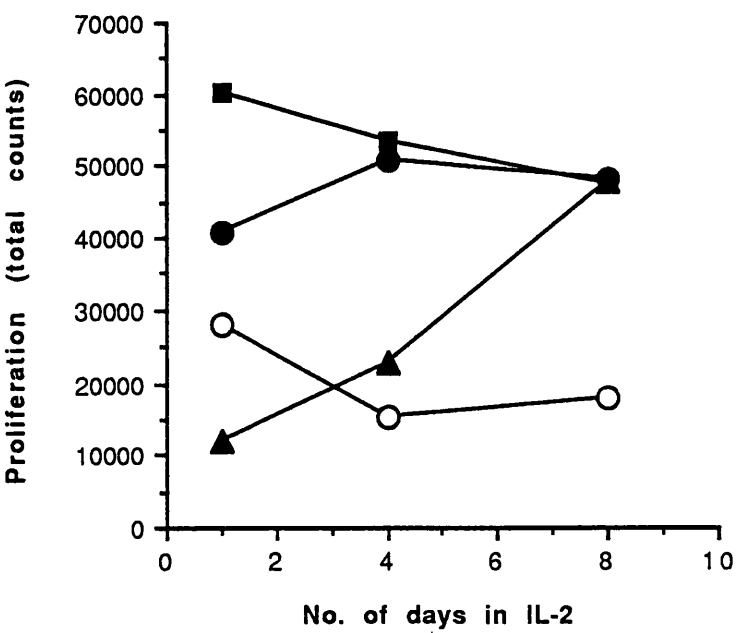


FIGURE 22a : DONOR C

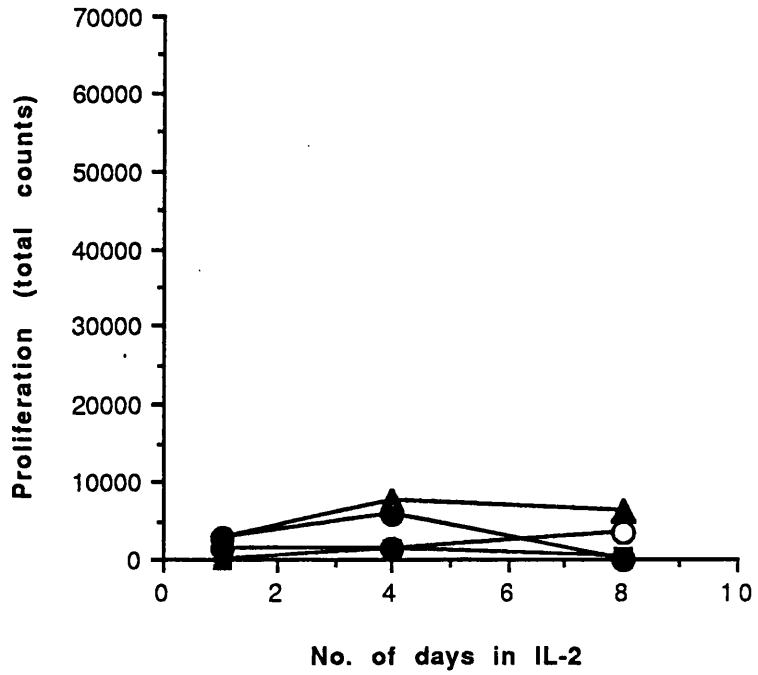


FIGURE 22b : DONOR C

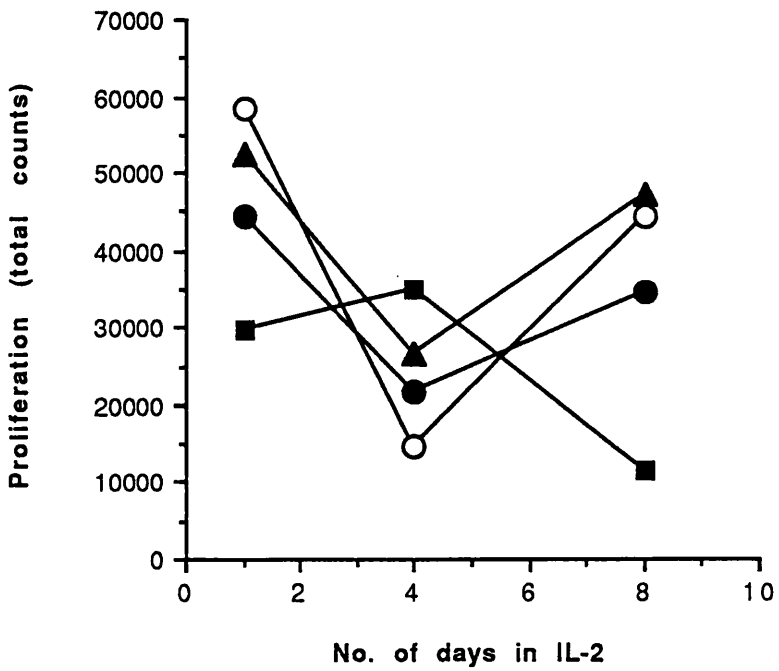


FIGURE 23b : DONOR D

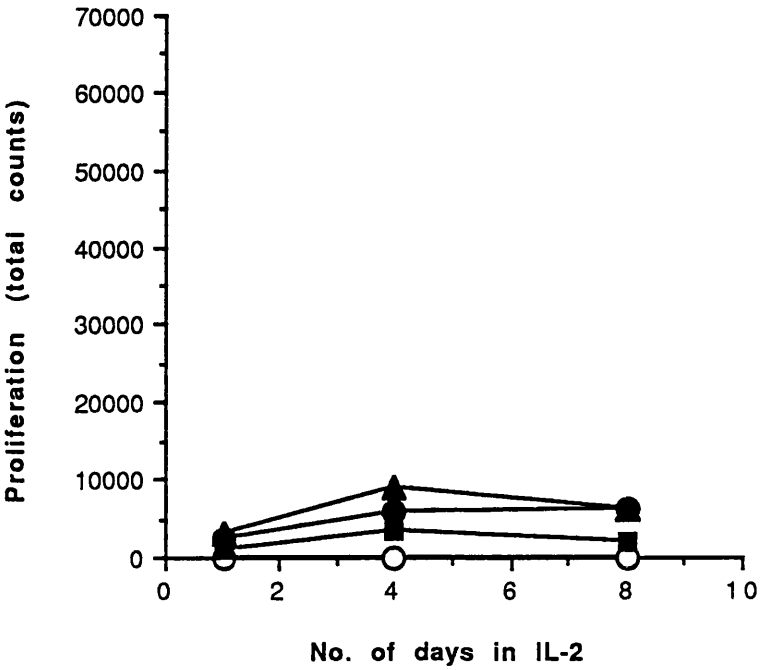
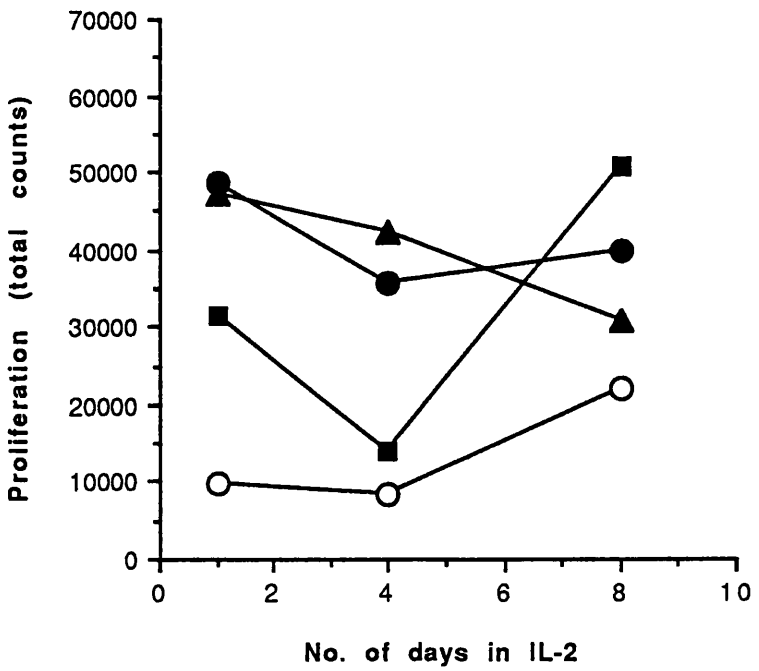


FIGURE 23b : DONOR D



3.8 : MITOGENIC EFFECTS OF ANTI-CD3 INDUCED LAK SUPERNATANTS IN THE PRESENCE OR ABSENCE OF EXOGENOUS ANTI-CD3 ON T-CELLS

Proliferation in response to the anti-CD3 induced LAK supernatants ranged from 48 cpm to a maximum of 32,276 cpm (Figures 24a to 27a) and was generally higher than the proliferation observed in response to the IL-2 induced LAK supernatants (Figures 20a to 23a), particularly for donors A and B (Figures 24a and 25a). Negligible proliferation was usually observed in those cells incubated with the supernatants generated from PBMC cultured in the absence of anti-CD3 (ie. 0ng/ml antibody), with proliferation ranging from 48 cpm to 170 cpm (with two exceptions: 4,004 cpm for the donor A supernatant generated with 0ng/ml anti-CD3 for 4 days and 24,612 cpm for the donor B supernatant generated with 0ng/ml anti-CD3 for 1 day). The maximal response for each donor was usually observed for cells activated with supernatants induced with high dose anti-CD3 (10 ng/ml or 100 ng/ml), with the exception of donor B. Overall, maximal proliferation ranged from 6,836 to 32,276 cpm.

In contrast to the IL-2 induced LAK supernatants, the addition of exogenous anti-CD3 (5 ng/ml) had very marginal effects on proliferation, with ^{3}H-thymidine incorporation ranging from 31 cpm to a maximum of 32,999 cpm (Figures 24b to 27b). Proliferation in the presence of anti-CD3 slightly increased for most of the anti-CD3 induced LAK supernatants, especially in the case of the supernatants produced in the absence of anti-CD3 stimulation. However, the same trends of proliferative response were maintained following the addition of exogenous antibody: the maximal proliferation observed for each donor, except for donor B as before, was again in response to activation with supernatants induced with high dose anti-CD3 (10 ng/ml or 100 ng/ml).

Consequently, the ability of anti-CD3 induced LAK supernatants to produce greater levels of proliferation than the IL-2 induced LAK supernatants may be due to the stimulatory effects of residual anti-CD3 antibody remaining in the supernatants from the LAK inductions, as not all of the antibody added will have been assimilated. It was therefore unlikely that adding exogenous anti-CD3 would potentiate proliferation owing to a saturation effect, except with those supernatants generated without anti-CD3 stimulation (ie. 0 ng/ml).

FIGURES 24a - 27a : Proliferation of peripheral T-cells in response to stimulation over 72 hours with LAK supernatants (25% v/v), induced with no anti-CD3 (open circles), 1ng/ml anti-CD3 (squares), 10ng/ml anti-CD3 (closed circles) or 100ng/ml anti-CD3 (triangles) for 1, 4 or 8 days. [*The proliferation in response to the following supernatants was not determined: C.C.1.10, C.C.1.100, C.C.4.100, C.C.8.100 and D.C.8.100].

FIGURES 24b - 27b : Proliferation of peripheral T-cells in response to stimulation over 72 hours with both LAK supernatants (25% v/v), induced with no anti-CD3 (open circles), 1ng/ml anti-CD3 (squares), 10ng/ml anti-CD3 (closed circles) or 100ng/ml anti-CD3 (triangles) for 1, 4 or 8 days, and exogenous anti-CD3 (5ng/ml). [*The proliferation in response to the following supernatants was not determined: C.C.1.100, C.C.4.100, C.C.8.100 and D.C.8.100].

FIGURE 24a : DONOR A

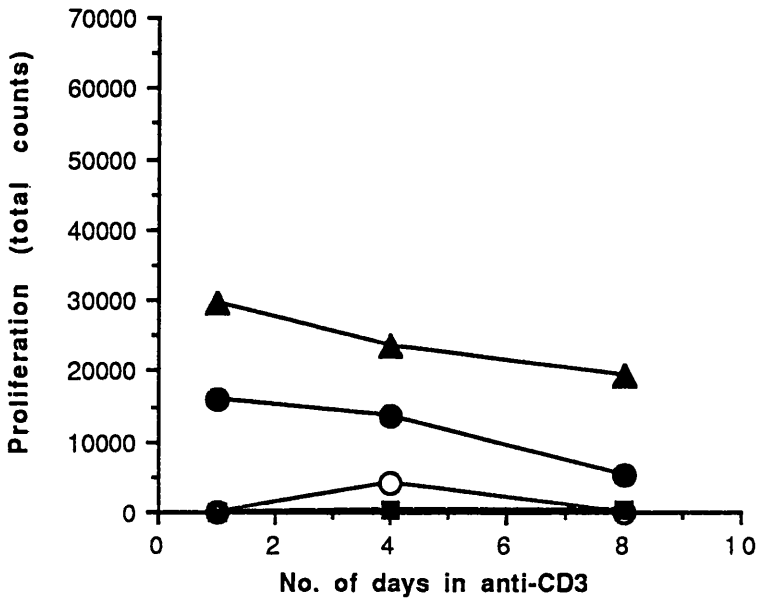


FIGURE 24b : DONOR A

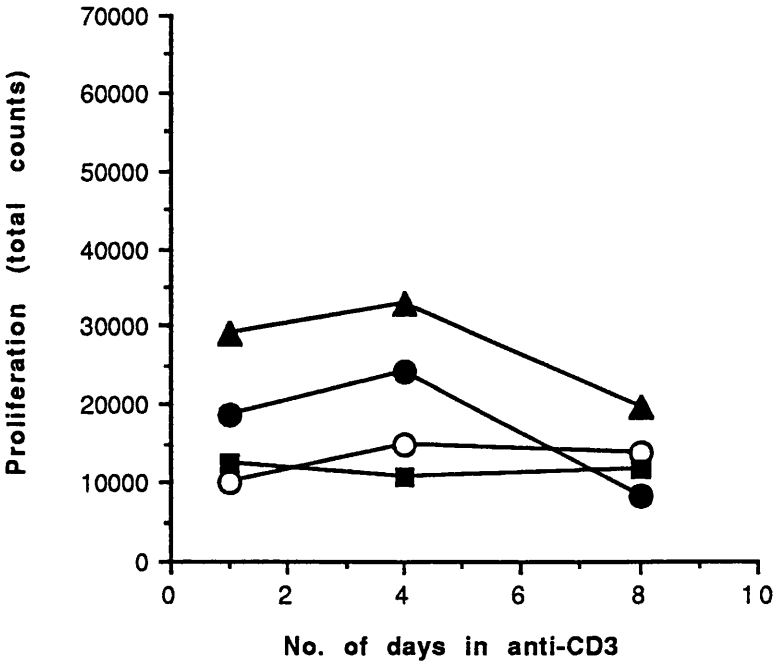


FIGURE 25a : DONOR B

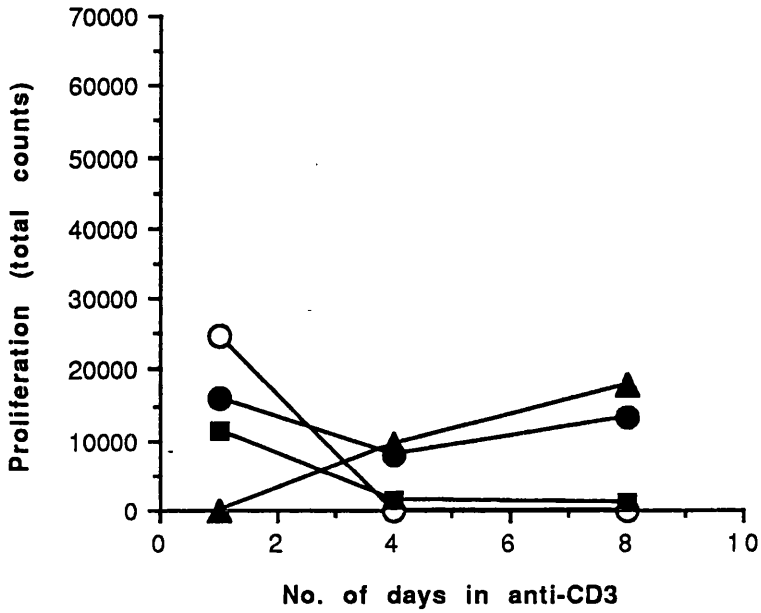


FIGURE 25b : DONOR B

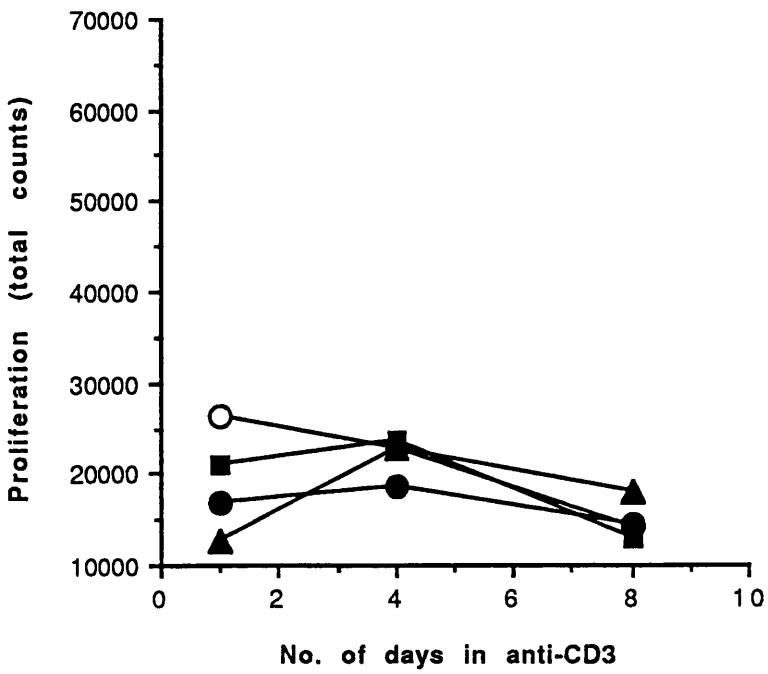


FIGURE 26a : DONOR C

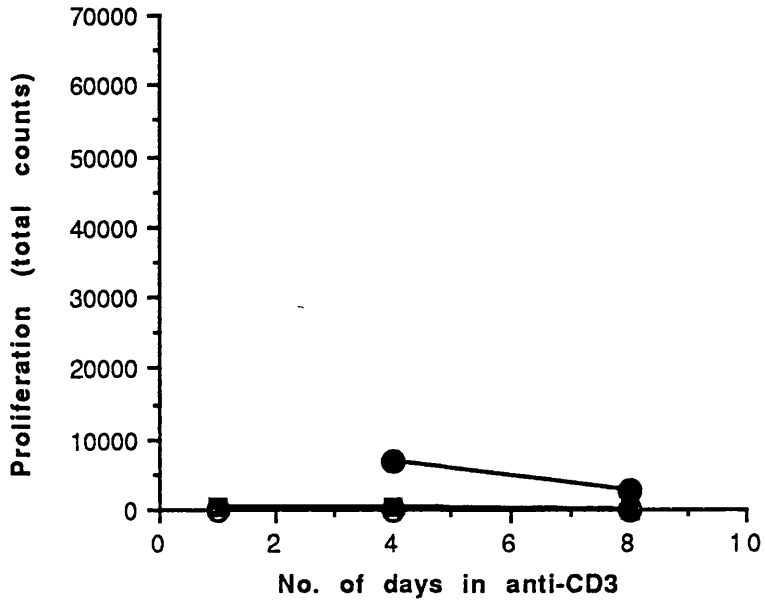


FIGURE 26b : DONOR C

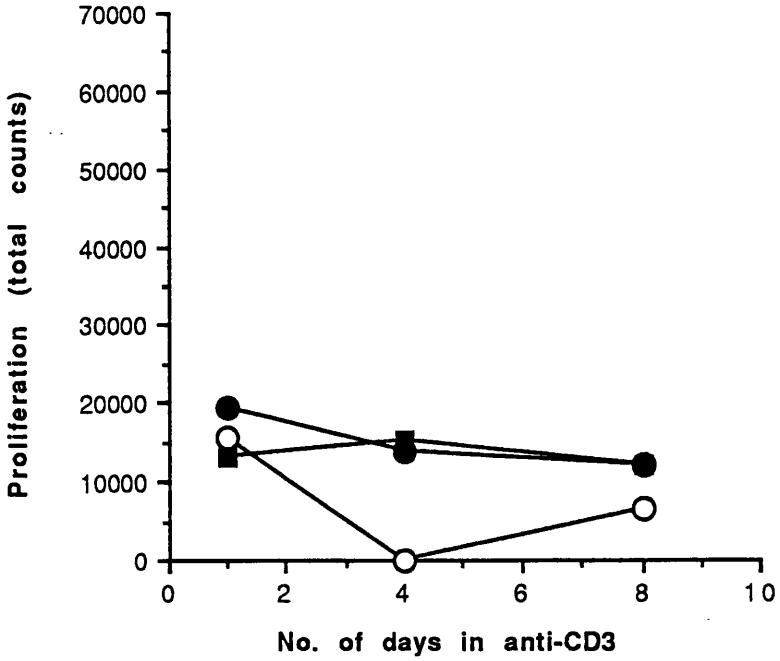


FIGURE 27a : DONOR D

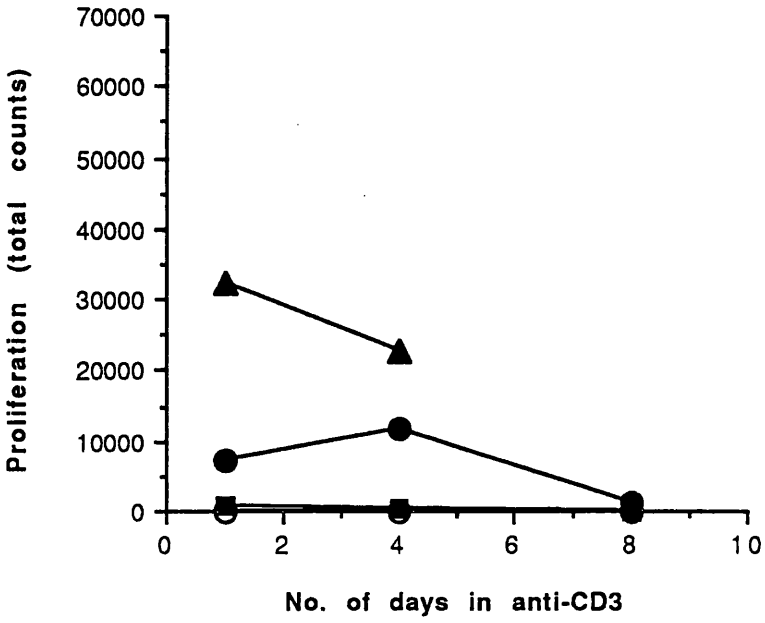
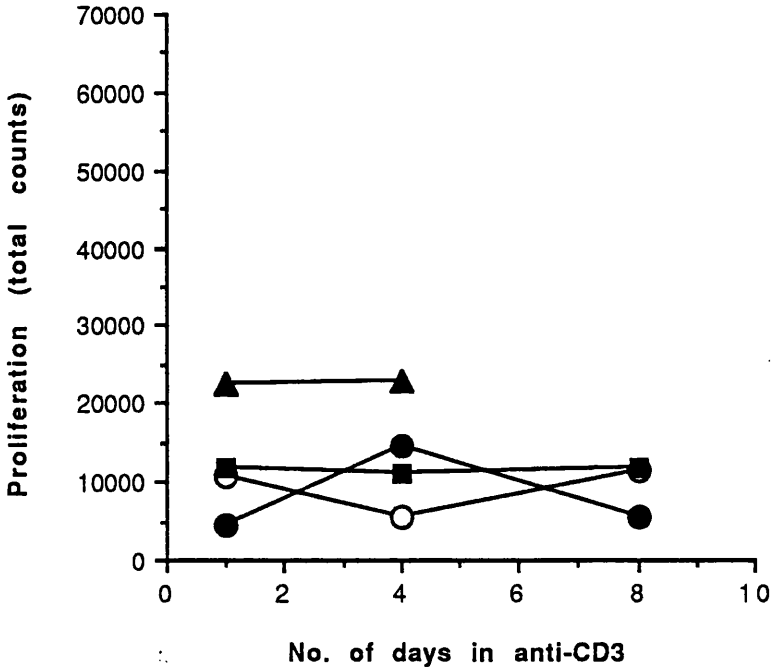


FIGURE 27b : DONOR D



DISCUSSION

4.1 : INDUCTION OF LAK ACTIVITY AND CYTOKINE SECRETION

In this study, the induction of LAK activity with either recombinant human IL-2 (rhIL-2) or monoclonal anti-CD3 antibody (SHL45.6) in vitro, from the PBMC of four normal individuals (donors A - D), was compared. The effects of varying type, level and duration of stimulus was investigated by comparing the percentage LAK cytotoxicity and the level of cytokine (namely TNFα, IFNγ and IL-1) secretion observed. Four donors were assessed in an attempt to allow for any variations between individuals. However, all donors were male and may not reflect the trends representative of both genders. All cells were cultured in AIM-V serum-free medium (**Jadus et al, 1988; Finkelstein et al, 1991**) to prevent any complications resulting from activation as a consequence of supplementing growth media with xenogeneic serum (**Grimm and Wilson, 1985**). Furthermore, AIM-V is specifically defined for the in vitro activation of human lymphocytes to be applied clinically (**Yang et al, 1990**) and therefore its use in this study was analogous to the clinical situation.

4.1a : CYTOLYTIC ACTIVITY

For those cells induced with rhIL-2, the maximal level of cytotoxicity observed against the colon adenocarcinoma cell-line SW742 for each donor was always demonstrated in LAK populations attained following four days in culture (Figures 1a-4a). In general, with a few exceptions, the LAK activity peaked on day 4 of stimulation and then declined with prolonged time in culture, irrespective of the level of rhIL-2 applied. Since **Rosenberg and Lotze (1986)** report that a 3 - 5 day incubation with IL-2-containing supernatants generated LAK cells, the trend of killing observed with duration in culture in this study suggests that the kinetics of IL-2 induced cytolysis in these experiments may be similar to that of previous studies. IL-2 induced LAK activity generally appeared to be a dose-responsive phenomenon, demonstrating greater cytotoxicity when activated with higher doses of rhIL-2 (1000 or 2000 IU/ml). In most cases, the maximal % LAK cytotoxicity was obtained when stimulated with 1000 IU/ml rhIL-2 for 4 days. These data were similar to the results of other research groups (**Limb et al, 1989; Saraya and Balkwill, 1993 ; Schoof et al, 1992; Jadus et al, 1988**).

Limb et al (1989) demonstrated that the incubation of mononuclear cells (MNC) with 1000 U/ml of IL-2 produced LAK activity against Daudi target cells, which peaked at 48 hours and was still maintained at 72 and 96 hours. Likewise, **Saraya and Balkwill (1993)** reported that maximal in vitro cytotoxicity against T24 target cells was observed in PBMC populations cultured for 3 days with 1000 U/ml of rhIL-2. In the study by **Schoof et al (1992)**, cells cultured with varying concentrations of IL-2

demonstrated a dose-dependent increase in cytotoxicity which reached a maximum level when activated by 1000 U/ml IL-2 for 72 hours, as also indicated in our study. An investigation (**Jadus et al, 1988**) to assess the ability of commercially available serum-free media to support LAK cell generation showed similar findings. Although cytolytic activity against Daudi was seen in all media formulations with stimulating levels of IL-2 at 10, 100 or 1000 U/ml, the use of 100 or 1000 U/ml was most effective. In order to generate maximum cytolytic activity, irrespective of which serum-free preparation was used, an IL-2 concentration between 100 and 1000 U/ml was required for the best responses after 7 days. They conclude, as we also observed, that stimulation with 1000 U/ml for 3 or 4 days produces optimal LAK cytotoxicity in vitro. Moreover, these results comply with the usual clinical strategy for LAK cell generation: activation of mononuclear cells with 1000 U/ml of rhIL-2 for 3 - 4 days (**Rosenberg et al, 1987; Schoof et al, 1992**).

Antibodies to CD3 have been shown to vary in their capacity to activate resting PBL to cytolytic status in the absence of exogenous IL-2. The anti-CD3 antibody RW24B6 (**Scott et al, 1988**) failed to activate PBL to proliferation or cytotoxicity in the absence of IL-2 when in soluble form. However, cross-linkage of RW24B6 to a solid-phase permitted PBL activation without the requirement for the addition of exogenous IL-2. Soluble RW24B6 synergized with exogenous IL-2 in the activation of PBL. The OKT3 anti-CD3 antibody has been shown to enhance IL-2 induced proliferation and LAK generation from PBL, when in soluble form (**Ochoa et al, 1987**). Likewise, **Jung et al (1987)** reported that soluble OKT3 stimulation of human PBMC results in the development of antibody-independent cytotoxicity in addition to antibody conjugate-dependent killing, with different sub-populations of lymphocytes eliciting the response. Cytotoxicity in response to 100ng/ml of OKT3 rose to maximal levels on the second and third days of stimulation. By day 4 of OKT3 treatment, the cytotoxicity declined. This lytic activity was found to be associated with a $CD4^{-}CD8^{-}$ population of cells which expressed activation markers (e.g. IL-2 receptor) but were largely $CD3^{-}$. Thus OKT3 stimulation was thought to indirectly activate these cells via the release of IL-2 by OKT3 stimulated $CD3^{+}$ cells. In a study to characterise OKT3 LAK effectors expanded in IL-2 (**Yang et al, 1990**) exposure to OKT3 at l0ng/ml for 48 hours produced optimal stimulation prior to the sequential addition of IL-2. Exposure to 100ng/ml of OKT3 for shorter periods had minimal effect on cellular expansion. They discovered that initial exposure to OKT3 upregulated Tac antigen and TNF receptor expression and therefore, may have rendered the cells more receptive to the effects of IL-2.

Consequently, the capacity of soluble anti-CD3 (SHL45.6), incorporating levels of stimulation of 1, 10 and 100ng/ml of antibody, to induce non-MHC restricted cytotoxicity was investigated in this study (Figures 5a-8a) . Contrary to the IL-2 data, which generally produced optimal killing following 4 days in culture, maximal cytotoxicity against SW742 cells for each of the donors was always observed 1 day after the onset of stimulation. With only one exception, the LAK activity observed always peaked on day 1 and then proceeded to decline with prolonged duration in culture. This agrees with the afore-mentioned studies which report maximal levels of activity around 2 days with a decreased cytotoxic capacity at the onset of day 4. In contrast to the data of **Jung et al (1987)**, 100ng/ml of anti-CD3 did not produce the greatest lysis in this study. In fact, the levels of killing obtained were usually optimal following stimulation with 10ng/ml anti-CD3 (donors A and D) or 1ng/ml of anti-CD3 (donors B and C) and therefore showed a greater resemblance to the results discussed by **Yang et al (1990)** : 48 hour activation with 10ng/ml. However, this may reflect the differences in either source of antibody or culture conditions, particularly with choice of media since both this project and the experiments by **Yang et al (1990)** used AIM-V to support the growth of the "LAK" cultures.

With the exception of donor C, the level of target lysis by effectors generated in the absence of either stimulant was low. The differences between the data from the IL-2 free inductions and the anti-CD3 free inductions was probably due to variations within each individual with time, since these experiments were conducted on consecutive samples of blood as opposed to one blood donation.

4.1b : CYTOKINE SECRETION

The IL-2 induced LAK cells were found to produce the cytokines TNFα, IL-1 and IFNγ as previously demonstrated, showing variances in the kinetics of cytokine secretion with stimulating dose of rhIL-2, duration in culture and individual response (Figures 1b-4b, 1c-4c and 1d-4d respectively).

In the absence of IL-2 stimulation, the PBMC from three of the four donors (donors A, B and D) produced little or no TNFα (6.2 U/ml to 31.7 U/ml). In contrast, the unstimulated cells of donor C produced unusually high levels of TNFα (117.9 U/ml to 144.8 U/ml), possibly as a result of activation during venupuncture or reflecting the possibility that donor C is a high TNFα producer. **Kovacs et al (1989)** failed to detect mRNA for TNFα in unstimulated cells and **Heslop et al (1988)** found that none of the six normal individuals they studied spontaneously produced TNFα, although three of the eight post-T-cell depleted allogeneic bone marrow transplant (TD-BMT) patients they examined, produced TNFα in the absence of IL-2. In comparison, four of the six

normals and seven of the eight patients produced TNFα in response to 200 U/ml IL-2. The study by **Schoof et al (1992)** also reports that no TNFα was produced by normal individuals in the absence of IL-2. **Limb et al (1989)** studied five donors who all produced low levels of TNFα (17 to 84 U/ml) over 72 hours in the absence of IL-2 whilst showing that in the presence of IL-2, the levels of TNFα could be elevated. As evident from our study, **Saraya and Balkwill (1993)** generally reported that in the absence of IL-2, no TNFα mRNA or protein was detected although one of their six donors also produced spontaneous TNFα (1100 pg/ml) in the absence of IL-2 stimulation.

In our study the levels of TNFα produced ranged from 6.2 U/ml to 144.8 U/ml. A similar trend was observed in three of the donors (donors A, B and D), demonstrating a time and dose-dependent association. Usually, at higher doses of rhIL-2 (1000 or 2000 IU/ml) TNFα production generally increased with the duration of culture, with maximal TNFα production being favoured by high dose IL-2 (1000 or 2000 IU/ml) in these three donors. **Heslop et al (1988)** reported TNFα production in response to 200 IU/ml as also observed in our experiments, although our stimulations were maximal at 1000 IU/ml. Similarly, **Limb et al (1989)** found that both donors in their study generally produced increased levels of TNFα (ranging from 37 to 300 U/ml) with maximal stimulation occurring at 1000 U/ml. Likewise, **Schoof et al (1992)** reported that maximal TNF production from normal individuals occurred following stimulation with 1000 IU/ml rhIL-2, with an IL-2 titration resulting in TNF concentrations ranging from 5 to 140 U/ml.

Both **Kovacs et al (1989)** and **Saraya and Balkwill (1993)** detected TNFα mRNA in response to 1000 U/ml rhIL-2. **Saraya and Balkwill (1993)** found that in all donors studied TNFα mRNA was detectable between 2 and 24 hours following stimulation and was continually expressed throughout the duration of culture (7 days). In the nine individuals studied by **Kovacs et al (1989)**, the kinetics of TNFα mRNA expression varied between individuals, reaching maximal level at any point between day 2 and day 7 of culture. Differences in the kinetics of TNFα produced in response to IL-2 activation has also been demonstrated: **Nedwin et al (1985)** found that maximal TNFα was produced following 48 hours of stimulation with 1000 U/ml IL-2, with further culture decreasing the level of TNFα. **Limb et al (1989)** demonstrated that irrespective of time in culture (24, 48, 72 or 96 hours) with 1000 U/ml IL-2, in all three donors studied the levels of TNFα measured was always greater than those in control samples. Of these donors, two individuals produced peak TNFα at 72 hours whilst the third individual produced maximal TNFα at 24 hours. Likewise, **Saraya and Balkwill (1993)** detected maximal TNFα in response to 1000 IU/ml rhIL-2 on day 4 of culture

in seven donors whilst the eighth donor studied showed maximal TNFα following seven days in culture (maximal TNFα values from 5300 to 10,000 pg/ml). Our data shows several similarities in that although donors A, B and D all produce maximal TNFα at 1000 IU/ml rhIL-2, their kinetics differ: donor A produced maximal TNFα on day 4 of culture whereas both donors B and D both reached maximal TNFα production following 8 days in culture with IL-2. The kinetics of TNFα production in donor C was more difficult to interpret owing to the high spontaneous TNFα secretion observed in this individual.

In conclusion, TNFα could be produced in donors A, B and D in response to 200, 1000 and 2000 IU/ml rhIL-2. However, the production of TNFα was generally found to be favoured by high dose IL-2 (1000 or 2000 IU/ml) with stimulation by 1000 IU/ml of rhIL-2 producing maximal levels of TNFα in all three of these donors. Generally, TNFα concentrations increased with time in culture, although this was not always the case. Extending their study to cancer patients, **Schoof et al (1992)** found that the production of TNFα as a secondary cytokine by the IL-2 (1000 IU/ml) activated PBMC of patients commencing LAK therapy had no apparent relevance to their clinical outcome: "responders" to LAK therapy produced 22+/-9 U/ml of TNF whilst "non-responders" showed no significant difference, secreting 20+/-6 U/ml of TNF. In contrast, the levels of IFNγ were found to be relevant to the clinical response.

With the exception of donor C, culture in the absence of rhIL-2 produced little or no IFNγ (0 to 20 U/ml). This observation is comparable to those of other studies: **Limb et al (1989)** report negligible amounts of IFNγ (2 to 18 U/ml) produced by mononuclear cells cultured for 72 hours without rhIL-2 whilst both **Schoof et al (1992)** and **Saraya and Balkwill (1993)** also failed to observe spontaneous production of IFNγ in unstimulated cells. **Heslop et al (1988)** showed the spontaneous production of IFNγ by the PBL from sixteen out of seventeen TD-BMT patients but found that only one of nine normal donors produced IFNγ in the absence of IL-2 stimulation. In contrast, all patients (17) and normals (9) produced IFNγ in the presence of IL-2. Furthermore, no appreciable IFNγ mRNA could be detected in unstimulated cultures **(Kovacs et al, 1989; Saraya and Balkwill, 1993).**

The kinetics of IFNγ secretion by PBMC in response to the stimulating dose of IL-2 and the duration of culture has been investigated in several studies with varying results. **Vilcek et al (1985)** found that 10 U/ml of crude IL-2 would produce IFNγ from PBMC whereas **Young and Ortaldo (1987)** reported that a minimum of 100 U/ml of crude IL-2 was required to produce IFNγ from LGL, although both these groups suggest that

a stimulating dose of 500 to 1000 U/ml of crude IL-2 would result in maximal IFNγ production. By examining the effects of a range of rhIL-2 concentrations (0, 1, 25, 50, 100 or 1000 U/ml) on IFNγ secretion, **Limb et al (1989)** found that a minimum of 25 U/ml of IL-2 was required for stimulation, with one donor showing a dose-dependent increase in IFNγ production whilst the second donor produced maximal IFNγ in response to 50 U/ml rhIL-2, which then declined as IL-2 concentration increased. This is of particular interest since our data for donor C demonstrates a dose and time-dependent increase in IFNγ production in response to rhIL-2. In contrast, the other three individuals (donors A, B and D) all produced maximal IFNγ when stimulated with 200 IU/ml rhIL-2 for 4 days (donors A and B) or 8 days (donor D), with an increase in rhIL-2 concentration leading to a fall in the levels of IFNγ. These observations suggest that the response to activation with IL-2 (i.e. the secretion of IFNγ) varies between individuals.

Heslop et al (1988) also report the production of IFNγ by the PBMC from TD-BMT patients and normal controls when activated with 200 IU/ml rhIL-2 for 2 days, finding a one to three log increase in IFNγ concentration. Unlike our study which indicates that IFNγ secretion is maximal with low dose rhIL-2, **Schoof et al (1992)** observed maximal IFNγ in response to 1000 IU/ml rhIL-2, although they also found a dose-dependent increase in IFNγ as demonstrated by our data for donor C. However, in comparing these results it should be considered that **Schoof et al (1992)** studied the responses of monocyte-depleted mononuclear cells whereas this study used whole PBMC for LAK generation and thus, the observed kinetics of IFNγ production via IL-2 stimulation may reflect the differences in the cell populations available to respond. Likewise, **Saraya and Balkwill (1993)** report IFNγ secretion in response to 1000IU/ml of rhIL-2 but they failed to study the effects of an IL-2 titration on IFNγ production, choosing to use 1000 IU/ml as the stimulating dose since this level of IL-2 was responsible for inducing maximal LAK cytotoxicity in vitro. Both of these groups observed maximal IFNγ following 4 days in culture, as also reflected in two of our four donors (donors A and B). The levels of IFNγ measured in this study ranged from 0 to 160 U/ml compared to 2 to 333 U/ml (**Limb et al, 1989**), 0 to 400 U/ml (**Schoof et al, 1992**) and a maximal range of 28 to 90 IU/ml (**Saraya and Balkwill, 1993**) in the other studies discussed.

In conclusion, low dose rhIL-2 (200 IU/ml) favours maximal IFNγ production from normal PBMC in this study, usually but not always, for a duration in culture of 4 days. Although 200 IU/ml rhIL-2 produced the highest levels of IFNγ in three of the donors studied (donors A, B and D), the fourth donor (donor C) displayed kinetics differing to

the others suggesting that IFNγ production in response to IL-2 stimulation is subject to individual variations. This concept is supported by the afore-mentioned data of **Limb et al (1989)** and the report by **Kovacs et al (1989)** of inter-individual variations in the kinetics of IFNγ mRNA production in reponse to IL-2. **Schoof et al (1992)** expanded their study to examine secondary cytokine production by LAK populations which were subsequently administered to cancer patients. They discovered that the levels of IFNγ in LAK culture supernatants from patients classified as "responders" or "non-responders" to LAK therapy varied: "responders" had lower levels of IFNγ (mean = 42+/-14 U/ml) compared to "non-responders" (mean = 232+/-94 U/ml). Not only does this data suggest variations in IFNγ production between individuals, but it also indicates that an individual's ability to secrete IFNγ in response to IL-2 may determine their therapeutic outcome to LAK treatment, and thus IFNγ production could potentially be an important therapeutic marker.

IL-1 secretion in the absence of rhIL-2 ranged from 0.06 ng/ml to 8.66 ng/ml for donors A, B and D. As also observed for TNFα, donor C produced unusually high levels of IL-1 spontaneously (8.42 ng/ml to 16.8 ng/ml) in the absence of IL-2 stimulation, probably as a result of cellular activation during venupuncture. **Kovacs et al (1989)** could not detect IL-1β mRNA or IL-1β protein in the unstimulated cells from cancer patients or normal volunteers. In a set of experiments to investigate the effects of time or dose of IL-2 on IL-1 production, **Limb et al (1989)** reported lower levels of IL-1α (2.5 U/ml to 28 U/ml over 72 hours of culture) present when compared to IL-1α production in IL-2 activated cells. **Saraya and Balkwill (1993)** failed to detect either IL-1α or IL-1β mRNA in unstimulated cells but demonstrated IL-1β protein in unstimulated cultures at a peak level of 100 pg/ml. At no point was IL-1α mRNA or protein detectable whether IL-2 was present or not.

Our data produced a range of IL-1 concentrations from 0.04 ng/ml to 20.48 ng/ml overall, in response to the addition of exogenous rhIL-2. Generally, the production of IL-1 followed a similar trend to that observed for IFNγ with maximal IL-1 secretion generally attained with low dose rhIL-2. Donors A and D produced maximal amounts of IL-1 when stimulated with (200 IU/ml) for 8 days. In this case donor B produced negligible amounts of IL-1 when compared to donors A and D (1.41 to 20.48 ng/ml). A dose and time-dependent response was again observed with donor C, as also apparent for IFNγ production: as both duration in culture and stimulating dose of IL-2 increased IL-1 secretion also increased. However high levels of IL-1 were also observed in the unstimulated cells from this donor. **Limb et al (1989)** studied the effects of IL-2 dose on the secretion of IL-1α by mononuclear cells. In the first donor

they observed peak IL-1α (116 U/ml) with 50 U/ml of IL-2 for 72 hours, with subsequent increases in IL-2 concentration resulting in a decline in IL-1α. The peak level of IL-1α (82 U/ml) was observed for the second donor with 1000 IU/ml IL-2 for 72 hours although low dose IL-2 (25, 50 or 100 U/ml) produced similar levels of IL-1α (56 to 70 U/ml). Using a stimulating dose of 1000 U/ml IL-2 in a parallel experiment, maximal IL-1α was produced following 72 hours in culture for two of the three donors studied, whereas the third donor produced maximal IL-1α after 96 hours in culture. **Kovacs et al (1989)** detected both IL-1α and IL-1β mRNA 2 to 18 hours after the onset of culture of PBMC, from cancer patients or normal volunteers with 1000 U/ml rhIL-2. All nine samples expressed IL-1β mRNA which returned to a baseline level at 48 hours. The expression of IL-1α mRNA was 5 to 50 fold lower than that of IL-1β and was detected during the first day of culture in 5 of 8 samples. One patient studied however expressed IL-1α mRNA on day 3 of culture whilst one normal volunteer expressed IL-1α RNA as late as day 7. The eighth sample had no detectable IL-1α mRNA present. **Saraya and Balkwill (1993)** likewise found early induction of IL-1β mRNA (2 to 24 hours) in response to 1000 IU/ml rhIL-2, with a decline in the expression of IL-1β transcript at 48 hours in all nine individuals examined. No IL-1β mRNA was detected if the duration of culture exceeded 3 days. In contrast to the data of **Kovacs et al (1989)** and **Limb et al (1989)**, neither IL-1α mRNA or IL-1α protein was detected in their study. The concentrations of IL-1β produced ranged from 90 pg/ml (0.09 ng/ml) to 1300 pg/ml (1.3 ng/ml), with maximal levels occurring during the first 2 days of stimulation. Our study demonstrates that high dose rhIL-2 (1000 or 2000 IU/ml) can stimulate IL-1 secretion, although the production of IL-1 in these experiments was favoured by low dose rhIL-2 (200 IU/ml) as previously described by **Limb et al (1989)**. Maximal IL-1 levels were usually detected later in culture (day 8) in contrast to the previous observations by the afore-mentioned researchers who generally detected peak IL-1 production in the first 4 days of culture, although **Kovacs et al (1989)** did find IL-1α mRNA expression on day 7 of stimulation in one sample. However, in comparing these studies it should be considered that our data measured total IL-1 whereas the other studies measured IL-1α or IL-1β as separate entities. This may account for some differences observed, particularly as the concentrations we report appear considerably higher than those of **Saraya and Balkwill (1993)**.

As an alternative to IL-2 stimulation, the ability of a bivalent monoclonal anti-CD3 antibody (SHL45.6) to induce cytokine secretion in the four donors (A - D) was assessed. The cells were stimulated for 1, 4 or 8 days with anti-CD3 antibody at 1, 10 or 100 ng/ml and, as before, control cultures were established in the absence of stimulant (i.e.. with 0 ng/ml anti-CD3). Anti-CD3 stimulation was also found to

produce TNFα, IFNγ and IL-1 (Figures 5b-8b, 5c-8c and 5d-8d respectively) although the pattern of secretion varied to that observed for the IL-2 activated cultures.

In the absence of anti-CD3 stimulation, donors A, B and C produced TNFα (ranging from 20.3 to 95.0 U/ml). No data was available for the spontaneous TNFα production by donor D in this set of experiments. The levels of TNFα secreted by donor A in the absence of anti-CD3 (20.3 to 22.9 U/ml) were similar to the concentrations observed in the absence of rhIL-2 (17.4 to 25.0 U/ml), although TNFα production peaked on day 4 in the anti-CD3 cultures compared to day 8 in the IL-2 cultures. However, donor B spontaneously produced greater quantities of TNFα in the anti-CD3-free cultures (30.5 to 62.3 U/ml) than in the IL-2-free cultures (8.7 to 8.8 U/ml), producing peak amounts of TNFα on day 1 whereas the TNFα concentrations in the absence of IL-2 stimulation remained steady with time in culture. The opposite was observed for donor C: culture in the absence of IL-2 produced 117.9 to 144.8 U/ml of TNFα, peaking on day 4, whilst a lower range of TNFα concentrations (62.0 to 95.0 U/ml) was observed in the absence of anti-CD3 with maximal TNFα attained following 1 day in culture. Generally, when compared to the other donors, donor C produced the greatest levels of TNFα spontaneously in either set of experiments, suggesting that perhaps donor C is a high TNFα producer. In the absence of either anti-CD3 or rhIL-2, similar TNFα production would be expected for a given donor, as observed for donor A. Major differences are apparent in donors B and C however, which may reflect the results of conducting the anti-CD3 experiments on a second blood donation from each donor rather than doing the IL-2 and anti-CD3 stimulations in parallel from the same aliquot of blood. Thus, the fluctuations in TNFα concentrations may be a consequence of intra-individual variations in vivo or differences in cellular activation during the withdrawal of blood (i.e.. at venupuncture).

Overall, TNFα concentrations ranged from 20.3 to 133 U/ml compared to 6.2 to 144.8 U/ml in the IL-2 experiments. TNFα was detected in all cultures stimulated with anti-CD3 and its production was generally favoured by high doses of antibody (10 or 100 ng/ml). Three of the four donors (donors A, C and D) produced maximal TNFα in response to 100 ng/ml of anti-CD3 for 1 day. In contrast, maximal TNFα production for donor B resulted from stimulation with 10 ng/ml of antibody for 8 days. Interestingly, the response of donor C to anti-CD3 was more consistent with the responses of the other donors (donors A and D) in contrast to the differences observed in response to IL-2 stimulation. The production of TNFα by donor A was fairly steady throughout the duration of culture. On the other hand, TNFα secretion by donor C usually peaked on day 1 and subsequently declined with prolonged time culture. Incubation with no or low dose anti-CD3 (1 ng/ml) produced a similar pattern of TNFα

secretion with time in donor B. However at greater doses of anti-CD3 (10 or 100 ng/ml) the production of TNFα by donor B increased with time in culture. Donor D probably shows a similar trend in TNFα secretion to donor A but insufficient data was available to confirm this.

In the cultures without anti-CD3, little (16 to 36 U/ml) or no IFNγ was detectable. The spontaneous production of low levels of IFNγ appeared to be random, as no pattern could be ascertained between donors or duration of culture. The levels of IFNγ spontaneously produced by the IL-2-free cultures (0 to 20 U/ml) was comparable to these values: in most cases the same donors produced spontaneous IFNγ at the same time points. Stimulating the PBMC of all four donors with anti-CD3 produced a wide range of IFNγ concentrations (0 to 125 U/ml), whilst activation with rhIL-2 produced levels of IFNγ ranging from (0 to 160 U/ml). As also observed for TNFα, the secretion of IFNγ was favoured by high dose anti-CD3 (10 to 100 ng/ml). Although the production of IFNγ by donors C and D was low overall (0 to 30 U/ml), with a range of concentrations comparable to the spontaneous levels produced, both these individuals produced maximal amounts of IFNγ when activated with 100 ng/ml of antibody for 4 or 8 days respectively. Donors A and B however produced maximal IFNγ in response to 10 ng/ml of anti-CD3 for 8 or 4 days respectively. The kinetics of IFNγ production due to anti-CD3 stimulation was generally unpredictable, although the more common trend was to either reach peak concentrations on day 4 and then decline or to rise in concentration with time in culture, peaking on day 8.

When compared to anti-CD3 stimulated cells, IL-1 production in the absence of antibody stimulation was usually negligible (0.24 to 5.21 ng/ml). IL-1 secretion in the absence of rhIL-2 ranged from 0.06 to 16.8 ng/ml, with the levels of IL-1 only exceeding 8.66 ng/ml in donor C. Whereas donor C appeared to secrete high spontaneous levels of IL-1 in the IL-2 experiments, the levels produced by donor C in the absence of anti-CD3 stimulation were consistent with those of the other three donors suggesting that the differences observed are probably a consequence of activation during venupuncture.

Activation with anti-CD3 produced a range of IL-1 concentrations between 0.24 and 26.09 ng/ml, which was similar to the IL-2 response (0.04 to 20.48 ng/ml). Whilst stimulation with IL-2 produced similar trends in both IL-1 and IFNγ production with dose, no similarities were observed with TNFα production. In contrast, the anti-CD3 induced secretion of IL-1, IFNγ and TNFα all exhibit a similar dose-response effect: IL-1 production is likewise favoured by stimulation with high-dose anti-CD3 (10 or 100 ng/ml). Incubation with 10 ng/ml of antibody induced maximal levels of IL-1 in

donors A, B and D whereas donor C produced maximal IL-1 in response to 100 ng/ml of antibody. Although the response to dose was fairly predictable, IL-1 production with time in culture was more variable. Donors A, B and C all produced greater quantities of IL-1 following 8 days in culture. However owing to insufficient data, the effects of time in culture on donor D could not be compared. Although not always the case, IL-1 secretion generally increased with time.

In summary, both rhIL-2 and monoclonal anti-CD3 (SHL45.6) induced LAK cytotoxicity and the secretion of TNFα, IFNγ and IL-1 in vitro, in all four donors studied. Maximal LAK cytotoxicity against SW742 tumour cells was usually observed with 1000 IU/ml of rhIL-2 for 4 days or with 10 ng/ml of anti-CD3 for 1 day. The LAK cytotoxicity induced with anti-CD3 was generally more consistent than IL-2 generated killing, when taking dose of stimulant, duration of culture and consistency between donors into consideration. Low dose IL-2 (200 IU/ml) favoured the production of IFNγ and IL-1 in vitro whereas stimulation with high dose IL-2 (1000 IU/ml or more) favoured the secretion of TNFα. This suggests that perhaps two distinct populations of cells within the PBMC were responding to IL-2, particularly as NK-cells, T-cells and monocytes are all responsive to IL-2 stimulation. In contrast, the production of all three cytokines (IFNγ, IL-1 and TNFα) was favoured by high dose anti-CD3 (10 or 100ng/ml) in vitro. This observation was perhaps to be expected since anti-CD3 stimulation drives a T-cell response and these cytokines are therefore likely to be from a single cellular source (i.e.. T-cell derived). This is particularly interesting since a recent study by **Gratama et al (1993)** suggests that activation of the immune system of cancer patients by continuous i.v. rhIL-2 therapy is dependent on dose and schedule of rhIL-2. They concluded that $CD56^{+}CD3^{-}$ NK-cells and $CD3^{+}$ T-cells were activated differentially in vivo by continuous i.v. infusion of rhIL-2, proportional to dose and duration of treatment.

Likewise, the production of these cytokines in response to anti-CD3 showed greater consistency than the IL-2 stimulated cultures. Activation with anti-CD3 produced similar trends in cytokine release by all four donors, with cytokine secretion usually being greatest in response to higher doses of antibody (10 or 100 ng/ml). IL-2 stimulation of secondary cytokine release was less consistent between donors, with donor C behaving very differently to the other three individuals studied. Furthermore, the LAK cytotoxicitity induced by IL-2 did not appear to correlate with the secretion of TNFα, IFNγ or IL-1, suggesting that the LAK cytotoxicity observed in vitro is not predictive of the in vitro production of these cytokines and vice-versa. On the other hand, some similarities were observed between the changes in LAK cytotoxicity and

cytokine production with time following anti-CD3 stimulation. Generally LAK cytotoxicity in vitro decreased with time. Likewise, TNFα concentrations in the cell supernatants either remained steady or decreased with time, indicating that the killing was probably greatest when TNFα concentration was high. As lysis subsided with time, the levels of IFNγ and IL-1 in the supernatants generally increased. Thus, since IFNγ and IL-1 production generally increased over time, the relationship between anti-CD3 induced LAK cytotoxicity and the production of these two cytokines was probably inverse. However, the interpretation of this data should be conducted with care since it is important to appreciate whether the cytokine measurements represent the total cytokine levels or the amount of biologically active cytokine present in the supernatants. In these experiments TNFα and IFNγ were measured by enzyme-linked immunosorbent assay (ELISA) whereas IL-1 was measured by bioassay. Thus, the total concentrations of TNFα and IFNγ were measured whilst biologically active levels of IL-1 were determined. Subsequently, although TNFα concentrations may have been high when killing was greatest, not all TNFα accounted for would have been biologically active.

Secondary cytokine production by lymphoid cells used in adoptive immunotherapy has been shown to influence the activation of immunocytes in vivo (**Silberstein et al, 1989**) and response of cancer patients to therapy (**Schoof et al, 1992; Osband et al, 1990**). **Schoof et al (1992)** discovered that LAK cell supernatants harvested from "non-responders" to LAK therapy contained a significantly higher level of IFNγ compared to "responders", whereas TNFα production was similar between the two groups. IFNγ therefore appears to play an important role in the host response to cellular immunotherapy. Similarly, a clinical study by **Osband et al (1990)** found that PBMC activated with soluble anti-CD3 produced various levels of IL-1 in culture supernatants. Cancer patients receiving activated cell populations which produced more than 500 pg/ml of IL-1 had a significantly longer survival interval, although the influence of IL-1 production on tumour regression was not reported. **Silberstein et al (1989)** observed that although IL-2 (1000 U/ml) did not affect the cytotoxic function of eosinophils from cancer patients or control subjects in vitro, LAK cell-conditioned medium (used at 25% v/v) enhanced eosinophil cytotoxicity. Thus treatment with IL-2/LAK therapy may cause physical changes in eosinophils in vivo, rendering them functionally activated. However, the involvement of IL-2 in regulating eosinophil activity is probably indirect, as an inducer of secondary cytokines (such as TNFα or TNFβ) which can enhance eosinophil function.

Although defined by their ability to mediate direct cytotoxicity against tumour cells in vitro, little evidence is available to suggest that LAK cells localise at the tumour site in vivo. **Rubin et al (1989)** showed that LAK cells did not accumulate at or infiltrate into metastatic deposits, supporting suggestions by **Rosenberg (1985)** that intravenously administered LAK cells traffic through the lungs and accumulate in the liver and spleen until cleared. Since LAK cells do not seem to be directly cytotoxic to tumours in vivo, then any anti-tumour effects they exert must be indirect by enhancing an existing anti-tumour response. As an important lymphoid organ, the spleen contains both T-cells and monocytes. Consequently, infiltrating LAK cells may be capable of potentiating the anti-tumour activity of pre-primed immunocytes present within the spleen, and thus mediating tumour regression indirectly. The proposition that T-cell or monocyte function can be altered by LAK cells via the release of secondary cytokines would seem feasible since eosinophil cytotoxicity can be altered in this way (**Silberstein et al, 1989**). Having established that LAK cells can modulate the effects of IL-2 on a T-cell mediated immune response using a murine model of delayed-type hypersensitivity (**McCulloch et al, 1991**), this project aimed to determine whether LAK-cell supernatants, obtained via stimulation of PBMC with either rhIL-2 or soluble anti-CD3, could alter T-cell activity in vitro. Using these supernatants at 25% (v/v), as also described by **Silberstein et al (1989)**, the affects on T-cell cytotoxicity and proliferation were determined.

4.2: EFFECTS OF LAK SUPERNATANTS ON T-CELL CYTOTOXICITY

In order to assess whether LAK cells can regulate T-cell cytotoxicity via the release of secondary cytokines, the effects of LAK-cell supernatants on a cytotoxic T-cell line (CTLL) were studied. Each of the LAK supernatants were screened for the ability to enhance the cytotoxicity of a given CTLL (ST-CTLL) against its specific target cells (SW-LCL), which express both EBV antigens and suitable MHC molecules. The killing observed was compared to the lysis obtained for controls: medium alone or rhIL-2 concentrations equivalent to the doses of rhIL-2 used for LAK cell generation (ie. 200, 1000 or 2000 IU/ml). An effector-to-target cell ratio of 1.5:1 was choosen from a titration assay since the degree of lysis at this ratio was detectable (around 40%) whilst being low enough for the cytotoxic capacity of the CTLL to be potentiated by the addition of the supernatants.

Initially, the ability of LAK supernatants to enhance CTLL killing over a 5 hour period was assessed by standard 5-hour chromium release assays. In the absence of CTLL, none of the LAK supernatants or rhIL-2 produced any target cell lysis, confirming that neither rhIL-2 or the LAK supernatants had any direct cytotoxic effect on SW-LCL

cells during the 5 hour incubation. Although fluctuations in killing were observed for both the IL-2 and the anti-CD3 induced LAK supernatants (ranging from 32% to 58%), there was no significant increase in cytotoxicity over controls (46% to 50%). An aberrant value was obtained for one of the supernatants but appeared to be the result of experimental error (Figures 9a and 9b). Therefore in conclusion, none of the LAK supernatants significantly enhanced the cytotoxic activity of the CTLL. However, the 5 hour duration of these assays may have been insufficient to permit the stimulation of the CTLL, as 5 hours may not have been enough time to allow for the synthesis of T-cell cytokines, cytotoxic mediators or adhesion molecules, all of which may have influenced the lytic ability of the CTLL. Consequently, similar assays were carried out over a 24 hour period. Since the use of chromium labelling for longer than 18 hours is toxic to cells, the 24 hour cytotoxicity assays were conducted using tritiated uridine (^{3}H-uridine) to label target cells.

Following activation with LAK supernatants (25% v/v) for 24 hours, the majority of IL-2 induced supernatants produced greater CTLL cytotoxicity (11.2 to 63.5%) than rhIL-2 controls (5.7 to 13.3%) or unstimulated CTLL (24.8%) in medium alone (Figures 11a-14d). The anti-CD3 induced LAK supernatants likewise produced greater CTLL cytotoxicity (28.1 to 69.0%) than any of the controls. However, both IL-2 and anti-CD3 LAK supernatants appeared to have direct cytotoxic effects on SW-LCL target cells over a 24 hour period, since the lysis observed in the absence of CTLL was greater than the degree of killing with CTLL present. For the IL-2 induced supernatants, killing in the absence of CTLL ranged from 25.1% to 71.1% as compared to 11.2% to 63.5% attained when CTLL were added. Similarly, lysis ranged from 37.3% to 76.5% for the anti-CD3 induced supernatants compared to 28.1% to 69.0% killing in the presence of CTLL. A similar phenomenon was detected with the rhIL-2 controls, with a reduction in lysis (12.8 to 15.3% killing falling to 5.7 to 13.3% lysis) occurring following the addition of CTLL to the assay (Figure 10). Thus a factor, or factors, within the LAK supernatants must be acting directly on the SW-LCL target cells.

The decline in killing observed on the addition of CTLL to the assay may be a consequence of the uptake of ^{3}H-uridine, released into the medium by dead target cells, by the CTLs themselves. However, this does not appear to be the case because if ^{3}H-uridine "turnover" was occurring, the plots expected would be less uniform when compared to those observed in the absence of CTLL. Thus, it seems likely that the CTLL are perhaps binding a factor present in these supernatants which can therefore no longer exert its effects on the SW-LCL target cells, although the CTLL are not becoming activated when they "absorb" this material from the LAK-cell supernatants

(otherwise the plots of cytotoxicity in the presence of CTLL would be more random and would not follow a similar trend in killing over time to the lysis observed in the absence of CTLL).

Since a similar phenomenon occurs in the controls (Figure 10), where adding CTLL to the various rhIL-2 concentrations results in a reduction in lysis, IL-2 itself may be a contributing factor to these events. Both B-cells (SW-LCL is an EBV-transformed B-cell line) and T-cells (such as ST-CTLL) can express IL-2 receptors and are therefore responsive to IL-2, although IL-2 promotes B-cell growth and may therefore not account for the lytic effects of these supernatants. Furthermore, there is no apparent association with the cytokines measured (ie. TNFα, IFNγand IL-1). However, these supernatants may contain a range of other cytokines not measured in this study, as outlined by **Saraya and Balkwill (1993)**: these include TGFβ, TNFβ, IL-6, IL-5, GM-CSF, M-CSF and gro. Of these cytokines, TGFβ is the most probable candidate for mediating these effects. TGFβ has been shown to have both growth enhancing and growth inhibiting properties, depending on the cell type it targets and the other cytokines present. T-cells have receptors for TGFβ, which are up-regulated following T-cell activation, and therefore have the capability to bind TGFβ if present in these supernatants. Furthermore, B-cells express receptors for TGFβ and are therefore subject to regulation by TGFβ, which has been shown to inhibit B-cell proliferation **(Kehrl et al, 1986)**. Although not generally cytotoxic, perhaps the cytostatic properties of TGFβ can result in the release of ^{3}H-uridine from target cells as a consequence of cell-death owing to the lack of proliferation. Alternatively, the differences in lysis observed in the presence or absence of CTLL may be due to the release of a mediator by activated CTLL, which can counteract the effects of the cytotoxic factor(s) present in these supernatants. However, there is no evidence to support either of these suggestions and in order to ascertain whether TGFβ or another cytokine is responsible for this phenomenon, antibody-blocking experiments to neutralise TGFβ/cytokine activity would have to be conducted.

In order to activate the CTLL with the LAK supernatants for longer than 5 hours, whilst overcoming the direct cytotoxic effects on target cells over 24 hours, a preactivation assay was carried out. CTLL were incubated with LAK supernatant, rhIL-2 or medium as before, for 19 hours prior to the addition of SW-LCL target cells. CTLL and targets were then co-incubated for a further 5 hours and the % lysis determined by ^{3}H-uridine release. In this experiment (Table 3), only 15 of the 96 supernatants produced killing (8.0 to 55.9%) in the presence of CTLL, with the remaining supernatants resulting in no or negligible killing (0 to 5.3%). Ten of these

supernatants were lytic (3.3 to 40.71%) to the target cells in the absence of CTLL, as was the 2000 IU/ml rhIL-2 control (32.6%): five samples resulting in greater cytotoxicity when CTLL were present whilst the other five produced greater lysis in the absence of CTLL. Conclusively, the enhancement of CTLL cytotoxicity, to levels (32.4 to 55.9%) greater than or similar to lysis with supernatants alone or IL-2 controls, was only observed in a few, random samples [D.I.1.2000, B.C.1.10, B.C.1.100, C.C.1.0 and D.C.1.100]. No patterns in cytokine concentrations (TNFα, IFNγ and IL-1) was apparent in these supernatants. Moreover, the killing observed was not dissimilar to the original 5 hour cytotoxicity assay. Therefore, it appears that secondary cytokines in these supernatants, produced by anti-CD3 or IL-2 induced LAK cells, cannot significantly enhance T-cell cytotoxicity in vitro.

4.3 : EFFECTS OF LAK SUPERNATANTS ON T-CELL PROLIFERATION

In order to determine whether the IL-2 or anti-CD3 LAK supernatants had any mitogenic effects on peripheral T-cells, the proliferation in response to the supernatants was determined by ^{3}H-thymidine incorporation. Generally, proliferation in response to IL-2 induced LAK supernatants was low (28 to 8,967 cpm) in all four donors (Figures 20a-23a). Variation between individuals was apparent and no proliferative trends were associated with duration of LAK induction or the dose of rhIL-2 used for LAK generation. The addition of exogenous anti-CD3 (5 ng/ml) greatly enhanced the proliferation (8,254 to 69,155 cpm) in all four donors, in a random fashion (Figures 20b-23b). However, the increases observed appeared to be associated only with exogenous anti-CD3 stimulation, showing no correlation with the parameters of LAK cell induction or the secondary cytokines present. This was supported by the fact that IL-2 itself (200, 1000 or 2000 IU/ml) produced negligible proliferation (1,238 to 1,553 cpm) whilst adding exogenous anti-CD3 enhanced proliferation (33,315 to 47,397 cpm), with proliferation increasing with IL-2 concentration (Figure 19). Thus, the mitogenic effects of IL-2 induced LAK supernatants on peripheral T-cells were poor.

Contrastingly, proliferation in response to the anti-CD3 induced LAK supernatants (Figures 24a-27a) was generally higher (48 to 32,276 cpm). However, the addition of exogenous anti-CD3 (5 ng/ml) had negligible effects on proliferation (31 to 32,999 cpm). The same trends in proliferative response were conserved following the addition of exogenous antibody (Figures 24b-27b). This suggests that residual, unassimilated anti-CD3 remaining in these supernatants after LAK induction had stimulatory effects on peripheral T-cells and, owing to a saturation effect, exogenous

antibody had no effect (except for those samples generated from anti-CD3 free LAK cultures).

4.4 ; CONCLUSIONS AND PROSPECTS

To conclude, this study demonstrated that either rhIL-2 or soluble anti-CD3 could induce LAK activity and the production of secondary cytokines (TNFα, IFNγ and IL-1). Each stimulant generated variations in lytic activities and cytokine concentrations with dose and duration in culture. Variable responses were observed between donors, particularly with IL-2 induced LAK activity which showed a poorer association with cytokine production compared to LAK cells generated by anti-CD3. The production of these cytokines and the lytic potential of the cells was more predictable with anti-CD3 stimulation. If the production of secondary cytokines by LAK cells has consequences on the clinical outcome of LAK therapy, as suggested by **Schoof et al (1992)** and **Osband et al (1990)**, then the cytokine profiles at LAK generation may be a crucial indicator of therapeutic benefit. This implies that if the cytokine profile cannot be predicted on the basis of the LAK cytotoxicity in vitro, then the patients' response to LAK therapy would be unpredictable. Clinical trials to date show individual variation in response to LAK therapy and this may be a consequence of varying ability to produce secondary cytokines during therapy, particularly as our normal volunteers demonstrated varying potential to secrete these cytokines during LAK induction.

The rationale behind LAK therapy is to potentiate the patient's on going immune response. Whenever LAK cells mediate tumour regression, the effects appear to be via an indirect mechanism, probably as a result of cellular activation via secondary cytokines produced by LAK cells. Although **Silberstein et al (1989)** demonstrated that LAK cell-conditioned medium could enhance eosinophil cytotoxicity, we were unable to see any significant effects of LAK supernatants on T-cell function in vitro. Irrespective of the duration of cytotoxicity assays, the LAK supernatants failed to significantly increase CTLL cytotoxicity: only five random samples increased lysis in the preactivation assay but even then, the killing observed was similar to the lysis observed in the 5-hour chromium release assay, which showed only fluctuations in killing compared to controls. Likewise, none of the LAK supernatants were particularly mitogenic, as peripheral T-cell proliferation only increased in those samples containing unassimilated anti-CD3 or following the addition of exogenous anti-CD3 to the cells.

From these results, it seems unlikely that LAK cells trafficking into the spleen following infusion would activate splenic T-cells and therefore potentiate an anti-

tumour response within the patient. However, these experiments have not considered that cell-to-cell between LAK cells and T-cells may be a prerequisite for T-cell activation, in addition to the effects of LAK cell-derived cytokines on T-cell function. Thus, it would be interesting to expand this study to examine LAK cell interaction with T-cells in vivo, using murine models of B16 melanoma. Two approaches could be used to determine whether T-cells played a role in vivo in the regression of the tumour: antibody-depletion of T-cells or T-cell subsets in immunocompetent mice (eg. C57/B6 mice) or T-cell/T-cell subset reconstitution of immunodeficient mice (eg. SCID mice) prior to LAK therapy. The results of such an experiment may enlighten us regarding the mechanisms by which the host might contribute towards tumour regression during LAK cell treatment. Thus, our knowledge of LAK cell functions in vivo could be greatly expanded as our comprehension of the mechanistics of LAK activity still remains a "jigsaw" with only some of the pieces in place.

For those cancer patients failing to respond to conventional therapy, it is imperative that current protocols for adoptive immunotherapy are continually improved whilst newer ideas for therapy are developed. Perhaps the concept of "lymphokine gene therapy" **(Russell, 1990; Rosenberg, 1991)** offers new hope to these patients, now that ethical approval has been given for clinical trials. There are two approaches to gene therapy, which aims to improve lymphokine delivery to the tumour. The first is "designer" TILs which have been genetically engineered, prior to the adoptive transfer, to be self-stimulating with the ability to enhance anti-tumour cytotoxicity in vivo. This approach by Rosenberg and his colleagues at the NCI has been used to selectively increase the local concentration of TNF at the tumour site and they have commenced a clinical study with TNF gene-modified TIL in patients with advanced cancer. Additional genes being studied for insertion into TIL to improve their anti-tumour activity includes IFNγ, IL-2, IL-6 and genes for chimeric T-cell receptors **(Rosenberg, 1991)**.

The alternative approach is tumour-cell-targeted lymphokine gene therapy which involves genetic modification of tumour cells to potentiate tumour immunogenicity and/or enhance the anti-tumour response by host effectors cells (Figure 28). Studies in mice and rats by various research groups have focused on this technique. So far insertion of genes for the following cytokines have been studied and show promise: IL-4 **(Platzer et al, 1992)**; IL-2 **(Russell et al, 1991)**; IL-7 **(Hock et al, 1991)**; IFNγ, TNF and GM-CSF **(Russell, 1990; Rosenberg, 1991)**.

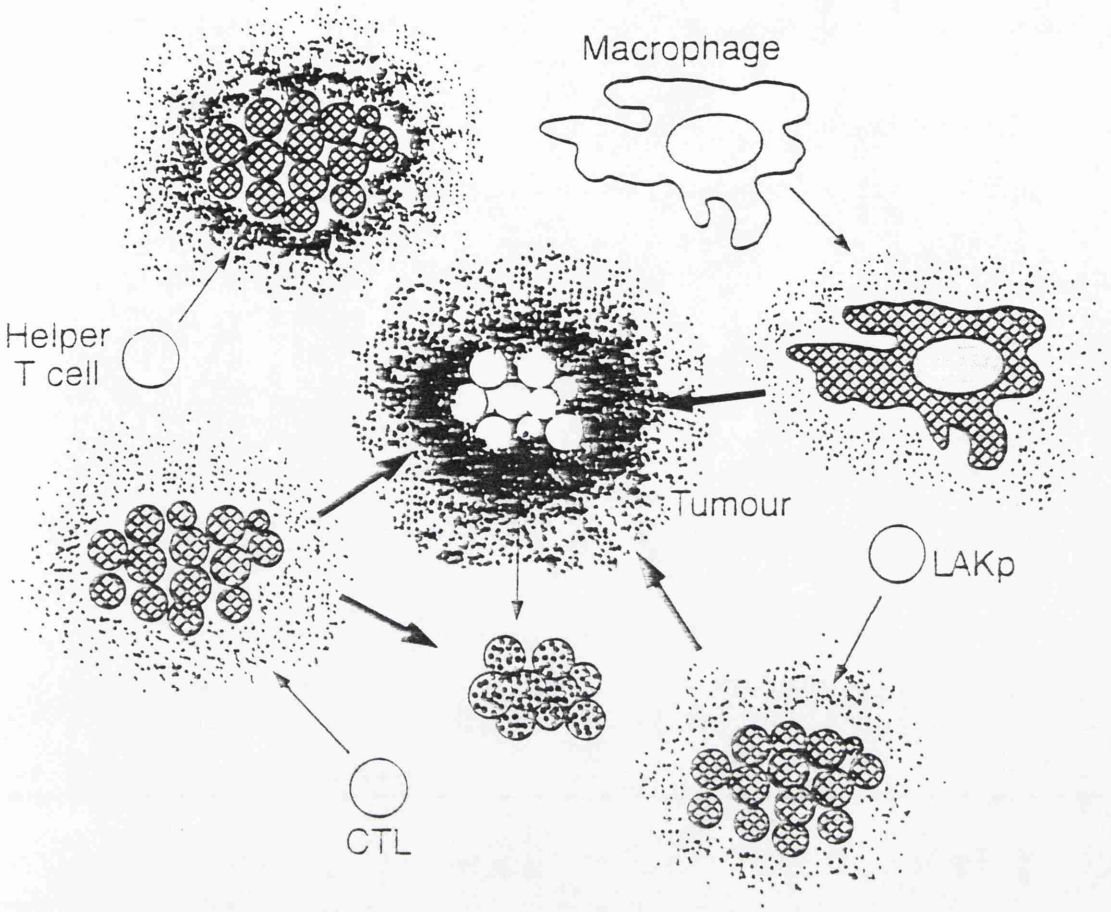


Fig. 28. *Rationale for tumour-cell-targeted lymphokine gene therapy. Tumour-cell-derived lymphokines will aid activation, proliferation, lymphokine secretion and tumour cell killing by host effector cells. CTL: cytotoxic T lymphocyte; LAKp: LAK cell precursor. For simplicity, neutrophils, eosinophils, basophils and B cells have been omitted. Small dots: interleukin molecules; cross hatching: activated cell; stippling: increased MHC expression; thin arrow: response to tumour derived interleukin; thick arrow: enhanced tumour cell killing.*

These techniques hold promise not only for active immunotherapy but also for the development of immune cells which may be more effective in adoptive immunotherapy. The last decade has shown variable successes with biological therapy but the continued development of therapeutic strategies of this nature may offer the hope of safe, effective treatment and the chance of survival to patients with advanced cancer.

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