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INDUCTION OF GRAFT-VERSUS-LEUKAEMIA ACTIVITY FOLLOWING BONE MARROW TRANSPLANTATION FOR CHRONIC MYELOID LEUKAEMIA

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ABSTRACT

This thesis describes the induction of in vitro graft-versus-leukaemia (GVL) activity following bone marrow transplantation (BMT) for chronic myeloid leukaemia (CML).

BMT is currently the only curative therapy for patients with CML but its success is limited mainly by the immunologically mediated complication of graft-versus-host disease (GVHD) which accounts directly or indirectly for approximately 50% of all transplant related deaths. Attempts to reduce the incidence and severity of GVHD by depleting the donor marrow of T cells have been successful but have been associated with a high incidence of leukaemic relapse. This suggests that the donor marrow exerts an as yet poorly defined GVL effect which is abrogated by the removal of donor lymphocytes.

The work detailed in this thesis was designed to induce in vitro GVL activity that might be separable from GVHD in man, as this approach could lead to improvements in clinical transplantation. The experiments involved incubating donor and recipient lymphocytes with supraphysiological concentrations of cytokines, in particular interleukin-2 (IL-2), and assessing the activity of these effector cells in cytotoxic and clonogenic assays. In addition the origin and activity of the adherent lymphokine activated killer (ALAK) cells in patients who had relapsed following BMT was studied.

The major conclusions to emerge from this work are:

1. Short term (18 hours) incubation of lymphocytes in alpha interferon enhances natural killer (NK) but not lymphokine activated killer (LAK) activity, whereas incubation with IL-2 increases both NK and LAK killing. Neither cytokine was capable of inducing GVL activity following short term incubation.

2. Long term (7 days) incubation of lymphocytes in IL-2 resulted in significant GVL activity in over half of the patients who were tested. This GVL activity was mediated by cells with the phenotype of NK cells and resulted in the killing of autologous and allogeneic CML cells. The effector cells mediating this GVL activity were able to kill clonogenic CML cells with relative sparing of donor marrow progenitor cells.

3. The ALAK cells in patients who relapsed following BMT were of donor origin in most patients but one patient had recipient cells which were part of the Ph-positive clone. The ALAK cells derived from some of the patients demonstrated in vitro GVL activity.

As the effectors mediating this activity were activated NK cells and not T cells, GVL and GVHD may be separable in man. Administration of cytokines following T cell depleted BMT could therefore in theory reduce the risk of GVHD without at the same time increasing the risk of leukaemic relapse.

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I owe a considerable debt of gratitude to the nursing staff in the Haematology Clinic and the senior house physicians on Dacie Ward all of whom helped in the collection of blood samples from patients and donors.

DECLARATION OF ORIGINALITY

This thesis is the result of my own work. The material contained in the thesis is not being presented either wholly or in part for any other degree or qualification.

The work was carried out between 1988 and 1990 in the LRF Centre for Adult Leukaemia at the Royal Postgraduate Medical School, London.

Apart from the karyotypic analyses described in Chapter 5 which were performed by Mrs Julie Bungey, all other technical work was performed by me.

PUBLICATIONS ARISING FROM THIS WORK

PAPERS

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ABSTRACTS

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Mackinnon S, Hows JM, Bridge J, Kaminski E, Goldman JM. Alpha interferon and interleukin 2 enhance in vitro cytotoxicity of blood mononuclear cells after bone marrow transplantation for chronic myeloid leukaemia. British Journal of Haematology 1989, 71 (suppl. 1): 9.

Mackinnon S, Tsakalakis C, Hows JM, Goldman JM. Interleukin-2 induces lymphokine activated killer cells with an in vitro graft-versus-leukaemia effect following BMT for chronic myeloid leukaemia. Experimental Hematology 1989, 17: 673.

Mackinnon S, Hows JM, Goldman JM. Induction of graft-versus-leukemia activity following BMT for CML. Blood 1989, 74 (suppl. 1): 184.

Mackinnon S, Hows JM, Goldman JM. In vitro induction of graft-versus-leukaemia activity following BMT for CML. British Journal of Haematology 1990, 74 (suppl. 1): 14.

Mackinnon S, Bungey J, Chase A, Paulsen A, Hows JM, Goldman JM. Adherent lymphokine activated killer cells may be of donor or recipient origin in patients who relapse after BMT for chronic myeloid leukaemia. Bone Marrow Transplantation 1990, 5 (suppl. 2): 46.

Mackinnon S, Hows JM, Goldman JM. Induction of in vitro graft-versus-leukemia activity following BMT for CML. Bone Marrow Transplantation 1990, 5 (suppl. 2): 26.

Mackinnon S, Hows JM, Apperley JF, Goldman JM. Induction of graft-versus-leukemia activity following BMT for CML: Selective inhibition of clonogenic leukemic cells with sparing of donor marrow progenitors. Experimental Hematology 1990, 18: (in press).

PRIZES

The research findings contained in this thesis were presented in part at The British Society for Haematology Annual General Meeting at Cambridge in March 1990. The presentation received second place in competition for The Vander Molen Prize for Leukaemia Research.

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LIST OF ABBREVIATIONS

aIFN	alpha interferon
ALAK	adherent lymphokine activated killer
BCG	Bacillus Calmette Guerin
BCR	breakpoint cluster region
BMT	bone marrow transplantation
CD	cluster differentiation
CFU-GM	colony forming unit granulocyte macrophage
CML	chronic myeloid leukaemia
CTL	cytotoxic T lymphocyte
FITC	fluorescein isothiocyanate
GVHD	graft-versus-host disease
GVL	graft-versus-leukaemia
HBSS	Hanks' balanced salt solution
HLA	human leukocyte antigen
IBMTR	International Bone Marrow Transplant Registry
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
LAK	lymphokine activated killer
LCL	lymphoblastoid cell line
LGL	large granular lymphocytes
LTN	leukaemia transplant number
MHC	major histocompatibility complex
MR	molecular mass
NK	natural killer

PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
Ph	Philadelphia
RPMI-AB	RPMI supplemented with 10% human AB serum
TCR	T cell receptor
TNF	tumour necrosis factor

CHAPTER 1

INTRODUCTION

CHRONIC MYELOID LEUKAEMIA

In 1845 several authors described a disease of the spleen associated with suppuration of the blood[1-3]. This is the condition now known as chronic myeloid leukaemia (CML), a myeloproliferative disorder of clonal origin that arises from the malignant transformation of a single pluripotent haemopoietic stem cell[4].

CML comprises about 20% of all cases of leukaemia with an annual incidence of 1 per 100,000 of the population. This incidence appears to be constant worldwide[5]. There is a slight male predominance (male:female ratio 1.4:1). The disease is seen in all age groups with a peak incidence in the fifth and sixth decades of life. Lack of concordance of CML in monozygotic twins suggests that CML is an acquired disorder, but in most cases there are no known predisposing factors. The incidence of CML was significantly increased in the survivors of the atomic bomb explosions at Hiroshima and Nagasaki who were exposed to high levels of radiation[6,7], and in a group of patients given radiotherapy for ankylosing spondylitis[8], but for most patients radiation plays no definite role in causation. There is no evidence that toxic chemicals or viruses are risk factors for CML. The majority of patients present with symptoms related to anaemia, splenomegaly or bleeding, but increasingly the diagnosis is made before the onset of symptoms - leukocytosis is recognised as the result of a routine blood test performed for totally unrelated reasons. The laboratory

findings are of a leukocytosis with a marked increase in myelocytes, neutrophils, eosinophils and basophils with a low neutrophil alkaline phosphatase score.

CML is usually a biphasic disease. After a median period of about three years the "benign" chronic phase evolves to a so-called blastic phase, sometimes preceded by a recognisable accelerated phase. This blastic or acute phase is the terminal stage of the disease, which is usually refractory to chemotherapy. Myeloblastic transformation has been notoriously difficult to treat, although return to chronic phase can be established after lymphoblastic crisis. The median duration of survival of untreated CML is 31 months[9].

PATHOGENESIS

There was little progress in the understanding of the pathogenesis of the disease until the discovery in 1960 that cells from the marrow of patients with CML were characterized by a deletion in the long arm of one of the group G chromosomes - the Philadelphia (Ph) chromosome[10]. This was therefore the first malignant disease to be associated with a consistent chromosomal defect. Over a decade later Rowley showed that this was not a deletion but a reciprocal translocation between the long arms of chromosomes 9 and 22, now usually designated t(9;22)(q34; q11)[11].

The majority (90%) of patients with CML have the Ph chromosome in all or almost all of their myeloid cells at the time of diagnosis. The position of the breakpoint on chromosome 9 is rather variable from patient to patient but the breakpoint on chromosome 22 is localised within a relatively short (5.8 kilobase) sequence of DNA that has been termed the breakpoint cluster region (BCR)[12]. A proto-oncogene, ABL, which bears homology with the transforming sequence of the Abelson strain of Moloney murine leukaemia virus, is normally located at the end of the long arm of chromosome 9. In CML the distal end of the long arm of chromosome 9, including the ABL oncogene, is translocated to chromosome 22 and the distal end of the long arm of chromosome 22, including a portion of the 3' (telomeric) end of the BCR gene, is translocated to chromosome 9; as a consequence a new hybrid gene consisting of the 5' end (centromeric) end of the BCR gene and the ABL oncogene is formed on the 22q- chromosome[13]. This hybrid gene is responsible for the production of a novel mRNA of 8.5 kb in length[14] and this in turn is associated with the production of a unique ABL-related protein of relative molecular mass (MR) 210 (P210)[15]. Unlike its normal counterpart which has a MR of 145, the P210 has tyrosine kinase activity[16], a function characteristic of the transforming sequences of other transforming retroviruses.

About 5% of patients have a disease that resembles CML but lacks the Ph chromosome. Some of these are clearly distinguishable from patients with Ph-positive disease on the basis of clinical features

and the morphology of their blood and marrow; their prognosis is probably poorer than that of patients with Ph-positive disease[17]. Other patients with Ph-negative disease are indistinguishable on clinical and haematological grounds from those with Ph-positive disease; in some of these the BCR gene is involved in a genomic rearrangement invisible at cytogenetic level but the same ABL-related P210 is produced as in Ph-positive CML[18]. The prognosis for some of the patients in this group is the same as for patients with Ph-positive disease[19,20]. Other patients sometimes erroneously diagnosed as CML have clinical features of chronic myelomonocytic leukaemia, an entity now included in the French-American-British classification of the myelodysplastic syndromes[21].

TREATMENT

Radiotherapy was the first beneficial treatment of this disease and was reported by Pusey in 1902[22]. Its value was reviewed 22 years later by Minot et al. and until the 1950s radiotherapy, either total body or splenic, was the mainstay of therapy[9]. Busulphan, introduced in 1953 by Galton[23], and hydroxyurea, reported in 1966 by Kennedy and Yarbro[24] can alleviate symptoms in chronic phase disease but both agents are responsible for only a moderate increase in survival, but with no reduction in the percentage of marrow cells which are Ph-positive. The administration of alpha interferon (aIFN) not only reduces the leukocytosis but may in some patients reduce the percentage of Ph-positive marrow cells,

although this reduction was not usually sustained[25]. There have been several attempts to prolong survival with more intensive chemotherapy regimens. In general, life was not appreciably extended, but in a subgroup of patients in whom a reduction in the proportion of Ph-positive metaphases was seen survival was thought to be improved[26].

In spite of the good control of symptoms with conventional cytotoxic drugs, the median duration of survival for patients aged less than 40 years is still only 4 years with almost all patients progressing to blast crisis and death[27]. This failure to alter the natural history of the disease has in the last 10 years encouraged study of the curative potential of high dose chemoradiotherapy followed by bone marrow transplantation (BMT).

BONE MARROW TRANSPLANTATION

The treatment of CML with cytotoxic agents is limited in part by their toxicity to host marrow. The availability of normal marrow for transplantation permits the administration of far higher and potentially curative doses of chemotherapy or chemoradiotherapy with reconstitution of the host's haemopoietic and immunological functions by the donor cells. In theory, therefore, ablation of the host marrow and transfusion of marrow from an human leukocyte antigen (HLA) identical donor could provide an opportunity for cure.

Initial studies were performed on patients in blast crisis of disease who had an identical twin donor and were therefore not at risk of developing graft-versus-host disease (GVHD). Syngeneic BMT was performed with a variety of chemoradiotherapy regimens in 8 patients who had entered blast crisis in the late 1970s[28]. The results of BMT in this late phase of the disease were generally poor; 3 patients died of leukaemia and 4 died of treatment complications. The eighth patient relapsed 10 months after BMT but was still in remission 4 years after a second syngeneic transplant[29]. The results of transplanting patients in blast crisis with allogeneic HLA-identical sibling donors were also disappointing[30].

As the transformed cells were extremely resistant to intensive treatment, various investigators speculated that the best chance of cure was to offer patients with identical twins BMT while they were still in chronic phase. This led to studies of the value of syngeneic BMT in chronic phase[28,31-34]. In the absence of GVHD the morbidity and mortality associated with the procedure were low but occasional patients died of "idiopathic" or cytomegalovirus-associated pneumonitis post-transplant. The probability of relapse after syngeneic transplantation seems rather variable. Based on experience obtained in Seattle[28,31,32] and the Hammersmith Hospital in London[34] the risk of relapse is no greater than that following HLA-identical sibling transplants for comparable patients. Others

have found that the risk is higher[35].

The success of syngeneic BMT in chronic phase and the finding that patients with acute myeloid leukaemia in remission who received an allogeneic transplant while in good clinical condition had fewer transplant-related deaths and a far lower incidence of relapse than did patients transplanted in relapse[36] encouraged physicians to offer allogeneic BMT to patients in chronic phase. A number of individual centres have now reported the results of transplant performed for CML in chronic phase using HLA identical sibling donors. The results of transplant in a relatively large series of patients have been reported by the International Bone Marrow Transplant Registry (IBMTR)[37] and many of the patients in the individual reports are included in the IBMTR analyses. In summary the probability of survival and relapse at 4 years are 55% and 19% respectively[37]. It should be noted however that this recent IBMTR analysis included some patients who had received T cell depleted bone marrow grafts. If these are excluded from the analysis, the probability of relapse is 9% and the probability of leukaemia-free survival is 47%. These results agree relatively well with those of the large series reported independently from Seattle, in which the actuarial probabilities of survival and of relapse were 49% and 20% respectively[30].

GRAFT-VERSUS-HOST DISEASE

Acute and chronic GVHD are among the principal causes of death in patients who receive transplants in chronic phase[30]. GVHD may lead directly to death or may contribute indirectly to death from other causes such as infection or interstitial pneumonitis. The survival of patients in chronic phase who develop grades II-IV acute GVHD is significantly worse than that of patients who do not[37]. Conversely in the recent IBMTR analysis of 405 CML patients allografted in chronic phase the probability of relapse was higher in those who did not develop chronic GVHD than in those who did, 24% vs 12% ($p < 0.004$)[37].

Most attempts to prevent GVHD have involved the use of immunosuppressive drugs given in the early post-transplant period. Methotrexate was initially used by the Seattle group starting within 24 hours of marrow transplantation and continuing for approximately 3 months[38], but GVHD remained a serious problem affecting 25-60% of patients. More recently cyclosporin A has been used for prophylaxis of GVHD. Although initial uncontrolled studies suggested that it was more effective than methotrexate[39], a controlled study in CML patients found no difference in the incidence of GVHD between patients given methotrexate and those given cyclosporin[40]. Because of these generally disappointing results, several attempts at combination therapy have been tried. No significant benefit was observed on combining methotrexate with

antithymocyte globulin when compared to methotrexate alone[41]. More recently cyclosporin combined with methotrexate has resulted in a significantly lower incidence of GVHD than can be achieved by using either agent alone[42]. Nevertheless even with the best of the above regimens a significant number of patients die from GVHD or its sequelae. In an attempt to reduce the incidence and mortality of GVHD the focus was switched in the early 1980s to immunological techniques of prophylaxis and in particular the use of monoclonal antibodies to deplete the donor marrow of T cells.

T CELL DEPLETION OF DONOR MARROW

Various methods of depleting donor marrow of T cells have been used. The most common and convenient technique is to incubate donor marrow in vitro with anti-T lymphocyte monoclonal antibodies and complement to induce T cell lysis. The Royal Free Hospital group in London used monoclonals with CD6 and CD8 specificities[43]. The monoclonal used by the University of California at Los Angeles group has an anti-CD2 specificity[44]. Much experience worldwide has been accumulated with the use of Campath-1, an IgM monoclonal antibody which fixes human complement and is active against an incompletely defined antigen present on T and B lymphocytes, natural killer (NK) cells and on some monocytes[45-47]. Other techniques for the elimination of T cells include soybean lectin agglutination in conjunction with E-rosette formation and counterflow elutriation[48,49]. In general these different methods

reduce the number of residual T cells in the marrow to <1% of the pretreatment levels.

The use of T cell depleted donor marrow is associated with a lower incidence and severity of GVHD, but the risk of graft failure is increased[47]. The graft failure may be due to survival in the host of chemoradioresistant immunologically competent cells which in the absence of T cells of donor origin are capable of mediating graft rejection[50]. The problem of graft failure has largely been overcome by increasing the intensity of the pretransplant conditioning regimen[51].

In CML patients the major remaining problem associated with the use of T cell depleted bone marrow is a substantial increase in the risk of relapse which is seen with all the different methods of T cell depletion. This increased risk of relapse applies both to patients transplanted in chronic phase[37] and to those transplanted in later phases. In a recent report from Seattle, 100% of patients transplanted in accelerated phase with T depleted marrow who survived long enough had evidence of recurrent leukaemia[52]; this contrasts with an actuarial risk of relapse of 55% for patients in acceleration transplanted with unmanipulated donor marrow[53]. One may conclude that depleting the donor marrow of T cells not only prevents GVHD but also abrogates a graft-versus-leukaemia (GVL) effect.

GRAFT-VERSUS-LEUKAEMIA ACTIVITY IN MAN

There is no totally satisfactory definition of GVL, but for operational purposes one might define GVL as any antileukaemic influence of the graft that is independent of the chemotherapy or chemoradiotherapy usually employed for cytoreduction and immunosuppression pre-transplant. If one accepts this definition then GVL probably plays a more important role in the cure of CML after BMT than in the acute leukaemias[54]. This conclusion derives in part from the observation that the actuarial relapse rate in patients who receive unmanipulated donor marrow is lower in CML transplanted in chronic phase than in acute myeloid leukaemia transplanted in first complete remission (10% versus 20%), whereas in transplants using T-depleted donor marrow cells, the reverse is true - the relapse rate is substantially higher in CML than in acute myeloid leukaemia (>60% versus 30%)[35,54,55].

More recently it has been shown that different methods of T cell depletion were associated with different frequencies of relapse. Use of antibodies reactive with T and NK cells or of broad specificity like Campath-1 increased the risk of relapse when compared to methods which only remove T cells from the donor marrow[35]. These data suggest that in some poorly defined manner preservation of NK cells in the donor marrow reduces the incidence of leukaemic relapse.

Attempts have been made to quantify this in vivo GVL effect in man. Not surprisingly they have produced widely varying results. One report suggested that GVL activity may represent 50% - 150% of the magnitude of the anti-leukaemia effect of conditioning therapy[56], while in another it was calculated to account for only the equivalent of a one-log cell kill[57].

It is assumed that lymphocytes in the donor marrow are capable of mediating GVHD on one hand and a GVL effect on the other[58]. Clinical data are inconsistent with the notion that the only component of the transplant procedure critical for cure is the chemoradiotherapy and that the graft functions only as haemopoietic rescue. Instead it seems likely that T cells in the donor marrow contribute to cure by more than one mechanism[59]. Evidence is accumulating that the GVL effect in man has at least 3 components: 1. Antileukaemia activity associated with clinically evident GVHD[60]; 2. Antileukaemia activity that can operate in the absence of clinically overt GVHD but is mediated by allogeneic T cells[35]; and 3. Antileukaemia activity mediated by MHC unrestricted NK cells[35].

EXPERIMENTAL GRAFT-VERSUS-LEUKAEMIA ACTIVITY

It could be argued that the increase in leukaemic relapse associated with T cell depletion should not have been unexpected and could have been predicted from research in animals. In animal

model systems GVHD may exert an anti-leukaemic effect[61]. In animals at least some of the cells responsible for GVL reactions are clearly distinct from those causing GVHD[61,62], but whether similar subpopulations are distinguishable in humans is currently unknown. However even in rodents it is not clear whether the same or different effector cells are responsible for the GVHD and the GVL effects[63]. In vivo GVL and GVHD reactivity are probably mediated by separate as well as overlapping populations[61].

Of considerable interest is the recent demonstration that the administration of GVL specific T cell clones to leukaemic mice can cause a significant GVL effect without evidence of GVHD[64]. Cytotoxic activity does not appear to be the sole or most important functional property of cells contributing to GVHD, but cytotoxic T cells are important effectors of the GVL reaction. Lymphokine production or recruitment of other cells may be of equal or greater importance in GVHD. Currently our knowledge is incomplete. Some T cells found in autologous BMT recipients may even alter their functional activity in response to changing environmental conditions. In one study Class II antigen-specific T cells in murine autologous BMT hosts appeared to be nonlytic suppressor cells[65]; however a subsequent study revealed that these CD4+ T cells reverted to a lytic phenotype in the absence of interleukin-2 (IL-2)[55].

If we cannot predictably modulate T cells to mediate a GVL effect,

given the complexity of their interactions and regulation, we may be able to exploit their effector molecules and/or the lymphokines they produce through recombinant DNA technology. Lymphokine activated killer (LAK) cells offer an alternative and promising source of GVL effector cells.

BIOLOGICAL RESPONSE MODIFIERS

The concept of enlisting the immune system in the treatment of malignancy is not new. The recognition that the immune system was responsible for allograft rejection suggested that it might be possible to elicit immune-mediated tumour rejection. Administration of sublethal numbers of syngeneic tumour cells to a variety of experimental animals led to the development of cells capable of rejecting a challenge by an otherwise lethal inoculum of the same tumour cells[66]. Similarly, transfer of lymphoid cells from an animal challenged by the tumour to a syngeneic tumour-bearing host could result in tumour regression. Additionally, the possibility that the immune system was involved in the early elimination of spontaneous malignancies (immune surveillance) has been supported by previous work[67].

The role of the immune system in controlling malignancies in man has been far more difficult to define. In 1969 Mathe et al. demonstrated that Bacillus Calmette Guerin (BCG) could prolong disease-free survival in childhood acute lymphoblastic leukaemia

following cytoreductive chemotherapy[68]. When administered intralesionally, BCG was shown to induce regressions of melanoma, and when administered systemically, was reported to prolong disease free survival in patients with resected malignant melanoma[69,70]. Many anecdotal reports of the antitumour effects of BCG appeared, but no controlled trials demonstrating significant antitumour effects have been published[71]. Other nonspecific immunostimulants (e.g. Corynebacterium parvum, levamisole) were evaluated, but the limited number of responses at a time of rapid advances in the area of chemotherapy led to a general loss of interest in this field[72].

In the 1970s leukocyte interferon, and later recombinant alpha interferon (aIFN), became available for laboratory and clinical evaluation[73]. Interferon was originally discovered in 1957 and described as a protein which "interfered" with viral growth[74]. It soon became apparent that there were several types of interferon and that they had many and varied biological properties. aIFN is reported to have a direct antitumour effect and an indirect action by enhancing NK activity. Early reports of tumour regression in response to leukocyte interferon were not always substantiated, but renal cell carcinoma, melanoma, lymphoma, hairy cell leukaemia and CML were reported to respond to aIFN[25,75].

The availability of an effective recombinant immunomodulator rekindled interest in immunotherapy and generated the term "biological response modifiers" which was defined as "those agents

or approaches that modify the host's biological response to tumour cells with resultant therapeutic effects"[76]. The cytokine which has proved to be of most interest in promoting antitumour activity in recent years is IL-2.

INTERLEUKIN-2

In 1965 Kasakura and Lowenstein and Gordon and MacLean independently demonstrated the release of a soluble mitogenic factor in mixed leukocyte cultures[77,78]. It is conceivable that the mitogenic activity observed was mainly due to the release of and the response to the lymphokine, which is now called IL-2. This molecule was "rediscovered" in 1976 and termed T cell growth factor for its ability to sustain the continuous proliferation of T lymphocytes[79]. In 1979, it was renamed as IL-2 by an international committee[80]. The IL-2 gene is located in the midportion of the long arm of the human chromosome 4, at band q26-28[81]. It exists as a single copy per haploid human genome, and is 5040 base pairs long and consists of 4 exons[82].

IL-2 is the most important cytokine known so far to activate NK cells. This 15 kd glycoprotein produced by activated T helper cells is an inducer of proliferation and functional activity of T-cells, NK cells, macrophages and B cells[83]. To exert its biological effects IL-2 must interact with a specific membrane IL-2 receptor (IL-2R). However there was an apparent discrepancy until recently,

since IL-2 responsive NK cells lacked the IL-2R Tac antigen (CD25), now known as the 55 kd alpha chain. Identification of the 75 kd IL-2R beta chain on NK cells has clarified this question and also contributed to the understanding of the requirements of high concentrations of IL-2 for NK activation[84]. The affinity of the alpha chain expressed on resting NK cells is about 100-fold less than that of the alpha/beta heterodimer high affinity IL-2R expressed on activated T-cells[85]. This has fuelled speculation that NK cells might serve as nonspecific auxiliary killer cells induced by immunocompetent T-cells at remote sites of activation with locally compartmentalised high concentrations of IL-2.

HUMAN CYTOTOXIC LYMPHOCYTES

Human cytotoxic lymphocytes can be divided into two major subgroups: (1) cytotoxic T cells (CTL) and (2) NK cells. Thymus derived CTL are defined by the expression of a specific recognition structure - the T cell receptor (TCR) and by rearrangement of the T cell receptor alpha/beta chain or gamma/delta chain genes[86]. Functionally they are characterised by antigen-specific cytotoxicity that is generally restricted by the major histocompatibility complex (MHC) and can be blocked by anticlonotypic monoclonal antibodies[87,88]. The bone marrow derived NK population is less precisely characterized, though comprising about 10% of peripheral blood lymphocytes (PBL)[89]. The TCR genes are in germline configuration and CD3 expression is absent, while

anti-CD56 and anti-CD16 monoclonal antibodies recognise these large granular lymphocytes (LGL)[89-91]. Their cytotoxic function is MHC unrestricted and directed against a broad range of target cells without prior sensitisation or activation[92].

The characteristics of these two populations of cytotoxic effector cells, however, seemed to be inconsistent with experimental data from IL-2 activated lymphocytes. These cells were reported to exhibit a unique phenotype as well as a potent lytic activity against NK-resistant tumour targets. Therefore a separate lineage of cytotoxic lymphocytes was proposed, the LAK cell lineage[93-95].

LYMPHOKINE ACTIVATED KILLER CELLS

Subsequent examination of these findings demonstrated that known cell types accounted for this LAK activity. It is now apparent that the term LAK cell is a definition of function rather than phenotype. Culture of PBL in the presence of high concentrations of IL-2 favours proliferation of CD3- lymphocytes with broad killer activity, while at lower doses an expansion of highly antigen specific CD3+ T cells is observed[96]. A small subset of CTL are capable of mediating MHC unrestricted killing, these cells often coexpressing the CD3 and CD56 antigens, but most of the MHC unrestricted killing of IL-2 induced PBL is mediated by LAK cells with the phenotype of activated NK cells[97].

The term LAK cells was introduced by Grimm and colleagues in

1982[93]. They reported that PBL stimulated by IL-2 were able to lyse autologous fresh tumour cells and a variety of tumour cell lines that were resistant to natural killer (NK) cell mediated cytotoxicity[98]. Subsequent experiments with adoptively transferred in vitro activated PBL in tumour-bearing mice supported the concept of antitumour activity in vivo[99]. Based on these experiments, studies in tumour patients with a combination therapy of high dose IL-2 and LAK cells were initiated in 1984[100]. Objective response rates of more than 25% in chemotherapy resistant tumours including renal cell carcinoma and malignant melanoma were reported, encouraging further clinical trials[101]. The initial enthusiasm was blunted because of considerable toxicity of IL-2/LAK treatment and short duration of most clinical responses in patients with advanced solid tumours. One way of avoiding treatment failure might be to use IL-2/LAK therapy in patients in good physical condition and with a minimal residual tumour load. Patients in clinical remission of disease following T cell depleted BMT for leukaemia might be ideal candidates for such a treatment strategy.

MECHANISMS OF TARGET RECOGNITION AND KILLING

Several membrane molecules have been implicated in NK/LAK cell activation and function. By means of Fc receptor (CD16) expression NK cells are involved in antibody dependent cellular cytotoxicity. Capping of the Fc receptor induces mRNA and protein synthesis in NK cells as well as triggering the lytic mechanisms[102]. However

soluble anti-CD16 monoclonal antibodies do not inhibit the killing of the standard NK target K562, a CML-derived cell line[103]. Triggering of NK activity has also been achieved with monoclonal antibodies against lymphocyte function associated antigens LFA-1 (CD11a/CD18 as alpha/beta subunits) and LFA-2 (CD2), suggesting that the intercellular adhesion molecule-1 (ICAM-1) and LFA-3 may be crucial target structures[104,105]. Another member of the LFA family, the complement receptor-3 (CR3) (CD11b/CD18 as alpha/beta subunit) is used by NK cells in complement mediated lysis via interaction with cell bound C3bi[106]. At the present time it appears that NK cells do not have a single predominant recognition receptor analogous to the TCR on T-cells. Rather they may interact with target cells using several surface molecules and their corresponding ligands. If this is correct one could speculate that lytic reactions could still develop even when a whole set of distinct receptor ligand systems is not available in a given effector-target cell interaction.

A crucial mediator of cytotoxic function is perforin as evidenced by experiments with anti-perforin monoclonal antibodies that block NK-dependent lysis of K562. Perforin is a homologue of the late complement components (C5-9), known as the membrane attack complex[107]. By assembling in a multimeric form, perforin binds to phosphorylcholine residues of the target cell membrane and induces pore-like membrane damage leading to osmotic cell lysis[108].

Additional components such as the cytokines tumour necrosis factor (TNF) or gamma interferon may also be involved in NK cell cytotoxic function[109].

ROLE OF NK CELLS IN DEFENCE AGAINST LEUKAEMIA

Abnormally low NK activity is associated with an increased incidence of malignant disease, especially leukaemia. This is well evidenced by patients with Chediak-Higashi syndrome, X-linked lymphoproliferative or combined immune deficiency syndrome, Sjogren's syndrome, and kidney allograft recipients with drug induced long term NK deficiency[110]. It is possible that in such patients the increased incidence of leukaemia is secondary to a primary defect in immune surveillance.

On the other hand, in previously healthy patients presenting with de novo acute leukaemia or CML the mechanism underlying the NK defect remains poorly characterised but there is evidence that the leukaemia itself may inhibit NK function. In patients with CML the NK cells are not part of the malignant Ph-positive clone[111]. At diagnosis patients with CML or acute leukaemia have deficient NK activity manifested by impaired tumour binding and lytic activity[112]. This defect in NK activity is, however, reversible by either incubating the NK cells in IL-2[112] or by treating the acute leukaemia patients with chemotherapy and achieving a clinical remission[113]. In CML the blast cells have IL-2 receptors which are

not present on their normal counterparts although IL-2 does not increase their proliferation[114,115]. Additionally patients with CML in chronic phase have increased circulating levels of soluble IL-2R; the levels rise further when the disease progresses to blast crisis[115]. The binding of IL-2 to its soluble IL-2R or CML blasts could provide a mechanism for "mopping up" circulating IL-2 and thereby preventing activation of the NK cells.

IMMUNE RECONSTITUTION FOLLOWING BMT

The recovery of a functional immune system following BMT is essential for the long term success of this treatment. The rate and degree of immunological recovery are influenced by a variety of factors including the type of GVHD prophylaxis and the severity and treatment of clinical GVHD[116-119]. Selective or nonselective depletion of T-cells from the transplanted marrow may obviate the need for GVHD therapies which are immunosuppressive but may be associated with the removal of functionally mature cells from the marrow graft. Some studies have suggested that immune compromise may result from a slower rate of engraftment of myeloid and/or lymphoid cells as well as from removal of immunocompetent donor derived cells transfused with the marrow graft in recipients of T cell depleted grafts[120-122]. Other studies in murine and in human systems have suggested that haemopoietic and immunological reconstitution in recipients of T cell depleted marrow is not impaired[120,123,124]. Treatment of bone marrow with monoclonal

antibodies such as Campath-1 removes not only T cells but also functionally mature B cells and NK cells[47,125,126], making it unlikely that either cellular or humoral donor immunity can be directly transferred with the marrow graft. The development of a donor derived immune system in recipients of marrow depleted of lymphocytes by this method therefore probably occurs from precursors of immunocompetent cells within the marrow graft.

NK cells are among the first lymphoid cells to recover after allogeneic BMT[127,128]. If the donor marrow has been depleted of T cells these regenerating cytotoxic cells are activated, spontaneously producing IL-2, gamma interferon and TNF[129-131] and behaving like cells with LAK activity with the ability to kill MHC nonidentical virus-infected and malignant target cells not susceptible to NK cells[127,132,133]. They respond to exogenous IL-2 with a further enhancement of cytokine production and a further increase in cytotoxic effector function[129,131,134]. The mechanisms by which these cells are generated is not clear but it is known that they also appear after autologous BMT but not after chemotherapy for haematological malignancy[135]; therefore induction does not require either alloreactivity between donor and host or removal of T cells from the donor graft. The appearance of NK cells with LAK activity post-BMT probably reflects the immune dysregulation that follows immune reconstitution of the host, treated with ablative chemotherapy and total body irradiation, with an incomplete "immune network" derived initially only from cells present in the

peripheral blood and marrow compartments. Immune regulation appears to be restored 3 to 4 months after BMT, since activation of cytotoxic effector cells has by then disappeared[127,129,132].

In contrast, the reconstitution of the T cells and B cells occurs over a longer period of time. B cells first appear within 6 weeks of the transplant but immunoglobulin production does not reach normal levels until 7 to 9 months following conventional BMT and 13 to 15 months following T cell depleted BMT[119]. The recovery of the T-cell populations is associated with a particularly low number of CD4+ helper cells in the first 6 months post-BMT, especially in recipients of T cell depleted marrow[119,126]. When this is coupled with a high of normal number of CD8+ cells it results in CD4:CD8 ratios that are below normal[128]. It has been suggested that the absolute deficiency of IL-2 producing CD4+ helper cells in T cell depleted BMT could result in immune dysregulation with loss of GVL activity with a resultant increase in leukaemic relapse[55].

These findings have prompted some workers to consider enhancing the potential GVL effect with biological response modifiers such as IL-2. It has been shown that IL-2 not only enhances LAK cell activity in vitro following BMT but also in vivo and that IL-2 infusions may be associated with secondary production of gamma interferon and TNF which may contribute to the antileukaemic activity[136,137].

SCOPE OF THE THESIS

The above studies prompted me to consider methods of enhancing GVL activity following BMT for CML. The aims of the work were therefore:

1. to determine whether GVL activity was inducible in vitro;

2. to phenotype the effector cell populations;

3. to assess whether any identifiable GVL activity was directed towards committed nondividing myeloid cells or clonogenic leukaemic progenitor cells;

4. to compare the effects of the LAK cells on both normal marrow and leukaemic cells;

5. to try to separate GVL activity from GVHD; and

6. to determine the origin and activity of adherent LAK (ALAK) cells in patients who relapsed post-BMT.

All studies were performed in vitro using peripheral blood mononuclear cells (PBMC) from either the donors pretransplant or the recipients post-BMT. Chapter 2 describes the effect of short term exposure of recipients' PBMC to IL-2 and aIFN and describes

their effect on NK and LAK activity as assessed in a chromium release assay system. Chapter 3 documents the NK, LAK and antileukaemic activity generated after 7 day incubation of the PBMC with IL-2 using a chromium release assay. In addition the phenotype of the effector cells, the contribution of the NK and T cell components to cytotoxic activity and the influence of pre- and post-BMT clinical parameters. Chapter 4 assesses the antileukemic activity of donor and recipient PBMC incubated with IL-2 for 7 days using a clonogenic assay. The influence of direct cell-cell contact and the differential killing of leukaemic and normal target cells is studied. In Chapter 5 I asked whether the ALAK cells of patients with CML who have relapsed post-BMT are of donor or recipient origin, whether they are part of the malignant clone and measure their antileukaemic activity in both cytotoxic and clonogenic assays.

CHAPTER 2

INDUCTION OF IN VITRO LAK ACTIVITY FOLLOWING BONE MARROW TRANSPLANTATION FOR CHRONIC MYELOID LEUKAEMIA: USE OF SHORT TERM INCUBATION WITH INTERLEUKIN-2 AND ALPHA INTERFERON

INTRODUCTION

The increased incidence of leukaemic relapse associated with T cell depletion of the donor marrow in allogeneic BMT recipients, initially reported in 1986[45], provided compelling evidence for the existence of a GVL effect in man. Although T cells were known to mediate GVL activity in these patients[60], it was thought that the NK population could also mediated antileukaemic effects[133]. Evidence from animals and man have demonstrated that NK cells can directly kill tumour cells and tumour cell lines[138]. NK cell function recovers rapidly after BMT and it has been suggested that augmentation of NK cell activity in the early post-transplant period with the recently available recombinant cytokines might provide a mechanism for enhancing GVL and therefore reducing the increase in leukaemic relapse associated with T cell depletion[127,134]. The cells responsible for this NK and LAK activity have been reported to be CD3- large granular lymphocytes[97,112].

The cytokines that were thought to be of most interest in CML were aIFN and IL-2. aIFN is effective in controlling the white cell count in CML and unlike conventional chemotherapeutic agents may lead to a reduction or even to the disappearance of the Ph chromosome in the bone marrow metaphases of a proportion of patients[25]. Although much is known about the mechanism of action of aIFN in experimental tumours[139] the basis of its beneficial effects in CML have not been fully characterised. It has been

reported that aIFN can enhance in vitro NK activity following BMT[140] and a similar effect was also observed using in vitro IL-2[132,134]. It seemed reasonable to postulate that aIFN- or IL-2-enhanced NK activity could be the basis of an in vitro GVL effect.

I therefore decided to investigate the in vitro effect of recombinant aIFN and IL-2 on the peripheral blood mononuclear cells of BMT recipients transplanted for CML using not only the standard cell lines but also the patients own pretransplant leukaemia as targets.

MATERIALS AND METHODS

STUDY POPULATION

Thirty-three patients were studied using IL-2 and 25 were studied using aIFN. Patients gave informed consent to being studied according to guidelines laid down by the Hammersmith Hospital ethical committee. All were in complete haematological and cytogenetic remission following BMT for CML having received allogeneic marrow from an HLA identical sibling donor. Pretransplant all patients were conditioned with cyclophosphamide (60 mg/kg/day x 2) and total body irradiation (6 x 2 Gy). Some patients also received additional chemotherapy, total lymphoid irradiation or splenic irradiation. GVHD prophylaxis was with either T-cell depletion of the donor marrow with the rat monoclonal

antibody Campath-1 or post-graft immunosuppression with cyclosporin with or without methotrexate. Patients were studied at intervals ranging from 4 weeks to 2 years post-BMT.

GENERATION OF CYTOKINE ACTIVATED CELLS

Peripheral blood was collected from the recipient following the transplant and PBMC were prepared by centrifugation on Lymphoprep (Nyegaard, Oslo). The low density cells were collected and washed twice with Ca^{2+}, Mg^{2+} free Hanks' balanced salt solution (HBSS) and resuspended in RPMI 1640 supplemented with 2 g/l sodium bicarbonate, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mmol/l L-glutamine and 10% heat-inactivated human AB serum (RPMI-AB).

For generation of cytokine activated cells, PBMC at a concentration of 1 - 2 x 10^6/ml were incubated for 18 hours in 5% CO_2 at 37°C in RPMI-AB alone or in RPMI-AB containing either 500 U/ml recombinant IL 2 (kind gift of Cetus, Emeryville, CA) or 500 U/ml recombinant aIFN 2a (kind gift of Roche, Welwyn Garden City, England). The cells were tested for cytotoxic activity after washing (see below).

TARGET CELLS

Target cells were (1) the NK-sensitive cell line K562[141], (2) an NK-resistant LAK-sensitive Epstein-Barr virus transformed B lymphoblastoid cell line (LCL)[142], (3) cryopreserved leukaemia cells

from the transplant recipients. The cryopreserved CML cells were thawed for 1 minute at 37°C in a water bath, diluted in HBSS and centrifuged on Lymphoprep. The low density cells were then collected, washed twice in HBSS and resuspended in RPMI-AB. All cells were labelled by incubation with 250 uCi ^{51}Cr (Na_2CrO_4) (Amersham, England) for 60 minutes. Following incubation the labelled cells were washed x3 in HBSS and resuspended in RPMI-AB.

CYTOTOXICITY ASSAY

Details of these assays have been described previously[127]. The target cell suspension at 5 x 10^4 cells/ml was dispensed in 100 ul volumes into U-shaped microtest plate wells. The effector cells were then added in a series of 5 different effector:target ratios, each in triplicate. The cells were incubated for 4 hours, after which half the supernatant was removed and ^{51}Cr release quantitated on a LKB Wallac 1260 Multigamma counter (Wallac, Turku, Finland). The percentage specific lysis for each target was calculated as follows:

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

where the maximum release was that obtained from target cells exposed to 1% Triton X-100 (New England Nuclear) and the spontaneous release was that observed when the target cells were incubated in RPMI-AB[143]. The spontaneous release was always less than 10% for the cell line targets and between 15 - 25% for the

cryopreserved CML targets. Cytotoxic activity was considered positive when the value of experimental ^{51}Cr release was significantly above that of spontaneous ^{51}Cr release according to the two-tailed Student's t-test.

RESULTS

IN VITRO RESPONSE TO aIFN

In the ^{51}Cr release assay effector cells from the 25 recipients always caused significant lysis of the NK-sensitive cell line K562. The data in Fig. 1 show the mean specific lysis $\pm$ one standard error of K562 cells. Incubation of the effectors in aIFN for 18 hours increased cell lysis ($p < 0.05$) compared to control effectors in the absence of aIFN. The addition of aIFN did not however increase lysis of the NK-resistant LAK-sensitive LCL target (Fig. 2). In 8 separate assays aIFN treated effectors failed to cause significant lysis of the recipients' leukaemia cells (data not shown).

IN VITRO RESPONSE TO IL-2

Effectors incubated in IL-2 always caused significant lysis of both the NK-sensitive K562 and the NK-resistant LCL cell lines. Culture of effectors in IL-2 caused a significant increase in the lysis of not only the K562 cells (Fig. 3) but also of the LCL cells (Fig.

4) when compared to controls. Although both NK and LAK killing was enhanced with IL-2, the effector cells again failed to cause significant killing of recipient CML cells in 10 separate experiments (data not shown).

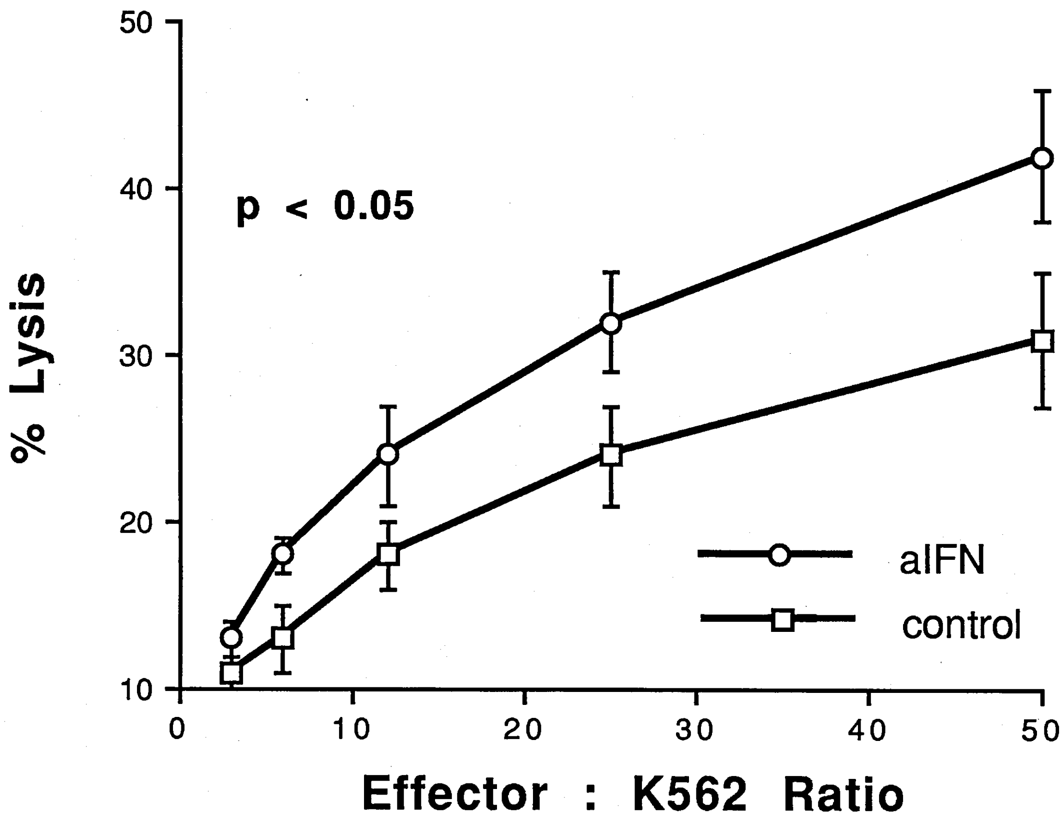


Figure 1. Results from 25 patients whose effector cells were added to K562 cells at E:T ratios of 50, 25, 12, 6 and 3 to 1. Each E:T ratio was set up in triplicate and the points plotted are the mean $\pm$ one standard error ^{51}Cr release. The graph shows dose-dependent killing of target cells with increased ($p < 0.05$) lytic activity seen in the effectors pretreated with aIFN.

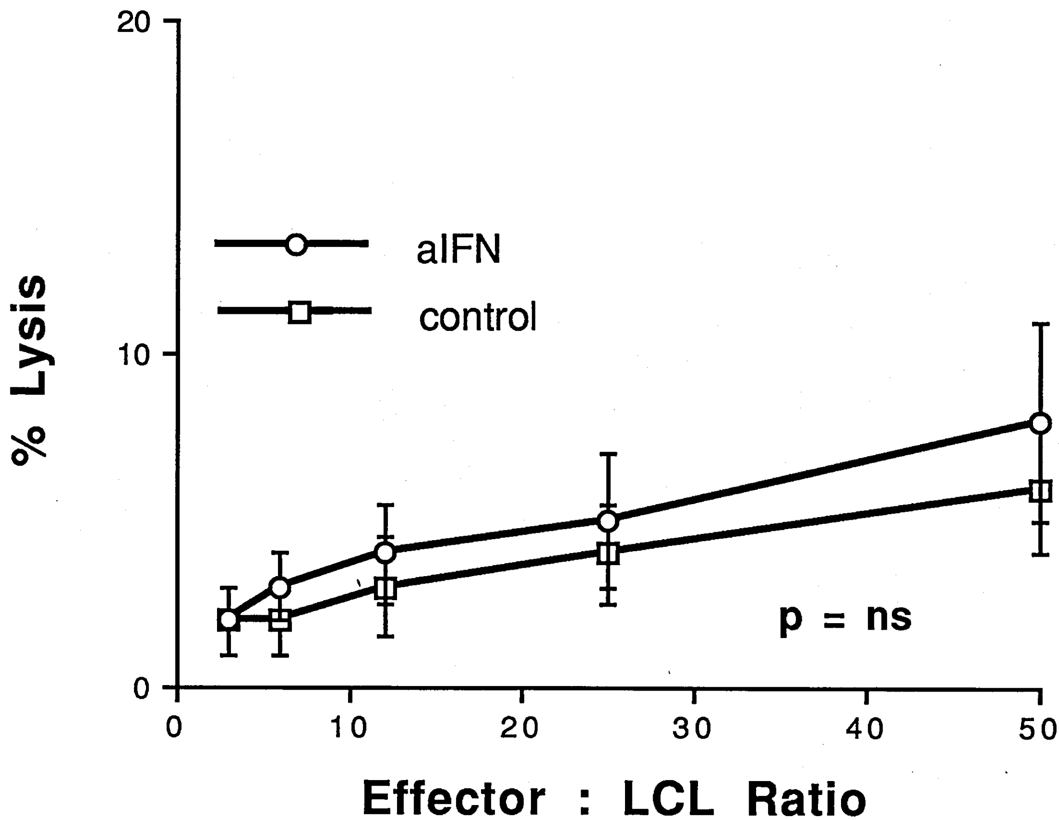


Figure 2. Results from 25 patients whose effector cells were added to LCL cells at E:T ratios of 50, 25, 12, 6 and 3 to 1. Each E:T ratio was set up in triplicate and the points plotted are the mean $\pm$ one standard error ^{51}Cr release. The graph shows poor killing of target cells by both control effectors and in effectors pretreated with aIFN.

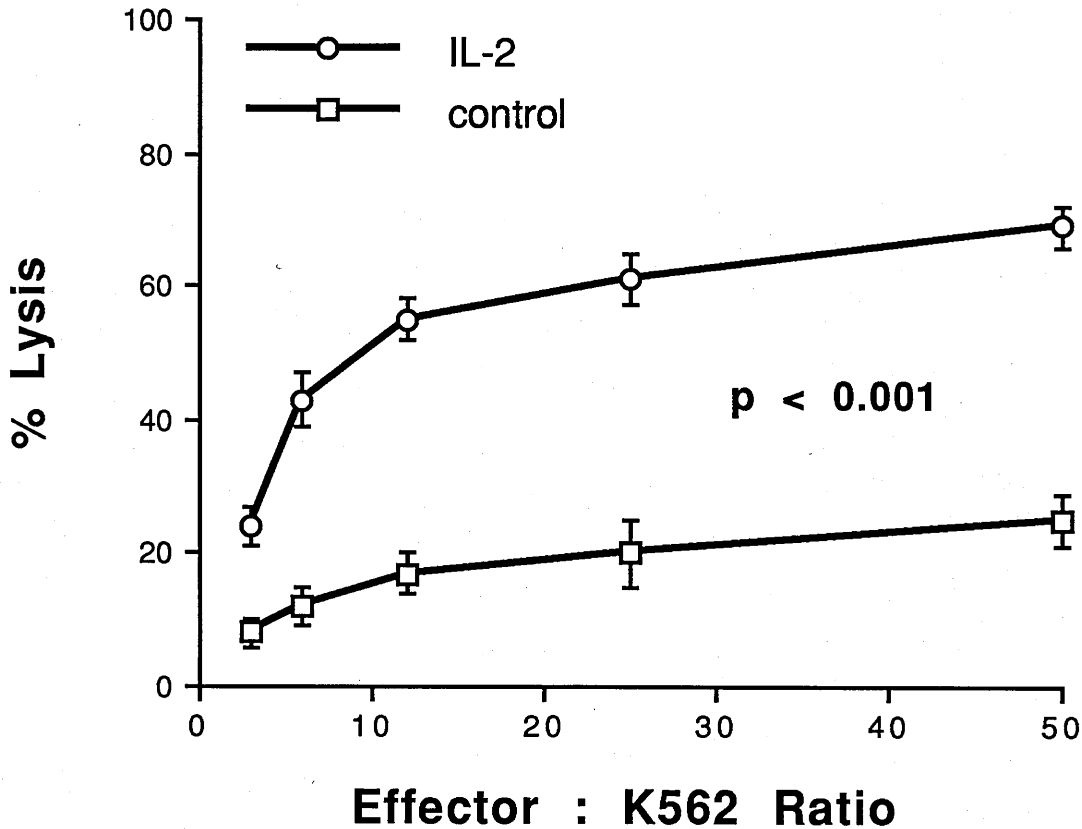


Figure 3. Results from 33 patients whose effector cells were added to K562 cells at E:T ratios of 50, 25, 12, 6 and 3 to 1. Each E:T ratio was set up in triplicate and the points plotted are the mean ± one standard error ^{51}Cr release. The graph shows dose-dependent killing of target cells with increased ($p < 0.001$) lytic activity seen in the effectors pretreated with IL-2.

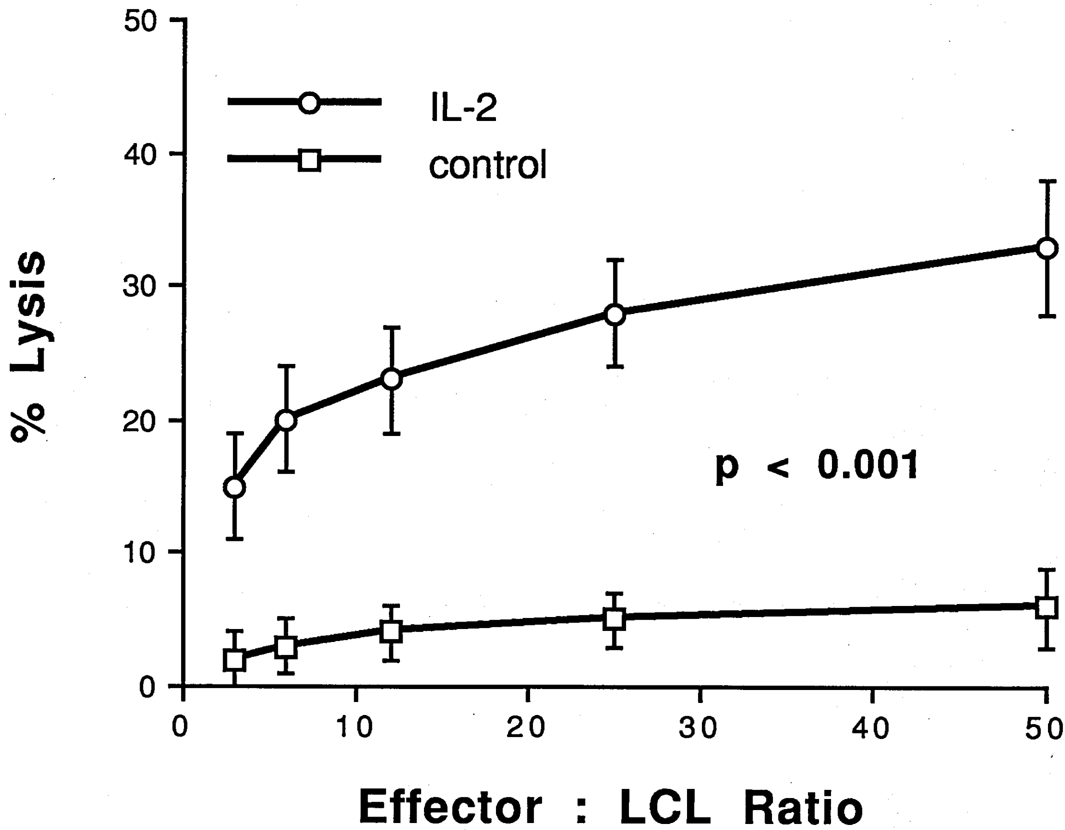


Figure 4. Results from 33 patients whose effector cells were added to LCL cells at E:T ratios of 50, 25, 12, 6 and 3 to 1. Each E:T ratio was set up in triplicate and the points plotted are the mean ± one standard error ^{51}Cr release. The graph shows dose-dependent killing of target cells with increased ($p < 0.001$) lytic activity seen in the effectors pretreated with IL-2 compared to poor lytic activity seen in the controls.

DISCUSSION

This study clearly indicates that although aIFN can enhance NK activity in BMT recipients, it does not increase LAK or leukaemic cell killing (Figs. 1 and 2). The results are in agreement with studies in hairy cell leukaemia, a disease highly responsive to in vivo aIFN, where it was shown that aIFN had no effect on LAK activity[144]. It is therefore unlikely that the administration of in vivo aIFN to T cell depleted BMT recipients would reduce the incidence of leukaemic relapse by a direct cytotoxic effect by the NK cells on the CML cells. Where aIFN has proved to be of clinical value post-BMT by reducing the percentage of Ph positive metaphases[145,146], it is probable that it mediates this effect via a different mechanism. Although the precise nature of its action in vivo remains obscure, evidence is accumulating that aIFN acts by inhibiting the proliferation of CML progenitors[147]. Recent studies have suggested that aIFN selectively inhibits late progenitors, a compartment which is greatly enlarged in patients with CML[148]. In doing so, a comparable inhibition of normal and CML progenitors will produce a greater absolute reduction of CML cells[149]. With prolonged in vivo administration of aIFN this effect could encourage the restoration of normal Ph negative haematopoiesis[149]. However most studies using aIFN in vitro have documented equivilant inhibition of both normal and leukaemic marrow progenitors and recent evidence has suggested that aIFN may enhance the attachment of CML progenitors to marrow stromal elements and thereby bring the

CML cells under the normal bone marrow homeostatic mechanisms[150].

Both NK and LAK activity are known to be increased following allogeneic BMT particularly in the first 3 months of immune reconstitution[119,126,128]. Studies performed with short term culture of lymphocytes in IL-2 (Figs. 3 and 4) indicated that NK and LAK activity can be further enhanced even at longer intervals from transplant. These findings suggest that although NK and LAK activity is supranormal in BMT recipients their lymphocytes are not maximally stimulated. Some workers have suggested that this increased LAK activity, as defined by increased killing of NK-resistant LAK-sensitive cell line targets, might be associated with a direct antileukaemic effect in transplant recipients[127]. The failure to cause significant lysis of the recipients' pretransplant CML implies that no direct comparison can be made between LAK and antileukaemic activity.

In summary short term incubation of lymphocytes from allogeneic BMT recipients with recombinant cytokines in vitro can increase NK and LAK activity but does not generate effectors capable of killing the patients' pretransplant CML cells. I therefore decided to study the effects of incubating the lymphocytes from transplant patients for a longer period (7 days) in IL-2 as this cytokine appeared to more efficacious than aIFN. These studies are described in Chapter 3.

CHAPTER 3

INDUCTION OF IN VITRO LAK ACTIVITY FOLLOWING BONE MARROW TRANSPLANTATION FOR CHRONIC MYELOID LEUKAEMIA: USE OF 7 DAY INCUBATION WITH INTERLEUKIN-2. CYTOTOXIC ASSAY.

INTRODUCTION

The failure of short term incubation of lymphocytes in IL-2 to produce killing of the patients' pretransplant leukaemic cells prompted me to investigate the use of a longer in vitro incubation of the PBMC with IL-2. This decision was based not so much on the hope that longer exposure to IL-2 might empirically produce LAK cells with more potent antileukaemic activity but rather on the results of the preliminary clinical trials in solid tumours. These studies had used LAK cells which had been generated by exposing leukapheresed PBMC to high concentrations of IL-2 for 5 - 7 days and had demonstrated objective regression of some advanced metastatic malignancies, particularly melanomas and bladder carcinomas[99-101].

I therefore designed studies to discover whether GVL activity could be induced in vitro following BMT for CML. As long term exposure to IL-2 could result in phenotypic changes in the effector cell population I set out to establish the phenotype of the effector cells. I wanted also to determine whether any such GVL effect was in theory separable from GVHD. I therefore undertook phenotypic and functional characterisation of IL-2 stimulated PBMC or LAK cells from recipients following BMT. This chapter describes an IL-2 inducible in vitro GVL effect mediated by MHC unrestricted cells.

MATERIALS AND METHODS

PATIENTS

Thirty-seven patients were studied in complete haematological and cytogenetic remission following BMT for CML. Twenty-six patients had received allogeneic marrow from HLA identical siblings, 7 allogeneic marrow from HLA matched unrelated donors and 4 syngeneic marrow from identical twin donors. Pretransplant all patients were conditioned with cyclophosphamide (60 mg/kg/day x 2) and total body irradiation (6 x 2 Gy). Some patients also received in vivo antilymphocyte monoclonal antibodies, additional chemotherapy, total lymphoid irradiation or splenic irradiation. GVHD prophylaxis consisted of T cell depletion of the donor marrow with the rat monoclonal antibody Campath-1 or post-graft immunosuppression with cyclosporin with or without methotrexate. Patients were studied at intervals ranging from 4 weeks to 10 years post-BMT. Each patient gave informed consent for these studies.

GENERATION OF LAK CELLS

Venous blood was collected from the recipient following the transplant and PBMC were prepared as described in Chapter 2. For generation of LAK cells, PBMC at a concentration of 1 - 2 x 10^6/ml were incubated for 7 days in 5% CO_2 at 37°C in RPMI-AB containing 500 U/ml recombinant IL-2. The cells were tested for cytotoxic

activity after washing (see below).

ELIMINATION OF T LYMPHOCYTE SUBPOPULATIONS

Washed LAK cells were incubated for 60 minutes on ice with an anti-CD3 monoclonal antibody (UCHT1, kindly provided by Dr Peter Beverley, ICRF, London) at saturating concentration[151]. The cells were washed and centrifuged, resuspended and incubated with sheep anti-mouse IgG coated magnetic beads (Dynal, Oslo) on ice for 40 min at a bead:cell ratio of 20:1. Following addition of 5 ml RPMI 1640 the resuspended preparation was placed in a magnetic field for 5 minutes. The cells in the supernatant were then withdrawn for use in the cytotoxicity assay.

SURFACE MARKER ANALYSIS

Phenotypic analysis was performed by indirect and two-colour direct immunofluorescence using standard techniques[152]. Samples were analyzed on a Becton Dickinson FACScan flow cytometer. Monoclonal antibodies used were purchased from Becton Dickinson (Mountain View, CA) and were directed against CD3 (Leu 4), CD4 (Leu 3), CD8 (Leu 2), CD16 (Leu 11) and CD56 (Leu 19) antigens.

TARGET CELLS

Target cells were (1) the NK-sensitive cell line K562, (2) an NK-

resistant LAK-sensitive Epstein-Barr virus transformed B lymphoblastoid cell line (LCL), and (3) cryopreserved leukaemia cells from the transplant recipients and from 37 different third party HLA-disparate patients with CML. Target cells were washed and labelled as described in Chapter 2.

CYTOTOXICITY ASSAY

Details of these assays have been described previously in Chapter 2. The percentage specific lysis for each target was calculated as follows:

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

Some results were expressed in lytic units defined as the number of effector cells/10^6 resulting in specific lysis of 60% of 5000 target cells[143]. The spontaneous release was always less than 10% for the cell line targets and between 15 - 25% for the cryopreserved CML targets. Cytotoxic activity was considered positive when the value of experimental ^{51}Cr release was significantly above that of spontaneous ^{51}Cr release according to the Student's t-test.

In cold target competition studies, unlabelled allogeneic normal bone marrow cells or allogeneic CML cells were mixed with ^{51}Cr labelled recipient CML cells at ratios of unlabelled (cold) to

labelled (hot) ranging from 1:1 to 20:1 before addition to the microtiter plates. Effector cells were added at a constant E:T ratio (50:1) to the various cold:hot (C:H) combinations, and the percentage of specific lysis was determined in the cytotoxicity assay described above. Results were expressed as the percentage of specific lysis at the indicated C:H ratios.

STATISTICAL METHODS

Differences in cytotoxic activity or phenotypic analysis were analyzed using the two tailed Student's t-test.

RESULTS

SURFACE MARKERS

The phenotype of the effector LAK cells is shown in Table 1. The predominant phenotype of the LAK cell population was CD56+, CD8+, CD4-, with a variable number of CD3+ cells. The CD8+ fraction was usually split between the CD3+/CD8^{bright} and CD3-/CD8^{dim} populations, with the CD8^{dim} cells coexpressing the CD56 antigen (Fig. 5). Following culture in IL-2 there was an increase in the expression of the CD56 antigen concurrent with a reduction in the expression of the CD16 antigen (Fig. 6) with the CD56 population being either CD16- or CD16^{dim} (Fig. 5). Although we observed an increasing number of CD3+ T-cells and a reduced number of CD56+ natural killer

cells with time from transplant, these changes were not statistically significant. Furthermore, there was no significant difference in the phenotype of the effectors which were able to lyse the recipients' leukaemic cells and those which were not killers (Table 1).

Table 1. Phenotype of LAK effectors from 29 patients. Results are shown for single and two colour fluorescence expressed as mean percent positive cells ± one standard deviation. Studies are subdivided on both time from BMT to time of assay and whether the patients' LAK cells were or were not able to kill their own pretransplant leukaemia.

Time from BMT/cytotoxicity	CD3	CD4	CD8	CD16	CD56	CD3/8	CD3/56	CD8/56	CD4/8	CD16/56
Less than 3 months (n = 8)	41±27	15±16	40±18	8±6	56±30	23±21	9±13	21±17	1±1	9±6
Less than 1 year (n = 13)	40±22	12±13	39±15	8±5	53±27	24±17	8±10	18±14	1±1	9±5
Greater than 1 year (n = 16)	52±23	10±8	50±17	12±9	46±19	35±20	12±6	15±8	2±2	12±9
Cytotoxic LAKs (n = 17)	47±25	13±13	43±19	8±4	46±24	31±21	9±9	15±12	1±1	8±4
Non-cytotoxic LAKs (n = 12)	47±22	9±6	48±13	14±10	53±20	32±19	11±8	19±9	2±2	14±10

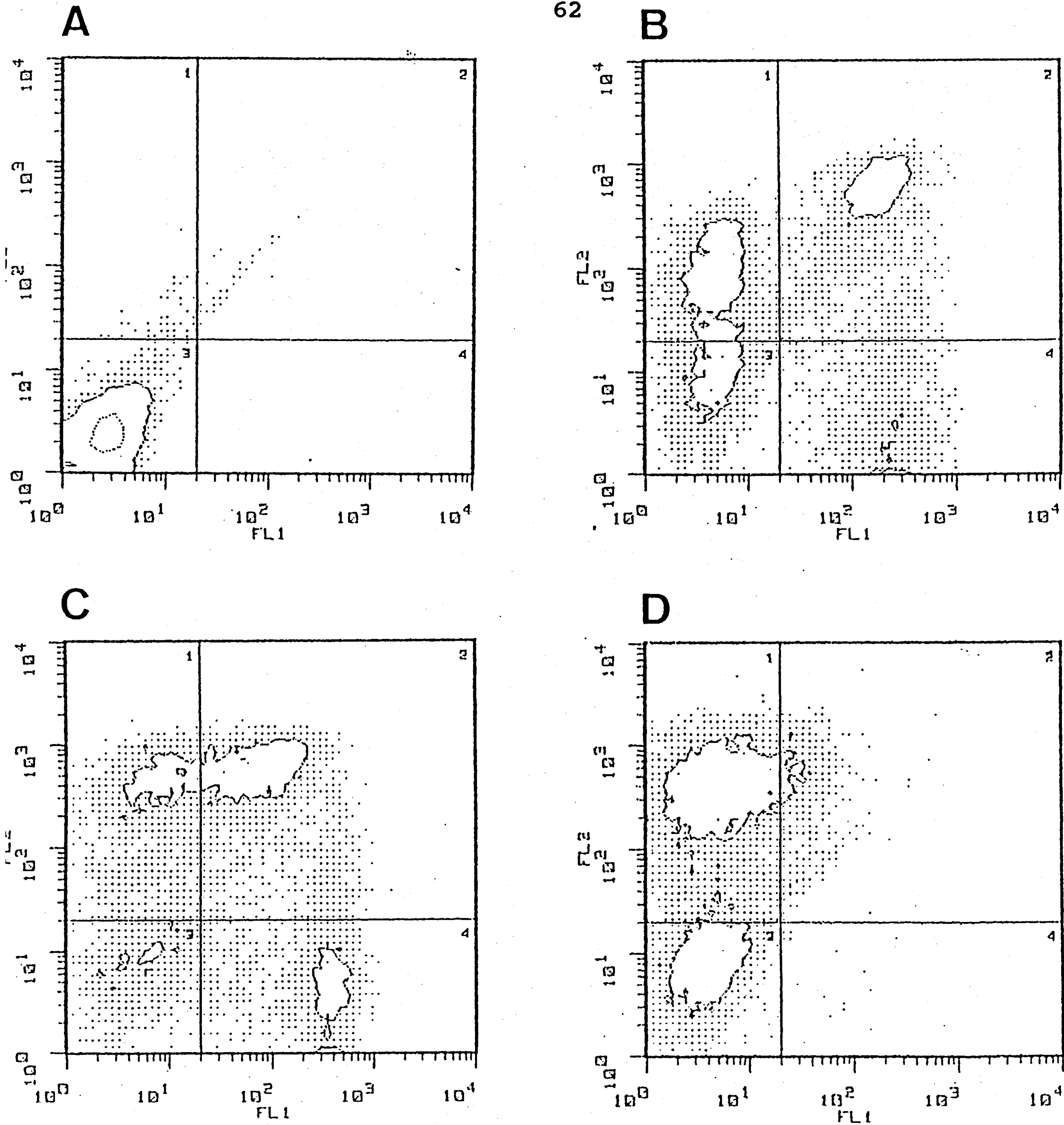


Figure 5. Flow cytometric analysis of day 7 LAK cells from a single patient. A. Histogram showing negative control staining with anti-mouse FITC and anti-mouse phycoerythrin (PE). B. Histogram showing: FL1 FITC anti-CD3; FL2 PE anti-CD8. The $CD8^{bright}$ population coexpress the CD3 receptor whereas the $CD8^{dim}$ cells are largely CD3-. C. Histogram showing: FL1 FITC anti-CD8; FL2 PE anti-Leu19 (CD56). The $CD8^{bright}$ cells are largely Leu19- whereas the $CD8^{dim}$ cells coexpress the Leu19 antigen. D. Histogram showing: FL1 FITC anti-CD16; FL2 PE anti-Leu19 (CD56). The Leu19+ cells are either CD16- or $CD16^{dim}$.

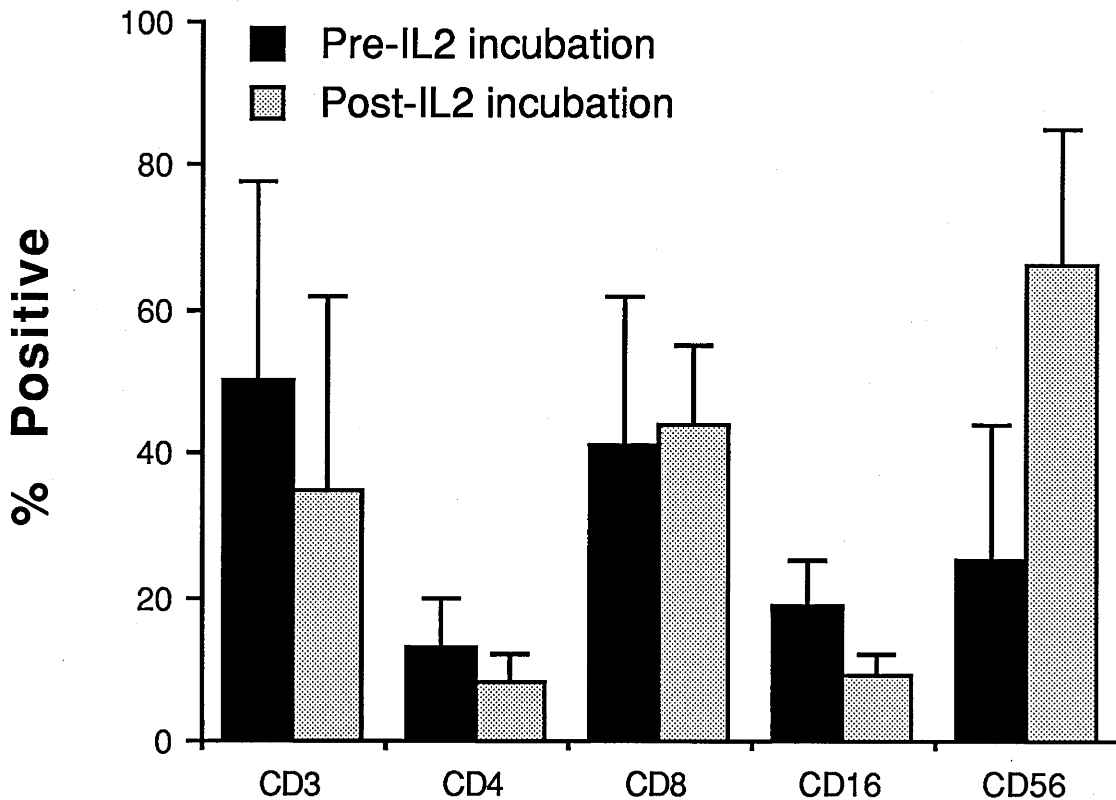


Figure 6. Surface marker analysis of peripheral blood mononuclear cells from 7 patients pre- and post-IL-2 incubation expressed as mean percentage $\pm$ one standard deviation. Following culture with IL-2 I saw a reduced number of T cells with an increased number of NK cells though these differences were not significant. There was a reduction ($p < 0.01$) in the proportion of CD56 cells which coexpressed the CD16 antigen following IL-2 incubation.

ACTIVITY OF EFFECTORS AGAINST CELL LINE TARGETS

The effectors generated after 7 days culture in IL-2 always caused significant lysis of the NK-sensitive cell line K562 and the NK-resistant LCL in 15 consecutive studies. However killing of the cell line targets did not predict killing of the patients' pretransplant leukaemia cells. Fig. 7 demonstrates the lytic activity against the NK-resistant LCL cell line targets of LAK cells from 7 patients where the effectors caused significant lysis of their pretransplant CML cells contrasted with the LAK cells from 8 patients which did not kill recipient leukaemic cells. There was no significant difference in the cell line lytic potential between LAK cells which were or were not CML lytic indicating that CML cells appear to be more resistant to lysis than the cell line targets. Fig. 7 also shows the lytic activity against the recipient CML cells is significantly ($p < 0.05$) less than that against the LCL cell line.

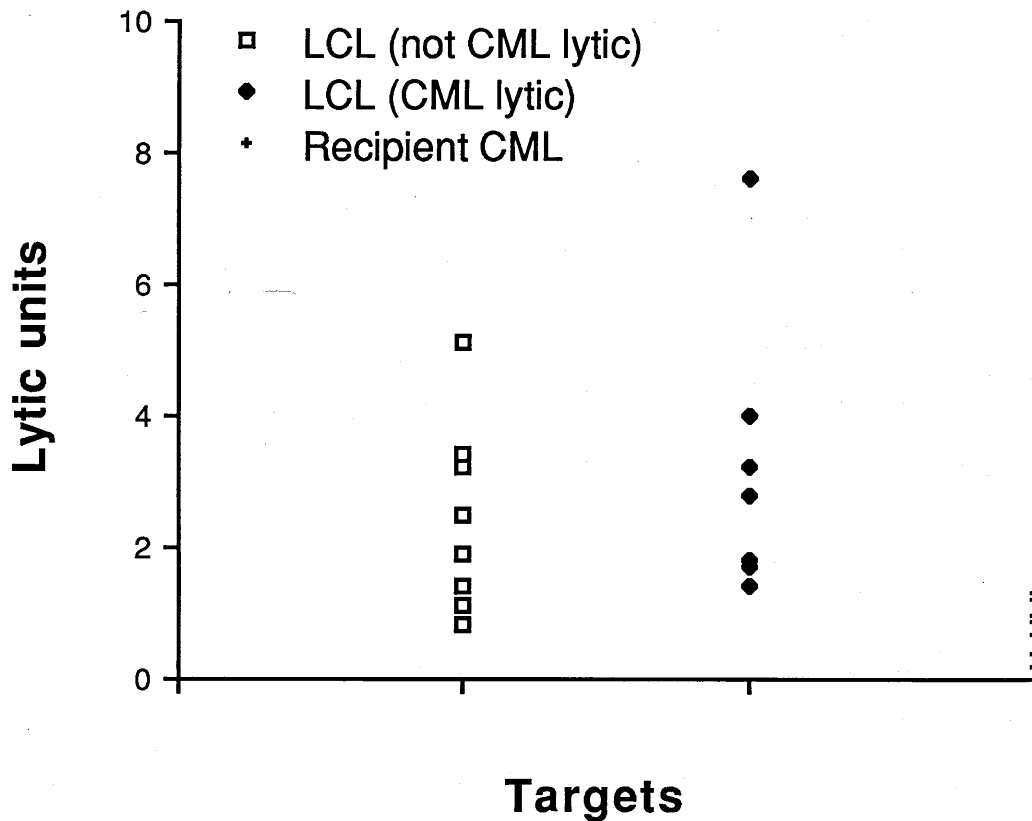


Figure 7. Lytic activity of LAK cells from 15 patients against the LCL cell line and recipient CML cells. All effectors resulted in significant ($p < 0.05$) lysis of the cell line target. The graph shows the LCL and CML lytic activity of 7 patients whose LAK cells demonstrated killing of their pretransplant leukaemic cells compared with 8 patients whose effectors failed to lyse their leukaemia. The data show no significant difference ($p = 0.84$) in the cell line lytic activity between those effectors which were or were not CML lytic indicating that failure to kill CML cells is due to target cell resistance to lysis. This point was reinforced when we compared the lytic activity against the LCL and CML targets in the 7 patients who showed significant lysis of the recipient leukaemia. Their LAK cells demonstrated significantly ($p < 0.05$) lower lytic activity for the CML cells.

CYTOTOXICITY OF EFFECTORS AGAINST CML CELLS

In the ^{51}Cr release assay LAK cells from 20 of 33 (61%) allogeneic and 2 of 4 syngeneic recipients killed recipient CML cells and in 22 of 37 (59%) cases also killed the HLA disparate CML cells. The data in Fig. 8 show the mean specific lysis for the leukaemic targets in the 22 positive assays. In 9 cases (2 syngeneic) where I demonstrated killing of the recipients' CML cells, the effector cells were depleted of CD3+ T-cells and assessed for GVL activity in the same experiment. In all experiments the T-depleted effectors lysed the recipients' and the 3rd party CML cells with a slight though not significant increase in specific lysis when compared to the non-depleted LAK cells (Fig. 9).

LAK CELL EFFICACY VERSUS LEUKAEMIC CELL SUSCEPTIBILITY

I analyzed the data to look for variability in both the LAK cell lytic potential and the leukaemic target cell susceptibility to killing among the 37 patients (Table 2). Patients were categorized into 4 groups. Patients in group 1 produced LAK cells with good lytic potential that killed more than one susceptible leukaemic target. Cells from patients in group 2 failed to lyse either target, but as these targets were not killed by effectors from any other patient, it cannot be determined whether this group represents poor effector lytic ability or resistant target cells. The LAK cells of the patients in group 3 were able to lyse 1 of the

2 CML targets. The targets which were resistant to lysis were not susceptible to killing from other effectors used in separate experiments. This suggests a differential sensitivity of the leukaemic cells to lysis in different patients. The LAK cells from patients in group 4 were unable to kill at least one leukaemic target which had been susceptible to lysis when tested with effectors from other patients indicating that the LAK cells from group 4 patients were less active. These data suggest that both effector and target cell factors are important in determining the outcome of an assay.

COLD TARGET COMPETITION EXPERIMENTS

The results of 6 separate experiments using cold allogeneic normal bone marrow cells and cold allogeneic CML cells mixed with hot recipient CML cells are shown in Figures 10A and 10B. In 3 studies both types of cold target blocked the killing of the recipient CML. In 2 experiments killing was not blocked with either cold target and in 1 study killing was blocked with the cold CML cells but not with the normal bone marrow.

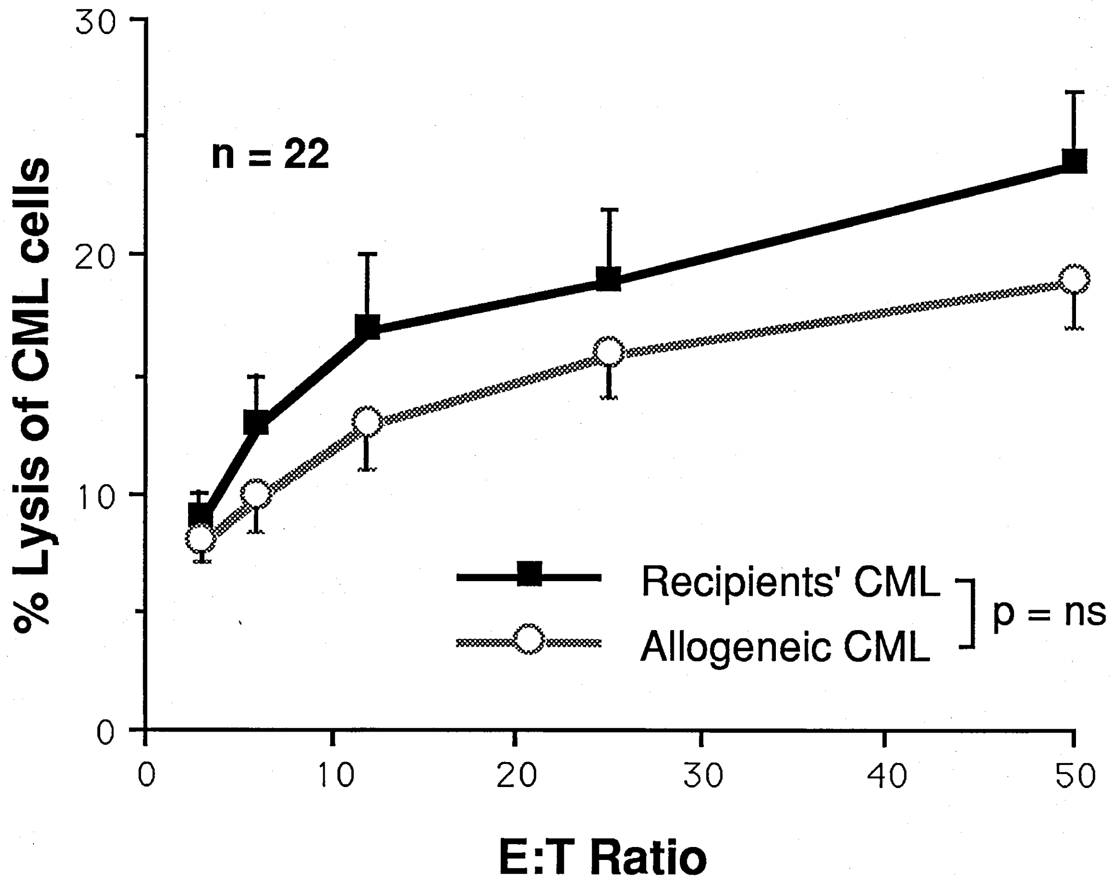


Figure 8. Results from all patients whose LAK cells demonstrated significant lysis of the CML targets. LAK cells were added to recipient CML and allogeneic CML cells at E:T ratios of 50, 25, 12, 6 and 3 to 1. Each E:T ratio was set up in triplicate and the points plotted are the mean ± one standard error ^{51}Cr release. The graph shows dose-dependent killing of both CML targets. There is no significant difference ($p = 0.37$) in the killing of the recipients' CML cells and the allogeneic CML cells according to the Student's t-test.

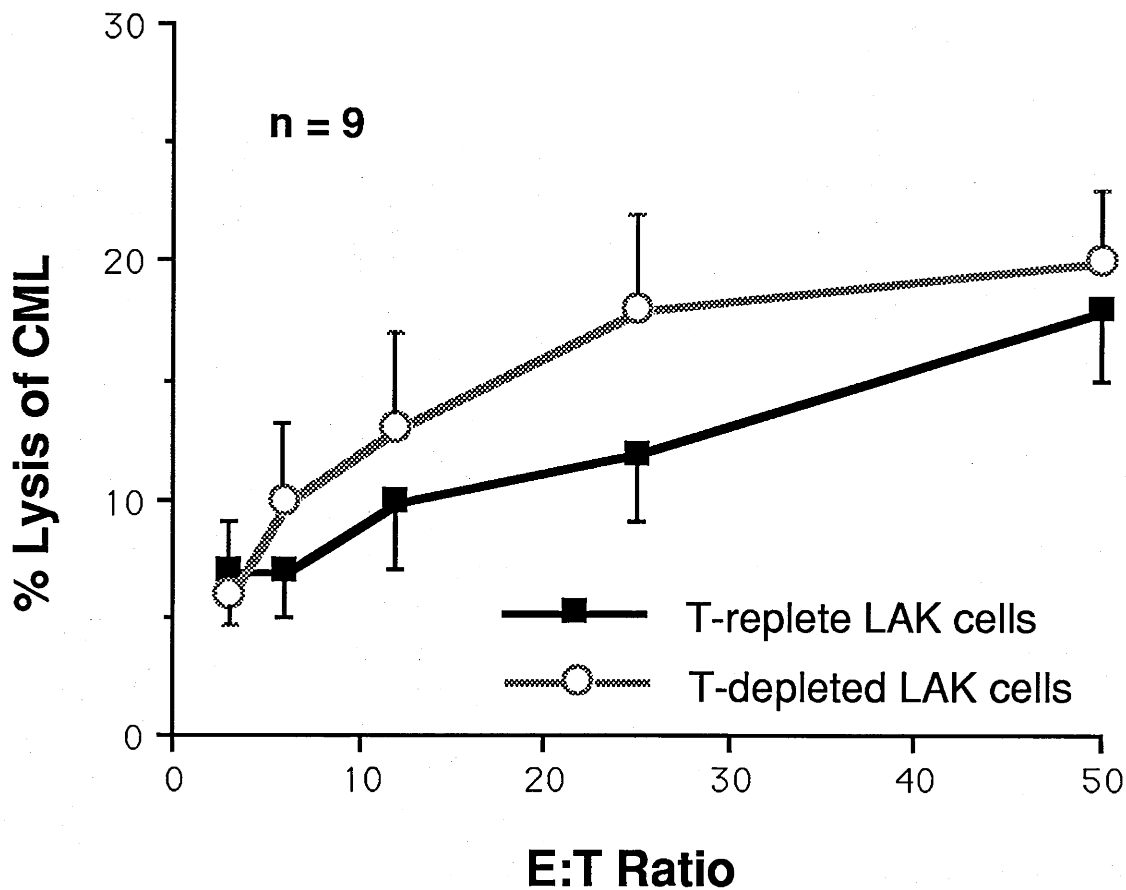


Figure 9. Results from all 9 patients whose LAK cells were T cell depleted and who demonstrated significant lysis of the CML targets. Unmanipulated and CD3 depleted LAK cells were added to recipient CML and allogeneic CML cells at E:T ratios of 50, 25, 12, 6 and 3 to 1. Each E:T ratio was set up in triplicate and the points plotted are the mean ^{51}Cr release. The graph shows that T cell depletion of the effectors did not reduce their ability to lyse the leukaemic cells. There was no significant difference ($p = 0.42$) in the killing of the recipients' and the allogeneic CML cells according to the Student's t-test.

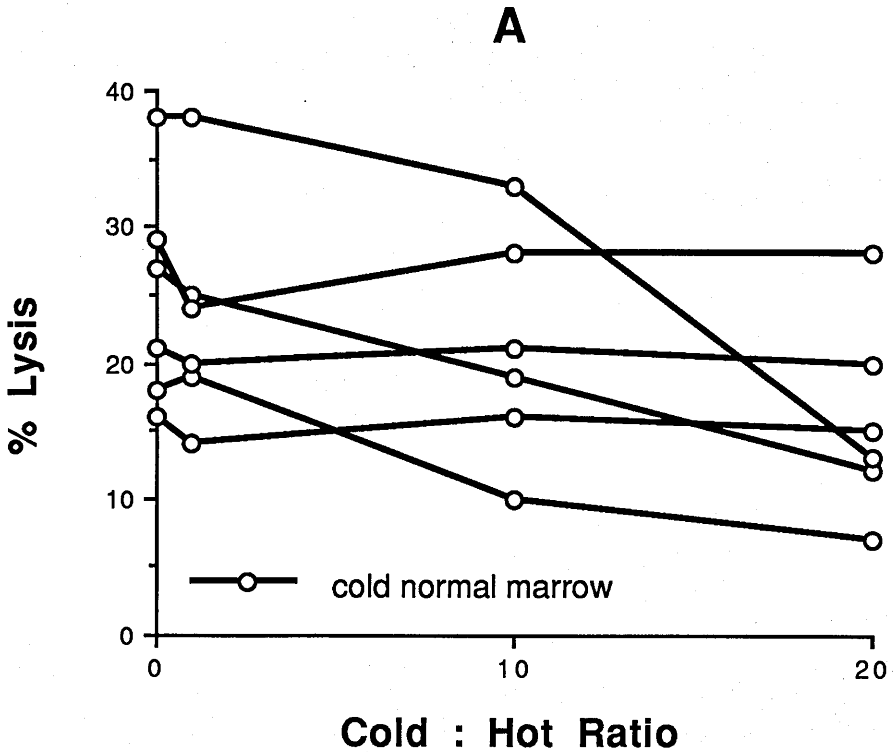
Table 2. The data are presented to show the relationship between the efficacy of the LAK effectors and the resistance to lysis of different leukaemic targets.

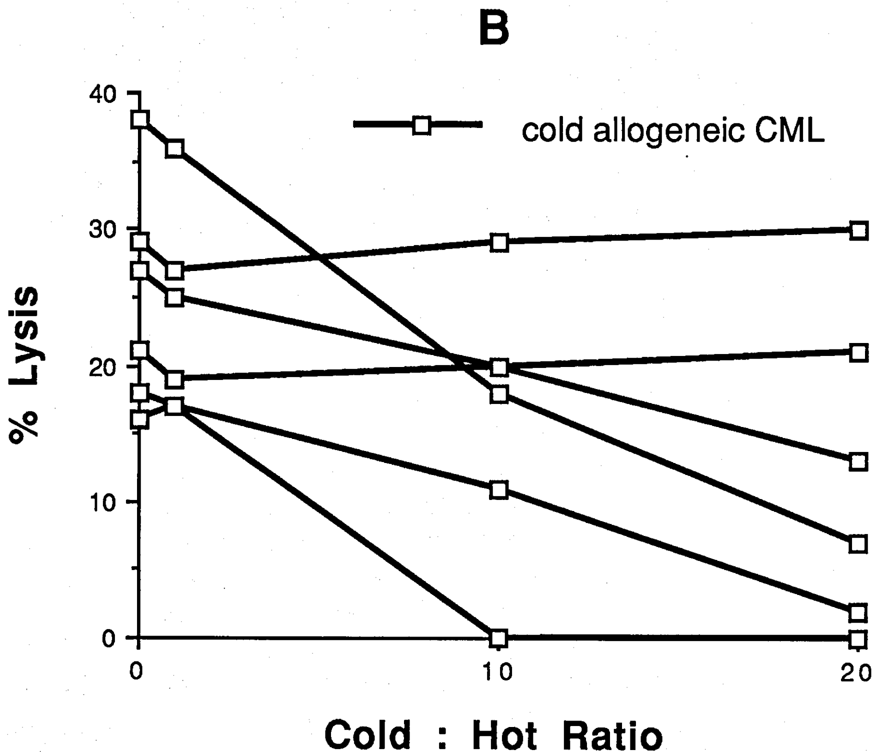
Targets killed by LAK cells from 37 different patients	Studies	Groups	Patient Numbers
Recipient +/Allogeneic +	15	1	15
Recipient --/Allogeneic --	4	2	4
Recipient +/Allogeneic --	5	3	11
Recipient --/Allogeneic +	6		
Recipient +/Allogeneic -	2	4	7
Recipient -/Allogeneic +	1		
Recipient -/Allogeneic -	4		

+ target killed by LAK cells

- target not killed by LAK cells from test patient but was killed by LAK cells from another patient

-- target not killed by LAK cells from test patient or by any other patients' LAK cells





Figures 10A and B. LAK cells from 6 different patients were used at a constant E:T ratio of 50:1 with cold normal bone marrow cells (Fig. 10A) and cold allogeneic CML cells (Fig. 10B) being added to hot recipient CML cells at cold:hot ratios of 1, 10 and 20 to 1. Each cold:hot ratio was set up in triplicate and the points plotted are the mean ^{51}Cr release. The graphs indicate that both normal marrow and allogeneic CML cold targets were capable of either blocking or not blocking the killing of the recipients' CML cells in different experiments.

CORRELATION OF CLINICAL AND IN VITRO DATA

There was no correlation between the ability of the patients' LAK cells to lyse their pretransplant cryopreserved CML cells and (a) the interval from transplant to the time of assay; (b) whether the donor marrow was T depleted or T replete and (c) the incidence and severity of acute and chronic GVHD (Table 3). None of the patients relapsed during the study period so we can make no comment about the relationship between in vitro GVL activity and the likelihood of relapse.

Table 3. Correlation of in vitro GVL activity with clinical parameters in 33 allograft recipients.

Clinical data	GVL Detected	GVL Absent
Patient no.	20	13
Median weeks from BMT to assay	65	58
T-cell depleted donor marrow	9	5
T-cell replete donor marrow	11	8
Acute GVHD: grades 0-I	15	9
grades II-IV	5	4
*Chronic GVHD: none	11	8
localised	5	3
generalised	3	2

* Only 32 patients were evaluable for chronic GVHD as one patient died within 3 months of the transplant.

DISCUSSION

Adoptive immunotherapy with LAK cells and IL-2 is a new approach in the treatment of malignant disease[99,100]. For haematological malignancies this strategy has been proposed for purging autologous bone marrow and as remission maintenance following conventional chemotherapy or BMT[153]. Although there is good evidence for the existence of enhanced natural killer and LAK cell activity following both autologous and allogeneic BMT, previous studies have in general used cell line targets rather than the recipient leukaemia cells[127,132,134,135], and LAK cells have not been shown to mediate in vitro GVL activity.

In the early phase of immune reconstitution post-BMT CD3-, CD16+, CD56+ large granular lymphocytes with NK activity form up to 45% of the lymphocyte compartment and CD3+ cells are slow to reappear[127,133]. Therefore one might have expected to generate LAK cells with a predominantly NK phenotype from the patients studied during the first 3 months post-BMT. In practice LAK cells were generated with surface markers not usually present in significant numbers in the peripheral blood of normal individuals, i.e. CD8+^{dim}/CD3-, CD8+^{dim}/CD56+, CD56+/CD16- and CD56+/CD16+^{dim}, phenotypes which have been described following culture of lymphocytes in IL-2 and in BMT recipients[154-158]. Although a greater percentage of CD56+/CD3- cells was found in patients studied within 3 months of BMT there was no significant difference when compared to the LAK

cell phenotype of patients studied at longer intervals from transplant. The inability to demonstrate phenotypic differences in the LAK cells which were or were not capable of mediating in vitro GVL activity could reflect the possibility that the GVL activity resides in a small subpopulation which I have not fully characterized. Alternatively it could reflect the variation in leukaemic target sensitivity which was seen in different patients (Table 2).

Conventionally LAK activity following BMT is tested using NK-resistant tumour cell line targets. Previous studies indicated that while fresh leukaemia cells are more resistant to killing by LAK cells than cell line targets there was a correlation between lysis of cell line targets and fresh leukaemic cells[113,159]. However my data indicate that LAK cells can in some cases kill substantial numbers of cell line target cells but induce no detectable lysis of the cryopreserved CML cells (Fig. 7). Thus correlating results using cell line targets and leukaemia cells is not always possible.

These experiments demonstrate that LAK cells generated from BMT recipients in remission whose lymphocytes are of donor origin[133,134] can kill recipient leukaemic cells in vitro in over half of the patients studied. This represents an inducible in vitro GVL effect. This effect was demonstrated in LAK cells from allograft recipients and also from 2 of the 4 syngeneic recipients. Although it is possible that killing of leukaemic cells following allogeneic

BMT could be mediated by MHC-restricted T cells, I have shown GVL activity in the syngeneic setting even when the syngeneic LAK cells were T cell depleted indicating that the killing was probably mediated by MHC unrestricted effectors. The same mechanism might operate in the autograft setting[135]. Furthermore although a subset of CD3+ cells is capable of MHC unrestricted killing[97], depletion of CD3+ cells from the effector population obtained from the allograft recipients did not reduce the observed antileukemic activity (Fig. 9), suggesting that the GVL activity was largely mediated by cells with the phenotype of CD3- natural killer cells. Additional evidence that this activity is MHC unrestricted comes from the observation that there was significant killing of allogeneic CML cells. Cold target competition studies failed to demonstrate a consistent pattern with both normal bone marrow cells and allogeneic CML cells capable of either blocking or not blocking the killing of the labelled recipient CML cells. When blocking occurred the effectors might have recognized receptors present on both leukaemic and normal cells; when there was a failure to block this could reflect a more resistant cold target cell. Results of these studies suggest that the LAK cells can recognize and interact with cell lines, leukaemic cells and normal bone marrow target cells.

Previous work in BMT recipients failed to show any correlation between LAK activity and the age of the patient, the speed of engraftment or the incidence of infections[132]. Our data also fail

to demonstrate any association between the presence or absence of in vitro GVL activity and time from BMT to assay, GVHD prophylaxis or the presence of acute or chronic GVHD. It could be argued that the lack of correlation results from culturing PBMC in supraphysiological concentrations of IL-2 which produces cells with activities that have little in vivo relevance. On the other hand it is possible that in vivo this could provide a mechanism for enhancing MHC unrestricted GVL activity. As the CD3- LAK cells kill the leukaemic cells these effectors could in theory be exploited to reduce leukaemic relapse without the morbidity and mortality of GVHD.

Our results indicate a potential role for LAK cell therapy with or without in vivo IL-2 following BMT. In the context of syngeneic or autologous BMT, GVL activity could perhaps be generated without the risk of initiating or enhancing GVHD. In allogeneic BMT, however, the use of LAK cell therapy with in vivo IL-2 could promote the growth of donor alloreactive T cells with resultant GVHD. This might be avoided by depleting donor marrow of T cells prior to the transplant or by depleting LAK cells generated in vitro of CD3 positive cells[160].

CHAPTER 4

INDUCTION OF IN VITRO LAK ACTIVITY FOLLOWING BONE MARROW TRANSPLANTATION FOR CHRONIC MYELOID LEUKAEMIA: USE OF 7 DAY INCUBATION WITH INTERLEUKIN-2. CFU-GM ASSAY.

INTRODUCTION

In Chapter 3 I demonstrated that LAK cells could kill CML leukaemia cells in a cytotoxicity assay. A question not addressed was whether the leukaemic cells killed were clonogenic or committed cells with no proliferative potential. If IL-2/LAK therapy was to be of clinical value LAK cells would need to target and kill clonogenic leukaemic cells and also not compromise the function of normal bone marrow cells.

There are conflicting reports regarding the role of NK cells in bone marrow graft rejection and the regulation of haematopoiesis[161]. NK cells have been reported to have lytic effects against normal bone marrow cells in vitro and in vivo via direct cell-cell contact or via the production of lymphokines such as gamma interferon or tumour necrosis factor (TNF)[109,153,162-171]. Because LAK activity is primarily derived from IL-2 activated NK cells, LAK cells could theoretically harm normal haemopoietic progenitors cells. Therefore limitations to the use of IL-2/LAK therapy following BMT might be either due to failure of LAK cells to inhibit the growth of recipient leukaemia cells and/or toxicity of LAK cells towards the normal donor marrow cells.

To investigate these possible limitations we analyzed the effect of both donor and recipient LAK cells on the colony growth of both normal donor marrow and the recipient CML cells in a semisolid

culture system. This Chapter describes an inducible GVL effect which inhibits the growth of clonogenic leukaemic cells with sparing of normal marrow progenitors.

MATERIALS AND METHODS

GENERATION OF LAK CELLS

Twenty-four patients were studied in complete haematological and cytogenetic remission following BMT for CML. Each patient gave consent for use of their blood in these studies. Fifteen patients had received allogeneic marrow from HLA identical siblings, 7 allogeneic marrow from HLA matched unrelated donors and 2 syngeneic marrow from identical twin donors. Venous blood was obtained from the donors prior to the transplant and from the recipients following the transplant. PBMC were prepared as described in Chapter 2 and LAK cells were generated by a method identical to that described in Chapter 3.

TARGET CELLS

Target cells in these assays were cryopreserved CML mononuclear cells from the transplant recipients and cryopreserved donor marrow mononuclear cells. Recipient leukaemic cells were collected from the peripheral blood or bone marrow prior to the transplant. Normal human bone marrow was obtained after informed consent from

the donors at the time of the bone marrow harvest by aspiration from the posterior iliac crests. The target cells were collected in HBSS with 10 units/ml preservative-free heparin. The target cell suspension was diluted in HBSS and centrifuged over Lymphoprep. The light density cells were collected, washed twice in HBSS and resuspended in RPMI-AB and cryopreserved until used in the CFU-GM assay.

SURFACE MARKER ANALYSIS

Phenotypic analysis was performed using two-colour direct immunofluorescence as described in Chapter 3.

ASSAY FOR GRANULOCYTE-MACROPHAGE PROGENITOR CELLS (CFU-GM)

The target cells were thawed for 1 minute at 37°C in a water bath, diluted in HBSS followed by centrifugation on Lymphoprep. The low density cells were then collected, washed twice in HBSS and resuspended in RPMI-AB. 1×10^5 mononuclear cells in RPMI-AB either of normal donor marrow or recipient CML origin were pre-incubated with or without the LAK cells at an effector:target ratio of 10:1 for 18 hours in liquid culture. After incubation the cells were resuspended and subsequently cultured in triplicate for CFU-GM in a methylcellulose culture system as described previously[172]. The methylcellulose based culture medium was prepared in bulk allowing the production of multiple identical 3.6 ml aliquots to

which was added 0.4 ml (4 x 10^5) target cells $\pm$ LAK cells. After mixing, 3 x 1 ml aliquots were decanted into sterile petri dishes and cultured at 37°C for 14 days. Each 1 ml aliquot contained 1% methylcellulose dissolved in 414 ul Iscove's modified Dulbecco's medium without supplements with L-glutamine (Gibco, Paisley, Scotland), 300 ul fetal calf serum (Sera-Lab, Sussex, England), 100 ul fraction V bovine serum albumin (Sigma, Poole, England), 1 ul 0.1M 2-mercaptoethanol (BDH, Poole, England), 10 ul 100 U/ml penicillin, 100 ug/ml streptomycin (Flow, Irvine, Scotland), 75 ul conditioned medium (culture supernatant from the 5637 bladder carcinoma cell line), and 100 ul of the target cell suspension.

In experiments designed to establish the role of effector-target cell-cell contact and to assess the possibility of nonspecific inhibition or stimulation of progenitor cell growth due to the presence of LAK cells or LAK-derived cytokines in the semi-solid culture medium, LAK cells at the same effector:target ratio were added to the progenitor cells immediately before plating. All LAK cells were irradiated (5 Gy) before use to prevent them from forming colonies[173]. CFU-GM colonies were defined as granulocytic, monocytic or mixed aggregates containing more than 20 cells. They were scored on day 14 under an inverted microscope. The number of colonies cultured from 10^5 normal marrow or CML mononuclear cells cultured in the absence of LAK cells was taken as 100%. In the cultures containing LAK cells the percentage of surviving progenitor cells was calculated by dividing the total number of

colonies by the number of colonies obtained in cultures free of LAK cells x 100%.

STATISTICAL METHODS

Differences in colony numbers in the CFU-GM assay were assessed for significance using the paired two-tailed Student's t-test.

RESULTS

SURFACE MARKERS

The surface marker analysis of the LAK cells from both the donors and recipients is shown in Table 4. I generated both CD56+, CD16+, CD3- NK cells and CD3+, CD56-, CD16- cytotoxic T cells. Although the LAK cells obtained from the recipients appeared to contain more NK and fewer T cells than those of the donors, these differences were not statistically significant.

TABLE 4

Phenotype of the LAK cells generated from donors and recipients expressed as mean percent values ± one standard deviation.

Percent cells positive for

	CD3	CD4	CD8	CD16	CD56
Recipients (n = 19)	45±25	11±13	48±19	7±5	53±25
Donors (n = 6)	57±7	24±8	34±8	10±7	39±10

CFU-GM ASSAY

LAK cells were generated from PBMC of 24 transplant recipients. The effect of LAK cell derived diffusible factors in the CFU-GM cultures was evaluated by comparing cultures grown in the absence of LAK cells with those cultures in which the LAK cells were not in contact with the CML progenitors during the liquid phase. LAK cells significantly ($p < 0.05$) increased colony numbers in 8 assays, significantly ($p < 0.05$) decreased colony numbers in 10 assays and made no difference in 6 assays in the absence of effector-target cell contact (Fig. 11).

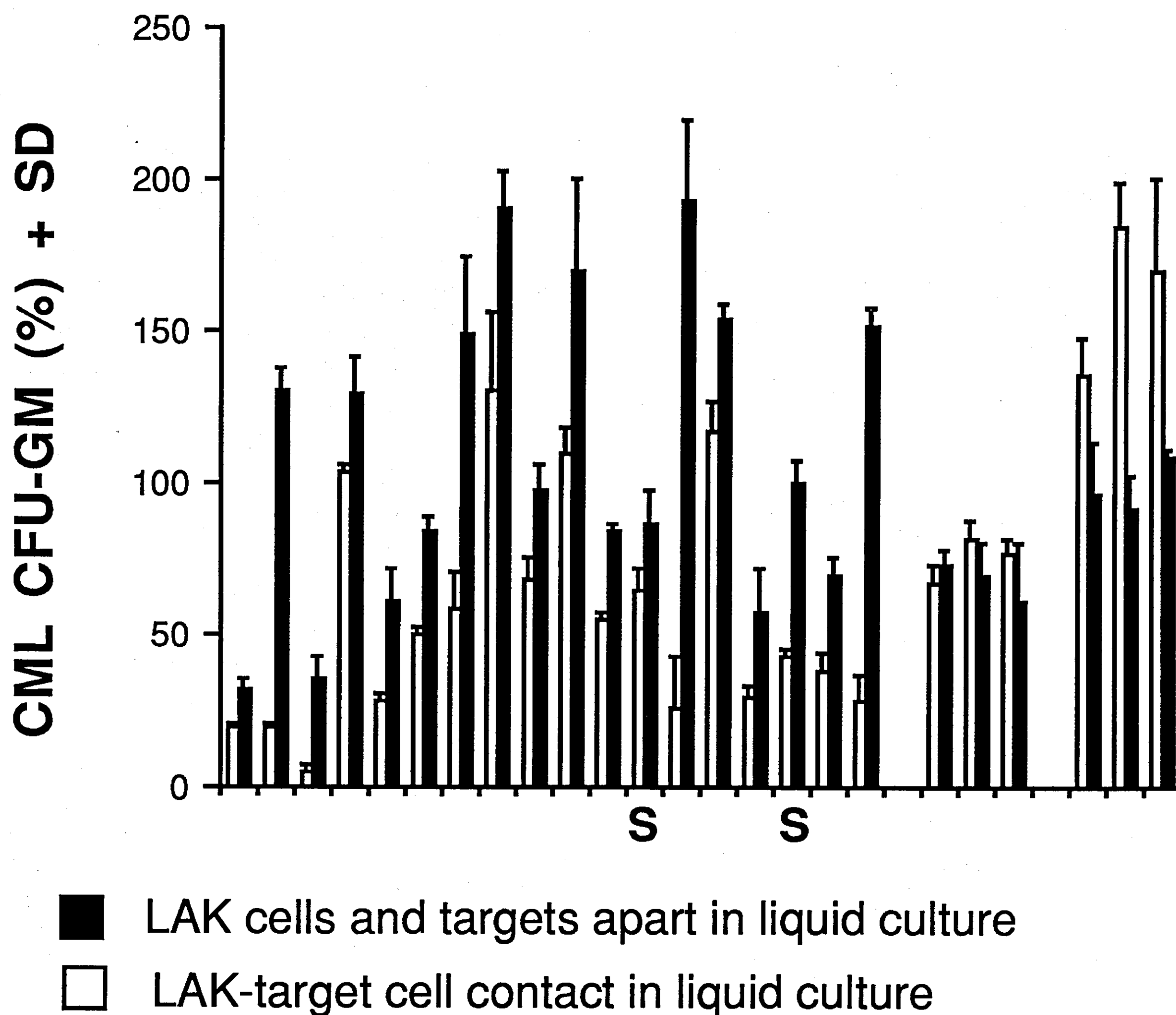
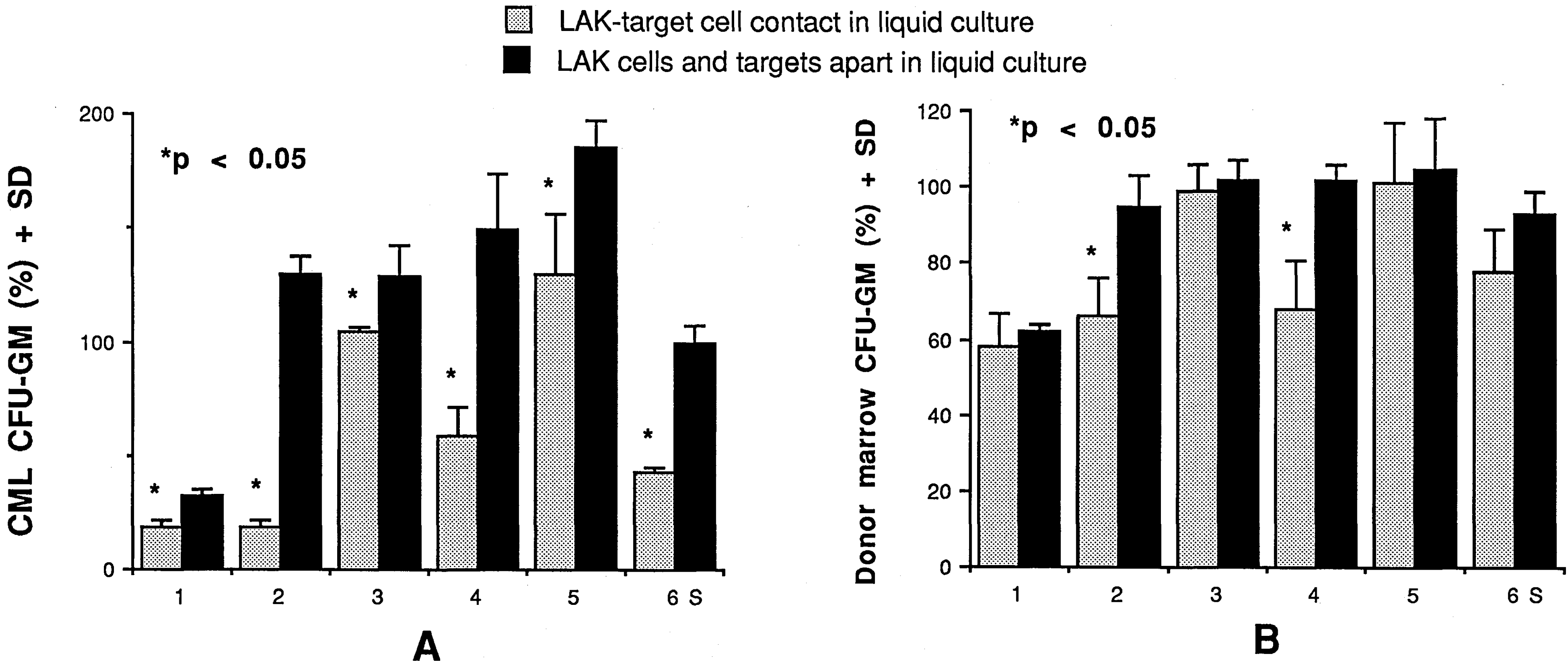


Figure 11. Activity of LAK cells generated from 24 transplant recipients using recipient CML cells as targets. Numbers of CFU-GM obtained in triplicate cultures are expressed as the mean percentage ± one standard deviation of colony numbers obtained in cultures without LAK cells. Experiments marked "S" involved syngeneic transplants.

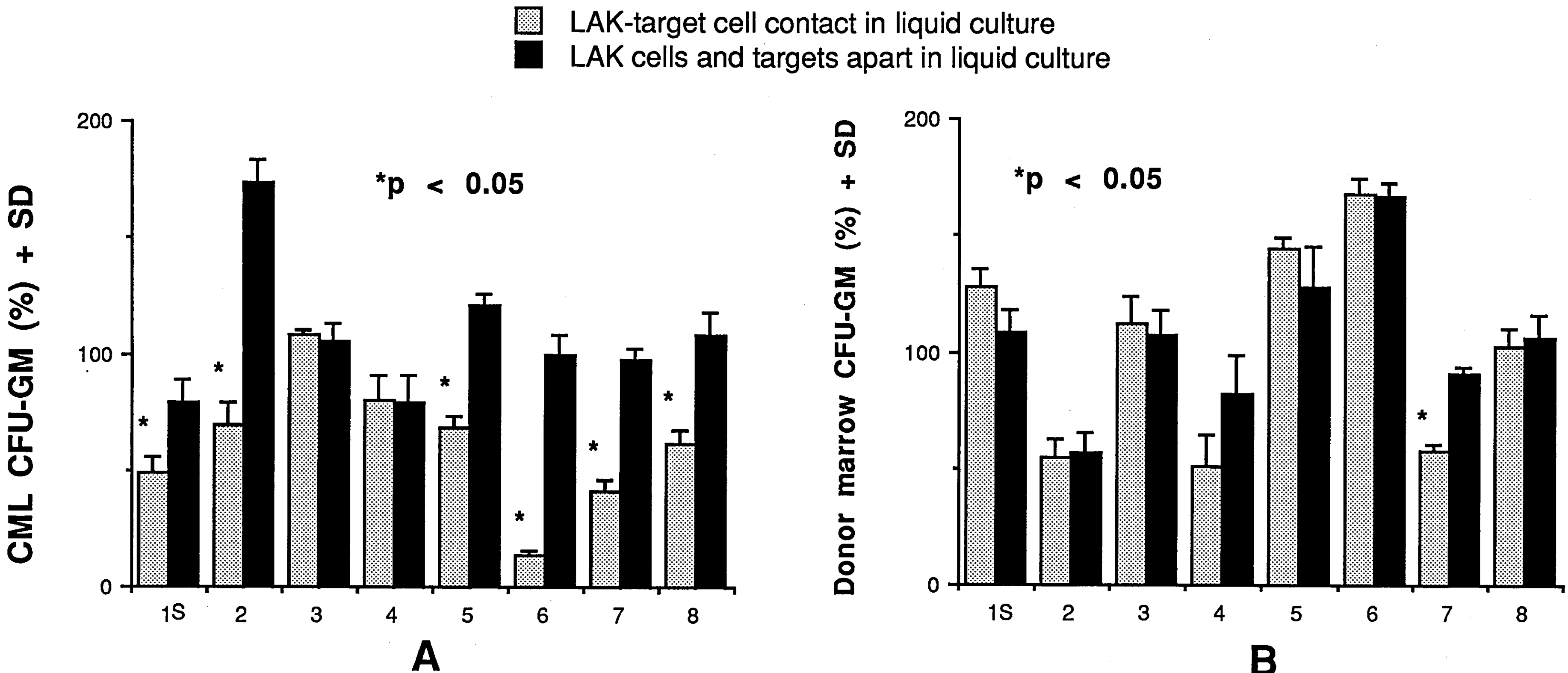
To assess any influence of effector-target cell contact independent of the effects of LAK cell cytokine release I compared experiments in which LAK cells were or were not in cell-cell contact with the CML progenitors in liquid culture prior to plating in the methylcellulose. These two sets of data are presented with the results expressed as mean percentages of the colony numbers obtained in cultures without LAK cells. I found significant ($p < 0.05$) inhibition of CFU-GM proliferation from CML cells in 18 (2 syngeneic) of the 24 (75%) experiments (Fig. 11). The mean ± SEM inhibition in these 18 studies was 47% ± 5 (range, 19 - 87%). No significant difference was seen on 3 occasions and significant stimulation ($p < 0.05$) of colony growth in 3 assays (Fig. 11).

I then compared the activity of LAK cells cultured from 1 syngeneic and 5 HLA-identical sibling transplant recipients incubated simultaneously with either donor marrow progenitors or recipient CML progenitors. LAK cells inhibited CML CFU-GM proliferation to a significant degree in all 6 experiments (mean ± SEM: 49% ± 9, range 19 - 85%) (Fig 12A), whereas proliferation of donor marrow CFU-GM was significantly inhibited in only 2 of the 6 experiments (31 and 33%) (Fig. 12B).



Figures 12A and B. Activity of LAK cells generated from 6 transplant recipients using donor marrow and recipient CML cells as concurrent targets. Numbers of CFU-GM obtained in triplicate cultures are expressed as a mean percentage ± one standard deviation of colony numbers obtained in cultures without LAK cells. Fig. 12A shows that LAK-CML progenitor cell contact resulted in a reduction in colonies ($p<0.05$) in all 6 experiments. Fig. 12B demonstrates that LAK-donor marrow cell contact resulted in a reduction ($p<0.05$) in colony numbers in only 2 of the 6 assays. When inhibition of donor marrow proliferation was observed it was much less than that seen with the corresponding patient's CML progenitors. Experiment 6 is syngeneic.

Similarly, the activity of LAK cells generated from 1 syngeneic and 7 sibling allogeneic bone marrow donors was assessed in analogous experiments. Of the 8 donors 7 were males or nulliparous females with no previous blood transfusions. Both donor marrow and recipient CML cells were used as concurrent targets. Donor LAK cells inhibited recipient leukaemia CFU-GM proliferation to a significant degree in 6 of 8 experiments (mean $\pm$ SEM: 52% $\pm$ 7, range 38 - 86%) (Fig. 13A), whereas proliferation of donor marrow CFU-GM was significantly inhibited (36%) in only one experiment (Fig. 13B).



Figures 13A and B. Activity of LAK cells generated from 8 marrow donors using donor marrow and recipient CML cells as concurrent targets. Numbers of CFU-GM obtained in triplicate cultures are expressed as a mean percentage ± one standard deviation of colony numbers obtained in cultures without LAK cells. Fig. 13A shows that LAK-CML progenitor cell contact resulted in a reduction in colonies ($p<0.05$) in 6 of 8 experiments. Fig. 13B demonstrates that LAK-donor marrow cell contact resulted in a reduction ($p<0.05$) in colony numbers in only 1 of the 8 assays. Experiment 1 is syngeneic.

DISCUSSION

This study demonstrates that LAK cells generated from either the bone marrow of the donor pretransplant or the recipient post-BMT inhibited the growth of recipient CML CFU-GM with sparing or relative sparing of donor marrow CFU-GM.

The effector cells responsible for this activity fell into two main populations: CD56+, CD16+, $CD8^{dim}$+, CD3- NK cells and CD3+, $CD8^{bright}$+, CD56-, CD16- T cells (Table 1). Immune reconstitution following BMT results in replacement of the recipients' NK and T cells with those of donor origin[119,134]. Therefore because all the effectors used in this study were donor derived and the targets were either autologous (donor marrow) or allogeneic (HLA-identical recipient leukaemia), it might be argued that any antileukemic activity could be due to a combination of MHC unrestricted NK cells and an allogeneic component mediated by donor derived T cells which recognise minor transplantation antigens. However it seems probable that allogeneic factors are not important in this assay system for three reasons: 1) the two patients studied following syngeneic BMT both showed inhibition of colony growth of the leukaemic marrow, 2) previous in vitro studies performed using a cytotoxic assay demonstrated that T cells were not important in mediating this in vitro GVL effect and that the LAK activity was present in the CD56+, CD16+, $CD8^{dim}$+, CD3- activated NK cells, and 3) effector cells generated from the male or female nulliparous

untransfused HLA identical sibling donors prior to the transplant could not have been primed to respond to any minor HLA antigen differences between donor and recipient. Furthermore in HLA-identical siblings the cytotoxic T lymphocyte precursor frequency in the graft-versus-host direction is extremely low, i.e. between 1 in 300,000 and < 1 in 1,000,000 making a specific allogeneic T cell cytotoxic effect unlikely[174].

The experiments demonstrated stimulation as well as inhibition of colony growth when LAK cells were added to the cultures immediately before plating in methylcellulose but not in cell-cell contact with the progenitor cells (Fig. 11). These effects were mediated by LAK-derived diffusible factors which we presume to be the cytokines reported by a number of investigators[109,166,168,170,175]. These apparently contradictory findings can be explained by the fact that LAK cells produce colony stimulating factors whose growth promoting activities may be masked by colony inhibiting activity due to concurrent release of TNF[170]. Therefore by assessing the net inhibition or stimulation of LAK cytokine release on the individual cultures, it was possible to define the role of LAK-target cell contact.

The experiments performed with pre-incubation of LAK cells and target cells together in liquid culture allowed me to determine the consequences of this cell-cell contact. Although significant inhibition of colony growth was seen in the majority of the

recipients' CML progenitors (Figs. 11, 12A and 13A), there was a similar effect in only a minority of cultures where normal donor marrow was the target, and then in an attenuated form (Figs. 12B and 13B). The mechanisms underlying this antileukemic activity with complete or relative sparing of normal marrow remain poorly characterized. Possible mechanisms include enhanced LAK cell recognition of leukaemic targets with resultant triggering of the perforin[107] mediated lysis or local release of cytokines such as TNF or gamma interferon which are known to affect leukaemic cells more than normal cells[176-178]. The studies in Chapter 3 using a ^{51}Cr release cytotoxicity assay demonstrated that the LAK cells are capable of lysing CML targets, suggesting that the reduction in colony numbers seen may be a reflection of progenitor cell death rather than merely an inhibition of cell division.

In conclusion, LAK cells may inhibit the growth of normal bone marrow cells to some extent but the in vitro data presented here indicate that the susceptibility of donor marrow is still considerably less than that of recipient CML progenitors. This might allow IL-2/LAK cell therapy following BMT to enhance antileukemic activity without compromising engraftment. In the context of syngeneic or autologous BMT, this GVL activity could be generated without the risk of initiating or enhancing GVHD. In allogeneic BMT, however, the use of IL-2/LAK cell therapy in vivo could promote the growth of donor alloreactive T cells with resultant GVHD. Animal data suggest that this might be avoided if

one could deplete the donor marrow of T cells prior to the transplant[160]. Whether this in vitro GVL activity can be exploited in the clinic is at present unclear but preliminary studies in which patients received IL-2 post-autografting indicate that LAK cell activity is indeed enhanced[136].

CHAPTER 5

ORIGIN AND FUNCTION OF ADHERENT LYMPHOKINE ACTIVATED KILLER CELLS IN PATIENTS WITH CHRONIC MYELOID LEUKAEMIA WHO RELAPSE FOLLOWING BONE MARROW TRANSPLANTATION

INTRODUCTION

The probability of relapse at 4 years is about 10% following allogeneic BMT for CML using unmanipulated donor marrow but GVHD remains a major complication[125]. T cell depletion of the donor marrow reduces the incidence and severity of GVHD but increases the probability of relapse to 50% or higher[37]. This observation provides support for the existence of a GVL effect in CML.

Some of the CML patients who relapse following BMT have "low grade" disease which is often asymptomatic without splenomegaly and requiring little or no treatment[179]. It could be argued that the apparent slow progression of disease reflects a reduced total myeloid cell mass at the time of detection of relapse after BMT and not an alteration in biological tempo. However in the majority of cases of newly diagnosed CML evidence of leukaemia is probably only present in the peripheral blood for 6 - 12 months prior to actual diagnosis[180]. Thus it is possible that even in patients who relapse post-BMT, leukaemic cell proliferation could still be restrained to some extent by a GVL effect mediated by lymphoid cells of donor origin.

A method has recently been developed for selective enrichment of recombinant IL-2 activated antitumour effector cells called adherent lymphokine activated killer (ALAK) cells from rat splenocytes[181] or from human peripheral blood mononuclear cells[182].

The method depends on the propensity of activated NK cells to adhere tightly to plastic in the presence of IL-2. Cultivation of these adherent cells with IL-2 for 14 days results in the rapid proliferation of cells with the morphological characteristics of large granular lymphocytes and a predominant CD56+/CD3- phenotype. ALAK cells derived from normal humans have significantly higher cytotoxic activity on a per cell basis and greater proliferative capacity than LAK cells prepared in a conventional fashion[181,182].

The ALAK population has not been studied in patients who have relapsed after BMT for CML. I wanted to determine whether these cells were of donor or recipient origin. I also wanted to assess their phenotype and whether the ALAK cells were capable of in vitro antileukaemia or GVL activity. If ALAK cells were capable of mediating an in vitro GVL effect this might support the hypothesis that the donor lymphoid cells inhibited leukaemic cell proliferation after transplant and this resulted in "low grade" chronic phase disease.

MATERIALS AND METHODS

PATIENTS

I studied in 7 patients following T cell depleted BMT for CML. Five patients were in haematological relapse, one was in cytogenetic relapse and the other patient was in complete cytogenetic remission at the time of study but had prior documentation of cytogenetic relapse. Four patients had received allogeneic marrow from HLA identical siblings, 2 received marrow from HLA matched unrelated donors and one patient received marrow from a one antigen mismatched family member. Pretransplant all patients were conditioned with cyclophosphamide (60 mg/kg/day x 2) and total body irradiation (6 x 2 Gy). Some patients also received additional chemotherapy, total lymphoid irradiation or splenic irradiation. Patients were studied at intervals ranging from 1 to 4 years following BMT.

GENERATION OF ALAK CELLS

Venous blood was collected from the patients following the transplant and PBMC were prepared by centrifugation on Lymphoprep (Nyegaard, Oslo). The low density cells were collected and washed twice with HBSS and resuspended in RPMI-AB and depleted of monocytes by plastic adherence.

For generation of ALAK cells, monocyte depleted PBMC at a concentration of 4 x 10^6/ml were incubated in a plastic culture flask (Falcon, Lincoln Park, NJ) for 24 hours in 5% CO_2 at 37°C in RPMI-AB containing 500 U/ml recombinant IL-2. After incubation the supernatant was decanted and all cells not firmly attached to plastic were removed by washing x3 with HBSS at 37°C. In distinction to the original method[181,182] the adherent cells were then refed with fresh RPMI-AB supplemented with 500 U/ml IL-2 rather than with autologous conditioned media and cultured for up to 14 days[111]. The ALAK cells were washed and then tested for phenotype and antileukaemic activity. Metaphases were prepared for cytogenetic studies (see below).

SURFACE MARKER ANALYSIS

Phenotypic analysis was performed by two-colour direct immunofluorescence using standard techniques[152] as described in Chapter 3.

ELIMINATION OF T LYMPHOCYTE SUBPOPULATIONS

T cell depletion of the ALAK cells was performed by the method described in Chapter 3.

ANTILEUKAEMIC ACTIVITY OF THE ALAK CELLS

ALAK cells were tested for activity using the ^{51}Cr release cytotoxic assay described in Chapter 2.

CYTOGENETIC ANALYSIS

T cell depleted ALAK cells were resuspended in RPMI-AB supplemented with 500 U/ml IL-2 and cultures harvested for cytogenetic analysis after 24 hours. Colcemid (Gibco, Paisley, Scotland) was added to the cultures at a final concentration of 0.1 ug/ml which were then left for 60 minutes at 37°C before harvesting. Thereafter the cells were centrifuged at 1000 rpm for 8 minutes. The supernatant was removed and the cell pellet was resuspended in 2 ml of 0.075 M hypotonic KCl and left at 37°C for 3 - 5 minutes. Cells were then centrifuged at 1000 rpm for 8 minutes and the supernatant removed. The resuspended cells were then fixed in freshly made 3:1 methanol:acetic acid solution. The fixed cells were left at -20°C overnight. They were then centrifuged and the supernatant removed. The pellet was then resuspended in fresh fix solution to a suitable dilution for slide making. Metaphase spreads were made as follows. The cell suspension was dropped onto a microscope slide and left on a 60°C hot plate for 1 hour. Metaphases were banded using a Trypsin-Giemsa method adapted from Seabright[183]. Between 9 - 41 metaphases were analyzed for each patient.

RESULTS

SURFACE MARKERS

Phenotypic analysis of cells from the 7 patients at day 14 of ALAK culture revealed that 49 $\pm$ 6% were CD56+/CD3-, 50 $\pm$ 11% were CD3+/CD56- with a few CD3+/CD56+ cells being found (5 $\pm$ 2%). The CD56+ cells were either CD16- (40 $\pm$ 6%) or $CD16^{dim}$ (9 $\pm$ 6%). Figure 14 which is a two-colour FACS analysis of ALAK cells from a single patient demonstrating that the CD3 and CD56 antigens were largely present on two different populations of cells.

CHROMOSOME ANALYSIS

Cytogenetic analysis of T cell depleted ALAK metaphases from the 7 patients are shown in Table 5. The patients' leukaemia transplant number (LTN), disease status, recipient/donor sex and number of metaphases studied are detailed. The ALAK cells from 6 patients were all Ph negative, and where donor and recipient were sex mismatched, ALAK cells were exclusively of donor origin. In patient LTN 102 the ALAK cells were of recipient origin in 8 of 9 metaphases. In addition all recipient cells had the Ph chromosome. The phenotype of the T cell depleted ALAK cells from patient LTN 102 are shown in Figure 15 and indicated that >97% of the cells were CD56+/CD3- activated NK cells. In patient LTN 135, where donor and recipient were both female, we were able to determine

whether the ALAK cells were of donor or recipient origin. By analyzing phytohaemagglutinin stimulated T cells from this patient post-BMT we were able to identify two populations of cells (donor and recipient) on the basis of chromosome 15 satellite polymorphisms (Fig. 16). The ALAK cells were all of recipient origin but lacked the Ph chromosome unlike the recipient myeloid cells which were Ph positive.

Table 5. Results of cytogenetic studies on the T cell depleted ALAK cells generated from the peripheral blood of the 7 CML patients.

LTN	Disease Status	Recipient/donor sex	ALAK metaphases no. analyzed	M/F	Ph+/total
62	CP	M/F	40	0/40	0/40
79	Rem	M/F	10	0/10	0/10
83	CP	M/F	40	0/40	0/40
102	CP	M/F	9	8/1	8/9
135	CP	F/F	41	0/41	0/41
138	CP	F/F	10	0/10	0/10
197	CyRel	F/M	10	10/0	0/10

LTN - Leukaemia Transplant Number. CP - Chronic phase. CyRel - Cytogenetic relapse.
Rem - Remission at time of study but had transient cytogenetic relapse.

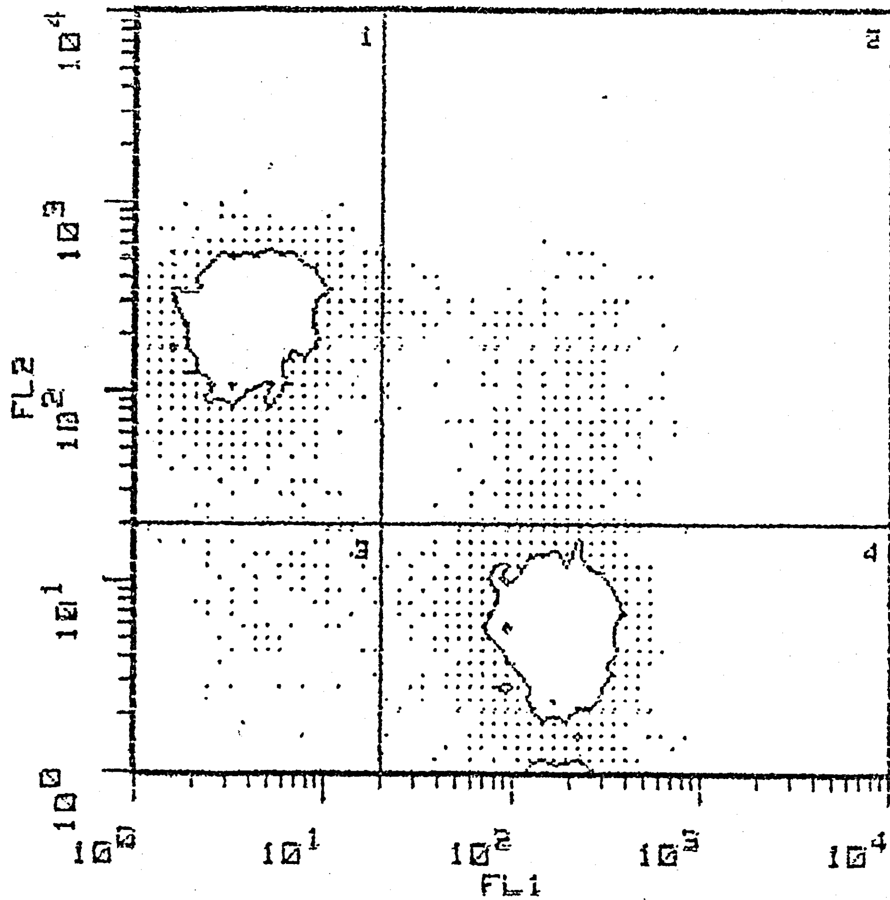


Figure 14. Two colour flow cytometric analysis of day 14 ALAK cells from a single patient. Histogram showing: FL1 FITC anti-CD3; FL2 PE anti-CD56. The CD3+ T-cells and CD56+ cells are largely separate populations with only a few T cells expressing both antigens.

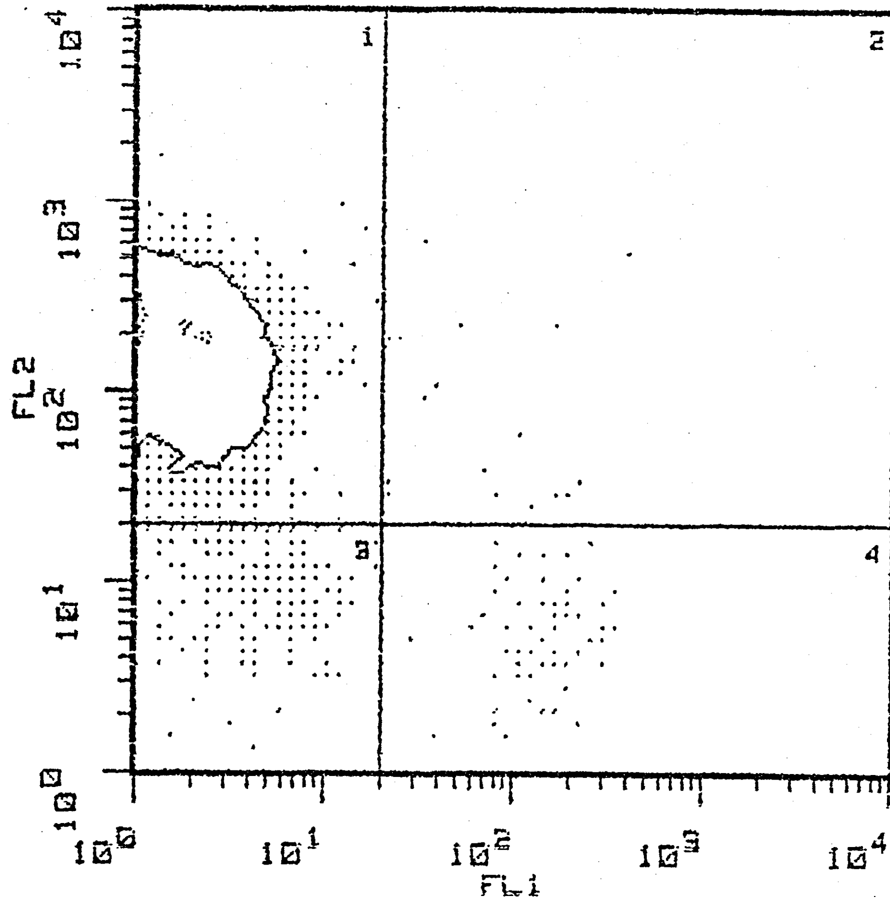


Figure 15. Two colour flow cytometric analysis of day 14 T cell depleted ALAK cells from a patient LTN 102. Histogram showing: FL1 FITC anti-CD3; FL2 PE anti-CD56. The figure shows that >97% of the cells are CD56+/CD3- activated NK cells.

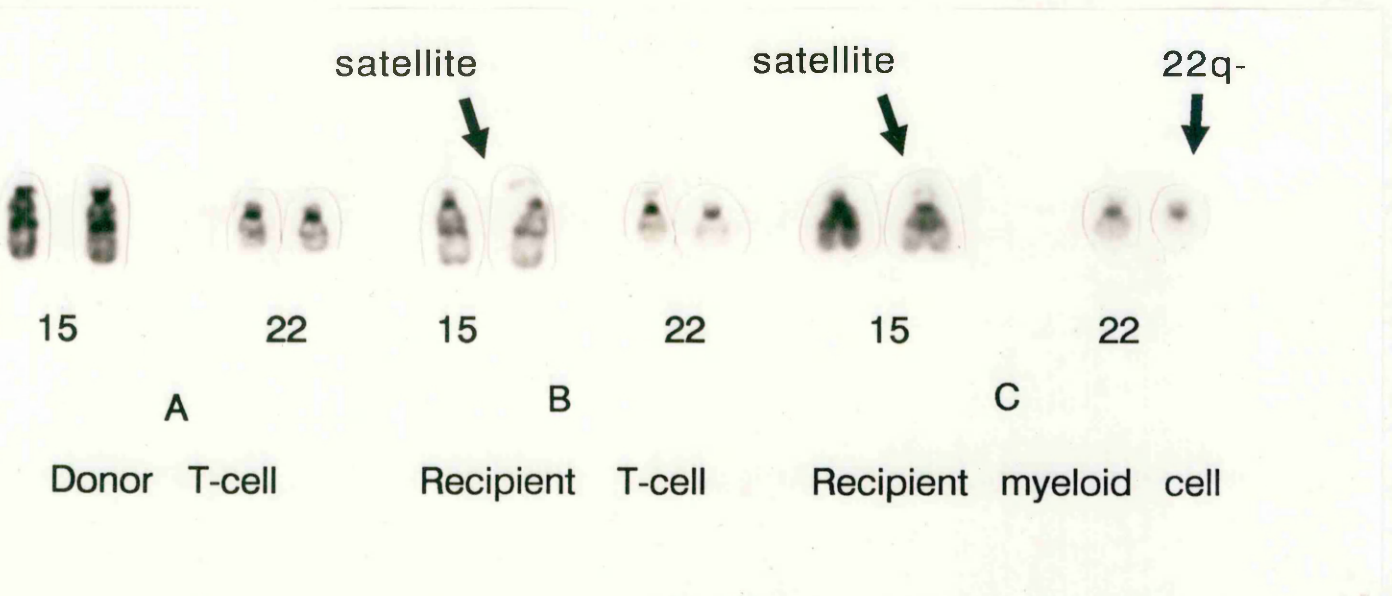


Figure 16. Photomicrographs of metaphase preparations. A. Donor T cell with small chromosome 15 satellites and normal chromosomes 22. B. Recipient T cell with one large chromosome 15 satellite and normal chromosomes 22. C. Recipient myeloid cell with one large chromosome 15 satellite, one normal chromosome 22 and the Ph chromosome.

ANTILEUKAEMIC ACTIVITY OF ALAK CELLS

T cell depleted ALAK cells significantly killed recipient CML cells in 5 of 7 patients studied (Fig. 17) with a mean ± SEM specific lysis of 28% ± 5 at an E:T ratio of 50:1. The recipient-derived Ph positive ALAK cells from patient LTN 102 were CML lytic. The ALAK cells from 2 patients (LTNs 83 and 135) did not kill their pretransplant CML cells.

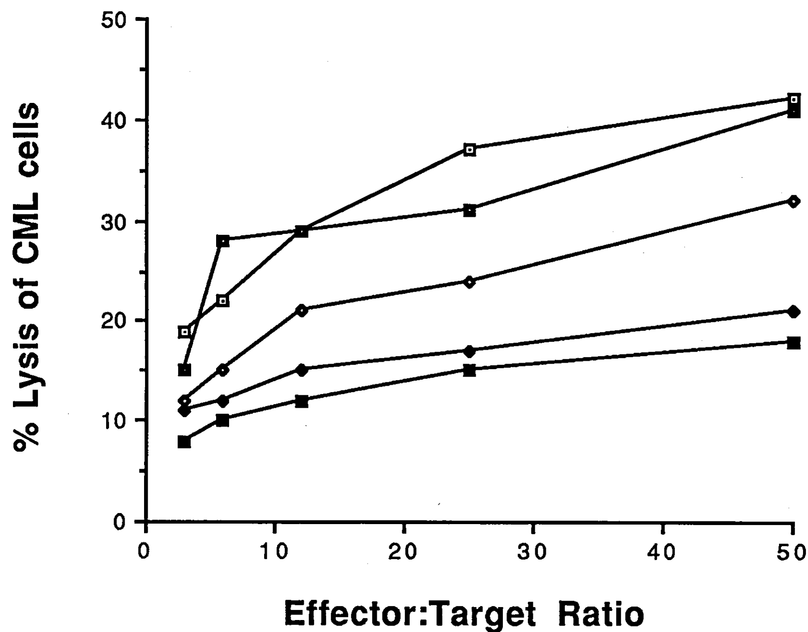


Figure 17. Results from the 5 patients whose T-depleted ALAK cells demonstrated significant lysis of the CML targets. ALAK cells were added to recipient CML cells at E:T ratios of 50, 25, 12, 6 and 3 to 1. Each E:T ratio was set up in triplicate and the points plotted are the mean ^{51}Cr release. The graph shows dose-dependent killing of the CML targets.

DISCUSSION

The phenotype of the ALAK cells generated from CML patients in relapse after BMT was different from that reported in previous studies on both normal subjects and in chronic phase CML patients who have not been transplanted[111,182]. Although we had a majority population of CD56+, CD3- activated NK cells as reported by others, we generated more CD3+ T cells. The reason for this difference is unclear but it could reflect disordered immune reconstitution following the transplant. One consequence of these findings was that we decided to T cell deplete the ALAK cells before performing cytogenetic analysis and testing for antileukaemic activity.

The ALAK cells were able to lyse recipient CML cells in 5 of the 7 patients. These results are similar to those seen in Chapter 3 using LAK cells from transplant patients in remission of disease. Somewhat surprisingly even the recipient Ph positive ALAK cells lysed the recipient CML cells. Thus in patients in haematological relapse post-BMT there remains a population of cells capable of being stimulated to produce an in vitro antileukaemic or GVL effect. Therefore it is possible that even in patients who relapse after T cell depleted BMT leukaemic cell proliferation may be restrained to some extent by a GVL effect mediated by lymphocytes of donor origin. The observation that cytogenetic relapse may on occasion be transient[45,184] or may persist without progression to haematological relapse[30] has been reported previously and provides

further support for this hypothesis.

In 2 patients the ALAK cells were of recipient origin and in one of these the ALAK cells were Ph positive and therefore part of the malignant clone, a finding not previously reported either before or following BMT. These results were obtained from a population of cells >97% CD56+, CD3-. NK cells were reported to be of donor origin in transplant recipients in remission[133,134]. Our results demonstrate that in all other patients studied the ALAK cells were Ph negative, and when the donor and recipient were sex mismatched, the ALAK cells were exclusively of donor origin. When T cells and B cells were studied in relapsed patients transplanted for CML or acute leukaemia similar findings were documented with those lymphocytes also being of donor origin[179,185].

In practice most of the patients studied progressed to haematological relapse in vivo even though some possessed donor NK cells capable of being stimulated to produce an in vitro GVL effect. CML transplant recipients are particularly prone to relapse when they receive a donor marrow depleted of T cells with a monoclonal antibody of broad specificity such as Campath-1[35]. Immune reconstitution in such patients is associated with a rapid recovery of NK cells but with a marked delay in the reappearance of the CD4+ helper T-cells[126] responsible for the production of IL-2 in vivo. Although donor NK cells activated in vitro can mediate GVL activity it is possible that a failure of the helper T cells

to fully activate the NK cells in vivo following T cell depleted BMT results in a loss of GVL activity resulting in an increase in leukaemic relapse. If such a mechanism was deficient in T cell depleted BMT recipients, the in vivo administration of IL-2 post-BMT might enhance GVL activity and reduce leukaemic relapse. Although this might be associated with augmentation of GVHD, murine studies indicate that this might not be a problem in the context of a T-cell depleted donor marrow[160].

CONCLUSIONS

The data presented in this thesis have demonstrated that it is possible to induce in vitro GVL activity following BMT for CML. This activity is MHC unrestricted and mediated by activated NK cells. The LAK cells not only lysed the CML cells but also prevented their proliferation. The growth of normal donor marrow CFU-GM was either not affected or inhibited to a lesser extent than that of the CML progenitors. In similar experiments in patients who had relapsed post-BMT the ALAK population was usually of donor origin and capable of mediating in vitro GVL activity.

It is tempting to speculate that this inducible in vitro GVL activity might have an in vivo counterpart, and that loss of in vivo NK activity following lymphocyte depletion of the donor marrow is responsible for the increased incidence of relapse. One can develop theories in which a number of separate small changes associated with lymphocyte depletion could lead to a large increase in post-BMT leukaemic relapse. Removal of NK cells as well as T cells from the donor marrow, impaired reconstitution of the IL-2 producing helper T cells and minimal residual CML leukaemia cells which release soluble IL-2R could all be factors which might act synergistically to increase relapse. However, apart from the results of the syngeneic transplants, which have a lower relapse rate than T cell depleted allografts for CML[35], there is scant evidence to support the concept of in vivo GVL mediated by NK

cells. In vivo the majority of the GVL effect is related to concurrent GVHD mediated by T cells. Furthermore the data presented in this thesis failed to show a correlation between induction of in vitro NK cell mediated GVL activity and the patients' clinical transplant parameters.

Although it might be construed as disappointing that the inducible in vitro GVL effect bears little relationship to what is happening in vivo, one need not view the results in that manner. There would have been little gained by reproducing the in vivo GVL effect with IL-2 in vitro as this would have been largely a T cell mediated effect and would therefore almost certainly result in an increase in GVHD with its associated morbidity and mortality when transferred to the clinic. By producing in vitro GVL activity mediated by activated NK cells, GVHD might be avoided after allogeneic BMT. There are murine data to support the view that IL-2 therapy post allogeneic BMT might not be associated with GVHD when the donor marrow is T cell depleted. However mice are not men and until the results of ongoing primate transplantation studies are available, it will not be ethical to use post-BMT IL-2/LAK therapy following allografting. However the results of the syngeneic studies presented in this thesis indicate that IL-2/LAK therapy post-autografting might reduce leukaemic relapse without any risk of GVHD. Preliminary studies have shown that post autologous BMT IL-2 infusion does enhance in vivo LAK activity but it is currently too early to determine whether this will result in

a reduction in leukaemia relapse.

Leukaemia patients in the early phase post-BMT could be seen as the ideal candidates for adoptive immunotherapy. The combination of a non-solid malignancy, minimal tumour load post-BMT, easy targeting of leukaemic cells either in the blood or bone marrow together with an already enhanced NK activity post-BMT should provide the ideal environment for in vivo IL-2/LAK therapy. Whether recombinant cytokines either alone or in combination will result in improved clinical results of BMT is currently not known but this question will only be answered by randomized prospective clinical trials.

BIBLIOGRAPHY

1. Craigie D. Case of disease of the spleen in which death took place in consequence of the presence of purulent matter in the blood. **Edin Med Surg J** 1845; **64**: 400-413.

2. Bennett JH. Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood. **Edin Med Surg J** 1845; **64**: 412-423.

3. Virchow R. Weisses blut. **Froriep's Notizen** 1845; **36**: 151-156.

4. Fialkow PJ, Gartler SM, Yoshida A. Clonal origin of chronic myelocytic leukemia in man. **Proc Natl Acad Sci USA** 1967; **58**: 1468-1471.

5. Gunz FW. The epidemiology and genetics of the chronic leukaemias. **Clin Haematol** 1977; **6**: 3-20.

6. Heyssel R, Brill B, Woodbury L. Leukemia in Hiroshima atomic bomb survivors. **Blood** 1960; **15**: 313-331.

7. Lange R, Moloney W, Yamawaki T. Leukemia in atomic bomb survivors. **Blood** 1954; **9**: 574-585.

8. Court Brown W, Abbatt J. Mortality from cancer and other causes after radiotherapy for ankylosing spondylitis. **Br Med J** 1965; **2**: 1327-1329.

9. Minot GR, Buckman TE, Isaacs R. Chronic myelogenous leukemia - age incidence, duration and benefit derived from irradiation. **JAMA** 1924; **82**: 1489-1494.

10. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. **Science** 1960; **132**: 1497-1501.

11. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. **Nature** 1973; **243**: 290-291.

12. Groffen J, Stephenson JR, Heisterkamp N, et al. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. **Cell** 1984; **36**: 93-99.

13. De Klein A, Guerts van Kessel A, Grosveld G, et al. A cellular oncogene is translocated to the Ph' chromosome in chronic myeloid leukaemia. **Nature** 1982; **300**: 765-767.

14. Collins SJ, Groudine MT. Rearrangement and amplification of c-abl sequences in the human chronic myelogenous leukemia cell line K562. **Proc Natl Acad Sci USA** 1983; **80**: 4813-4817.

15. Konopka JB, Watanabe SN, Witte ON. An alteration of the human c-abl protein in K562 leukemia cells unmasks associated tyrosine kinase activity. **Cell** 1984; **37**: 1035-1042.

16. Ben-Neriah Y, Daley GQ, Mes-Masson AM, et al. The chronic myelogenous leukemia specific p210 protein is the product of the bcr/abl hybrid gene. **Science** 1986; **223**: 212-214.

17. Pugh WC, Pearson M, Vardiman JW, Rowley JD. Philadelphia chromosome negative chronic myelogenous leukaemia: a morphological reassessment. **Br J Haematol** 1985; **60**: 457-468.

18. Ganesan TS, Rassool F, Guo AP, et al. Rearrangement of the BCR gene in Philadelphia chromosome negative chronic myeloid leukemia. **Blood** 1986; **68**: 957-960.

19. Morris CM, Reeve AE, Fitzgerald PH, Hollings PE, Beard MEJ, Heaton DC. Genomic diversity correlates with clinical variation in Ph'-negative chronic myeloid leukaemia. **Nature** 1986; **320**: 281-283.

20. Dreazen O, Klisak I, Rassool F, et al. Do oncogenes determine clinical features in chronic myeloid leukaemia?. **Lancet** 1987; **i**: 1402-1405.

21. Bennett JM, Catovsky D, Flandrin G, Galton DAG, Gralnick HR, Sultan C. The French-American-British Cooperative Group: proposals for the classification of the myelodysplastic syndromes. **Br J Haematol** 1982; **51**: 189-199.

22. Pusey WA. Report of cases treated with Roentgen rays. **JAMA** 1902; **38**: 911-919.

23. Galton DAG. Myleran in chronic myeloid leukaemia. Results of treatment. **Lancet** 1953; **i**: 208-213.

24. Kennedy BJ, Yarbro KW. Metabolic and therapeutic effects of hydroxyurea in chronic myeloid leukemia. **JAMA** 1966; **195**: 1038-1043.

25. Talpaz M, Kantarjian HM, McCredie KB, et al. Chronic myelogenous leukemia: hematologic remission and cytogenetic improvements induced by recombinant alpha A interferon. **N Engl J Med** 1987; **314**: 1065-1069.

26. Cunningham I, Gee T, Dowling M, et al. Results of treatment of Ph'+ chronic myelogenous leukemia with an intensive treatment regimen (L-5 protocol). **Blood** 1979; **53**: 375-395.

27. Sokal JE, Baccarini M, Tura S, et al. Prognostic discrimination among younger patients with chronic granulocytic leukemia: relevance to bone marrow transplantation. **Blood** 1985; **66**: 1352-1357.

28. Fefer A, Cheever MD, Greenberg PD, et al. Treatment of chronic granulocytic leukemia with chemoradiotherapy and transplantation of marrow from identical twins. **N Engl J Med** 1982; **306:** 63-68.

29. Fefer A, Clift RA, Doney K, et al. Syngeneic and allogeneic bone marrow transplanation for chronic granulocytic leukemia. **Pro Am Soc Clin Oncol** 1984; **3:** 205.(Abstract)

30. Thomas ED, Clift RA, Fefer A, et al. Marrow transplantation for the treatment of chronic myelogenous leukemia. **Ann Intern Med** 1986; **104:** 155-163.

31. Fefer A, Cheever MA, Greenberg PD. Identical twin (syngeneic) marrow transplantation for hematological cancers. **JNCI** 1986; **76:** 1269-1273.

32. Fefer A, Cheever M, Thomas ED, et al. Disappearance of Ph'positive cells in four parients with chronic granulocytic leukemia after chemotherapy, radiation and bone marrow transplantation from an identical twin. **N Engl J Med** 1979; **300:** 333-337.

33. Goldman JM, Johnson SA, Catovsky D, et al. Identical twin bone marrow transplantation for patients with leukemia and lymphoma. **Transplantation** 1981; **31:** 140-141.

34. Jones L, Thein SL, Jeffreys AJ, Apperley JF, Catovsky D, Goldman JM. Identical twin marrow transplantation for 5 patients with chronic myeloid leukaemia: role of DNA fingerprinting to confirm homozygosity. **Europ J Haematol** 1987; **39:** 144-147.

35. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions following bone marrow transplantation in humans. **Blood** 1990; **75:** 555-562.

36. Thomas ED, Buckner CD, Clift RA, et al. Marrow transplantation for acute nonlymphoblastic leukemia in first remission. **N Engl J Med** 1979; **301:** 597-599.

37. Goldman JM, Gale RP, Horowitz MM, et al. Bone marrow transplantation for chronic myelogenous leukemia in chronic phase: increased risk of relapse associated with T cell depletion. **Ann Intern Med** 1988; **108:** 806-814.

38. Storb R, Epstein RB, Graham TC, et al. Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. **Transplantation** 1970; **9:** 240-246.

39. Powles RL, Clink HM, Spence D, et al. Cyclosporin A to prevent graft-versus-host disease in man after allogeneic bone marrow transplantation. **Lancet** 1980; **i:** 327-329.

40. Storb R, Deeg HJ, Thomas ED, et al. Preliminary results of prospective randomized trials comparing methotrexate and cyclosporin for prophylaxis of graft-vs-host disease after HLA identical marrow transplantations. **Transplant Proc** 1983; **15:** 2620-2623.

41. Doney K, Buckner CD, Thomas ED, et al. Allogeneic marrow transplantation for chronic granulocytic leukemia. **Exp Hematol** 1981; **9:** 966-971.

42. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporin compared with cyclosporin alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. **N Engl J Med** 1986; **314:** 729-735.

43. Prentice HG, Blacklock HA, Janossy G, et al. Depletion of T lymphocytes in donor marrow prevents significant graft-versus-host disease in matched allogeneic leukaemic transplant recipients. **Lancet** 1984; **i:** 472-475.

44. Mitsuyasu RT, Champlin RE, Gale RP, et al. Treatment of donor bone marrow with monoclonal anti-T-cell antibody and complement for the prevention of graft-versus-host disease: a prospective randomized double blind trial. **Ann Intern Med** 1986; **105:** 20-26.

45. Apperley JF, Jones L, Hale G, et al. Bone marrow transplantation for patients with chronic myeloid leukaemia: T-cell depletion with Campath-1 reduces the incidence of graft versus host disease but may increase the risk of leukaemic relapse. **Bone Marrow Transplantation** 1986; **1:** 53-68.

46. Apperley JF, Mauro F, Goldman JM, et al. Bone marrow transplantation for chronic myeloid leukaemia in chronic phase: importance of a graft versus leukaemia effect. **Br J Haematol** 1988; **69:** 239-245.

47. Hale G, Cobbald S, Waldmann H for Campath-1 users. T-cell depletion with Campath-1 in allogeneic bone marrow transplantation. **Transplantation** 1988; **45:** 753-758.

48. Cunningham I, Castro-Malaspina H, Flomenberg N, et al. Improved results of bone marrow transplantation (BMT) for chronic myelogenous leukemia using marrow depleted of T cells by soybean lectin agglutination and E-rosette depletion. In: Gale RP, Champlin RE, eds. Progress in Bone Marrow Transplantation. New York: Alan Liss Inc., 1987: 359-363.

49. de Witte T, Hoogenhout J, de Pauw B, et al. Depletion of donor lymphocytes by counterflow centrifugation successfully prevents graft-versus-host disease in matched allogeneic marrow transplantation. **Blood** 1986; **67:** 1302-1308.

50. Butturini A, Seeger RC, Gale RP. Recipient immune competent T lymphocytes can survive intensive conditioning for bone marrow transplantation. **Blood** 1986; **68:** 954-956.

51. Burnett AK, Hann IM, Robertson AG, et al. Prevention of graft-versus-host disease by ex vivo T-cell depletion: reduction in graft failure with augmented total body irradiation. **Leukemia** 1988; **2:** 300-303.

52. Clift RA, Martin PJ, Fisher L, Buckner CD, Thomas ED. Allogeneic marrow transplantation for CML in accelerated phase - risk factors for survival and relapse. **Blood** 1987; **70 (supplement 1):** 291.(Abstract)

53. Speck B, Bortin MM, Champlin R, et al. Allogeneic marrow transplantation for chronic myelogenous leukaemia. **Lancet** 1984; **i:** 665-668.

54. Goldman JM. Biological difference in the three major leukaemias. **Lancet** 1988; **ii:** 164-165.

55. Marmont AM, Gale RP, Butturini A, et al. T-cell depletion in allogeneic bone marrow transplantation: progress and problems. **Haematologica** 1989; **74:** 235-248.

56. Butturini A, Bortin MM, Gale RP. Graft-versus-leukemia following bone marrow transplantation. **Bone Marrow Transplantation** 1987; **2:** 233-242.

57. Schultz FW, Vriesendorp HM, Hagenbeek A. On the quantitative role of graft-versus-host disease in decreasing the leukemia relapse rate after allogeneic bone marrow transplantation. **Submitted.**

58. Gale RP, Reisner Y. Graft rejection and graft versus host disease: mirror images. **Lancet** 1986; **ii:** 862-863.

59. Butturini A, Gale RP. The role of T cells in preventing relapse in chronic myelogenous leukemia. **Bone Marrow Transplantation** 1987; **2:** 351-354.

60. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft versus host disease in recipients of allogeneic marrow grafts. **N Engl J Med** 1979; **300:** 1068-1073.

61. Truitt RL, Shih CY, Lefever AV. Manipulation of graft versus host disease for a graft versus leukemia effect after allogeneic transplantation in AKR mice. **Transplantation** 1986; **41:** 301-310.

62. Bortin MM, Truitt RL, Rimm AA, Bach FH. Graft-versus-leukemia reactivity induced by alloimmunisation without augmentation of graft-versus-host reactivity. **Nature** 1979; **281:** 490-491.

63. Boranovic M. Graft versus leukemia in bone marrow transplantation. **Blut** 1989; **57**: 57-63.

64. Truitt RL, Lefever AV, Shih CC-Y. Graft versus leukemia reactions: experimental models and clinical trials. In: Gale RP, Champlin R, eds. Progress in bone marrow transplantation. New York: Alan R Liss, 1987: 219-232.

65. Shih CC, Truitt RL. A class II antigen-specific T cell clone can suppress graft-versus-host reactivity in murine allogeneic bone marrow chimeras. **Transplant Proc** 1987; **19**: 2664-2667.

66. North RJ. The murine antitumor immune response and its therapeutic manipulation. **Adv Immunol** 1984; **35**: 89-155.

67. Herberman RB, Ortaldo JR. Natural killer cells: their roles in defenses against disease. **Science** 1981; **214**: 24-30.

68. Mathe G, Amiel JL, Schwartzenberg L. Active immunotherapy for all. **Lancet** 1969; **i**: 697-699.

69. Morton DL, Eilber FR, Malmgren AR. Immunological factors which influence response to immunotherapy in malignant melanoma. **Surgery** 1970; **68**: 658-664.

70. Gutterman JU, Mavligit GM, McBride C, et al. Active immunotherapy of recurrent malignant melanoma with systemic BCG. **Lancet** 1973; **i**: 1208-1212.

71. Nathanson L. Use of BCG in the treatment of human neoplasms: a review. **Sem Oncol** 1984; **1**: 337-350.

72. Mathe G. Active immunotherapy. **Adv Cancer Res** 1971; **14**: 1-36.

73. Streuli M, Nagata S, Weissmann C. At least three human type alpha interferons: structure of alpha 2. **Science** 1980; **209**: 1343-1347.

74. Isaacs A, Lindenmann J. Virus interference. I. The interferon. **Pro Royal Soc London** 1957; 259-267.

75. Quesada JR, Reuben J, Manning JT, Hersh EM, Gutterman JU. Alpha interferon for induction of remission in hairy-cell leukemia. **N Engl J Med** 1984; **310**: 15-18.

76. Mihich E, Feffer A. Biological response modifiers: subcommittee report. **National Cancer Institute Monograph** 1983; **63**: 1-252.

77. Kasakura S, Lowenstein L. A factor stimulating DNA synthesis derived from the medium of leucocyte cultures. **Nature** 1965; **208**: 794-795.

78. Gordon J, MacLean LD. A lymphocyte stimulating factor produced in vitro. **Nature** 1965; **208:** 795-796.

79. Morgan DA, Ruscetti FW, Gallo RC. Selective in vitro growth of T lymphocytes from normal human bone marrows. **Science** 1976; **193:** 1007-1008.

80. Aarden LA, Brunner TK, Cerottini J-C, et al. Revised nomenclature for antigen-nonspecific T cell proliferation and helper factors. **J Immunol** 1979; **122:** 2928-2929.

81. Seigel LJ, Harper ME, Wong-Staal F, Gallo RC, Nash WG, O'Brien SJ. Gene for T-cell growth factor: location on human chromosome 4q and feline chromosome B1. **Science** 1984; **223:** 175-178.

82. Fujita T, Takaoka C, Matsui H, Taniguchi T. Structure of the human interleukin 2 gene. **Proc Natl Acad Sci USA** 1983; **80:** 7437-7441.

83. Sondel PM, Hank JA, Kohler PC, Chen BP, Minkoff DZ, Molenda JA. Destruction of autologous human lymphocytes by interleukin 2-activated cytotoxic cells. **J Immunol** 1986; **137:** 502-511.

84. Siegel JP, Sharon M, Smith PL, Leonard WJ. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. **Science** 1987; **238:** 75-78.

85. Smith KA. Interleukin-2: inception, impact, and implications. **Science** 1988; **240:** 1169-1176.

86. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. **Nature** 1988; **334:** 395-402.

87. Hercend T, Meuer S, Brennan A, et al. Identification of a clonally restricted 90 kD heterodimer on two human cloned natural killer cell lines. Its role in cytotoxic effector function. **J Exp Med** 1983; **158:** 1547-1560.

88. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. **J Exp Med** 1983; **157:** 705-719.

89. Hercend T, Griffin JD, Bensussan A, et al. Generation of monoclonal antibodies to a human natural killer clone. Characterization of two natural killer-associated antigens, NKH1A and NKH2, expressed on subsets of large granular lymphocytes. **J Clin Invest** 1985; **75:** 932-943.

90. Ritz J, Campen TJ, Schmidt RE, et al. Analysis of T-cell receptor gene rearrangement and expression in human natural killer clones. **Science** 1985; **228:** 1540-1544.

91. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. **J Immunol** 1983; **131:** 1789-1796.

92. Barlozzari T, Reynolds CW, Herberman RB. In vivo role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti- asialo GM1-treated rats. **J Immunol** 1983; **131:** 1024-1027.

93. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenonenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. **J Exp Med** 1982; **155:** 1823-1841.

94. Grimm EA, Robb RJ, Roth JA, et al. Lymphokine-activated killer cell phenomenon. III. Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells. **J Exp Med** 1983; **158:** 1356-1361.

95. Grimm EA, Ramsey KM, Mazumder A, Wilson DJ, Djeu JY, Rosenberg SA. Lymphokine-activated killer cell phenomenon. II. Precursor phenotype is serologically distinct from peripheral T lymphocytes, memory cytotoxic thymus-derived lymphocytes, and natural killer cells. **J Exp Med** 1983; **157:** 884-897.

96. Talmadge JE, Phillips H, Schindler J, Tribble H, Pennington R. Systematic preclinical study on the therapeutic properties of recombinant human interleukin 2 for the treatment of metastatic disease. **Cancer Res** 1987; **47:** 5725-5732.

97. Phillips JH, Lanier LL. Dissection of the lymphokine activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. **J Exp Med** 1986; **164:** 814-825.

98. Lotze MT, Grimm E, Mazumder A, Strausser JL, Rosenberg SA. Lysis of fresh and cultured autologous tumor by human lymphocytes cultured in T cell growth factor. **Cancer Res** 1981; **161:** 4420-4425.

99. Rosenberg SA, Mule JJ, Spiess PJ, Reichert CM, Schwartz S. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high dose interleukin 2. **J Exp Med** 1985; **161:** 1169-1188.

100. Rosenberg SA, Lotze MT, Muul LM, et al. Observations on the systemic administration of autologous lymphokine activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. **N Engl J Med** 1985; **313:** 1485-1492.

101. Rosenberg SA. The development of new immunotherapies for the treatment of cancer using interleukin-2. A review. **Ann Surg** 1988; **208:** 121-135.

102. Anegon J, Cuturi MC, Trinchieri G, Perussia B. Interaction of Fc receptor (CD16) ligands induces transcription of interleukin 2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. **J Exp Med** 1988; **167:** 452-472.

103. Lanier LL, Phillips JH. Evidence for three types of human cytotoxic lymphocytes. **Immunol Today** 1986; **7:** 132-134.

104. Schmidt RE, Bartley G, Levine H, Schlossman SF, Ritz J. Functional characterization of LFA-1 antigens in the interaction of human NK clones and target cells. **J Immunol** 1985; **135:** 1020-1025.

105. Siliciano RF, Pratt JC, Schmidt RE, Ritz J, Reinherz EL. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. **Nature** 1985; **317:** 428-430.

106. Ross GD, Cain JA, Lachmann PJ. Membrane complement receptor type three (CR3) has lectin-like properties analogous to bovine conglutinin as functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. **J Immunol** 1985; **134:** 3307-3315.

107. Zalman LS, Brothers MA, Chiu FJ, Muller-Eberhard HJ. Mechanism of cytotoxicity of human large granular lymphocytes: relationship of the cytotoxic lymphocyte protein to the ninth component (C9) of human complement. **Proc Natl Acad Sci U S A** 1986; **83:** 5262-5266.

108. Tschopp J, Schafer S, Masson D, Peitsch MC, Heusser C. Phosphorylcholine acts as a Ca2+-dependent receptor molecule for lymphocyte perforin. **Nature** 1989; **337:** 272-274.

109. Degliantoni G, Murphy M, Kobayashi M, Francis MK, Perussia B, Trinchieri G. Natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and NK cytotoxic factor. Relationship with tumor necrosis factor and synergism with immune interferon. **J Exp Med** 1985; **162:** 1512-1530.

110. Lotzova E. Role of natural killer cells in defense against leukemia: therapeutic considerations. **Nat Immun Cell Growth Regul** 1988; **7:** 170-179.

111. Verfaillie C, Miller W, Kay N, McGlave P. Adherent lymphokine-activated killer cells in chronic myelogenous leukemia: a benign cell population with potent cytotoxic activity. **Blood** 1989; **74:** 793-797.

112. Lotzova E, Savary CA, Herberman RB. Induction of NK cell activity against fresh human leukemia in culture with interleukin 2. **J Immunol** 1987; **138:** 2718-2727.

113. Adler A, Chervenick PA, Whiteside TL, Lotzova E, Herberman RB. Interleukin 2 induction of lymphokine-activated killer (LAK) activity in the peripheral blood and bone marrow of acute leukemia patients. **Blood** 1988; **71:** 709-716.

114. Visani G, Delwel R, Touw I, Bot F, Lowenberg B. Membrane receptors for interleukin 2 on hematopoietic precursors in chronic myeloid leukemia. **Blood** 1987; **69:** 1182-1187.

115. Motoi T, Uchiyama T, Hori T, Itoh K, Uchino H, Ueda R. Elevated serum-soluble interleukin-2 receptor (Tac antigen) levels in chronic myelogenous leukemia patients with blastic crisis. **Blood** 1989; **74:** 1052-1057.

116. Witherspoon RP, Matthews D, Storb R, et al. Recovery of in vivo cellular immunity after human marrow grafting. Influence of time postgrafting and acute graft-versus-host disease. **Transplantation** 1984; **37:** 145-150.

117. Witherspoon RP, Deeg HJ, Lum LG, et al. Immunologic recovery in human marrow graft recipients given cyclosporine or methotrexate for the prevention of graft-versus-host disease. **Transplantation** 1984; **37:** 456-461.

118. Noel DR, Witherspoon RP, Storb R, et al. Does graft-versus-host disease influence the tempo of immunologic recovery after allogeneic human marrow transplantation? An observation on 56 long-term survivors. **Blood** 1978; **51:** 1087-1105.

119. Keever CA, Small TN, Flomenberg N, et al. Immune reconstitution following bone marrow transplantation: comparison of recipients of T-cell depleted marrow with recipients of conventional marrow grafts. **Blood** 1989; **73:** 1340-1350.

120. Wimperis JZ, Brenner MK, Prentice HG, et al. Transfer of a functioning humoral immune system in transplantation of T-lymphocyte-depleted bone marrow. **Lancet** 1986; **1:** 339-343.

121. Daley JP, Rozans MK, Smith BR, Burakoff SJ, Rappeport JM, Miller RA. Retarded recovery of functional T cell frequencies in T cell- depleted bone marrow transplant recipients. **Blood** 1987; **70:** 960-964.

122. Saxon A, Mitsuyasu R, Stevens R, Champlin RE, Kimata H, Gale RP. Designed transfer of specific immune responses with bone marrow transplantation. **J Clin Invest** 1986; **78:** 959-967.

123. Shalit M, Ayalon M, Weiss L, Leshem B, Kedar E, Slavin S. Bone marrow transplantation with T-cell-depleted grafts. I. Reconstitution of immunohemopoietic functions in lethally irradiated mice transplanted with unseparated or T-cell-depleted syngeneic bone marrow grafts. **Transplantation** 1986; **42:** 118-122.

124. Brenner MK, Wimperis JZ, Reittie JE, et al. Recovery of immunoglobulin isotypes following T-cell depleted allogeneic bone marrow transplantation. **Br J Haematol** 1986; **64:** 125-132.

125. Goldman JM, Apperley JF, Jones L, et al. Bone marrow transplantation in patients with chronic myeloid leukemia. **N Engl J Med** 1986; **314:** 202-207.

126. Parreira A, Smith J, Hows JM, et al. Immunological reconstitution after bone marrow transplant with Campath-1 treated bone marrow. **Clin Exp Immunol** 1987; **67:** 142-150.

127. Rooney CM, Wimperis JZ, Brenner MK, Patterson J, Hoffbrand AV, Prentice HG. Natural killer cell activity following T-cell depleted allogeneic bone marrow transplantation. **Br J Haematol** 1986; **62:** 413-421.

128. Ault KA, Antin JH, Ginsburg D, et al. Phenotype of recovering lymphoid cell populations after marrow transplantation. **J Exp Med** 1985; **161:** 1483-1502.

129. Welte K, Keever CA, Levick J, et al. Interleukin-2 production and response to interleukin-2 by peripheral blood mononuclear cells from patients after bone marrow transplantation: II. Patients receiving soybean lectin-separated and T cell-depleted bone marrow. **Blood** 1987; **70:** 1595-1603.

130. Brenner MK, Reittie JE, Grob JP, et al. The contribution of large granular lymphocytes to B cell activation and differentiation after T-cell-depleted allogeneic bone marrow transplantation. **Transplantation** 1986; **42:** 257-261.

131. Heslop HE, Price GM, Prentice HG, et al. In vitro analysis of the interactions of recombinant IL-2 with regenerating lymphoid and myeloid cells after allogeneic marrow transplantation. **J Immunol** 1988; **140:** 3461-3466.

132. Keever CA, Welte K, Small T, et al. Interleukin 2-activated killer cells in patients following transplants of soybean lectin-separated and E-rosette depleted bone marrow. **Blood** 1987; **70:** 1893-1903.

133. Hercend T, Takvorian T, Nowill A, et al. Characterization of natural killer cells with antileukemia activity following allogeneic bone marrow transplantation. **Blood** 1986; **67:** 722-728.

134. Leger O, Drexler HG, Reittie JE, Secker-Walker L, Prentice HG, Brenner MK. Interleukin 2 enhances cytotoxic cell function in vitro after T-cell depleted marrow transplantation. **Br J Haematol** 1987; **67**: 273-279.

135. Reittie JE, Gottlieb D, Heslop HE, et al. Endogenously generated activated killer cells circulate after autologous and allogeneic bone marrow transplanation but not after chemotherapy. **Blood** 1989; **73**: 1351-1358.

136. Gottlieb DJ, Prentice HG, Heslop HE, Bello-Fernandez C, Galazka AR, Brenner MK. Effects of recombinant interleukin-2 administration on cytotoxic function following high-dose chemo-radiotherapy for hematological malignancy. **Blood** 1989; **74**: 2335-2342.

137. Heslop HE, Gottlieb DJ, Bianchi AC, et al. In vivo induction of gamma interferon and tumor necrosis factor by interleukin-2 infusion following intensive chemotherapy or autologous marrow transplantation. **Blood** 1989; **74**: 1374-1380.

138. Reid LM, Minato N, Gresser I, Holland J, Kadish A, Bloom BR. Influence of anti-mouse interferon serum on the growth and metastasis of tumor cells persistently infected with virus and of human prostatic tumors in athymic nude mice. **Proc Natl Acad Sci U S A** 1981; **78**: 1171-1175.

139. Knight E, Anton ED, Fahey D, Friedland BK, Jonak G. Interferon regulates c-myc expression in Daudi cells at the post-transcriptional level. **Proc Natl Acad Sci USA** 1985; **82**: 1151-1154.

140. Hokland M, Jacobsen N, Ellegaard J, Hokland P. Natural killer function following allogeneic bone marrow transplantation. Very early reemergence but strong dependence of cytomegalovirus infection. **Transplantation** 1988; **45**: 1080-1084.

141. Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. **Blood** 1975; **45**: 321-334.

142. van de Griend RJ, van Krimpen BA, Bol SJL, Thomson A, Bolhuis RLH. Rapid expansion of human cytotoxic T cell clones: growth promotion by heat-labile serum component and by various types of feeder cells. **J Immunol Meth** 1984; **66**: 285-298.

143. Pross HF, Maroun JA. The standardization of NK cell assays for use in studies of biological response modifiers. **J Immunol Meth** 1984; **68**: 235-249.

144. Griffiths SD, Cawley JC. Alpha interferon and LAK cell activity in hairy-cell leukemia. **Leukemia** 1988; **2**: 377-381.

145. Newland AC, Jones L, Mir N, Slocombe GW. Alpha 2 interferon in chronic myeloid leukaemia following relapse post-allogeneic transplant [letter]. **Br J Haematol** 1987; **66:** 141-143.

146. Borgies P, Ferrant A, Delannoy A, Van-den-Berghe H, Michaux JL. Interferon alpha induced and maintained complete remission in chronic granulocytic leukemia in relapse after bone marrow transplantation. **Bone Marrow Transplant** 1989; **4:** 127-128.

147. Dowding CR, Gordon MY, Goldman JM. Increased DR antigen expression by committed hemopoietic progenitor cells but not primitive progenitor cells in chronic myeloid leukemia. **Exp Hematol** 1988; **16:** 171-175.

148. Eaves AC, Cashman JD, Gabourg LA, Kalousek DG, Eaves CJ. Unregulated proliferation of primitive CML progenitors in the presence of normal adherent cells. **Proc Natl Acad Sci USA** 1986; **83:** 5306-5310.

149. Galvani DW, Cawley JC. Mechanism of action of alpha interferon in chronic granulocytic leukaemia: evidence for preferential inhibition of late progenitors. **Br J Haematol** 1989; **73:** 475-479.

150. Dowding C, Siczkowski M, Osterholz J, Gordon MY, Goldman JM. Interferon alpha increases glycosaminoglycan synthesis by normal marrow stromal cells. **Br J Haematol** 1990; **74 (suppl. 1):** 3.(Abstract)

151. Burns GF, Boyd AW, Beverley PCL. Two monoclonal anti-human T lymphocyte antibodies have similar biologic effects and recognize the same cell surface antigen. **J Immunol** 1982; **129:** 1451-1457.

152. Lanier LL, Engleman EG, Gatenby P, Babcock GF, Warner EL, Herzenberg LA. Correlation of functional properties of human lymphoid cell subsets and surface marker phenotype using multiparameter analysis and flow cytometry. **Immunol Rev** 1983; **74:** 143-160.

153. van den Brink MRM, Voogt PJ, Marijt WAF, van Luxemburg-Heys SAP, van Rood JJ, Brand A. Lymphokine activated killer cells selectively kill tumor cells in bone marrow without compromising bone marrow stem cell function. **Blood** 1989; **74:** 354-360.

154. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. **J Immunol** 1986; **136:** 4480-4486.

155. Perussia B, Fanning V, Trinchieri G. A human NK and K cell subset shares with cytotoxic T cells expression of the antigen recognized by antibody OKT8. **J Immunol** 1983; **131:** 223-231.

156. Gottschalk LR, Kaizer H, Gebel HM. Characterization of peripheral blood CD8/11 cells in bone marrow transplant recipients. **Transplantation** 1988; **45**: 890-894.

157. Ellis TM, Fisher RI. Functional heterogeneity of Leu19"bright"+ and Leu19"dim"+ lymphocyte activated killer cells. **J Immunol** 1989; **142**: 2949-2954.

158. Gottschalk LR, Bray RA, Kaizer H, Gebel HM. Two populations of CD56 (Leu-19)+/CD16+ cells in bone marrow transplant recipients. **Bone Marrow Transplantation** 1990 (in press).

159. Oshimi K, Oshimi Y, Akutsu M, et al. Cytotoxicity of interleukin-2 activated lymphocytes for leukemia and lymphoma cells. **Blood** 1986; **68**: 938-948.

160. Malkovsky M, Brenner MK, Hunt R, et al. T-cell depletion of allogeneic bone marrow prevents acceleration of graft versus host disease induced by exogenous interleukin 2. **Cell Immunol** 1986; **103**: 476-480.

161. Trinchieri G, Murphy M, Perussia B. Regulation of hematopoiesis by T lymphocytes and natural killer cells. **CRC Critical Rev Oncol Hematol** 1987; **7**: 219-265.

162. Takahashi M, Oshimi K, Saito H, Mizoguchi H. Inhibition of human granulocyte-macrophage formation by interleukin 2-treated lymphocytes. **Exp Hematol** 1988; **16**: 226-230.

163. Degliantoni G, Perussia B, Mangoni L, Trinchieri G. Inhibition of bone marrow colony formation by human natural killer cells and by natural killer cell-derived colony-inhibiting activity. **J Exp Med** 1985; **161**: 1152-1168.

164. Mangan KF, Hartnett ME, Matis SA, Winkelstein A, Abo T. Natural killer cells suppress human erythroid stem cell proliferation in vitro. **Blood** 1984; **63**: 260-269.

165. Holmberg LA, Miller BA, Ault KA. The effect of natural killer cells on the development of syngeneic hematopoietic progenitors. **J Immunol** 1984; **133**: 2933-2939.

166. Pistoia V, Ghio R, Nocera A, Leprini A, Perata A, Ferrarini M. Large granular lymphocytes have a promoting activity on human peripheral blood erythroid burst-forming units. **Blood** 1985; **65**: 464-472.

167. O'Brien T, Kendra J, Stephens H, Knight R, Barrett AJ. Recognition and regulation of progenitor marrow elements by NK cells in the mouse. **Immunology** 1983; **49**: 717-725.

168. Herrmann F, Schmidt RE, Ritz J, Griffin JD. In vitro regulation of human hematopoiesis by natural killer cells: analysis at a clonal level. **Blood** 1987; **69:** 246-254.

169. Hansson M, Beran M, Andersson B, Kiessling R. Inhibition of in vitro granulopoiesis by autologous allogeneic human NK cells. **J Immunol** 1982; **129:** 126-132.

170. Pistoia V, Zupo S, Corcione A, et al. Production of colony-stimulating activity by human natural killer cells: analysis of the conditions that influence the release and detection of colony-stimulating activity. **Blood** 1989; **74:** 156-164.

171. Niemeyer CM, Sieff CA, Smith BR, Ault KA, Nathan DG. Hematopoiesis in vitro coexists with natural killer lymphocytes. **Blood** 1989; **74:** 2376-2382.

172. Voogt PJ, Fibbe WE, Veenhof WFJ, et al. Cell-mediated lysis of human hematopoietic progenitor cells. **Leukemia** 1987; **1:** 427-431.

173. Zarcone D, Tilden AB, Lane VG, Grossi CE. Radiation sensitivity of resting and activated nonspecific cytotoxic cells of T lineage and NK lineage. **Blood** 1989; **73:** 1615-1621.

174. Kaminski E, Sharrock C, Hows J, et al. Frequency analysis of cytotoxic lymphocyte precursors - possible relevance to HLA-matched unrelated donor bone marrow transplantation. **Bone Marrow Transplantation** 1988; **3:** 149-165.

175. Broxmeyer HE, Lu L, Platzer E, Feit C, Juliano L, Rubin BY. Comparative analysis of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells. **J Immunol** 1983; **131:** 1300-1305.

176. Broxmeyer HE, Williams DE, Lu L, et al. The suppressive influences of tumor necrosis factors on bone marrow hemopoietic progenitor cells from normal donors and patients with leukemia: Synergism of tumor necrosis factor and interferon gamma. **J Immunol** 1986; **136:** 4487-4495.

177. Munker R, Koeffler P. In vitro action of tumor necrosis factor on myeloid leukemia cells. **Blood** 1987; **69:** 1102-1108.

178. Peetre C, Gullberg U, Nilsson E, Olsson I. Effects of recombinant tumor necrosis factor on proliferation and differentiation of leukemic and normal hemopoietic cells in vitro. **J Clin Invest** 1986; **78:** 1694-1700.

179. Hughes TP, Economou K, Mackinnon S, et al. Slow evolution of chronic myeloid leukaemia relapsing after BMT with T-cell depleted donor marrow. **Br J Haematol** 1989; **73:** 462-467.

180. The Italian Cooperative Study Group on chronic myeloid leukaemia. Timing of the haematologic diagnosis of Ph+ chronic myeloid leukaemia. **Eur J Haematol** 1987; **38:** 75-79.

181. Vujanovic NL, Herberman RB, al-Maghazachi A, Hiserodt JC. Lymphokine-activated killer cells in rats. III. A simple method for the purification of large granular lymphocytes and their rapid expansion and conversion into lymphokine-activated killer cells. **J Exp Med** 1988; **167:** 15-29.

182. Melder RJ, Rosenfeld CS, Herberman RB, Whiteside TL. Large-scale preparation of adherent lymphokine-activated killer (A-LAK) cells for adoptive immunotherapy in man. **Cancer Immunol Immunother** 1989; **29:** 67-73.

183. Seabright M. A rapid banding technique for human chromosomes. **Lancet** 1971; **2:** 971-972.

184. Arthur CK, Apperley JF, Guo AP, Rassool F, Gao LM, Goldman JM. Cytogenetic events after bone marrow transplantation for chronic myeloid leukemia in chronic phase. **Blood** 1988; **71:** 1179-1186.

185. Lawler SD, Baker MC, Harris H, Morgenstern GR. Cytogenetic studies on recipients of allogeneic bone marrow using the sex chromosomes as markers of cellular origin. **Br J Haematol** 1984; **56:** 431-443.