CLINICAL AND IMMUNOLOGICAL ASSESSMENT OF HIGH DOSE COMBINATION THERAPY AND AUTOLOGOUS BONE MARROW TRANSPLANTATION IN THE HAEMATOLOGICAL MALIGNANCIES.

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Summary

The studies described were performed on samples obtained from patients undergoing high dose therapy and autologous bone marrow transplantation at University College and Middlesex Hospital School of Medicine between 1986 and 1989. Since 1981 a total of 227 patients have undergone autologous bone marrow transplantation for the haematological malignancies acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), Hodgkin's disease (HD) and Non-Hodgkin's Lymphoma (NHL) and are available for analysis of clinical results. The clinical results obtained in each patient group are described in the first chapter.

Considerable literature exists on the influence of allogeneic bone marrow transplantation on immune recovery. Immune function in these circumstances is influenced by the immune suppression which these patients receive and also by graft versus host disease. The aim of this thesis is to evaluate the recovery of immune function following different forms of myeloablative therapy with re-infusion of autologous cryopreserved bone marrow. The major theme of the work is to compare in vivo immune responses with in vitro assays of immune function using peripheral blood before and after transplantation.

In vivo studies:

The responses to skin testing with recall antigens were used to assess T cell responses pre and post ABMT in 69 patients - 20 with with AML, 7 with ALL,

10 with NHL and 32 with Hodgkin's disease. Responses were markedly depressed in all patients immediately after ABMT but rapidly returned to normal, in most cases by two to three months after ABMT. In patients with Hodgkin's disease the response was markedly depressed before ABMT when the patients had active disease. Responses recovered to normal only in those patients who achieved good responses to the high dose therapy.

B and T cell responses were assessed by measurement of the specific immunoglobulin response against influenza virus using a killed influenza vaccine administered on the day of return of autologous marrow after high dose therapy. A total of 45 patients were vaccinated in the study - 12 patients with AML in first complete remission, five patients with ALL in first complete remission, eight patients with NHL and 20 patients with Hodgkin's disease. All patients with AML, NHL and HD mounted a brisk response, although this response was significantly reduced compared to the normal controls. The peak response was not delayed compared to the controls despite the recent administration of very high dose chemotherapy. In contrast, although the numbers are small, in the patients with ALL the responses were barely detectable, with no early peak response seen in any of the five patients. It was also found that a number of patients given recombinant GM-CSF developed antibodies against this recombinant protein and the specificity of this antibody response was determined.

In vitro studies:

Peripheral blood mononuclear cells were obtained from 100 patients prior to ABMT and at regular intervals following haematological recovery. Fluorescent activated cell sorter analysis of the cell surface phenotype of these cells was carried out by using available monoclonal antibodies. In 36 of these cases double labelling studies were performed with particular emphasis on the phenotype of the early recovering cells. There was a rapid recovery of NK and B cells after ABMT, but total T lymphocyte numbers did not return to normal until nine months after ABMT and there was a reversal of the normal CD4/CD8 ratio for up to one year after ABMT. The early regenerating CD4 cells were found to co-express CD29 and $CD45R_0$ which suggests that these cells are derived from previously activated or memory cells.

In vitro T cell proliferative responses were investigated in 91 patients by assessment of the mitogenic response to anti T cell monoclonal antibodies and the lectins PHA-P and Con A and in 25 patients using a mitogenic combination. Abnormal proliferative responses were obtained for 6-9 months post ABMT and this abnormality was not corrected by the addition of exogenous recombinant IL-2, although each agonist resulted in an increased expression of the IL-2R (CD25).

In vitro B cell functions were assessed in 91 patients by measurement of the response to polyclonal activators. Specific IgG production against influenza virus was assessed using in vitro assay in the 45 patients who had received the

influenza vaccine. In vitro antibody production was absent in samples from peripheral blood in the first three months after ABMT. This non responsiveness was not altered by the addition of exogenous IL-2 or GM-CSF. After the first three months responses remained reduced until one year after ABMT and the responses were particularly slow to recover in the patients with ALL.

These studies show that after autologous bone marrow transplantation, the defect in immune function rapidly corrects in vivo when remission is obtained even in heavily pre-treated patients. This is in keeping with the fact that these patients have few complications associated with depressed immunity.

The immune defect after ABMT is therfore influenced by the underlying disease, the type of therapy given and the response to that therapy. The persisting abnormalities detected in the peripheral blood may not be a true reflection of the in vivo immunological status and may not have physiological relevance.

Chapter 1.

Clinical Studies on the Use of Autologous Bone Marrow Transplantation in the Haematological Malignancies

1.1. Acute Myeloid Leukaemia

Conventional therapy for acute leukaemia has changed over the last fifteen years with the aim now being curative and not palliative. It is now generally accepted that a proportion of patients with newly diagnosed with acute myeloid leukaemia (AML) can achieve long term disease free survival (DFS) by intensive induction and consolidation chemotherapy. Complete remission (CR) can be achieved in approximately seventy percent of patients. However, more than fifty percent of these patients will relapse from CR such that the fraction of patients remaining in CR averages 30% in most studies (Rees et al 1986, Priesler et al 1987). Following relapse long term DFS in AML is rare (Zander et al 1988). Current conventional chemotherapy in adults with AML therefore results in a high remission rate with a subsequent high relapse rate. The use of maintenance therapy has not been shown to improve DFS (Priesler et al 1987) and this has lead to a search for an effective post remission therapy in AML.

Following the first report of successful allogeneic bone marrow transplantation for patients with AML in first CR (Thomas et al 1975) allogeneic bone marrow transplantation has become an established form of therapy for patients with

AML who have an HLA identical sibling. Transplantation in first CR of AML with marrow from an HLA identical sibling donor results in long term DFS in approximately 50% of patients. (Appelbaum et al 1988, Clift et al 1987). Single centre studies (Clift et al 1987) and data from registries (Gratwohl et al 1989) have suggested that long term survival is improved in patients treated in first remission by ablative chemo-radiotherapy and allogeneic bone marrow transplantation and this has been confirmed recently in prospective comparative studies in which actuarial event free survivals of approximately 50 % have been reported. These prospective studies have been performed to establish the role of bone marrow transplantation versus more conventional therapy for patients who lack donors and have demonstrated that marrow transplantation resulted in increased DFS (Appelbaum et al 1988, Zander et al 1988). These studies, however, are open to the criticism that patients with matched donors are lost from the non-transplant arm of the study and that a more informative study would be to randomize patients with donors to receive a bone marrow transplant in first CR or at the time of relapse. Such a study would require a large number of patients at a single institution and has not been performed.

Allogeneic transplantation is generally only applicable to the younger patient with an HLA-identical sibling. Reports from the Seattle Marrow Transplant Team suggest that the good results with allografting are mainly restricted to those patients under the age of 30 years (Clift et al 1987) and yet the majority of patients with AML are over the age of 50 years. In the MRC AML 8th trial 73 % of patients were less than 60 years but only 34 % were less than 40

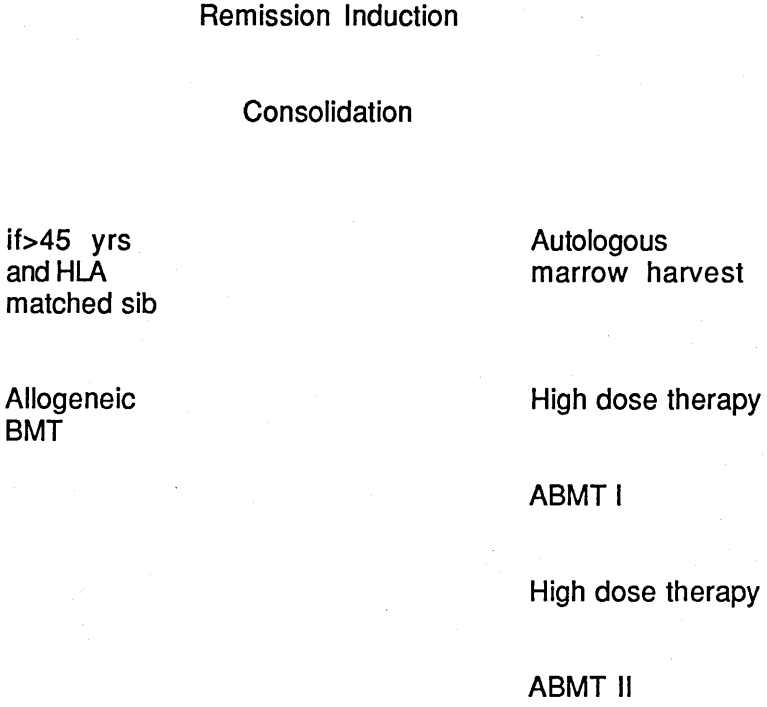
years and only 21 % were less than 30 years (Rees et al 1986). Furthermore since only about 1 in 4 patients in most Western societies have a matched sibling, allogeneic trans-plantation, if only offered to those less than 30 years old having a matched sibling, will be available to less than 10 % of patients with AML.

The majority of patients, therefore, do not have an HLA identical sibling donor or are too old to be considered as candidates for allogeneic bone marrow transplantation. An alternative approach has been to use high dose chemotherapy with rescue using autologous bone marrow harvested and cryopreserved while the patient is in remission. Autologous bone marrow transplantation (ABMT) is usually associated with a lower frequency of transplant associated mortality and morbidity but a higher frequency of relapse of leukaemia after transplantation (Linch and Burnett 1986). This may occur from the persistence of leukaemia in the patient, in the cryopreserved bone marrow or both. ABMT has resulted in long term DFS in up to 60% of patients with AML in first CR and several studies report survival comparable to that achieved with allogeneic bone marrow transplantation (Gorin et al, 1989, Yeager et al 1986). Autografts performed in second or subsequent remissions are more easy to evaluate as there is no long term survival in patients receiving chemotherapy alone in these groups of patients. Results of autografting in AML beyond first remission have been disappointing with survival of only 10 - 15% at two years (Yeager et al 1986).

1.2 Results of ABMT in Acute Myeloid Leukaemia by the Bloomsbury Transplant Group at University College and Middlesex School of Medicine.

Seventy two adult patients have now been treated using an identical protocol (Gribben et al 1989 a). The median follow up of this group is 32 months (range 7 - 103 months). All patients with AML in first complete remission were entered into the programme if they gave informed consent, had a good performance status (Karnofsky >70 %), had no HLA-matched donor if under 40 years of age and were aged 16-60 years. It must be noted that because some patients were referred to the transplant centres already in remission, an element of patient selection by the primary physician on other undefined criteria cannot be excluded. There were 34 males and 38 females with a median age of 40 years (range 16 to 57 years). Six patients had a preceding myelodysplastic syndrome (MDS) which in all cases had progressed to acute myeloid leukaemia at the time of commencement of induction therapy. The patients were all induced with a combination of daunorubicin, cytosine arabinoside and 6-thioguanine (DAT). Some patients who failed initial induction therapy received second-line induction therapy with other agents such as amsacrine, mitozantrone and etoposide. Patients received two courses of consolidation therapy with daunorubicin and cytosine arabinoside after the achievement of a complete remission. The treatment plan is shown in figure 1.1

Figure 1.1. Treatment plan.



The median time to achieve remission for the whole group was 53 days (range 20 to 338 days) and the median time from attainment of remission to ABMT was 157 days (range 23 -365 days).

All patients had a marrow aspirate performed prior to harvesting to confirm that they remained in remission by morphological and karyotypic analysis. It is of interest that one patient had morphological evidence of early relapse (Blast cells 10% of cellularity) at the time of harvest, but progressed to ABMT and regenerated normally. He refused to progress to the planned second ABMT,

but remained in remission for thirteen months after ABMT (Gribben 1987 a). Bone marrow was harvested under general anaesthesia and cryopreserved (Linch et al 1982). No in vitro purging was undertaken in an attempt to reduce minimal residual disease.

The high dose chemotherapy (UCH1 protocol) given is shown in figure 1.2.

Figure 1.2

UCH1 protocol

Drug	Dose	Days				
		- 4	- 3	- 2	- 1	0
BCNU	300 mg/m^2	*				
Cyclophosphamide	1.5 Gm/m^2	*	*	*		
Mesna		*	*	*		
Adriamycin	50 mg/m^2	*				
Cytosine arabinoside	200 mg/m^2	*	*	*	*	
6 thioguanine	200 mg/m^2	*	*	*	*	
Marrow return						*

The autologous marrow was thawed rapidly at the bedside and re-infused on day 0. Following satisfactory haematological regeneration from the first procedure (neutrophils $\geq 0.5 \times 10^9$/l and unsupported platelet count of $\geq 50 \times 10^9$/l by Day 42) patients in whom there was no contra-indication to further intensive therapy were advised to proceed as soon as possible to a second ABMT following

re-harvest of bone marrow followed by a second cycle of the same high dose chemotherapy. No further therapy was given while patients remained in remission. All patients were treated in single rooms with simple reverse barrier nursing procedures without laminar flow facilities. Blood products were not irradiated (Gribben et al 1987 b) and were not screened for cytomegalovirus . Platelets were given prophylactically when the count was below 20 x 10^9/ l. No prophylactic antibiotics were given during the period of neutropenia but empirical antibacterial therapy was given for febrile neutropenic episodes.

For all 72 patients the median number of days to achieve a total neutrophil count of ≥0.5 x 10^9/l was 19 days (range 10-49) and the median number of days to achieve an platelet count of ≥ 50 x 10^9/l, unsupported by platelet transfusion, was 34 days (range 13 - 300 days). The median number of days, following the second ABMT procedure to achieve a total neutrophil count of ≥0.5 x 10^9/l was 27 days (range 12-48 days) and platelet count of ≥ 50 x 10^9/l , unsupported by platelet transfusion, was 39 (range of 21-98 days). Paired analysis of the times for haematological recovery after the first and second procedures in the 26 patients who received two grafts showed a highly significant delay (p= 0.0005) for both neutrophil and platelet recovery after the second procedure.

There were 4 procedure related deaths from the first ABMT procedure (5.5 %) and 9 patients relapsed within 90 days of the first procedure. Of the remaining 59 patients, 43 (73%) achieved full haematological reconstitution by day 42.

These 43 patients were eligible to proceed to the planned second ABMT however only 26 patients actually received the second cycle of chemotherapy and ABMT, including two patients who had regenerated between 42 and 49 days after the first procedure.

All patients developed fever and required antimicrobial therapy. There were no cases of pneumonitis during a first procedure. The causes of the procedure related deaths which occurred after a first procedure were cerebral haemorrhage (2 cases), hepato-renal failure (1 case) and Aspergillus pneumonia (1 case). There were two cases of pneumonitis during a second procedure which were both due to cytomegalovirus, neither of which were fatal. There was a single death after a second procedure due to a Varicella pneumonia. Overall in 98 procedures in 72 patients there have been 5 procedure related deaths. Therefore the risk of a procedure related death is 5% per procedure or 7% overall per patient. There has been a single case of late pneumonitis (> 3 months) which occurred in a patient who had received a double graft but this resolved completely with a short course of oral steroid therapy. The cause of this pneumonitis was ascribed to the cumulative dose of BCNU received. There has been no long term morbidity from the procedure, except for the one case referred to above and all the survivors have a Karnofsky status of 100 %. There has been no case of cataract development in any patient.

The median follow up for the whole group is 31 months and at 5 years the projected overall survival is 58 % with a projected leukaemia free survival of 52 % (figure 1.3).

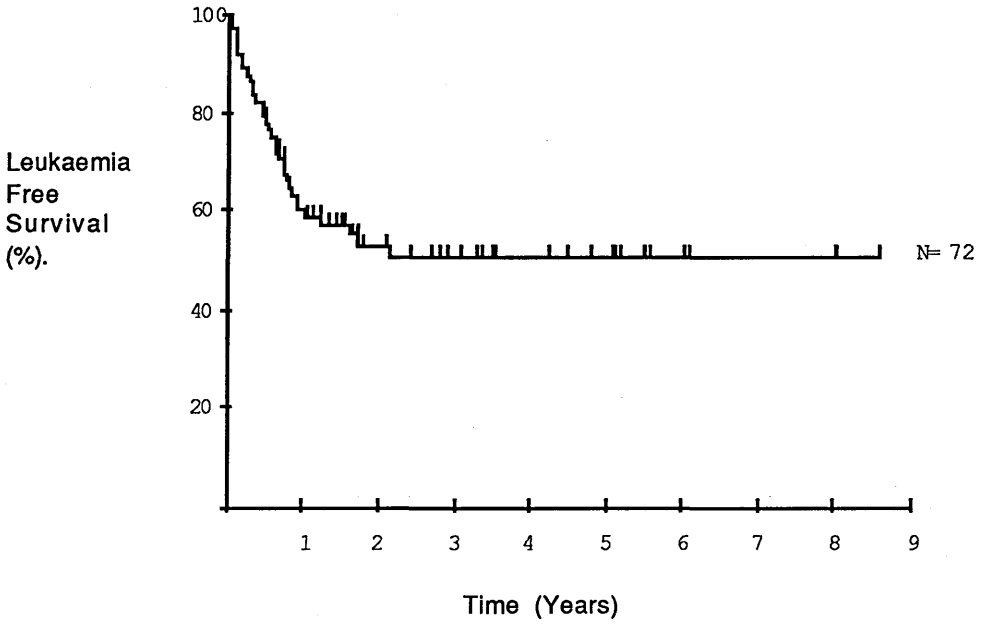


Figure 1.3.

Disease free survival after ABMT in 72 patients with AML in first remission.

As can be seen in figure 1.4. the outcome in the six patients with a known preceding myelodysplastic state has been poor with only 2 out of 6 remaining in remission at 6 months and 22 months post ABMT. All of these patients had progressed to acute myeloid leukaemia and received induction and consolidation therapy to achievement of remission before ABMT

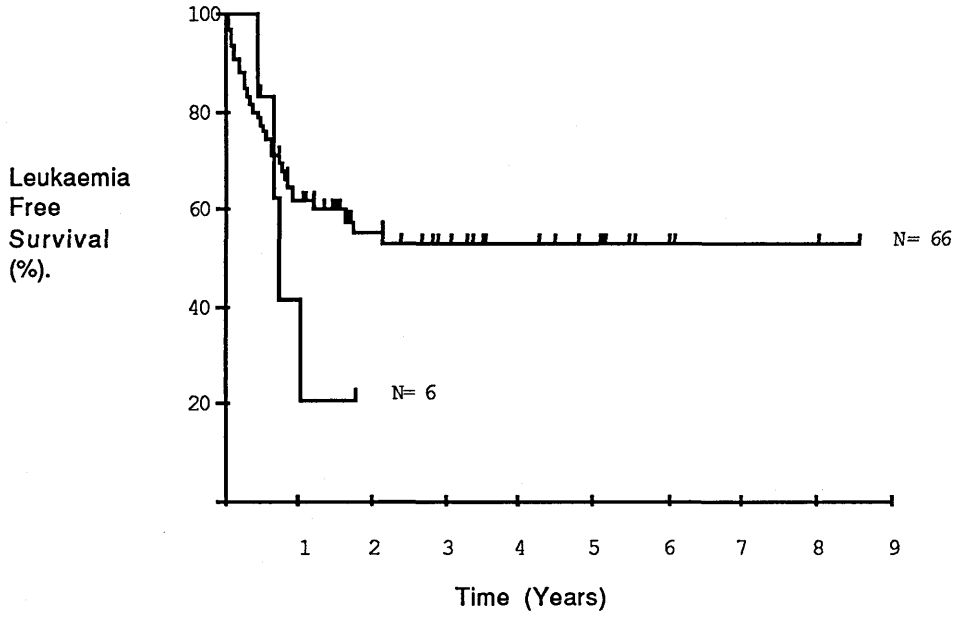


Figure 1.4. Disease free survival after ABMT in patients with AML in first remission comparing those patients who had a preceding myelodysplastic disorder with those who had de novo AML

There have been no relapses later than 26 months after ABMT. In the 25 patients with an apparently primary disorder who completed the double autograft protocol the projected overall survival at 3 years was 76%, with a projected DFS of 66% as shown in figure 1.5. This figure compares those patients who completed a double graft with only those patients who would have been eligible for a second graft, but did not proceed to the second graft.

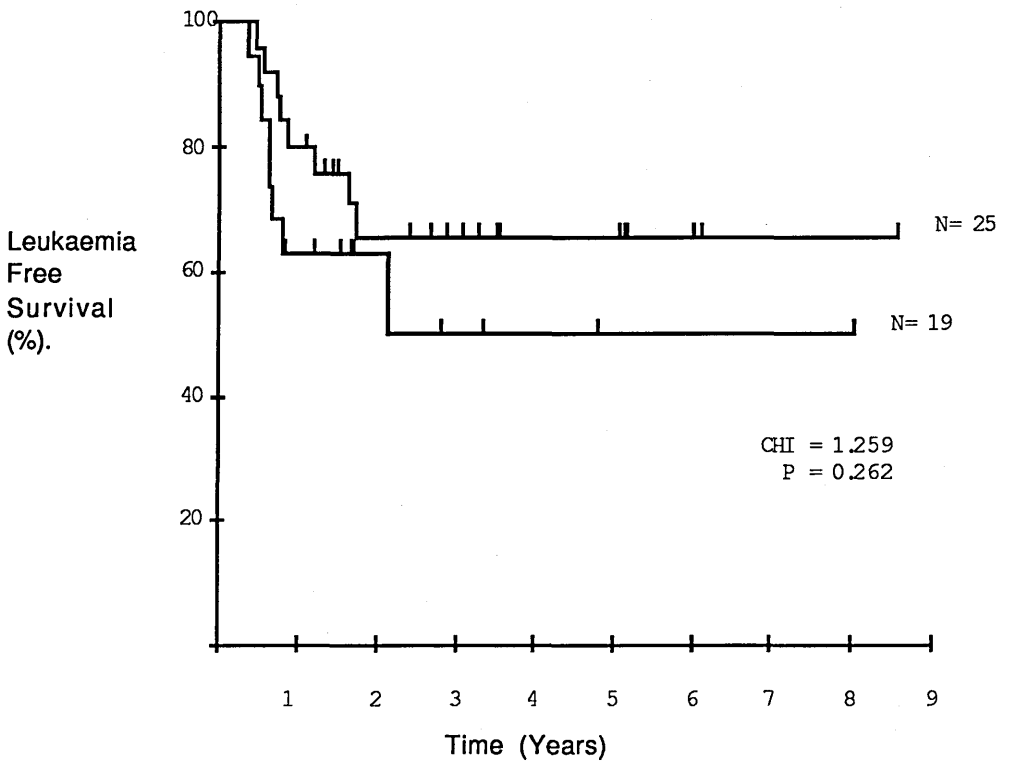


Figure 1.5.. Disease free survival comparing those patients who received only a single or double autograft.

Prognostic factors for outcome of ABMT which have been analyzed by univariate analysis included age, sex, FAB type, interval from diagnosis to remission, interval from remission to transplant and the number of consolidation courses given. Increasing age was associated with a worse outcome but none of the other factors reached statistical significance. In particular when the patients are divided into two groups, those grafted within 6 months of remission (n=41) and those grafted between 6 and 12 months into remission (n=25) there is no significant difference between the two groups in terms of Leukaemia free survival (54% versus 51%).

The chemotherapy autografting procedure used in this study has resulted in a low early procedure related mortality (7%) compared to the 20% procedure related deaths in the first 100 days after allogeneic BMT reported by Seattle (Clift et al 1987). In addition, in allograft recipients there is also a 10% incidence of late procedure related deaths (Clift et al 1987) compared to no late non-leukaemic deaths following ABMT in this study. There has been almost no significant long term morbidity from cataracts or pneumonitis observed with this regime and this contrasts with the sequelae of conventional allografting protocols. The early morbidity with our protocol is however considerable compared to conventional therapy as evidenced by the fact that only 44% of patients alive in remission after the first procedure proceeded to a second autograft as originally planned.

The rationale of the double autograft programme is based on two premises. Reharvesting of the bone marrow after completion of the first course of chemotherapy when leukaemic contamination is likely to be at a nadir effects an "in vivo" purge and the two stage process allows the overall dose of drug which is delivered to be increased without a corresponding increase in toxicity.

The advantage of decreased toxicity compared with allogeneic BMT is balanced by the increased relapse rate seen after ABMT. However the projected leukaemia free survival at 5 years for the two groups is very similar with a DFS of 52 % in this study compared with 45 % in a recent report from Seattle (Clift et al 1987) and 48 % reported by the IBMTR (Advisory Committee of the IBMTR, 1989) for allogeneic BMT in AML in first remission.

The key question in this study is whether survival has been improved compared with conventional chemotherapy alone. Unlike the comparison of transplant series, the effect of selection of patients for transplantation and the effect of the delay between remission and transplantation ("time censoring") needs to be considered when these results are compared with those of conventional chemotherapy. As this group of patients had been in remission for a median of 5 months prior to ABMT their outcome has been compared to that of patients in the 9 th MRC AML trial who maintained first remission for a minimum of 6 months. This shows that compared with the projected DFS of 52 % at 5 years reported here the projected DFS at 4 years with conventional therapy in the 9 th MRC AML trial of those in remission for a minimum of 6 months is 40 %. (personal communication Dr R Gray). This difference is small but the apparent absence of relapse later than 2.5 years from ABMT contrasts with the pattern of ongoing relapse seen with the results of long term follow up of patients treated with chemotherapy alone . Comparison of patients in different studies however cannot justify a definite conclusion and the results of large prospective randomized trials are needed.

1.3. Acute Lymphoblastic Leukaemia

The success of chemotherapy in the treatment of acute lymphoblastic leukaemia (ALL) in children (Riehm et al 1980) has not been equalled in the treatment of adults with this disease who have long term survival of only some 20% (Marcus et al 1986, Hoelzer et al 1988). The transition between adult and children with this disease appears to be early in adolescence (Crist et al 1988).

In order to improve on these results in adults with ALL many centres recommend allogeneic bone marrow transplantation either in first remission or at later stages in the disease (Thomas et al 1985). The results of allogeneic transplantation in adults with this disease are difficult to ascertain as many centres report the results of adults and children together, but the results in adults appear to be less satisfactory because of the increased procedure related mortality in adults.

As with AML, most patients do not have an HLA matched sibling donor and cannot undergo allogeneic bone marrow transplantation. We have therefore offered high dose therapy with chemotherapy and subsequent chemo-radiotherapy with autologous bone marrow rescue in adults with ALL who do not have an HLA matched sibling donor.

1.4. Results of ABMT in Acute Lymphoblastic Leukaemia by the Bloomsbury Transplant Group at University College and Middlesex School of Medicine.

Thirty one adult patients with ALL have received high dose therapy and ABMT, seventeen patients in first remission and fourteen patients beyond first remission. There were 13 males and 18 females. The median age was 23 years (range 14-47 years). All patients were initially treated with one of the Medical Research Council United Kingdom Acute Lymphoblastic Leukaemia (UKALL) protocols and received intrathecal methotrexate and cranial irradiation unless total body irradiation (TBI) was planned as part of the conditioning for ABMT within three months.

Originally the chemotherapy given was identical to that given to patients with AML (figure 1.2) and it was planned that all patients would receive two consecutive grafts as described for patients with AML. Subsequently twelve patients received a TBI containing regimen and in all cases TBI was delivered from a Cobalt source as a single fraction to a dose of 9.5 - 10.5 Gray at a slow dose rate of <5 cGy/minute. Autologous bone marrow was harvested and cryopreserved in the usual manner (Linch et al 1982) and in addition two patients with T cell ALL had their marrow treated in vitro with the monoclonal antibody CAMPATH-1 during marrow processing. The patients were treated in single rooms without the use of filtered air. Sterile food and gut sterilization were not used. Patients conditioned with TBI received irradiated blood

products, but these were not used in those patients conditioned with chemotherapy only.

There were seven early procedure related deaths due to cardiac arrest (one case), intracerebral haemorrhage (one case), bacterial sepsis (one case), aspergillus pneumonia (one case) and idiopathic interstitial pneumonitis (three cases).

The time taken for haematological recovery did not vary significantly between the different regimens used, but as with AML there was a statisitically significant delay in engraftment following the second ABMT procedure in those patients who underwent a second graft. The median survival for those patients treated beyond first remission was 178 days and for those patients treated in first remission with chemotherapy alone was 272 days. For seven patients treated in first remission with Melphelan and TBI three have relapsed and four patients remain in remission from 130 to 840 days after ABMT.

The results of intensive therapy and ABMT in ALL patients beyond first remission have not been encouraging with long term survival less than twenty per cent. The long term survivors are all notable in that they had very long duration of first remission before relapse. However, using an identical chemotherapy to that used in AML, the results in first remission of disease have been disappointing (Gribben et al 1987 c). The EBMTG results have suggested an advantage for TBI containing regimens in this disease (Gorin et al 1989) and we have therefore treated seven patients in first remission of

disease with the protocol which produced excellent results in the Newcastle study (Proctor et al 1988). This group is too small to be analyzed separately but the preliminary results are encouraging.

The procedure related mortality in this group of patients was higher than that found in a similar group of patients with AML treated with an identical regimen and there have been no procedure deaths in the seven patients treated in first remission with Melphelan and TBI. No reason for this higher mortality is apparent.

Overall, therefore, the results of ABMT in ALL have been disappointing and the role of ABMT in the management of acute lymphoblastic leukaemia in adults must remain controversial at the present time.

1.5. Hodgkin's disease

The majority of patients with Hodgkin's disease can now be cured using combination chemotherapy or local or extended field radiotherapy (Tura et al 1986). It is established that chemotherapy is the treatment of choice for patients with advanced disease, although there is controversy regarding the optimum regimen. Despite these advances there remains a group of patients who fail to respond to first-line therapy and who have a very poor prognosis (Gribben et al 1987 d). The prognosis of those patients who relapse after first line chemotherapy is not necessarily unfavourable because durable remissions can be obtained in some patients using alternative salvage chemotherapy regimens (Canellos et al 1972, Santora et al 1982). Patients who fail to achieve a second CR or who have a further relapse also have a very poor prognosis (Gribben et al 1987 d). Patients with primary resistant disease or those who fail salvage chemotherapy are therefore candidates for alternative forms of therapy. In view of the dose dependent response rate of Hodgkin's disease (Frei and Canellos 1980) these patients are eligible for high dose therapy necessitating rescue with autologous bone marrow transplantation.

We used the experience obtained by the British National Lymphoma Investigation (BNLI) to assess which patients might be candidates for ABMT (Gribben et al 1987 d). Since 1970 over 600 patients with advanced Hodgkin's disease have been treated with MOPP type regimens and this large data base served as a tool for the design of future studies. Various prognostic factors can be defined at presentation including age, histological subtype, stage,

haemoglobin, lymphocyte count, ESR and serum albumin (Vaughan Hudson et al 1983,1987). Age is one of the strongest prognostic indicators with patients over the age of 60 years faring most poorly (Vaughan Hudson et al 1983). This group will generally not be suitable for autografting and must be excluded form further analysis. Using the remaining factors it was not possible to define at presentation a sizable group of younger patients who will do sufficiently badly to merit ABMT at presentation or in first complete remission . Once a CR has been obtained the prognostic influence of the presenting prognostic features is considerably reduced. Obviously, determination of what is a sufficiently poor survival to merit progression to ABMT is somewhat subjective. We have suggested that a projected five year survival of less than 35% is a reasonable starting point for the design of a randomized study. Since the earlier morbidity and mortality of ABMT is high we also proposed that no patient should receive an ABMT if the two year projected survival is more than 65%.

1.6 Results of ABMT in Hodgkin's disease by the Bloomsbury Transplant Group at University College and Middlesex School of Medicine.

By December 1989 74 patients with Hodgkin's disease had been treated with high dose therapy and ABMT at University College and Middlesex Medical School and the one year follow up has been reported on 44 of these patients (Gribben et al 1989 a). All patients had active disease at the time of ABMT despite receiving at least two courses of standard regimen treatments at standard

dosages or following hybrid therapy and were therefore considered to be refractory to treatment. The histological diagnosis of each patient was reviewed by an independent histopathologist. The median age of these patients was 28 years (range 18 - 52 years). Thirty seven patients had extra nodal involvement at some stage during their clinical course but only four patients had previous bone marrow involvement. At the time of ABMT no patient had bone marrow involvement, but 21 patients had extra nodal disease, particularly involving the lung. Four patients had failed to respond to front line alternating therapy and progressed immediately to ABMT. All other patients had received at least two regimens of chemotherapy and 36 patients had received radiotherapy in addition. Forty one patients had never achieved CR. The median time from diagnosis to ABMT was 19 months (range 7 - 195 months). All patients were treated using the BEAM protocol which consists of BCNU 300 mg/m^2 day 1, Etoposide 100 or 200 mg/m^2 and Ara C 200 or 400 mg/m^2 on days 2-5 and melphelan 140 mg/m^2 on day 6. All drugs were given by intra venous infusion. The marrow was returned on day 7, 24 hours after completion of the melphelan infusion.

36% of patients achieved CR within three months of the procedure. These patients received no further therapy as consolidation or maintenance. 51% achieved a partial response as defined by a > 50% reduction in tumour mass at the time of ABMT. Three patients initially assessed as a PR had slow resolution of their residual mass and achieved CR with no further therapy. Six further patients had a further decrease in the size of residual mediastinal mass over 14 - 46 months without having received further therapy and may not have active

disease. The overall survival of patients following ABMT for HD is shown in figure 1.6.

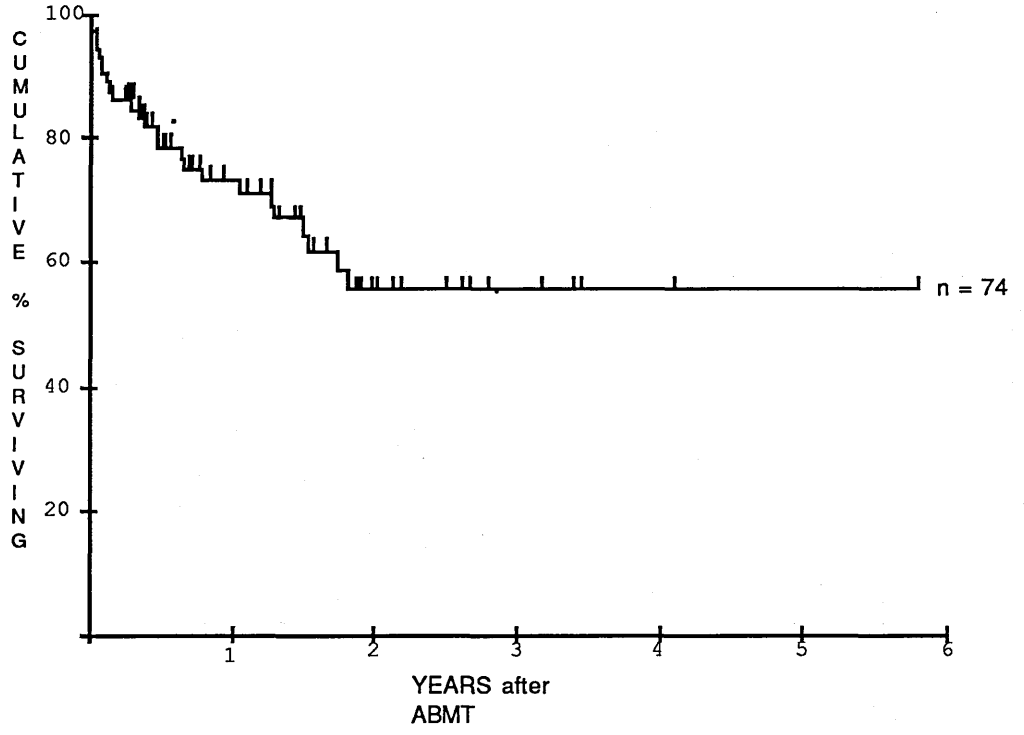


Figure 1.6. Overall survival after ABMT in Hodgkin's disease.

Eleven patients who had a partial response to ABMT as assessed by a residual mass on CT scan received subsequent radiotherapy; a full dose mantle was planned in eight and an inverted Y in two patients. Full dose was given to seven patients, one with major delays because of marrow suppression. Myelosuppression limited radiotherapy dosage in three patients. One patient receiving a mantle developed moderate pneumonitis. Of the 11 radiotherapy recipients four obtained a CR although one later relapsed, three have continued

in stable PR and four had disease progression (McMillan et al 1989). Although seven of eleven of these patients appear to have responded favourably, this must be viewed with caution as two patients initially assessed as a PR have obtained a late CR and a further four patients have had progressive improvement in CT scan abnormality over periods of one to three years without any subsequent radiotherapy. It is possible that some patients with residual mediastinal masses may not have active disease (Radcliffe et al 1988)

Univariate and multivariate analysis were performed to assess the prognostic value of age, sex stage at diagnosis and at ABMT, histology, size of largest tumour mass at diagnosis and at the time of ABMT, extra-nodal disease, previous radiotherapy and whether the patient had ever achieved CR prior to transplantation. The size of the tumour mass at ABMT was the only significant factor (χ^2=12.48) identified.

Fourteen of these patients received recombinant derived Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) as part of a phase II study to determine the effect of this growth actor on haematological regeneration after ABMT. This resulted in more rapid engraftment of myeloid precursors, but had no effect on platelet regeneration (Devereaux et al 1989). An ongoing study involves randomization of patients with Hodgkin's disease to receive either GM-CSF or placebo following re-infusion of bone marrow. This study has not yet been completed and the randomization code remains intact, so that those patients who have recently received GM-CSF cannot definitely be identified.

High dose therapy with the use of additional radiotherapy where applicable has achieved a CR rate of 50% in a group of heavily pre-treated patients with active Hodgkin's disease. Only three of these patients have subsequently relapsed. The prognosis of this group of patients with conventional dose therapy is poor with a 5 year survival of <20% and high dose therapy with ABMT may represent an advance over conventional salvage therapy. A prospective randomized study is now under way to establish whether high dose therapy with ABMT is superior to other salvage therapy in these patients with Hodgkin's disease.

1.7. Non Hodgkin's Lymphoma

Despite the success of combination chemotherapy in the treatment of diffuse intermediate and high grade non Hodgkin's lymphomas, only approximately 50% of these patients are curable on first line therapy. Those who fail to respond to first line treatment or who relapse from first remission have very poor prognoses and are only very rarely cured by alternative salvage therapy (Gribben et al 1987 d). The rationale for the use of high dose therapy and ABMT was from observations made from pilot studies by allogeneic transplantation in identical twins in lymphoma (Appelbaum et al 1981) of treatment in Burkitt's lymphomas, particularly in the paediatric age group (Philip et al 1986).

This led initially to the situation in which transplantation was used as salvage therapy for relapsed disease. This was similar to the early pioneering work from Seattle in transplantation for acute leukaemias. Once it had been established that the procedure could be carried out relatively safely and was more efficacious in disease in remission rather than in those who had already relapsed, bone marrow transplantation was used more frequently early in the course of disease. This has been justifiable because of the overall poor prognosis of patients with leukaemia, few of whom are cured by chemotherapy alone. Traditionally, patients achieving CR on induction therapy for advanced intermediate and high grade therapy have not then received bone marrow transplantation, with its attendant related risks, as consolidation therapy

before relapse of disease on the grounds that many of these patients have already been cured by first line chemotherapy.

However, many centres are now performing bone marrow transplantation procedures as consolidation therapy in first remission of advanced non Hodgkin's lymphomas. This area is fraught with many difficulties. Overall, about 65% of adult patients who achieve first CR and who are in the age group to be eligible for dose escalation and marrow transplantation are cured by their first line therapy. The identification of those patients with non Hodgkin's lymphomas who are at risk of relapse has been difficult. Those factors which predict for poor prognosis are predictive largely for those patients who will fail to achieve remission. After remission has been achieved the value of these predictive factor has largely disappeared. (Gribben et al 1987 d). Despite these difficulties, however, a considerable number of patients are now being transplanted for non Hodgkin's lymphomas in first remission. By analysis of the characteristics of these patients we can predict that many of these patients had already been cured by chemotherapy and may needlessly have been exposed to the risks of the transplant procedure. The setting for the correct use of bone marrow transplantation in such patients must be clearly established.

1.8. Treatment of Non Hodgkin's lymphoma.

Although there is general consensus on the first line treatment of patients with AML, ALL and HD, this is not the case in NHL. The reasons for this are largely the complexities of assessment of responses to therapy because of the

heterogeneity of the histological types of NHL and their impact on response to treatment. In terms of the first line treatment of NHL, clinical staging does not dictate the treatment strategy in most patients. If pathological staging is carried out, the incidence of disseminated disease is nearly 80%. However, even in patients with aggressive histologies, excellent results may be achieved by localized radiotherapy in patients who have Stage I disease confirmed by pathological staging. All patients with aggressive histology beyond pathological stage I should receive combination chemotherapy. Prognostic features identifying those patients less likely to achieve complete remission include age, male sex and presence of B symptoms, low albumin and raised ESR at presentation, bulk abdominal disease, low performance status and raised LDH.

1.9. First line treatment regimens for NHL.

In the fourteen years since the publication of De Vita (De Vita et al 1975) that the diffuse NHL's were potentially curable, there have been a large number of different combinations chemotherapy protocols developed. The major studies published during this time are shown in Table 1.1. In the early 1970's the powerful anti-lymphoma effect of the anthracyclines was appreciated and CHOP became the standard treatment at many centres. The complete remission rates reported were between 44% and 70% with approximately 30% of patients having long term disease free survival (Gams et al 1985, Armitage et al 1984). In subsequent studies a number of other agents were added to the basic CHOP agents. It was noted in the BACOP study that the incidence of CNS disease was high and high dose methotrexate was added to provide CNS prophylaxis

producing the M-BACOD regimen. More recently the dose of methotrexate has been considerably reduced without loss of efficacy.(Cannellos et al 1987). Further agents were added and the original COP-BLAM regimen was constructed to intensify treatment and increase the use of non-myelosuppressive drugs to augment tumour kill. Following the apparent improvement of efficacy using infusions of vincristine and bleomycin, COP-BLAM 3 was initiated and produced excellent responses (remission rate 84%) regardless of stage, histology, the presence of bulk disease or B symptoms

Several centres have now reported on the use of sequential different regimens such as ProMACE/MOPP (Longo et al, 1987). There have been few studies alternating two different non-cross resistant chemotherapy regimens largely because there is no obvious alternative which is equally effective as CHOP. In an attempt to solve this there has been a development of so-called 'hybrid' regimens introducing multiple agents from the beginning of treatment in one complex continuous sequence without the use of separate different regimens with gaps in between. Examples of these regimens are a hybrid ProMACE/MOPP, ProMACE-cytaBOM. A particularly interesting and novel strategy is the use of weekly rotating drug schedules such as occurs in MACOP-B which has been designed in accordance with the principles of the Goldie-Coldman hypothesis. The Vancouver group (Klimo and Connors 1985) have produced a preliminary report of excellent results using this sort of hybrid regimen although these have not been easily produced in a randomized study organized by the South West Oncology (SWOG) group (Fisher et al 1987).

Table 1.1.

Selected combination chemotherapy regimens in advanced diffuse non Hodgkin's Lymphoma.

	CR rate	DFS	Reference
MACOP - B	84%	69%	Klimo & Connors, 1987
ProMACE - CytaBOM	83%	70%	Longo et al, 1987
ProMACE - MOPP	77%	58%	Longo et al, 1987
M - BACOD	72%	48%	Skarin et al, 1983
COP - BLAM I	72%	55%	Lawrence et al, 1982
CHOP	47%	35%	Gams et al, 1986
MOPP	41%	35%	De Vita et al, 1975

1.10. Salvage chemotherapy for Non Hodgkin's Lymphoma.

This increase in the success rate of modern combination chemotherapy and hybrid regimens produces a cure rate in only 50% of patients with intermediate and high grade NHL. Patients who relapse from these front line regimens are usually refractory to drug treatment at the time of relapse and the prognosis is even worse in those patients with primary resistant disease. For this reason a number of salvage regimens have been developed, most notably at the MD Anderson Hospital in Houston. The first such regimen was IMVP16 (Cabinillas et al, 1982). Overall IMVP16 produced a remission rate of 37% but with a median remission survival of this group of only 15 months. The addition of methyl gag to this regimen (MIME) produced a remission rate of 33% as salvage in the diffuse large cell lymphomas (Cabinillas et al 1987), but a a lower remission rate of 11% in the follicular small cleaved cell types. The disease free survival of those who achieved CR was not improved by the addition of methyl GAG to the original IMVP16 regimen. In this study (Cabinillis et al, 1987) it was noted that the remission rate was highest in those patients who had ceased first line therapy more than six months prior to relapse and the commencement of MIME. The most recent reported study from this same group has been with the DHAP regimen (Velasquez et al, 1988). This regimen utilizes the reported synergistic effect of cisplatin and high dose cytosine arabinoside with the concurrent use of dexamethasone both as an anti-tumour agent and as an anti emetic. The DHAP regimen also produced an overall remission rate of 31% with a median relapse free survival in these patients of 15 months. Although the reported follow up in this report is short, there is no

apparent plateau in the survival curves. However, this regimen appears to be more effective than MIME in salvage of patients with primary resistant disease and in those with lower grade histology.

It also seems likely that the use of more drugs and more intensive regimens for first line treatment either as alternating sequential regimens or as "hybrid" regimens will mean that the tumour will be even more resistant to salvage therapy at the time of relapse. There may therefore be no effective salvage for patients who fail these high drug intensity regimens such as proMACE-cytaBOM or MACOP-B. If further studies confirm that these regimens truly do not produce more effective long term disease free survival than more simple regimens, then there may be an argument for saving some drugs to be used as potential salvage agents at the time of relapse.

1.11. Autologous bone marrow transplantation in Non Hodgkin's Lymphoma.

An alternative form of salvage therapy is the use of dose escalation using high dose combination chemotherapy or in combination with total body irradiation (TBI) followed by rescue with autologous bone marrow (ABMT). The results from selected series of ABMT studies are shown in Table 1.2.

Table 1.2.

Results of selected studies of autologous bone marrow transplantation for non Hodgkin's lymphomas.

centre	No	CR%	OS	Reference
	1. Chemosensitive disease			
	Pr to induction therapy			
France, multicentre	17	75	75 (2yr)	Philip et al, 1988
Bloomsbury	15	66	62 (3yr)	Gribben et al, 1989 c
	Chemosensitive relapse			
Boston	24	85	65 (3yr)	Takvorian et al,1987
Europe/USA	44	86	38 (2yr)	Philip et al,1987
EBMTG	75	40	28 (3yr)	Goldstone et al 1989
	2. Chemoresistant disease			
Bloomsbury	29	10	7 (2yr)	Gribben et al, 1989 c
Europe/USA	56	34	6 (2yr)	Philip et al,1987
EBMTG	194	34	15 (3yr)	Goldstone et al 1989

1.12. Allogeneic bone marrow transplantation in Non Hodgkin's Lymphoma.

It remains extremely difficult to evaluate the usefulness of allogeneic bone marrow transplantation in the non Hodgkin's lymphomas. There have been no randomized studies between autologous and allogeneic bone marrow transplantation and from EBMTG data there are many differences between those patients who have received allogeneic as opposed to autologous transplantation (Gribben et al 1987 e). The majority of patients who received an allogeneic transplant for NHL were poor prognosis patients in complete remission of disease at the time of BMT, whereas at that time 87% of patients with NHL who received an autologous transplant were treated in relapse. Since multivariate analysis of the autograft data had shown that disease status at the time of ABMT was the major determinant to predict outcome, one can only attempt to compare the two types of BMT procedures in those patients who were in complete remission at the time of transplant. There are also major differences between these two groups of patients. In those receiving allogeneic transplantation 64% had lymphoblastic lymphoma, but only 45% of patients who received ABMT in first remission had this histological type. There are other factors which have to be considered as to why patients received allogeneic transplantation in NHL other than that fact that these patients had a HLA identical sibling donor. Whereas only 11% of those patients transplanted in first remission with autologous marrow had previous bone marrow involvement, this rises to 55% in those patients who received allogeneic BMT. Virtually all patients who received allogeneic marrow were conditioned using a TBI containing regimen,

whereas only 27% of patients in first remission who received autologous marrow were conditioned with TBI. Despite the differences in patient selection, there was no difference in the overall or disease free survival of those patients treated in first remission with either autologous or allogeneic bone marrow (Gribben et al 1987 e).

The salvage of patients who have bone marrow involvement at relapse is difficult. Clearly some such patients may be best treated by allogeneic transplantation, but we restrict allogeneic transplantation in NHL to those patients under 25 years of age who had bone marrow involvement but little other disease. Clearly, the results from the Boston group of purging the bone marrow of patients who had obvious disease at the time of ABMT are very encouraging (Takvorian et al 1987) and potential methods for purging marrow of residual disease are currently under investigation, so that purging of disease from involved marrow and use of purged autologous marrow may be feasible in many of these patients. Finally, it should also be mentioned that there are a small but growing number of patients with NHL with bone marrow involvement who have been rescued after high dose therapy with stem cells collected from peripheral blood (J Armitage, personal communication).

1.13. Results of ABMT in Non Hodgkin's Lymphoma by the Bloomsbury Transplant Group at University College and Middlesex School of Medicine.

At our own centre fifty adult patients with diffuse non Hodgkin's lymphoma of intermediate and high grade histologies as defined by the International Working Formulation have been treated with high dose combination chemotherapy with a minimum follow up of one year (Gribben et al 1989 c). All patients had failed to achieve CR on conventional first line therapy or had relapsed and had active disease at the time of ABMT. The median age of the patients was 38 years (range from 18-57 years); 30 were male and 20 female. All patients referred were accepted for ABMT if they were less than 60 years of age and had failed to achieve CR on conventional dose chemotherapy or had subsequent relapsed providing that there was no bone marrow involvement at this time and there was no major cardiac, renal or liver impairment. ABMT was performed within three weeks of a decision to proceed to this form of therapy. Twenty seven patients (54%) had extra nodal disease at some stage in their disease, but only two patients had previous bone marrow involvement and two further patients had previous central nervous system (CNS) involvement. All patients had received a regimen containing adriamycin at some stage and twenty patients (40%) had also received localized radiotherapy. Thirty four patients (68%) had never achieved CR. Nineteen of these patients had primary refractory disease, defined as no response (NR) to first line regimens or initial partial response (PR) but subsequent progressive disease while still receiving treatment. A further fifteen patients had only a partial response,

defined as achieving a reduction in tumour mass greater than 50% (but not CR) and still responding to conventional dose chemotherapy. Nine of the patients who achieved PR went on immediately to ABMT after failing first line therapy (although three patients received prophylactic cranial radiotherapy) and another six received alternative conventional therapy (systemic chemotherapy or local radiotherapy excluding prophylactic cranial radiotherapy) and failed to attain a CR but continued to show response to therapy, before going on to ABMT. Sixteen patients achieved CR from initial therapy and subsequently relapsed. Five patients achieved a partial response (greater than 50% reduction in tumour mass) to the first three to four courses of salvage therapy and then went on to ABMT. These patients were defined as "responding relapse" (RR). Ten patients who had achieved a CR but did not respond to salvage therapy were defined as "non responding relapse" (NRR). One patient could not be classified for response as he did not receive any further therapy after relapse but proceeded immediately to ABMT.

Overall therefore, twenty patients had disease which was still responsive to chemotherapy at conventional dosage, twenty nine patients had disease which was resistant to chemotherapy at conventional dosage and one patient could not be classified by this criteria.

There was no evidence of bone marrow involvement in any of the patients at the time of ABMT by histological examination only of iliac crest trephine biopsies. No immunological assessment of potential marrow involvement was carried out. Bone marrow was harvested under general anaesthetic and cryopreserved as

previously described (Linch et al, 1982). Bone marrow was not "purged" by any method in an attempt to remove residual lymphoma. Unless there was already such a line in place, all patients had a Hickman central venous catheter inserted at the time of bone marrow harvest.

The first eight patients were treated with the UCH-1 regimen, consisting of BCNU 300 mg/m^2 on day 1, Cyclophosphamide 1.5 Gm/m^2 on days 1-4 and Ara C 200 mg/m^2 on days 2-5 and it was initially planned that all patients would electively undergo a double ABMT procedure. Three patients received a second procedure on this protocol using the UCH II regimen, in which the high dose cyclophosphamode is replaced by methotrexate 1 Gm/m^2 on day 1, the second time. This approach was felt to result in excessive toxicity which would limit applicability to older patients and was therefore replaced by the BEAC regimen which differed from UCH-1 regimen by the addition of etoposide 75 mg/m^2 on days 1-4, and the BEAM regimen which was identical to that given to the patients with Hodgkin's disease. Two patients who had no response to BEAM underwent a second ABMT with one patient subsequently receiving a second procedure conditioned with cyclophosphamide and total body irradiation and the other patient receiving a second BEAM regimen.

All patients were treated in single rooms with simple reverse barrier nursing procedures and without laminar flow facilities. Blood products were not irradiated and were not screened for Cytomegalovirus. No prophylactic antibiotics were given during the period of neutropenia.

All patients were assessed for response by computerized axial tomography (CT) scan at three months after ABMT in addition to clinical and radiological review.

Twelve patients (24%) achieved CR within three months of ABMT. These patients received no further therapy as consolidation or maintenance until relapse of disease. Five of these patients remain in continuous complete remission at 19 - 43+ months post ABMT. Seven patients relapsed from CR, all within two years of the procedure. Six of these patients have died of progressive disease. The seventh patient remains in stable non-progressive disease at 37 months after relapse and is maintained on therapy consisting of α-Interferon, chlorambucil and prednisolone.

Seventeen patients had a partial response to high dose therapy. Three of these patients received radiotherapy to sites of residual disease and two patients entered CR. One patient who had no response to the first procedure using BEAM and was assessed as a partial response to her second ABMT with BEAM, died at 27 months post ABMT of pulmonary toxicity and was found to have no evidence of lymphoma at an extensive post mortem examination. The overall complete remission rate of the whole group is therefore 30% i.e. 15 patients. Five patients assessed as PR on the basis of an abnormal CT scan have shown no progression of their disease despite receiving no further therapy for periods of 16 - 68+ months post ABMT and a total of 20 patients (40%) have thus had a good response (CR or lack of progression of residual radiological abnormality for more than one year) to the procedure.

Twelve patients had no response to ABMT and eleven have died of progressive disease within one year of the procedure. One patient remains alive with relapse of disease at sixteen months after ABMT. One of these patients initially received BEAM and received a second procedure after conditioning with cyclophosphamide/TBI but died during the second aplastic period of progressive disease and sepsis.

The overall survival of all patients is shown in figure 1.7.

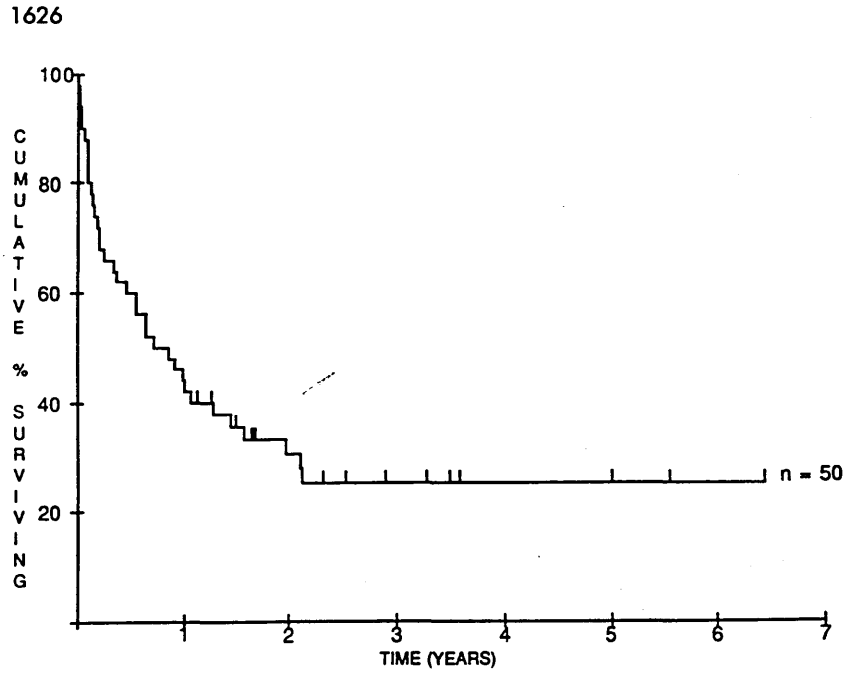


Figure 1.7. Overall survival after high dose therapy and ABMT in 50 patients with non Hodgkin's lymphoma

Using statistical analysis, the influence of sex, age at diagnosis and at ABMT, histology, stage at the time of diagnosis and at ABMT, presence of extra nodal disease previously or at ABMT, size of greatest tumour mass at diagnosis and at ABMT, response status (as defined by response to conventional dose therapy), administration of previous radiotherapy, time to recovery of granulocytes or platelets and the high dose regimen used were assessed to determine their significance as prognostic factors with regard to response to high dose therapy and overall survival after ABMT. The response status of patients at the time of ABMT (χ^2= 20.8, $p < 0.001$) was the only factor which had statistical significance, on univariate and multivariate analysis, as a predictive factor for survival after ABMT and in particular size of mass at ABMT ($p = 0.39$), presence of extra nodal disease ($p = 0.47$) and use of previous radiotherapy ($p = 0.84$) were not significant factors on analysis. The overall survival of patients dependent on response status at the time of ABMT is shown in figure 1.8.

The major procedure-related toxicities were significant marrow suppression in all patients and febrile episodes which occurred in 92% of patients. Nine patients (18%) died of sepsis during the aplastic phase and one further patient who died at 27 months after the procedure, while in CR, must also be considered a toxic death producing an overall toxic related death rate 20%. There is a marked difference in the procedure related death rate in those patients who still had disease responsive to conventional dose therapy (PR and RR) compared with those who had disease resistant to conventional dose therapy. All nine deaths occurring within three months of the procedure

occurred in those patients who had chemotherapy resistant disease and the procedure related death rate in this group is therefore 31%. Only one treatment related death occurred in those patients who still had chemotherapy responsive disease and this occurred at 27 months in the one patient who had a double procedure with BEAM. The treatment related mortality in the patients with chemotherapy responsive disease is therefore 5%. No other patients had failure of marrow engraftment. The median period after re-infusion of autologous marrow to achieve a neutrophil count of 0.5 x 10^9/l was 21 (range 13 - 40) days and to achieve a platelet count greater than 50 x 10^9/l without transfusion was 24 (range 14 - 54) days. No patient remains transfusion dependent following the procedure. Mucositis was troublesome in all of the patients, particularly in those treated with the BEAM regimen, but resolved in all cases within a few days of neutrophil recovery. Herpes simplex infections were documented or suspected on clinical grounds in 78% of patients and in all cases responded to intravenous acyclovir.

In the 15 patients who had a partial response to initial therapy, 10 patients (67%) achieved CR and a further three patients have non progressive disease on CT scan as the only abnormality. One patient who had no response to the procedure received a second procedure after a second BEAM and died in CR at 27 months of presumed chemotherapy induced pulmonary toxicity. This patient represents the only procedure related mortality which occurred in this group of patients. It must be acknowledged that many of the patients with persistent residual masses after initial chemotherapy were not re-biopsied. Active disease at the time of the ABMT was not therefore unequivocally proven.

However their prompt response to intensive therapy (reduction in mass) is, we believe,indicative of disease and not fibrosis. Even in the three patients in this group who did not achieve CR but have shown no progression of residual masses, all had a greater than 50% reduction in the tumour mass following ABMT.

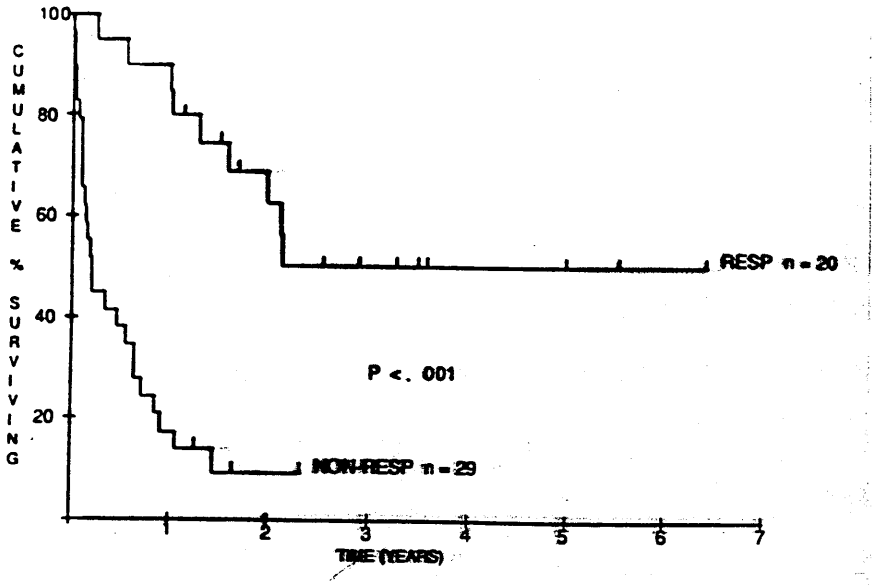


Figure 1.8. Overall survival after ABMT of patients with non Hodgkin's lymphoma depending on status at ABMT. Twenty patients had disease responsive to conventional therapy and 29 patients had disease unresponsive to conventional therapy

Of the 20 patients who still had chemotherapy responsive disease the procedure had low toxicity and a high response rate in agreement with the excellent results reported from the Boston group (Takvorian et al 1987) who electively treated only small bulk disease, which was still responsive to chemotherapy, with cyclophosphamide and total body irradiation (TBI) and rescue using bone marrow purged of residual disease using the monoclonal antibody B1. Although a number of their patients had obvious bone marrow involvement with lymphoma at the time of ABMT and these may have benefited from the purging procedure, the patients who had chemotherapy responsive disease and reported here had comparable results, despite a greater tumour burden at the time of ABMT and did not receive purged marrow. This suggests that the role of patient selection is as important as purging of bone marrow in such patients.

Ten of our patients had resistant relapse at the time of ABMT. Only two patients achieved CR and in both cases this was of short duration. Two patients in this group died of bacterial septicaemia during the aplastic period. All ten patients in this group died within 18 months of the procedure.

Nineteen patients had primary refractory disease. Only one patient achieved CR and relapsed after seven months and died of progressive lymphoma. Five patients achieved a partial response, two of whom have non-progressive changes present on CT scan at 24 and 32 months after ABMT and represent the only long term survivors in this group of patients. Seven of these patients died of sepsis during the aplastic period resulting in a treatment related mortality rate in this group of 37%.

In none of the cases was the bone marrow purged with either monoclonal antibodies or chemotherapy to attempt removal of residual disease in the bone marrow. Only two patients relapsed after ABMT at sites not previously involved; one patient relapsed in the bone marrow and one in the CNS. This suggests that re-infusion of residual lymphoma from the bone marrow is not a major problem in those patients who have no histological evidence of marrow involvement at the time of marrow harvest. Until there are more effective methods of elimination of residual tumour from the patient the precise role of bone marrow purging will be difficult to elucidate.

There is no significant difference in survival between those patients who were deemed to have achieved a CR and those who achieved a PR after ABMT despite the fact that 42% of these complete remissions have been maintained. This illustrates some of the difficulties in defining response in patients with malignant lymphoma. Firstly, the definition of CR is dependent on the sensitivity of the investigations used to detect residual tumour. Those patients who relapse shortly after the procedure may not have achieved CR post ABMT. All patients in this study were assessed by CT scan but Nuclear Magnetic resonance (NMR) and surgical restaging were not performed. Secondly, it is often difficult to assess the significance of residual masses remaining after therapy, particularly in those patients presenting with large mediastinal or retroperitoneal masses. Five patients in this study who were defined as PR on the basis of residual masses detected on CT scan have had resolution or non progression of these masses at periods from 16 - 68+ months after high dose therapy. Such masses may not represent active disease (Surbonne et al 1988.)

Two further patients initially assessed as achieving PR received localized radiotherapy to sites of residual disease and subsequently achieved CR, so that all the long term survivors in the PR group may have no active disease.

These findings, from a single centre, confirmed previous reports from multi-centre studies (Philip et al 1987, Goldstone et al 1989) that those patients who have no response to chemotherapy at conventional doses have little further response to dose escalation which may also be associated with high and perhaps unacceptable levels of morbidity and procedure related mortality. This differs from the results obtained in resistant Hodgkin's disease, where the response to BEAM followed by ABMT was not affected by the response status to conventional dose therapy. (Gribben et al 1989 a)

In those patients with NHL who have disease still responsive to conventional dose chemotherapy however, dose escalation is associated with good responses and low levels of morbidity and mortality. This is in agreement with the findings reported from the Boston group (Takvorian et al 1987) for those patients with chemotherapy responsive, low bulk disease and from the multi-centre French study (Philip et al 1988) of patients who achieved a partial response to first line therapy. Although as described above, poor responses have been reported to conventional dose salvage regimens, a number of the patients in these studies were in the category of patients who have fared equally badly with high dose therapy and ABMT. For this reason a prospective randomized study of high dose therapy versus best conventional salvage therapy in these chemotherapy responsive patients should now be carried out.

However, the use of recombinant haemopoietic growth factors may allow improved salvage therapies to be developed without the use of autologous marrow transplantation. Until this trial has been carried out it remains unclear if the potentially lethal procedure with high dose therapy requiring ABMT has a role in the management of patients with non-Hodgkin's lymphoma. No randomized studies have been undertaken of allogeneic transplantation versus either autologous transplantation or chemotherapy in the non Hodgkin's lymphomas. Although many necessary studies may now never be performed, it is essential that dose escalation requiring marrow rescue be compared in prospective randomized studies against conventional dose salvage therapy to determine the use of this approach.

1.14. Late complications of Autologous Bone Marrow Transplantation.

ABMT is therefore used increasingly in the management of the haematological malignancies and also in a variety of other solid tumours. The morbidity and mortality of ABMT is largely due to infectious complications, treatment related toxicities and relapse of disease. Although most of these infections occur during the neutropenic period these patients remain at increased risk of infection particularly during the first year after transplantation. Herpes zoster infections are particularly common in patients with malignancies and in those receiving chemotherapy, including ABMT. Herpes zoster infection occur after allogeneic bone marrow transplantation in one third to one half of patients,

making it one of the most common late complications of allogeneic BMT. In a recent study of one hundred and fifty patients undergoing ABMT 28% developed zoster infections after ABMT at a median of five months after ABMT with 91% occurring in the first year. Although there were only a small number of patients with lymphoma, the incidence of VZ was higher in patients with Hodgkin's disease and Non-Hodgkin's lymphoma (46%) than in those with acute leukaemia (23%) (Schuchter et al 1989). The overall incidence of zoster after ABMT is therefore similar to that reported after allogeneic BMT and the incidence, median time of onset and the morbidity appear to be similar in the two groups. The rate of dissemination to varicella and the mortality of VZ infections is higher in those patients receiving allogeneic bone marrow transplantation as opposed to those receiving ABMT.

1.15. Late complications after ABMT at University College and Middlesex School of Medicine.

The results described in each of the patient groups have focussed on patient selection, the early toxicities of the procedure, response to high dose therapy and subsequent disease free survival following ABMT. An important consideration is the quality of life and the longer term complications of high dose therapy and ABMT.

Only two cases of Cytomegalovirus pneumonitis have been seen in the entire population of patients autografted at this centre and both patients responded to Gancyclovir and intravenous gammaglobulin therapy. No cases of Pneumocystis

carinii have been seen. One patient developed pneumonitis with lung biopsy changes of fibrosis at one year after double ABMT for AML in first CR, but this responded well to a short course of steroids. Varicella pneumonia was fatal in one case, but this occurred during the neutropenic period. Herpes zoster infections have been seen in the first year after ABMT in forty two patients, but the 28 of these patients had Hodgkin's disease which had not responded to high dose therapy. It is noteworthy that the incidence of herpes zoster infection in this population of patients is lower than that reported from Johns Hopkins (Schuchter et al 1989) despite the fact that our own patient group comprises more patients with NHL and Hodgkin's disease, who were reported to be at particularly high risk.

Overall therefore these patients are not subject to the opportunistic infections which are seen in bone marrow allograft recipients. It therefore seemed important to evaluate the immune function of these patients following ABMT

Chapter 2.

Effects of Bone Marrow Transplantation on Immune Function.

Intensive chemotherapy required for the treatment of refractory malignancies is ablative to the lymphoid as well as the myeloid component and is therefore associated with severe immune depression. In allogeneic transplantation this immune suppression is secondary not only to the preparative chemotherapy and radiotherapy used but also to the host - graft relationship and the immunosuppressive therapy given to prevent graft versus host disease (GVHD). Syngeneic bone marrow transplantation is also associated with delay in immune reconstitution and this provided evidence that the preparative regimen itself is in some way responsible for the immune dysfunction which follows bone marrow transplantation. The use of high dose therapy with autologous bone marrow transplantation as rescue allows evaluation of the immune suppressive effects of high dose therapy uncomplicated by the presence of GVHD or immune suppressive therapy to prevent GVHD

2.1. Immune function after allogeneic bone marrow transplantation.

The high dose chemo/radiotherapy required for the treatment of refractory malignancies and the immune suppression required to prevent allogeneic graft rejection is associated with immunosuppression and an altered immune

reconstitution such that after bone marrow transplantation the recipient is affected by a functional cellular and humoral immune deficiency which lasts for up to one year (Witherspoon et al 1984 a). This immune deficiency is worsened and prolonged by the presence of GVHD (Witherspoon et al 1984 b). Immunological impairment is one of the major causes of morbidity and mortality after allogeneic bone marrow transplantation as the associated immune deficiency is associated with opportunistic infections which may be life threatening. Although the period of greatest risk is during the first 100 days the patients with GVHD remain at risk for periods beyond one year.

Studies in patients receiving allogeneic grafts have shown that the re-population of the immune system is dependent on the proliferation, maturation and differentiation of donor cells, exactly analogous to re-population of the haemopoietic system. Immunological reconstitution depends on the emergence of different components of the immune system such that the patients progress from an immunological state of relatively primitive systems with the early emergence of natural killer (NK) activity (Ault et al 1985, Reittie et al 1989) and phagocytic functions followed by a more orderly re-establishment of lymphoid cells having firstly a excess of suppressor and specific cytotoxic function and subsequently T cells responsible for regulatory control functions (Keever et al,1989).

2.2. B cell function after Allogeneic Bone Marrow Transplantation.

Following allogeneic bone marrow transplantation serum levels of IgG and IgM return to normal 3 to 4 months after grafting but IgA levels remain low for years (Gale et al 1978). Marrow recipients have severely depressed responses to immunization with new antigens (Witherspoon et al 1981), but may have normal responses to recall antigens within the first three months (Lum LG, 1987). It is likely that the source of these antibodies are B cells of donor origin and not surviving host B cells since host ABO isohaemagglutinins are absent by three months after BMT (Witherspoon et al 1981). The adoptive transfer of antibody responses from donor to recipient has been performed for recall antigens has been performed after BMT with both whole marrow and marrow depleted of T cells. (Wimperis et al 1986) and the requirements for the primary transfer of antibody responses have been established (Wimperis et al, 1990).

B cell phenotyping data is scanty after BMT. CD20 cells return rapidly but CD5+ B cells have been reported during the early post transplant period (Ault et al 1985) suggesting that more immature forms of B cells are present in the circulation in the early post transplant period. In vitro assays of Ig production from enriched B cell populations are consistently markedly reduced even in the presence of normal T cells (Witherspoon et al 1982) and similarly in vitro assays of B cell activation with Staphylococcal aureus Cowan I remain abnormal in the early post transplant period despite the addition of normal T cells

(Matsue et al 1987). These B cell defects return to normal by one year in healthy bone marrow recipients, but remain abnormal beyond this period in those patients who have chronic GVHD (Lum et al 1986).

2.3. T cell function after Allogeneic Bone Marrow Transplantation.

Consistent phenotypic and functional abnormalities have been described after allogeneic bone marrow transplantation. A number of studies have shown that the CD8+ cells quickly return to normal or even above normal levels but the CD4+ cells remain low for periods beyond six months after BMT, so that there is a reversal of the normal CD4/CD8 ratio (Reviewed by Lum LG, 1987). These changes are most pronounced in the early recovery period and in patients with acute and chronic GVHD (Friedrich et al 1982). It has also been noted that the CD3+ cells have decreased CD5 expression. The majority of these CD3+, CD5- cells co-express CD8 (Bierer et al 1989). These changes have largely reverted to normal by one year after BMT, but again those patients with GVHD have prolongation of the reversed CD4/CD8 ratio (Witherspoon et al 1984).

In vivo delayed type hypersensitivity reactions are impaired during the first three months after BMT and similarly, in vitro assays of T cell proliferative function show persistent defects to a range of mitogens during this early post transplant period (Witherspoon et al 1984).

Consistent phenotypic and functional abnormalities of T cells have therefore been described after BMT. These abnormalities are most pronounced in the early recovery period and in allogeneic patients with acute or chronic GVHD and may be increased by concomitant viral infections particularly with cytomegalovirus.

2.4 Immune function after Autologous Bone Marrow Transplantation

The immune deficit following allogeneic transplantation has been extensively investigated. The recovery of immune function after ABMT provides a model for the assessment of the effect of high dose therapy alone on immune reconstitution without the concomitant effects of GVHD, but as yet few studies have been published on immune function after ABMT

2.5. B cell function after autologous bone marrow transplantation.

Following ABMT serum levels of IgM and IgG either remain at the lower limits of normal (Armitage et al 1986) or rapidly return to normal levels by three months after ABMT (Baumgartner et al 1988, Pedrazzini et al 1989).

Very few studies have been reported on B cell function after ABMT. Kiesel et al assayed the proliferative responses to anti μ, SAC and low molecular weight B cell derived growth factor (BCGF) and found absent responses in 9 out of 22 patients and defective responses in a further 8 patients. 19 of these 22 patients had normal in vitro IgG secretion. The authors postulated that the in vivo serum IgG may be produced from the stimulation of a small number of re-infused pre-committed memory B cells while in parallel immature B cells may develop from autografted haemopoietic progenitor cells (Kiesel et al 1988).

Following ABMT with bone marrow purged with the monoclonal antibody B1, Pedrazzini et al reported in vitro impairment of proliferative responses to anti Ig, Epstein Barr virus (EBV), Interleukin 2 (IL-2) or low molecular weight BCGF for up to two years post ABMT. It is noteworthy that in this study serum Immunoglobulin levels were below the normal range for IgM for 3 months, IgG for 6 months and IgA for 2 years (Pedrazzini et al 1989). This may be explained by the fact that in these studies there has clearly been re-infusion of only a very small number of pre-committed memory cells because of the purging with B1.

B cell phenotyping data after ABMT is also scanty. In this same study Pedrazzini reported that CD20+ cells were detected immediately at the first evidence of engraftment and that 50% of patients had reached normal numbers by 3 months post ABMT (Pedrazzini et al 1989).

2.6. T cell function after Autologous Bone marrow transplantation.

There have been a number of reports confirming that a similar pattern of phenotypic abnormalities of T cells is seen after ABMT as is seen after allogeneic bone marrow transplantation. There is a rapid regeneration of CD8+ cells with a more slow recovery of CD4+ cells, resulting in a reversal of the normal CD4/CD8 ratio. (Armitage et al 1986, Olsen et al 1988, Bengtsson et al 1988, Pedrazzini et al 1989,).

Functionally, there is defective IL-2 production by T cells (Bosly et al 1987). This has been attributed to the low numbers of CD4+ cells, but perhaps may also be due to a decreased responsiveness of these cells to endogenous IL-2 (Lopez-Botet et al 1987, Cayeux et al 1989). The precise nature of the T cell defect remains unknown.

These studies suggest that similar defects occur in vitro after autologous and allogeneic bone marrow transplantation, but that these changes tend to be of shorter duration. These studies have failed to explain why the autografted patients are not prone to the same opportunistic infections that are seen after allogeneic BMT.

Chapter 3.

Lymphocyte ontogeny and function

Lymphocytes can be functionally subdivided into distinct populations by their expression of unique cell surface and molecular markers. Human T cells were classically defined by their expression of sheep red blood cell (sRBC) receptors and their ability to modulate immune responses. B cells were identified by their expression of cell surface or cytoplasmic immunoglobulin (Ig). During the past decade, the development of monoclonal antibodies (mAbs) directed against T cell molecules expressed on the surface of human lymphocytes has led to great advances in defining the phenotypic and functional characteristics of these cells. mAbs have been also been useful in the determination of cellular lineages and in identifying normal stages of lymphoid generation.

3.1. Normal B Cell Ontogeny.

Normal B cell ontogeny can be conveniently divided into stages including pre-B cells, resting B cells, activated/proliferating B cells, differentiating B cells and plasma cells whciich are secreting immunoglobulin (figure 3.1.). These stages can be defined by the expression of unique cytoplasmic and cell surface antigens. In this regard, human B cell antigens can be subgrouped into broad categories including: pan B cell; resting B cell; activation antigens; and plasma cell antigens.

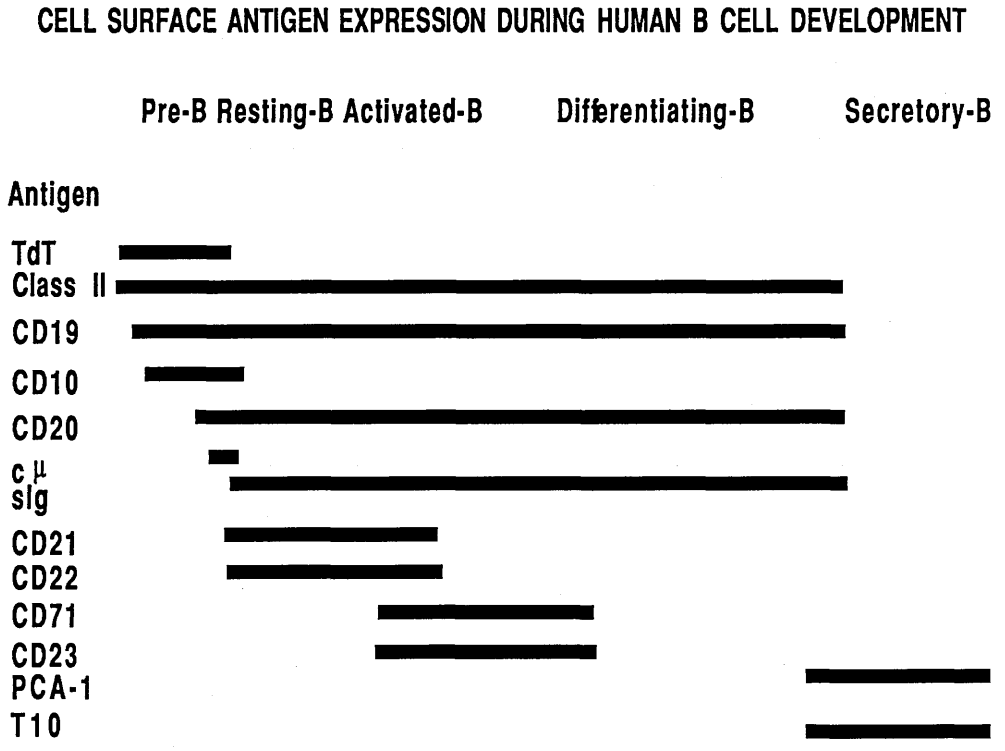


Figure 3.1.

B cell development.

Pre-B cell differentiation takes place in the foetal liver and bone marrow prenatally and in the bone marrow in adults. The earliest pre-B cells have been defined by their expression of cell surface antigens including MHC class II (HLA-DR) and CD19, and rearrangements of immunoglobulin μ heavy chain genes (Brouet et al 1979, Korsmeyer et al 1981, Korsmeyer et al 1983, Nadler et al 1983, Nadler et al 1984). CD19 is the most reliable cell surface marker of B lineage at the pre-B cell level. In addition to these cell surface proteins, a B cell restricted antigen, CD22 is present in the cytoplasm

(cCD22) at the earliest stages of pre-B cell differentiation then exported to the cell surface at the mature resting B cell stage when sIgD is first expressed (Dorken et al 1986, 1987). Following the expression of CD19 and cCD22 cells express CD10 (cALLa). Pre-B cells next express the pan-B cell restricted antigen CD20 (Nadler 1981, Stashenko et al 1980). The last stage of pre-B cell ontogeny appears with the expression of cytoplasmic Ig heavy chain without the expression of light chains. Several other antigens are expressed during pre-B cell development including CD9, CD24, CD45R (leukocyte common antigen), CD74 and CDw78 (Funderdud et al 1989, Kersey et al 1981, Hercend et al 1981)

As pre-B cells mature, they are exported to the peripheral blood and lymphoid tissues where they reside until activated by antigen (mature B cells). Mature resting B cells express sIgM/D and HLA-DR, CD19, CD20, CD24, CD22 but no longer express CD9, CD10, and CD34. Coincident with the maturation to the sIg+ stage, cells express the CD11a/CD18 LFA-1 complex (Campana et al 1986). Resting B cells also express CD44 which has been shown to be involved in lymphocyte recirculation and homing. In addition, resting B cells express CD21 which is the receptor for the C3d cleavage fragment of complement and for Epstein Barr virus (EBV) and CD35 which is the C3b complement receptor (Iida et al 1983, Fingeroth et al 1984).

A subset of mature B cells have recently been described which express the T cell associated antigen CD1. CD1 is a member of the immunoglobulin supergene family and shares homology to both MHC class I and class II. CD1 exists as 3

molecules, CD1a (49 kD), CD1b (45 kD), and CD1c (43 kD) which are non-covalently associated with beta$_2$-microglobulin. Approximately 50% of normal adult splenic B cells and a proportion of peripheral blood B cells are CD1c+, and following activation CD1c is up-regulated (Small et al 1989). B cells acquire several other antigens which are present until the plasma cell stage (CD37, CD39, CDw75, CD76) (Ling et al 1987, Dorken et al 1989, Erikstein et al 1989).

Following triggering with antigen or various polyclonal mitogens, resting B cells are activated and subsequently proliferate. This activation of resting B cells is accompanied by a sequence of cell surface antigenic changes. Within 24 hours of activation, resting B cells begin to lose sIgD, CD21, and CD22, and this process is complete by 72 to 96 hours (Boyd et al 1985a). As these antigens are lost, a number of B cell restricted and associated antigens sequentially appear (Kehrl et al 1984, Boyd et al 1985 b). These activation antigens are excellent candidates for growth factor receptors, molecules which regulate proliferation and differentiation, structures involved in cell-cell interaction and molecules which play a role in the localization and binding of activated B cells within a micro-enviroment. The majority of these activation antigens demonstrate peak expression by 72 hours and are no longer expressed on the cell surface at 120 hours.

With terminal differentiation there is the gradual loss of the B cell activation antigens as well as pan-B cell antigens including Ia, CD19, CD20, and CD24 (differentiating B cell). This terminal differentiation stage is also

characterized by the appearance of several other antigens including CD38 and PCA-1 which are expressed on plasma cells (Terhorst et al 1981, Stamenkovic et al 1989, Anderson et al 1983)

Immunoglobulin gene rearrangements.

A vast variety of possible immunoglobulin molecules exist each corresponding to a unique antigenic epitope. This divergency is though to be produced by genetic recombination within the DNA of B lymphocytes. The germline DNA contains segments coding for different subunits of the immunoglobulin molecule. For the immunoglobulin heavy chain, these consist of a very large number of many different variable (V) segments, a smaller number of different diversity (D) segments, a few joining (J) segments, and a constant region for each subclass of immunoglobulin.

The usual mechanism for achieving recombination of the immunoglobulin gene DNA includes bringing together a single V, D, and J segments with the appropriate constant region segment and excising the unused segments. Therefore, the numbers of potential recombinants of V, D, and J segments are enormous. Light chain genes have a similar mechanism but lack D segments. A functional rearrangement of a heavy or light chain gene prevents rearrangements of another gene of the same chain type (allelic exclusion), whereas an abnormal heavy or light chain rearrangement which cannot be transcribed will allow the other heavy chain allele, or one of the other light chain alleles, respectively, to attempt rearrangement. This functional allelic

exclusion ensures that only one immunoglobulin specificity is allowed for any one B cell. The heavy chain gene undergoes rearrangement first, followed by the kappa, and then the lambda light chain genes.

3.2. Normal T Cell Ontogeny.

A large number of mAbs have been developed which define cell structures which are expressed on the surface of human T cells. These mAbs have been used to characterize the stages of T cell ontogeny and differentiation, identify subsets of functionally distinct T cells, and elucidate the function of some of these cell surface antigens (Haynes et al 1989).

During embryonic and early post-natal life, bone marrow precursor cells migrate to the thymus. The thymic micro-enviroment provides a setting for the processing and eventual development of functionally competent T cells. These cells are subsequently exported into peripheral lymphoid tissues and the circulation. A sequence of changes in cell surface antigens identified by mAbs are observed to accompany intrathymic differentiation (Reinherz and Schlossman 1980) The cells in the earliest stage (stage I) of intrathymic differentiation, which constitute 10% of the thymic lymphocytes, express CD2 (E rosette receptor), CD71 (the transferrin receptor), CD38, and CD7 (Terhorst et al 1981).

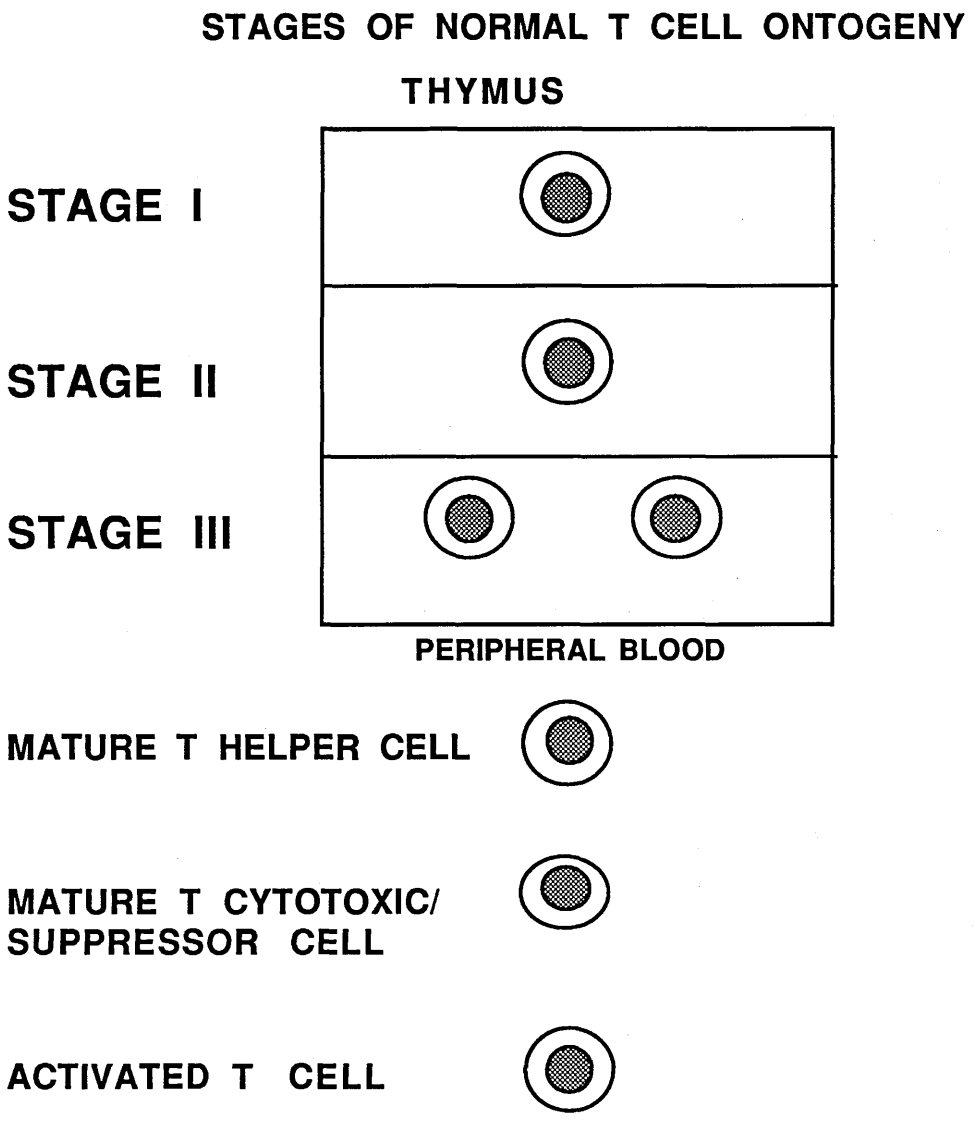


Figure 3.2.

Normal T cell development.

At the next stage of differentiation (stage II) thymocytes are characterized by the loss of CD71, and the acquisition of CD1 (Terhorst et al 1981, Amiot et al 1987) and co-expression of CD4 and CD8 (McMichael et al 1987). The

population, co-expressing CD1, CD2, CD4, CD7, CD8 and CD38, constitute 70% of thymocytes.

With further maturation (Stage III) cells lose CD1 and acquire mature T cell antigens CD3, CD5, and CD6 (Campana et al 1987). In parallel with the expression of CD3, cells express the T cell antigen receptor (TCR). CD3 is a complex of 5 chains - γ, δ, ε, ζ and η, which are non-covalently associated with the T cell antigen receptor(Acuto and Reinherz 1985) . The TCR can exist as an $\alpha\beta$ heterodimer and unlike the antigen receptor for B cells, sIg, the CD3/TCR complex recognizes antigen in the context of MHC. A second T cell receptor, is also associated with CD3 termed $\gamma\delta$ (Brenner et al 1986). Cells which express the $\gamma\delta$ TCR appear earlier in ontogeny than $\alpha\beta$ TCR cells, are CD4 and CD8 negative, and are associated with natural killer cell activity (non-MHC restricted cytotoxicity).

When cells leave the thymus, they no longer express CD38 and are segregated into cells expressing CD4 or CD8, constituting 60-70% and 30-40% of peripheral blood T cells, respectively. More recently, a series of mAbs have been developed which further subdivide CD4 helper cells into inducers of help (CD45R) and the inducers of suppression (CD29) (Morimoto et al 1985a , 1985 b). Peripheral T cells, when activated by antigen, mitogen, anti-CD2, anti-CD3, or anti-TCR mAbs, undergo additional changes in cell surface antigens. During the first 2 days after activation, T cells express CD25 - the IL-2 receptor (Uchiyama et al 1981) and CD26. The transferrin receptor

(CD71), CD70, CD9 (Hercend et al 1981), as well as CD38 reappear by 4 days of in vitro culture and HLA-DR antigens are present by 6 to 8 days.

T Cell Receptor Gene Rearrangements.

As described above the T cell receptor (TCR) is composed of several subunits including a heterodimer composed of the α and β chains or γ and δ chains in associated with the CD3 complex. The α, β and γ chains genes undergo rearrangements in the same way as immunoglobulin genes. V, D, and J DNA segments have been identified for the β chain gene, and so far, V and J segments for the others. There is also a temporal progression of rearrangement and gene expression, starting with γ chain gene rearrangement, followed by β chain gene rearrangement, and γ chain gene mRNA expression, followed by β chain mRNA expression, and finally α chain gene rearrangement, and α chain mRNA expression. It may be possible to utilize this progression to assign a stage within early T cell differentiation to a particular T cell lymphoma.

3.3. T

Lymphocyte cell surface antigens - structure and function

Monoclonal antibodies reactive with a number of lymphocyte surface proteins have recognised a variety of cell surface proteins which exhibit some homology with the immunoglobulins and are known as the immunoglobulin supergene family. This group include the T cell receptor, MHC class I and class II molecules, CD2, CD3, CD4 and CD8 each of which is critical to T cell activation.

In general the function of these molecules seems to be to mediate adhesion between cells

The T cell receptor and CD3

T cell receptors (TCR) are heterodimer glycoproteins expressed on the membrane of T cells in association with the CD3 complex. The TCR-CD3 complex contains at least seven polypeptide chains; two TCR chains and the γ, δ, ε, η and ζ CD3 chains

The classical TCR $\alpha\beta$ (TCR2) is involved in specific recognition by T cells of antigens on the surface of other cells. Initiation of an immune response against antigen requires recognition by the TCR of both antigen and MHC class II determinants on the surface of the antigen presenting cell, a phenomenon known as MHC restriction.

Recently a second TCR was described, namely the TCR $\gamma\delta$ (MB Brenner et al 1986) which is also expressed in association with the CD3 complex. The TCR $\gamma\delta$ (TCR 1) is mainly expressed on CD4-, CD8- double negative T cells, which can be found in low numbers in the adult thymus, spleen, lymph node and peripheral blood. However, in the early stages of thymic ontogeny $\gamma\delta$ bearing cells account for the majority of T cells. There is evidence that there is a limited variability in the expressed TCR $\gamma\delta$, but the function of the TCR $\gamma\delta$ bearing cells in unknown. The genomic organization and basic structure of the TCR α, β, γ and δ genes is now well characterized .

CD2

Originally described as the sheep red blood cell receptor, an alternative name was LFA-2. It exists on the cell surface as a 50 kD monomer, primarily on T cells, though it is also found on NK cells. Its natural ligand is LFA-3 (CD58), which is another member of the immunoglobulin supergene family (Selvaraj et al 1987). The virtual restriction of CD2 to T cells, with an almost ubiquitous expression of LFA-3 suggests that this may allow adhesion of T cells to any cell type requiring immune surveillance. The molecule is known to undergo conformational change on binding with the mAb 9.6 exposing a new epitope recognised by the mAb 9.1(CD2R). Stimulation of T cells with both antibodies induces antigen presenting cell independent T cell activation (Meuer et al 1984), but it is as yet unclear what the physiological significance of this pathway may be.

CD4 and CD8.

CD4 and CD8 are expressed on mutually exclusive populations of peripheral T cells, although as described above, during T cell ontogeny cells co-express CD4 and CD8. T cells do not recognize antigens in isolation but only in the context of an MHC molecule. Most antigens must be processed in order to bind to MHC molecules to be presented to T cells. Several pathways of processing have been identified and processing of antigen could potentially play a role in the triggering of a particular functional subset of T cells by targeting the processed peptide to a particular MHC class. T cells expressing CD4 almost invariably

recognise foreign antigens as peptides presented by self MHC class II molecules to which they bind (Doyle and Strominger 1987) whilst CD8+ T cells recognise foreign antigen presented as peptides by self MHC class I molecules and there is direct evidence for binding of CD8 to MHC class I (Rosenstein et al 1989).

CD4

CD4 is a monomeric 55 kD cell surface glycoprotein. There are four extracellular domains each homologous to the immunoglobulin V regions. There is homology between human and murine CD4 which is most marked in the intracytoplasmic domain, suggesting a functional role for this part of the molecule. The cytoplasmic tails of CD4 contain serine residues that are phosphorylated via protein kinase C (PKC) as a consequence of antigen or mitogen stimulation. PKC activation results in the down modulation and internalisation of CD4.

The molecule was originally described as a marker for T cells with helper function and CD4 expression is predominantly associated with helper T cell function. However, expression of CD4 is most strictly associated with recognition of antigen in association with self MHC class II molecules. The stringent association of CD4 expression with MHC class II recognition of target cells has led to the suggestion that CD4 increases the avidity of cell - cell interaction by direct binding of CD4 to determinants of the MHC class II structure. This binding function of CD4 may not be its only function. Recent

evidence suggests that CD4 may associate with TCR/CD3 (Anderson et al 1988) and be involved in signal transduction (Janeway 1989). CD4 regulatory function may be through its association with a T cell specific protein tyrosine kinase ($p58^{Lck}$) (Rudd et al 1988).

Whilst most helper T cells are CD4+, it is clear that not all CD4+ T cells are capable of providing help to B cells to produce antibodies. CD4+ cells may be cytotoxic, suppressor or T cells that activate macrophages and share the ability to mediate their effects by secreting IL-2 (Janeway 1989). Similarly most cytotoxic T cells express CD8 but CD8+ T cells can provide T helper function and some can secrete IL-2.

Recognition of other surface proteins has helped to delineate functional subsets of CD4 cells. Helper T cells have the phenotype CD4+, CD45R-, CD29+. (Morimoto 1985a) Suppressor inducer cells are CD4+, CD45R+, CD29- (Morimoto 1985 b). Suppressor effector cells are CD4+ and are induced after activation with anti CD3 with IL-2 playing a major role in their differentiation.

T cell dependent B cell activation requires direct interaction between B cells and activated T cells, presumably involving specific cell surface receptors which have not yet been recognized. It is possible that CD45R- CD4+ cells may express interaction molecules at a higher density. There is evidence that CD4 cells may function directly also as suppressor effector cells and that CD45R+ CD4+ cells can directly suppress B cell responses in poke weed mitogen

cultures. This suppressor activity can be abrogated by irradiation of the CD4 cells. CD45R+ CD4+ cells induce suppressor CD8 cells more effectively than CD45R- CD4+.

CD4 also serves as the receptor of Human Immunodeficiency Virus I.

CD8

CD8 has been characterised as a 32-43 kD protein which is present on the cell surface as an $\alpha\beta$ heterodimer or $\alpha\alpha$ homodimer.

The molecule originally described a subset of T cells delineated as cytotoxic/suppressor, but this is now known to be an over-simplification. Experimental evidence suggests that the CD8 molecule may have several roles to play on the surface of T lymphocytes. In conjunction with other accessory molecules it may enhance TCR/antigen interaction by facilitating adhesion between the T cell and antigen presenting cell. As with CD4, CD8 has been implicated in functioning also as a signal transduction molecule.(Emmrich et al 1986). Like CD4, the CD8 antigen is coupled to a protein tyrosine kinase ($p58^{Lck}$) (Barber et al 1989).

CD19

CD19 is a 95 kD glycoprotein which is a member of the Ig supergene family, with three extracellular Ig-like domains (Tedder et al 1989). In vitro studies

with normal mature B cells and pre-B cells demonstrate that anti-CD19 mAbs inhibit pre-B and mature B cell proliferation (Pezzutto et al 1987).

CD20

CD20 is a 35 kD nonglycosylated phosphoprotein. In vitro B cell functional studies suggest that CD20 is involved in the regulation of B cell activation. Different mAbs directed against CD20 can both inhibit B cell activation/proliferation or induce B cells to go from the G_0 phase of the cell cycle to the G_1 phase, depending on the source and state of activation of B cells (Clark et al 1985, Tedder et al 1985). From the cDNA, it is hypothesized that CD20 has a very small extracellular domain, several hydrophobic regions which traverse the cell membrane multiple times and a large cytoplasmic domain (Tedder et al 1988, Einfeld et al 1988, Stamenkovic et al 1988).

CD25

CD25 is the 55 kD Tac antigen which is the low affinity IL-2 receptor. It is encoded by a single gene with at least two forms of mRNA present. Originally it was believed that the CD25 antigen was present only on activated T cells, but it is now clear that it is also present on activated B cells and monocytes.

CD 29

This is the common β chain of the very late antigen (VLA) and is a member of the integrin supergene family. The putative function of CD29 is to act as an adhesion molecule and perhaps also to modulate signal transduction.

CD56

This pan NK antigen is also expressed on a subset of T cells and IL-2 activated T cells that mediate MHC unrestricted cytotoxicity (Lanier et al 1986) It is present on the cell surface as a 175 kD isoform of the adhesion molecule N-CAM.(Lanier et al 1989)

CD45 - The Common Leucocyte Antigen

CD 45 is a family of major membrane glycoproteins that range from 180 - 240 kD and are found on all haemopoietic cells except the erythroid series. Eight isoforms of CD45 have been described in mammals which are expressed differentially on leukocyte sub-populations (Thomas & Lefrancois 1988). These isoforms differ both in their pattern of glycosylation and in their primary structure as a result of alternative splicing of mRNA (Streuli et al 1987). The CD45 molecule has three distinct segments; a heavily glycosylated cysteine rich extracellular segment, a 22 residue transmembrane segment and a large carboxy terminal cytoplasmic residue which is the same in each isoform.

There are several potential phosphorylation sites for PKC within the cytoplasmic domain and it is proposed that phosphorylation of CD45 on key tyrosine residues may regulate its activity (Clark and Ledbetter 1989). The fact that CD45 is a phosphatase suggests that it may function by interacting with other membrane associated proteins (Charbonneau et al 1988). CD45 is potent regulator of lymphocyte signal transduction and affects signalling in a number of ways. When CD3, CD2 or CD28 receptors on resting human T cells are cross linked with mAbs, there is an increase in cytoplasmic free Ca^{++} (iCa^{++}) which is abolished if these same receptors are cross-linked to CD45 (Ledbetter et al 1988). Similar results are obtained when CD45 is brought into close proximity with receptors which can transmit a signal resulting in increased iCa^{++} in resting B cells such as surface Immunoglobulin and CD19. (Ledbetter et al,1988.). CD45 functions most effectively when in close proximity to other membrane associated proteins. It is possible that CD45 modifies signal transduction in T cells directly by dephosphorylating membrane associated cytoplasmic proteins required for T cell activation. When CD45 is brought into close proximity with CD4 it enhances rather than inhibits CD4 mediated signal transduction.(Ledbetter et al 1988). Together these data suggest that CD45 can regulate pathways dependent on phosphoinositol hydrolysis. CD45 can also inhibit signalling associated with with CD40 which activates cells independently of a rise in iCa^{++}. CD45 can therefore regulate signal transduction through receptors that either do or do not depend on Ca^{++} mobilisation (Ledbetter et al 1988). These multiple functions may be facilitated by the differential expression of the different isoforms of CD45 on resting, memory and activated T and B lymphocytes.

3.4. Cell surface changes on activation

During cell differentiation there are changes in the expression of surface membrane proteins which may modify the cell's ability to interact with other cells or its environment. Cellular adhesion plays a crucial role in the induction, expression and regulation of the immune response. The LFA-1 molecule has been shown to be of particular importance. Monocyte and T cell LFA-1 contribute to the adhesive interaction between these cell types. LFA-1 is a cell surface glycoprotein which has been implicated in adhesion of lymphocytes, macrophages and granulocytes. LFA-2 is also known as CD2 and is restricted to T lymphocytes. LFA-3 is a widely expressed glycoprotein which has been shown to be the ligand for CD2 .(Selvaraj et al 1987). LFA-3, CD2 and LFA-1 may also function in cell activation. T cells can be activated by binding of mAbs to CD2 (Meuer et al 1984) and LFA-3 can participate in this activation (Bierer et al 1987) Binding of mAb to LFA-3 can induce IL-1 secretion by monocytes and thymic epithelium. The role of other adhesion molecules and their interaction with their natural ligands is an area of great interset at the present time.

There have been a number of studies which have shown that there are alterations in the cell surface expression of a number of surface proteins on T cells which have undergone primary antigenic stimulation. These memory T cells are capable of mounting a rapid anamnestic response. CD29, CD45R and $CD45R_0$ (UCHL1) have each been reported to be altered in expression on the subset of cells which mount secondary in vitro responses to recall

antigens.(Morimoto et al 1985, Smith et al 1986). The CD45 molecule undergoes important changes with cell activation, so that CD45R is down-regulated while $CD45R_0$ is up-regulated (Sanders ME et al 1988.). The enhanced expression of adhesion molecules on previously activated memory cells could then be critical to their ability to respond rapidly to subsequent stimulation. The activation of T cells induces a wide variety of cell surface molecules. Some, such as CD25 or HLA class II molecules are induced only transiently and are useful markers of recently activated cells. Others have stable expression after activation and therefore serve as markers of previously activated (memory) cells. LFA-3, $CD45R_0$ and CD29 are particularly useful markers of prior activation.

3.5. In vitro activation of T cells

The development of mAbs to T cell surface proteins has provided useful tools for can be used to understand the activation pathways within T cells. The CD3/TCR complex is thought to represent the initial pathway which in the presence of a second signal from an alternative pathway will result in T cell activation and proliferation. The monoclonal antibody UCHT1 is an IgG2a antibody directed against the CD3 complex. It induces an accessory cell dependent T cell proliferation. The function of the accessory cell involves production of IL-1 and interaction of the Fc receptor with the Fc part of the monoclonal antibody promoting cross linkage of the TCR/CD3 complex. Activation of resting T lymphocytes to proliferation and terminal differentiation is a multi-step process involving synthesis of IL-2 and expression of the IL-2 receptor. IL-1

is necessary for the the synthesis of IL-2. Early studies of T cell triggering relied exclusively on the mitogens PHA and Con A. These also activate cells through the CD3/TCR complex, but since the introduction of monoclonal antibodies, the signalling can be studied more directly. As described above stimulation of T cells with the antibodies 9.6 in combination with 9.1 induces antigen presenting cell independent T cell activation via the CD2 receptor. (Meuer et al 1984). Because this pathway does not involve CD3 it is known as the alternative pathway of T cell activation and proliferation. Although CD3/TCR and CD2 are not physically associated they do interact (Bockenstedt et al 1988).

Chapter 4

General materials and methods

4.1. Media

RPMI 1640 with L glutamine and 20 mM Hepes buffer, Gibco (Paisley). Penicillin (50 U/ml) and streptomycin (50 mg/ml) were added to the medium before use. Foetal calf serum (FCS) was obtained from Gibco (Paisley). Batches were screened for their ability to stimulate optimal cell growth in in vitro liquid culture systems. FCS was heat inactivated before use by heating to 56^0C for 30 minutes, aliquoted and stored at -70^0C before use.

Ficoll Hypaque.

Ficoll Hypaque obtained from Lymphoprep (Upsala, Sweden) was used throughout.

Buffers.

The following buffers were used:

Bicarbonate buffer pH 9.6 was made by mixing 1.59 g Na_2CO_3, 2.9 g $NaHCO_3$ and 0.2 g NaN_3 with 1 litre of distiled water.

Phosphate/citrate buffer pH 5 was made by adding 25.7 mls of 0.2 M Na_2HPO_4 and 24.3 mls of 0.1 M citric acid to 50 mls of distiled water.

Phosphate buffer pH8 was made by titrating 0.2 M NaH_2PO_4 with 0.2M Na_2HPO_4 and diluting to 0.1 M with distiled water.

Monoclonal antibodies used

Antigen	Antibody	Isotype	Source
CD2	9.6	Mouse IgG1	Gift from Dr Linda Terry, New York.
	9.1	Mouse IgG1	Gift from Dr Linda Terry, New York.
CD3	UCHT1.	Mouse IgG1.	Gift from Prof PCL Beverley
CD4	T4 .	Mouse IgG1.	CoulterClone(Coulter,Hialeah,FL, USA)
CD8	T8	Mouse IgG1	Coulter Clone (Coulter, Hialeah, FL, USA)
	UCHT4.	Mouse IgG1.	Gift from Prof PCL Beverley
CD14	UCHM1.	Mouse IgG1	Gift from Prof PCL Beverley
CD20	B1	Mouse IgG2a	Gift from Dr LM Nadler, Boston.
CD25	2AS	Mouse IgG1	Gift from Prof PCL Beverley
CD29	4B4.	Mouse IgG1.	Coulter Clone (Coulter, Hialeah, FL, USA)
CD45R	2H4	Mouse IgG1.	Coulter Clone (Coulter, Hialeah, FL, USA)
CD45R0	UCHL1.	Mouse IgG2a	Gift from Prof PCL Beverley
CD56	NKH1	Mouse IgG1.	Coulter Clone (Coulter, Hialeah, FL, USA)
TCR$\gamma\delta$	Identi-T TCRδ1	Mouse IgG1	T cell sciences (Cambridge, MA, USA)

4.3. Lectins

The following lectins were used

Phytohaemagglutin PHA-P (Sigma) salt free, lyophilised powder.

Conconavalin A - Con A (Sigma) purified salt free, lyophilised powder.

Phytolacca americana - Pokeweed Mitogen (Sigma) partially purified TCA precipitate, salt free, lyophilised powder.

All mitogens were dissolved in PBS and sterilized by membrane filtration before use.

4.4. Isolation of peripheral blood mononuclear cells (PBM).

Peripheral blood samples were taken into preservative free heparin at 2 U/ml. The blood was diluted approximately one in two with RPMI and layered on to an approximately equal volume of lymphoprep. The tubes were centrifuged at 100 g for 20 minutes at room temperature with no braking force applied. Low density cells were harvested from the interface between the medium and the lymphoprep using a plastic pasteur pipette. The cells were washed twice and resuspended in RPMI .

4.5. Cell counting and Viability.

10µl of cell suspension was mixed with 10µl of 0.25% Trypan Blue solution (Flow Laboratories) and counted microscopically in a new, improved Neubeaur cell counting chamber. Viable cells remain unstained whereas the dead cells took up the blue dye.

4.6. Preparation of AET treated sheep red blood cells.

402 mg of 2-aminoethylisothioronium bromide hydrochloride (AET) (Sigma) was added to 10ml double distiled water, the pH adjusted to pH 9.0 and the solution sterilized by membrane filtration. Sheep red blood cells (SRBC) (Tissue Culture Services Limited) were washed three times in RPMI and one volume of washed cells incubated with four volumes of AET at 37^0C for 30 minutes.. The cells were washed four times in RPMI and resuspended at a final haematocrit of 4%.

4.7. Rosetting procedures.

T cells were prepared from PBM's by rosetting with sheep red blood cells (SRBC). Cell suspensions at 10^7/ml were mixed with an equal volume of 4% AET treated SRBC and 20% final volume FCS. The cells were then pelleted by centrifugation at 400 g for five minutes and kept on ice for 60 minutes. The pellet was then gently resuspended and re-layered onto lymphoprep as previously described for whole blood separation. The E- cells were collected

from the interface. The E+ cells were separated from the rosetted SRBC by lysis with red blood cell lysis reagent (Ortho Diagnostic Systems Ltd, High Wycombe, Bucks). The E+ and E- cells were washed three times in RPMI and counted and assessed for viability before use.

4.8. Removal of adherent cells

5 mls of PBMC suspended at 3 x 10^6/ml in RPMI (20% FCS) was incubated at 37^0C in a plastic petri dish for one hour . The non adherent cells were removed by gentle washing with RPMI. Non adherent cells were washed three times with RPMI before use. This process results in partial depletion of monocytes.

4.9. Immune rosetting with magnetic beads.

Cells were incubated with 50µl of mouse monoclonal antibodies (at optimal concentration) in tubes and left on ice for 20 minutes. The cells were then washed three times with RPMI kept on ice. 10 µl per 10^6 cells of magnetic microbeads coated with rabbit anti mouse IgG (Dynabeads, Dynal, Oslo, Norway) was added and incubated at 4^0C for 30 minutes with occasional gentle mixing. The tubes were placed in a Dynamagnet (Dynal) overnight and cells stained with monoclonal antibody adhered via the magnetic beads to the Dynamagnet The unbound cells remained in the supernatant medium. The supernatant medium was collected, the cells washed and counted.

4.10. Freezing and Thawing of Cells.

For freezing, cells at 10^8 cells/ml were resuspended in freezing medium consisting of RPMI with 20% v/v FCS and 10% v/v dimethyl sulphoxide (DMSO) (BDH) and aliquotted at 1 ml/vial into freezing vials. These were left overnight in a -70^{0}C freezer in a sealed polystyrene box before transfer to a liquid nitrogen storage tank.

Cells were thawed rapidly in a 37^{0}C water bath, transferred to a 20 ml universal tube and slowly diluted with RPMI with 20% v/v FCS. The cells were washed once in RPMI and the cell viabilities determined as described above.

4.11. Direct and Indirect Immunofluorescence and Flow Cytometric Analysis.

For direct fluorescence aliquots of 2 x 10^5 cells were stained with 50 µl of monoclonal antibodies directly conjugated to fluorescein (green fluorescence) or phycoerythrin (red fluorescence). The cells were incubated with antibody at the appropriate concentration on ice for 30 minutes and washed three times with RPMI kept on ice. Care was taken to centrifuge the plates containing the cells at 4^{0}C to prevent capping of the antigen on the cell surface.

For indirect fluorescence, aliquots of 2 x 10^5 cells were stained with 50 µl of monoclonal antibodies (at the appropriate concentration) in V bottomed flexible

microtitre plates and left on ice for 20 minutes. The cells were then washed three times with RPMI kept on ice. 50 µl of fluoresceinated goat anti mouse Ig was added to each well. The cells were further incubated for 20 minutes and washed a further three times with RPMI.

Flow cytometric analysis was performed initially using a FACS IV (Becton Dickinson, Oxnard, California) and subsequently using an EPICS (Coulter, Hialeah, FL, USA)

4.12. Proliferation Assays.

Radiolabelled precursors of DNA were used to assess cell proliferation. Cells at the required concentration were plated out in quadruplicate in round bottomed microtitre plates in RPMI supplemented with 10% v/v FCS and the compound under assessment for growth stimulation. Cells were cultured for 72 hours in a fully humidified atmosphere of 5% CO_2 at 37^0C. 1 µCi of tritiated thymidine was added to each well for the last eight hours of culture. The cells were harvested onto Whatman fibreglass filters using an Automash (Dynatech). Counts were assessed using a scintillation counter for ^3H and results are expressed as median counts per minute.

4.13. ELISA Method for the Measurement of non specific Immunoglobulin.

Non specific Ig was measured by an enzyme linked immunoabsorbent assay (ELISA) using the sandwich technique. Rabbit anti human IgG Fc fragment (1 in 500) and sheep anti human μ chain (1 in 200) were diluted in bicarbonate buffer pH 9.6. 100μl of each was added per well to flat bottomed microtitre plates and incubated for 1 hour at 37^0C. The plates were washed 4 times in PBS using an automatic plate washer (Automash 2000, Dynatech).

Unknown samples were added in 100 μl volumes to the wells. A standard Ig solution was also added at duplicate serial doubling dilutions starting at 500 ng/ml of IgG or 5 μg/ml of IgM. The plates were incubated for one hour at 37^0C and washed as before. The plates were then incubated with 60 μl of alkaline phosphatase conjugated anti human IgG (Sigma a-3150) or IgM (Sigma a-3151) for a further 90 minutes. The plates were washed twice with PBS and once with de-ionised water. 100 ml of the substrate *p* - nitrophenyl phosphate (Sigma) at 1 mg/ml diluted in bicarbonate buffer pH 9.4 and incubated at 37^0C for 90 minutes. Absorbence at 405 nm was measured using a Dynatech automatic plate reader and the optical densities related to the concentration Ig by reference to the standard preparation.

4.14. ELISA Method for the Measurement of Specific Antibodies

a) Detection of Specific Antibodies against Influenza Virus

Flat well EIA microtitre plates (Linbro, Flow laboratories) were coated with 100 µl of purified Influenza Virus at a concentration of 100 µg/ml in phosphate buffered saline (PBS) at pH 8.6. A standard solution of human IgG was also set up on each plate in serial doubling dilutions starting at a concentration of 500 ng/ml. The plates were incubated at 37^0 C for six hours and washed three times PBS containing 1% bovine serum albumin (BSA). The plates were further incubated for one hour with 200 ml/well of PBS - 1% bovine serum albumin (BSA) to block non specific binding sites. After washing, 50 µl of cell culture supernatant or patients' serum diluted one in four in PBS was added to each well in quadruplicate and incubated for a further 90 minutes. The wells were then washed as before and allowed to dry. The plates were then incubated with 60 µl of alkaline phosphatase conjugated anti human IgG (Sigma a-3150) for a further 90 minutes. The plates were again washed and 100 µl of the substrate *p*-nitrophenyl phosphate (Sigma) at 1 mg/ml added to each well and the plate was incubated at 37^0C for 90 minutes. To ensure that rh GM-CSF binding had occurred in each plate a positive control was set up using supernatant from the cell culture of cells which were known to respond to that virus (JG) or serum from the same individual. Absorbence at 405 nm was measured using a Dynatech automatic plate reader and the optical densities related to the concentration log IgG by reference to the standard preparation with one unit defined as the absorbence corresponding to 1 ng/ml of the IgG standard.

b) Detection of Specific Antibodies against Granulocyte Macrophage Colony Stimulating Factor

Flat well EIA microtitre plates (Linbro, Flow laboratories) were coated with 60 µl of rh GM-CSF at 2 mg/ml in phosphate buffered saline (PBS) at pH 9.0. A standard solution of human IgG was also set up on each plate in serial doubling dilutions starting at a concentration of 500 ng/ml. The plates were incubated at 37^0 C for six hours and washed three times PBS containing 1% bovine serum albumin (BSA). The plates were further incubated for one hour with 200 ml/well of PBS - 1% bovine serum albumin (BSA) to block non specific binding sites. After washing, 50 ml of patients' serum, diluted one in four in PBS, was added to each well in quadruplicate and incubated for a further 90 minutes. The wells were then washed as before and allowed to dry. The plates were then incubated with 60 ml of alkaline phosphatase conjugated anti human IgG (Sigma a-3150) for a further 90 minutes. The plates were again washed and 100 ml of the substrate *p*-nitrophenyl phosphate (Sigma) at 1 mg/ml added to each well and the plate was incubated at 37^0C for 90 minutes. To ensure that rh GM-CSF binding had occurred in each plate a positive control was set up using a monoclonal anti GM-CSF antibody (provided by Dr D Warren) which was detected as before but with the use of an alkaline phosphatase conjugated anti mouse IgG. Absorbence at 405 nm was measured using a Dynatech automatic plate reader and the optical densities related to the concentration log IgG by reference to the standard preparation with one unit defined as the absorbence corresponding to 1 ng/ml of the IgG standard.

Chapter 5.

Skin Response to Recall Antigens.

T cell in vivo responses can be assessed by the measurement of cutaneous testing to antigen. This can be assessed by using new antigens such as Dinitrochlorobenzene which measures the body's capacity for immunization. However, by introducing an antigen to which the body has already been exposed, the capacity of the T lymphocytes to respond to an antigen already in the T cell memory repertoire can be assessed. These 'recall antigens' are assessed by measuring the delayed cutaneous hypersensitivity at 48 hours by measuring the diameter of induration after subcutaneous injection of the appropriate antigen. Cutaneous tests therefore represent a simple and quick technique, with rapid interpretation.

5.1. Method.

The Multitest CMI (Merieux, France) is an applicator consisting of eight heads with nine tines on each head loaded with seven different recall antigens and a glycerin control. The antigens were applied to the patients' back. The skin was first cleaned with disinfectant solution, the skin stretched and the Multitest applied maintaining firm pressure for five seconds to ensure equal intradermal penetration of each of the eight heads.

The antigens consist of :-

1. Tetanus antigen 550000 U/ml,
2. Diphtheria antigen 1.1 MU/ml,
3. Streptococus antigen (group C) 2000 U/ml,
4. Tuberculin antigen 300,000 U/ml,
5. Glycerine control 70% w/v,
6. Candida albicans antigen 2000 U/ml,
7. Trichophytonantigen 150 U/ml,
8, Proteus mirabilis antigen 150 U/ml.

Reading of the tests was performed after 48 hours. Positivity was defined only as induration and not erythema. The glycerin control serves as a means of comparison to assess the positivity of weak reactions. Using calipers provided with the Multitest the score for each antigen was assessed by measurement of the induration of two perpendicular diameters. The CMI score is the sum of all positive reactions read at 48 hours.

Results

A normal response range was established by assessing the CMI scores in six normal individuals on a total of 20 occasions over a one year period. There was no significant increase in score in any of the normal individuals with repeated testing. The normal range established for the CMI score was 36-48. (Mean 42 $\pm$ 2 SD of results obtained). Responses were assessed in a total of 69 patients

before and at intervals after ABMT - 20 patients with AML, 7 with ALL, 10 with NHL and 32 with Hodgkin's disease.

5.2. Acute Myeloid Leukaemia.

The skin responses to recall antigens were assessed in twenty patients with acute myeloid remission in first CR before commencement of high dose therapy and at intervals after ABMT.

The responses elicited in six patients immediately before and at intervals after single ABMT are shown in figure 5.1. The responses before and at intervals after double ABMT in a further 14 patients are shown in figure 5.2.

Even though these patients had all received recent chemotherapy, the responses were normal before ABMT. There was no significant difference in response after the second procedure in those patients who had a double procedure compared to the responses elicited in those patients who only had a single procedure ($p = 0.42$).

The response to the recall antigens was markedly impaired in all of the patients following high dose therapy and ABMT. However, three of eight patients had responses in the normal range by three months after ABMT and a further three patients had normal responses by three months after the second procedure. The responses were normal in virtually all patients by six months after the second procedure.

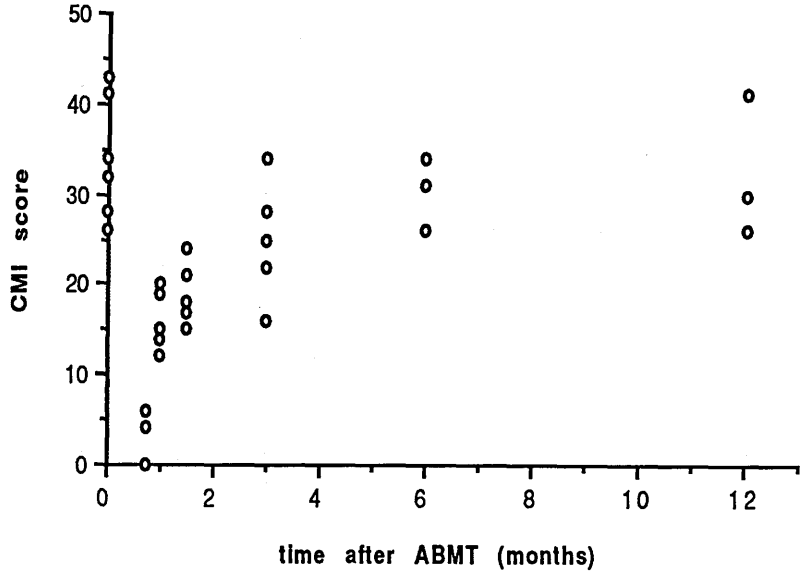


Figure 5.1.

Response to recall antigens assessed by CMI score in patients undergoing single ABMT in first CR of AML

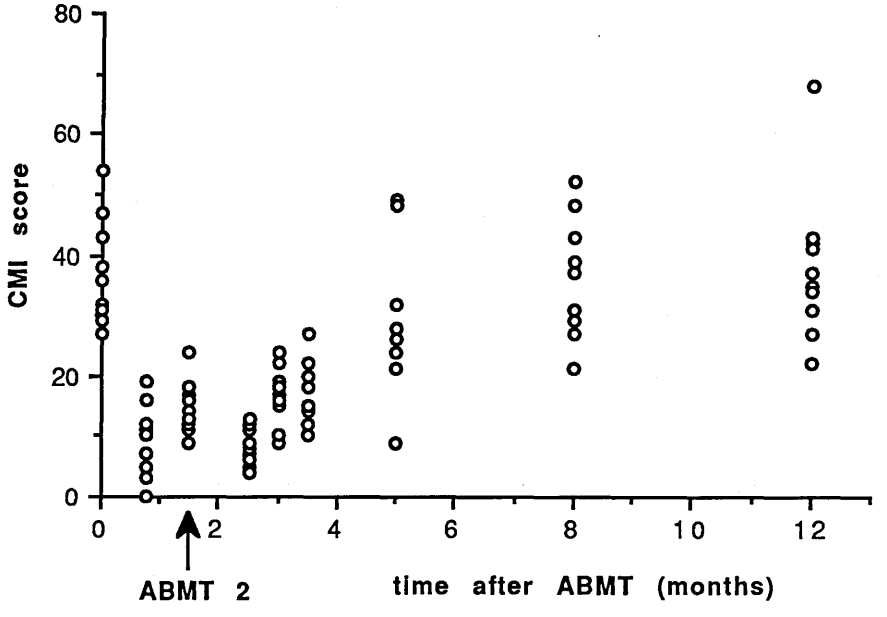


Figure 5.2.

Response to recall antigens assessed by CMI score in 14 patients undergoing double ABMT in first CR of AML

5.3. Acute Lymphoblastic Leukaemia.

The skin response to recall antigens was tested in seven patients with ALL before and for twelve months after ABMT. The first two patients received chemotherapy alone and received two consecutive autografts. In these two patients the time after ABMT relates to the time after the second graft. The subsequent six patients received ABMT following melphelan and TBI, although follow up skin testing was possible in only five of these patients. Figure 5.3 shows the responses in these patients.

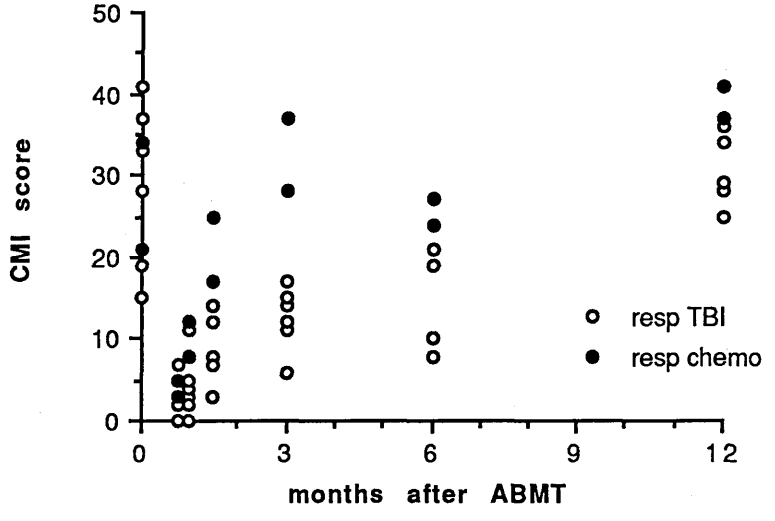


Figure 5.3.

Response to recall antigens assessed by CMI score in patients undergoing ABMT in first CR of ALL. Two patients received chemotherapy alone and five patients received TBI.

Before ABMT the responses in the patients with ALL were significantly reduced compared to the normal controls ($p<0.05$) and to the patients with AML ($p<0.05$). Although conclusions cannot be drawn because of the small numbers of patients, both patients who received chemotherapy alone had a return to their pre-transplant response by three months after the second procedure. In contrast, the patients who received TBI continued to have depressed responses compared to pre ABMT for one year after ABMT.

5.4. Non Hodgkin's Lymphoma

The skin response to recall antigens was tested in ten patients with NHL prior to ABMT. The response was heterogeneous in this group of patients. Those patients who had no response to first line therapy and who had bulky disease at the time of ABMT had the lowest score in skin testing - these patients also had the poorest response to the high dose therapy. Because of the very poor response to ABMT and subsequent high mortality of this group of patients only four patients were available for longer term assessment of response.

As can be seen in figure 5.4., the response was markedly depressed following ABMT, but in the four patients who had a good response to the ABMT, there was a return to normal response by three months after ABMT.

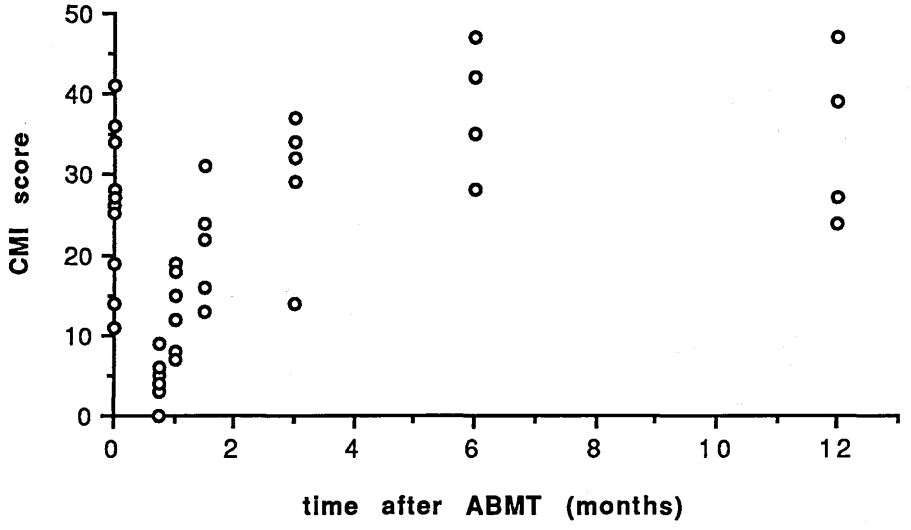


Figure 5.4.

Response to recall antigens assessed by CMI score in patients undergoing ABMT for NHL. Because of the poor outcome in this group of patients, only four patients were available for long term follow up.

Figure 5.5 shows the responses in AML, ALL and NHL patients expressed as mean ± standard deviation. There is no difference in responses in the AML and the small number of NHL patients. The response in the patients with ALL is significantly reduced compared to AML and NHL patients until one year after ABMT.

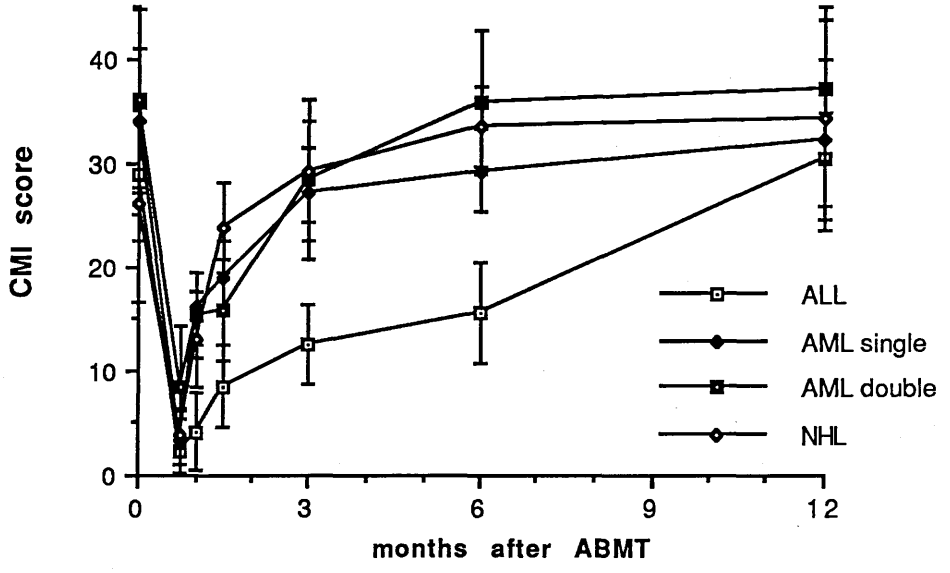


Figure 5.5.

CMI score skin responses to recall antigens expressed as mean ± standard deviation in AML, ALL and NHL patients after ABMT.

5.5. Responses in patients with Hodgkin's disease.

Responses to recall antigens were assessed in 32 patients with Hodgkin's disease. The most striking feature in Hodgkin's disease is that all patients had a marked abnormally reduced response prior to ABMT (mean 27% of normal). All patients had active disease at the time of ABMT and this illustrates the immune suppressive effect of Hodgkin's disease.

Figure 5.6 shows the responses before and after ABMT in this group of patients. Although responses rapidly return to the pre-tranplant level, often when the patient has few circulating lymphocytes, many patients continued to have markedly reduced responses beyond one year after ABMT. In this group of patients there was a marked variation in response to the recall antigens at each time period.

Figure 5.7. shows the response to the recall antigens assessed by the response the patient was subsequently found to achieve after ABMT. Fifteen patients achieved CR and seventeen patients did not achieve CR after ABMT. Those patients who achieved CR after ABMT had significantly higher scores than those who did not achieve CR ($p<0.005$) and this difference was apparent by as early as two months after ABMT. In those patients who did achieve CR only half had responses within the normal range at six months after ABMT. One patient in this group had markedly reduced responses at six months after ABMT but had a normal response at one year after ABMT. In those patients who did not achieve CR the response to recall antigens never returned to normal.

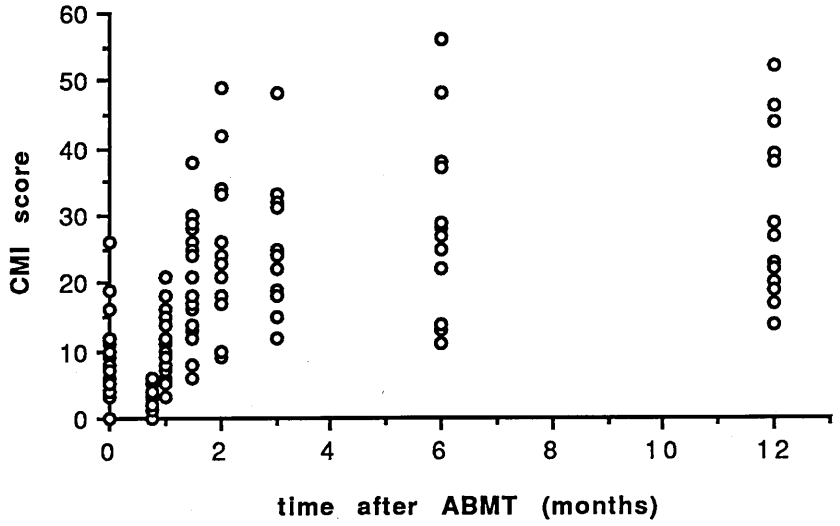


Figure 5.6.

Response to recall antigens assessed by CMI score in patients undergoing ABMT for Hodgkin's disease.

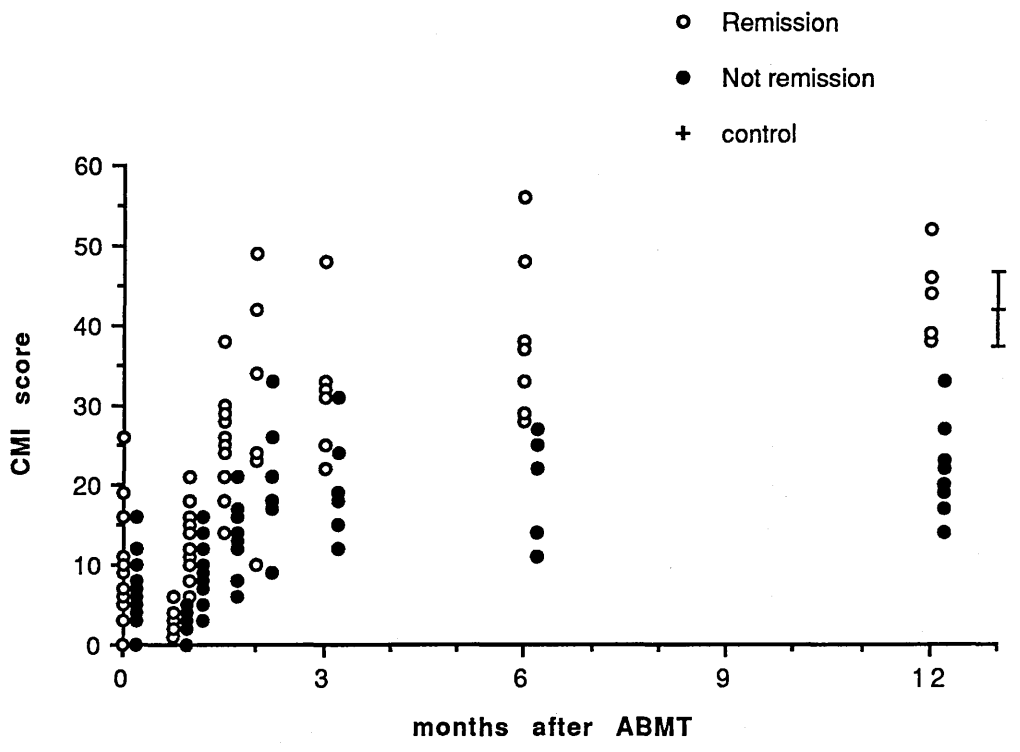


Figure 5.7.

Response to recall antigens assessed by CMI score in patients undergoing ABMT for Hodgkin's disease. The open circles show the response in those patients who were subsequently found to have achieved CR after ABMT (n=15) and the solid circles show the responses of those who did not achieve CR after ABMT (n=17).

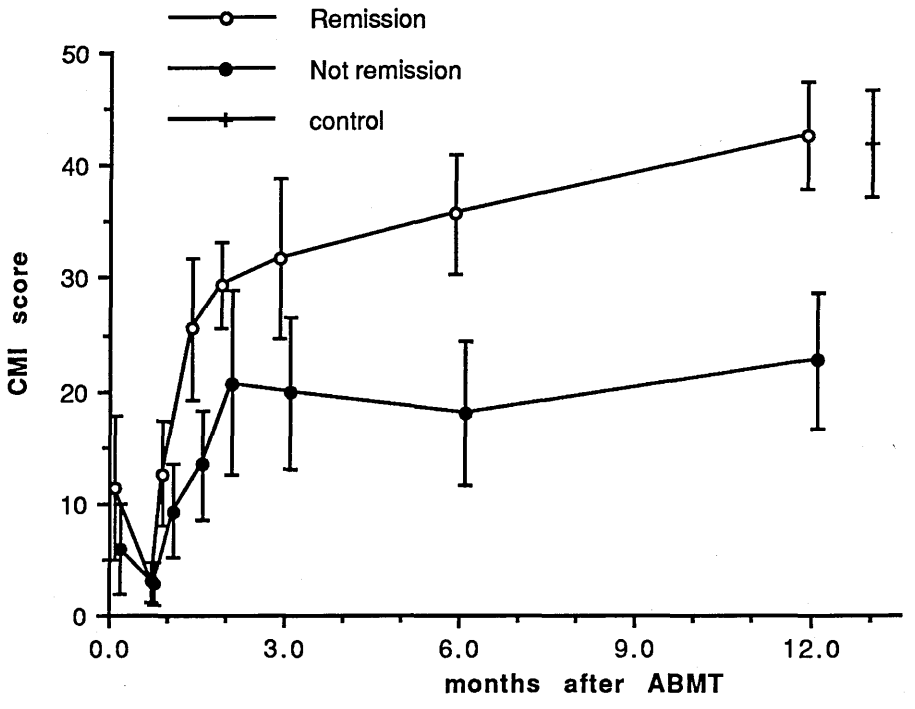


Figure 5.8.

Response to recall antigens assessed by CMI score in patients undergoing ABMT for Hodgkin's disease. The responses are significantly reduced in those patients who did not achieve CR after ABMT compared to those who achieved CR after ABMT ($p>0.005$). The normal range is shown on the right.

5.6. Conclusions.

Following ABMT the skin response to recall antigens was markedly depressed in all cases studied. This decreased responsiveness rapidly reversed with the majority of patients having a normal response by three to six months after ABMT. Patients with ALL, who received total body irradiation had significantly reduced response compared to those who had received chemotherapy alone for up to one year after ABMT. The responses were markedly reduced in patients with Hodgkin's disease at the time of presentation for ABMT, reflecting the immune suppressive effect of active Hodgkin's disease. The responses returned to normal rapidly in those patients who achieved a good response to the high dose therapyn given.

The duration of this depressed response after ABMT may be therefore be affected by the patient's disease type, the bulk of disease present, the form of high dose therapy given, whether chemotherapy alone or chemotherapy in combination with TBI, or the response of the disease has to the high dose therapy.

Chapter 6.

B cell responses in vivo after Autologous Bone Marrow Transplantation. Specific Anti Influenza Immunoglobulin Production In Vivo

Introduction

6.1. Influenza Viruses

Influenza A viruses are classified into subtypes on the basis of two antigens; haemagglutinin (H) and neuraminidase (N). Three subtypes of haemagglutinin are recognized (H_1, H_2, H_3) and two subtypes of neuraminidase (N_1 and N_2) are recognized among influenza A viruses that have caused widespread human disease. Immunity to these antigens, especially the haemagglutinin antigen, reduces the likelihood of infection and the severity of disease if infection occurs. However, over time there may be enough antigenic variation (antigenic drift) within the same subtype that infection or vaccination with one strain may not induce immunity to distantly related strains of the same subtype. Although influenza B viruses have shown more antigenic stability than A viruses, antigenic variation does occur. For these reasons, epidemics of respiratory disease caused by new variants of influenza viruses continue to occur.

6.2. Influenza Vaccines.

The antigenic characteristics of current strains provide the basis for the selection of strains in each years vaccine. The Influenza vaccine is made from highly purified, egg grown viruses that have been rendered noninfectious by inactivation. Most vaccines available have been chemically treated to reduce the incidence of febrile reactions in children and contain highly purified haemagglutinin and neuraminidase antigens prepared from strains of influenza virus currently recommended by the World Health Organization. Vaccination causes minimal systemic upset or febrile reactions. 90% of vaccinated young adults develop haemagglutinin-inhibition antibody titres. Titres are maximal 10 - 21 days after vaccination and persist for up to nine months. Elderly patients and certain individuals with chronic diseases may develop lower levels of antibody after vaccination. Because the vaccine contains only non-infectious viruses, it cannot cause influenza. There are occasional reports of respiratory disease after vaccination which most probably represent coincidental illness unrelated to the vaccine. The most frequent side effect, occurring in one third of patients, is soreness around the vaccination site which may last for one to two days. Fever, myalgia and other systemic symptoms occur infrequently and occur six to twelve hours after vaccination. Immediate, presumably allergic reactions occur extremely rarely after influenza vaccination and probably result from hypersensitivity to some component of the vaccine, especially the egg protein. Although the current vaccines contain only a small amount of egg protein, the vaccine is contra-indicated in those who have anaphylactic hypersensitiviy to egg. Unlike the 1976 swine influenza vaccine, subsequent

vaccines prepared from other viral strains have not been associated with an increased frequency of Guillain Barre syndrome. There are no reported adverse effects reported with the virus and any other drugs.

6.3. In vivo antibody production against Influenza vaccination.

Patients undergoing autologous bone marrow transplantation were vaccinated using the killed Influenza vaccine on the day of marrow return. Serum samples were obtained at twice weekly intervals. Specific serum anti-Influenza IgG levels were measured by ELISA as described in the methods section.

6.4 Results

Patients undergoing ABMT were vaccinated with Influenza vaccine (Merieux, France) on the day of bone marrow return as shown in figure 6.1. A total of 45 patients were vaccinated in the study. - 12 patients with AML in first CR, five patients with ALL in first CR, eight patients with NHL and 20 patients with Hodgkin's disease. All the patients with AML, NHL and NHL received high dose therapy consisting of chemotherapy only, as previously described. In the AML patients the Influenza vaccine was given on the day of the marrow return with the second graft. All five patients with ALL received high dose therapy combination of high dose melphelan and total body irradiation (TBI).

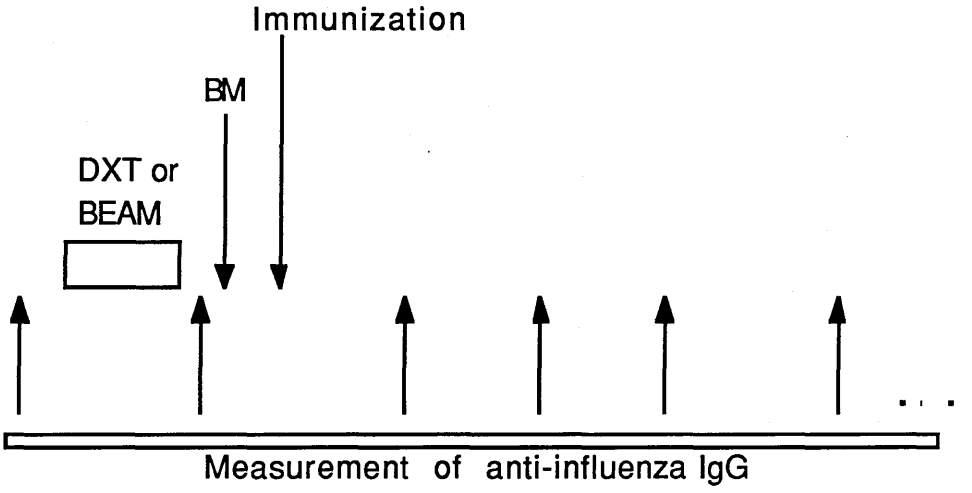


Figure 6.1.

Responses were assessed before high dose therapy and immediately prior to ABMT. Immunization was performed on the day after bone marrow return. IgG responses to Influenza were assessed before and at intervals after ABMT

A normal response range was established by measuring the response after vaccination of 12 normal individuals who were in the same age range as the the patients. All 12 of the normal volunteers mounted a brisk response following vaccination with a peak level of IgG detected at 20 to 30 days post vaccination. In all cases with the controls and the patients the maximal response was mounted against the Influenza A H_3N_2 component of the vaccine and the results shown are for specific IgG against the X87 (A H_3N_2) component of the vaccine only. Ten patients with acute leukaemia - five with AML and five with ALL who did not progress to ABMT were vaccinated with influenza vaccine to assess the response in patients who had previously received chemotherapy. There was no

significant difference in peak response or day to peak response in these patients compared to normal controls (Table 6.1).

	No.	Day	ng/ml	± SD	p value
Controls	12	28	137	25.8	
AML	5	24	122	42.4	0.766
ALL	5	28	118	30.6	1.467

Table 6.1.

Peak response and day to peak response in normal controls and patients with acute leukaemia who did not undergo ABMT

Responses in patients with acute leukaemia.

Seventeen patients with acute leukaemia were given the Influenza vaccine on the day after marrow recovery. Twelve patients with AML were immunized on the day of return of the second marrow after the second course of high dose therapy. Five patients with ALL were immunized on the day after marrow return after high dose chemotherapy and total body irradiation. The responses elicited in patients with acute leukaemia are shown in Figure 6.2.

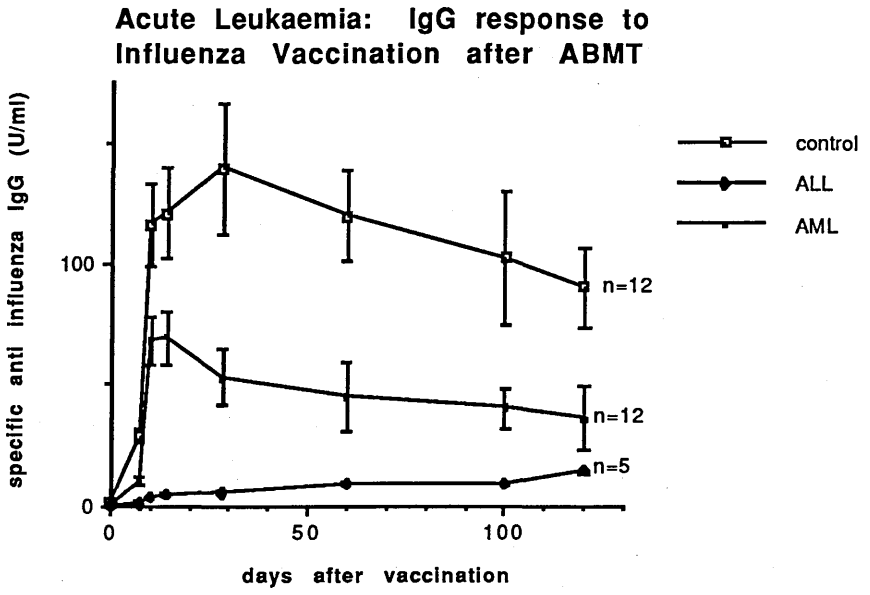


Figure 6.2.

In vivo IgG response after vaccination with Influenza vaccine in 12 normal adult control individuals, 12 adult patients with AML in first remission undergoing second ABMT and five adult patients with ALL in first remission undergoing ABMT after TBI. The results are shown as mean ± SD.

All patients with AML mounted a brisk response although the peak response was significantly reduced compared to the normal controls. The time course of the response was not delayed compared to the controls despite the recent administration of very high dose chemotherapy.

In contrast, although the numbers are small, in the patients with ALL the responses were barely detectable, with no early peak response seen in any of

the five patients vaccinated. The subsequent small rise in titre occurring after three months may be due to subsequent exposure to the virus in the community.

Responses in patients with Hodgkin's disease.

Twenty patients with Hodgkin's disease received the Influenza vaccine on the day after marrow return. The response in patients with Hodgkin's disease is shown in figure 6.3.

HD: IgG Response against Influenza Vaccination

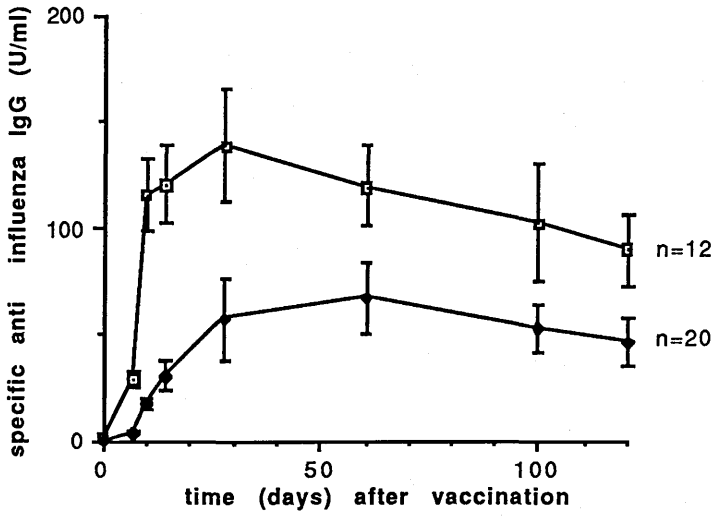


Figure 6.3.

In vivo IgG response after Influenza vaccination in 12 normal adult control individuals and in 20 patients with Hodgkin's disease undergoing ABMT.

The specific anti Influenza IgG response in patients with Hodgkin's disease was brisk, although the peak response was also suboptimal compared to normal controls. Compared to the normal controls and the patients with AML there was a delay in the peak response after vaccination in the HD patients, the peak response occurring 60 days after vaccination. This may be correlated with the more rapid regeneration of lymphocytes seen in the AML patients after ABMT compared to those patients with HD. After the first six weeks after ABMT there was no significant difference in response in the AML and HD patients.

Responses in patients with Non Hodgkin's Lymphoma.

Eight patients with NHL were vaccinated on the day of return of their autologous bone marrow. The responses elicited in these patients with NHL are shown in figure 6.4. The responses show a similar pattern to that seen in patients with AML with a brisk if suboptimal response compared to the normal controls and no delay in peak IgG response compared to normal controls.

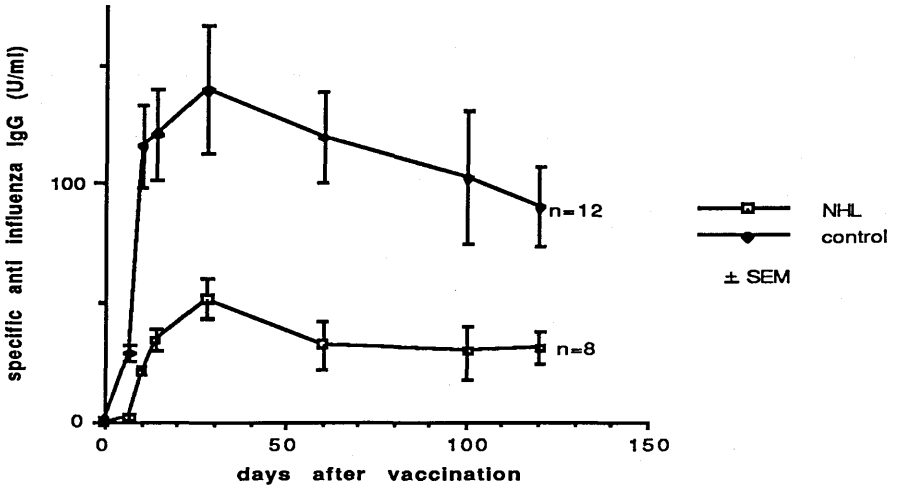


Figure 6.4.

In vivo IgG response after Influenza vaccination in 12 normal adult control individuals and in 8 patients with Non Hodgkin's Lymphoma undergoing ABMT.

6.5. Conclusions.

Despite the very recent administration of very high dose chemotherapy, all the patients with AML, NHL and Hodgkin's disease mounted a brisk humoral response against the Influenza vaccine with significant no delay in the time to peak response after vaccination. There was no evidence that patients with refractory Hodgkin's disease had reduced responses compared to those patients who subsequently achieved CR with high dose therapy. In contrast, all the

patients with ALL had very poor IgG responses after vaccination. This may be due to differences in the patient populations, but since responses were not significantly reduced in the ALL patients who did not progress to ABMT, this more likely reflects the different effects of the conditioning with TBI compared to chemotherapy alone.

Chapter 7.

Development of Antibodies to recombinant derived Granulocyte Macrophage Colony Stimulating Factor

7.1. Introduction

Granulocyte-macrophage colony stimulating factor (GM-CSF) is an important molecule in the regulation of growth and differentiation of haemopoietic progenitor cells. The naturally occurring protein has been isolated and partially sequenced and the gene coding for GM-CSF has been cloned (Wong et al 1985, Cantrell et al 1985). The availability of recombinant human (rh) GM-CSF has made it possible to explore a number of potential therapeutic indications for this protein and encouraging preliminary results showing that rh GM-CSF accelerates neutrophil recovery in neutropenia have been reported. (Groopman et al. 1987, Brandt et al.1988, Antin et al 1988, Antman et al 1988, Devereaux et al. 1989.)

rh GM-CSF is available for use in man in several forms. rh GM-CSF with the identical amino acid sequence to the naturally occurring protein has been produced in mammalian cells (Chinese Hamster Ovary (CHO) cells) and is variably glycolysated on both O-linked and N-linked sites giving rise to a heterogeneous population of proteins with molecular weights of 18-30 kD (Wong GC et al 1985) and in E. coli resulting in a non glycosylated product with a molecular weight of 14.1 kD. A further form of rh GM-CSF has been produced in yeast with a single amino acid difference from the naturally

occurring protein with the substitution of a leucine for a proline at position 23 of the amino acid sequence to remove a Kex protease site (Gillis et al. 1987) . This product is N-linked glycosylated resulting in three molecular species of molecular weight 15.5, 16.8 and 19.5 kD (Cantrell et al 1985). The non glycosylated rh GM-CSF (produced in E. coli) and partially glycosylated rh GM-CSF (produced in yeast) appear as efficacious as the fully glycosylated product which is produced in mammalian cells (Mayer et al.1987). All three forms of rh GM-CSF have been used in phase I and II clinical trials.

7.2. Patients

Four patients with solid tumours which were resistant to chemotherapy received yeast derived rh GM-CSF for ten days in phase 1 studies at a time when they were receiving no chemotherapy or radiotherapy. Twelve patients receiving high dose combination chemotherapy and ABMT for resistant Hodgkin's disease received either yeast (13 patients) or E. coli (3 patients) derived rh GM-CSF (Devereaux et al 1989). In the ABMT recipients only a test dose of rh GM-CSF of 15 μg/ m^2/hour for one hour was given prior to the ablative chemotherapy and treatment by continuous infusion was started after the ablative therapy and 24 hours following infusion of autologous bone marrow. rh GM-CSF was given at a dose of 100 - 400 μg/ m^2/day. It was planned that all patients should receive at least ten days therapy although the treatment schedule was curtailed in two patients.

Since it had been shown that these patients could mount a humoral immune response against influenza vaccine, the sera of these patients was screened for the development of antibodies against recombinant GM-CSF.

The majority of the results in this chapter have recently been published (Gribben et al 1990). I acknowledge the help of Mr HM Jones for help with the marrow cultures and Dr NSB Thomas for help with the Western blotting.

Serum was obtained three times weekly until patients were discharged from hospital. Sera from twenty patients other with Hodgkin's disease undergoing autologous bone marrow transplantation but not receiving rh GM-CSF were examined as controls.

7.3. rh GM-CSF

rh GM-CSF containing a single amino acid substitution from the naturally occurring protein were obtained from Immunex Seattle,USA (yeast derived). rh GM-CSF, with no amino acid substitutions, expressed in mammalian cells was obtained from Sandoz, Basel, Switzerland and in E coli from Hoechst/Behringwerke,Marburg, W Germany.

7.4. Assays for the detection of antibodies to GM-CSF.

ELISA.

Antibody to rh GM-CSF was detected by a solid phase immunoassay using a modification of the method of Voller (Voller et al 1976) as described in the general methods section.

Western Blotting.

For western blotting, rh GM-CSF proteins were diluted 1 in 2 in SDS gel sample buffer at 30 μg/ml,50 μg/ml and 100 μg/ml for the E.coli, yeast and mammalian derived products respectively and electrophoresed on 15% acrylamide gels (Laemmli U K. 1970) . Proteins were then transferred onto nitrocellulose (HybondC Extra, Amersham Intl.) by semi-dry blotting (Atto, Japan) at 0.5A for 45 min in 25 mM Tris, 197 mM Glycine, 20% methanol. After blotting, the filter was immersed in blocking solution (10% BSA, phosphate buffered saline (PBS)) and agitated for >1 hr at room temperature. The filter was then incubated overnight at 4^{o}C with the serum samples at 1 in 10 dilution in 10% BSA in PBS, 0.05% Tween-20 (Sigma)). After three washes the filter was incubated for 1 hr at room temperature with [^{125}I] mouse anti human antibody (20mCi/mg; Amersham Intl.) at 1mCi/ml in 10% BSA in PBS, 0.05% Tween-20). The filter was again washed with three changes of PBS, 0.05% Tween-20, dried and exposed to X-Ray film (Amersham Intl.). As a positive control, the filter was incubated with anti

GM-CSF monoclonal antibody, washed and incubated with [^{125}I] sheep anti mouse antibody (20mCi/mg; Amersham Intl.) at 1mCi/ml in 10% BSA in PBS, 0.05% Tween-20).

7.5. Marrow cultures

10 mls of marrow was aspirated at the time of bone marrow harvest from a normal donor. The mononuclear cell fraction was obtained by centrifugation over Ficoll gradient and washed in RPMI (Gibco). The cells were incubated with AET treated sheep red blood cells and the E^- fraction obtained and washed. The resulting cell fraction was then depleted of adherent cells by incubating in plastic plates at 37^{o}C for one hour. The non adherent cells obtained were cultured in 0.9% agar containing 25% foetal calf serum and 10% human serum with rh GM-CSF at 12.5 ng/ml (optimal concentration) and 1.25 ng/ml (suboptimal concentration) or rh G-CSF at 2.5 x 10^3 U/ml with or without sera obtained from patients pre and post infusion of rh GM-CSF to assay for the presence of neutralizing antibodies. The cells were cultured at a final concentration of 2 x 10^4/ml for twelve days.

7.6. GM-CSF assays.

GM-CSF concentration in patients' sera were assayed using a commercially available ELISA (Medical Resources Ltd, Surrey Hills, NSW, Australia) (Cebon et al. 1988) and are expressed with reference to a standard curve obtained using E. coli derived rh GM-CSF.

7.7. Production of Deglycosylated rh GM-CSF.

The deglycosylated forms of the mammalian derived rh GM-CSF were produced as follows. N linked sugars were cleaved by digesting 20 mg rh GM-CSF with 1U glycopeptide-N-glycosidase (PNGaseF) (*ex Flavobacterium meningosepticum*, Boehringer Mannhein, West Germany) in 100 mM $NaPO_4$, 25 mM EDTA, 0.5% (v/v) Triton X 100, 0.1% (w/v) SDS, 1 mM DTT pH 7.0 (Buffer A) at 37^0C for 18 hours. O-linked sugars were removed by the following procedure: 50 mg GM-CSF was digested with 0.05 U Neuraminidase (*ex Vibrio cholerae*, Boehringer Mannhein, West Germany) and 5 mU N-acetyl-b-D-glucosaminidase (EC 3.2.130; *ex Diplococcus pneumoniae;* Boehringer Mannhein, West Germany) in 50 mM $Na_3C_6H_5O_7$ pH 5.0, 2 mM $CaCl_2$ at 37^0C for 18 hours. Following dialysis against 100 mM $NaPO_4$ pH 7.0, the sample was boiled for 5 minutes in buffer A and then 20 mg was digested with 4 mU endo-a-N-acetylgalactoaminidase (EC3.2.1.97; *ex Diplococcus pneumoniae;* Boehringer Mannhein, West Germany) at 37^0C for 18 hours.

7.8. Results

Detection of antibodies to rh GM-CSF.

Of sixteen patients who received rh GM-CSF in Phase I/II clinical studies in our institution four patients who had received yeast derived rh GM-CSF developed serum antibodies reactive to this protein (Table 7.1.).

Table 7.1. .

Peak Level of Anti rh GM-CSF IgG (U/ml) detected and day of peak level

Patient	Source of GM-CSF		
	Yeast	E. coli	CHO cell
1	26.9 day 17	24.7 day 17	0
2	36.3 day 10	29.8 day 10	0
3	29.4 day 16	22.7 day 16	0
4	16.1 day 13	21.1 day 13	0

Anti rh GM-CSF antibody was found in four patients. The peak level of antibody and the day on which this occurred following initiation of rh GM-CSF administration is shown. There was full cross reactivity against the E coli derived rh GM-CSF not no cross reaction against the mammalian derived product. The units used approximate to ng/ml as defined by the absorbence obtained from the standard curve.

None of the three autograft recipients who received E. coli derived rh GM-CSF developed detectable antibodies. No antibodies were detected in any of twenty autologous bone marrow transplant recipients who did not receive rh GM-CSF. The development of these antibodies was rapid and, as shown in figure 7.1, could be detected in each case by seven days after the commencement of the rh GM-CSF treatment.

The ABMT recipients had all received a test dose of rh GM-CSF one week before commencing therapy, but no test dose had been given to the patients in the phase I study. In the recipients of very high dose therapy and ABMT it is noteworthy that the antibodies were produced at detectable levels at a time when there were no circulating leucocytes.

7.9. Specificity of anti GM-CSF antibodies.

These antibodies were initially detected by specific solid phase ELISA using yeast derived rh GM-CSF as the coating antigen. The serum from these patients showed no reactivity against plates coated with irrelevant protein (influenza virus). The antibodies from all four patients showed full cross reactivity against the E. coli derived GM-CSF but no reactivity with the mammalian derived rh GM-CSF (Table 7.2).

An attempt was made to detect the presence of IgM antibodies as it was felt that this may represent a primary immune response. However this was not possible since in each instance it proved impossible to block non specific binding of IgM to the ELISA plates under the assay conditions used.

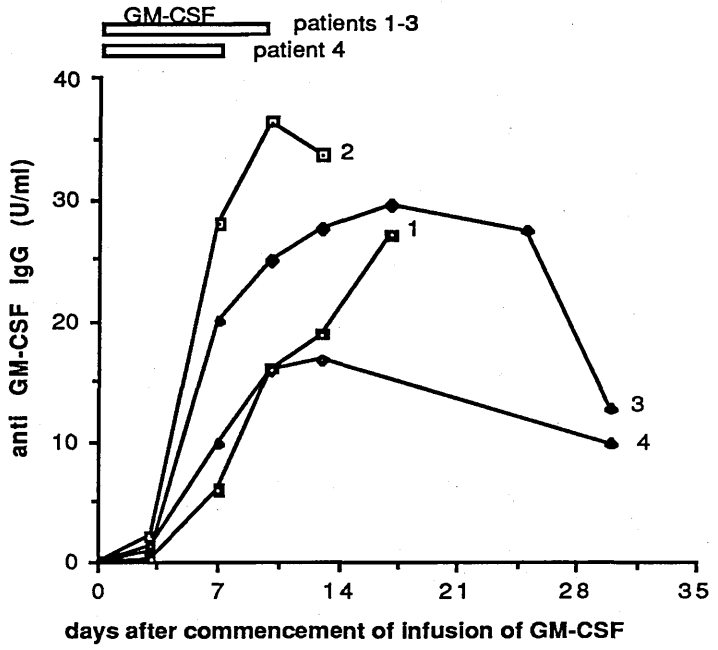


Figure 7.1.

The time course for the production of specific IgG against the yeast derived rh GM-CSF after commencement of continuous infusion in the four patients who developed antibodies against GM-CSF. The rh GM-CSF infusion was for ten days in three patients and for seven days in the fourth patient .

IgG response to rh GM-CSF in different glycosylation states.

	Anti rh GM-CSF (U/ml)			
	all patients pre	patient 1 post rh GM-CSF	patient 3 post	ELISA optical density readings on the same plate using a murine monoclonal anti GM-CSF
Source of rh GM-CSF				
E coli	0	30.6	22.7	0.678
Yeast	0	29.7	24.9	0.702
Mammalian	0	0	0	0.639
Mammalian N cleaved	0	0	0	0.624
Mammalian O cleaved	0	25.9	21.3	0.648
Mammalian O and N cleaved	0	29.4	25.1	0.671
No GM CSF	0	0	0	0.075

Table 7.2.

The reactivity of Sera from patients 1 and 3 against different forms of rh GM-CSF

In the same experiments a mouse monoclonal anti GM-CSF reacted equally well with all three forms of rh GM-CSF. The serum from these patients showed no reactivity against uncoated plates and plates coated with irrelevant protein

(influenza virus) and the reactivity was inhibited by the addition of excess yeast derived rh GM-CSF to the serum (figure 7.2)

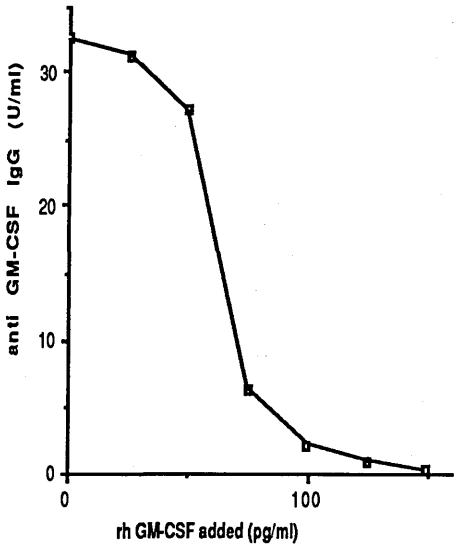


Figure 7.2.

The addition of free yeast derived rh GM-CSF inhibits binding of the specific IgG (U/ml) in the solid phase ELISA with yeast derived material as the coating antigen. The results shown were obtained from the post rh GM-CSF serum of patient 2. Inhibition of binding was similarly produced by the addition of E. coli and O-deglycosylated and fully deglycosylated CHO derived rh GM-CSF but not with glycosylated CHO derived rh GM-CSF or N-deglycosylated rh GM-CSF.

The specificity of the antibodies was confirmed by Western blotting of samples from two patients (patient nos. 1 and 2) from whom sufficient sera were available. The electrophoretic patterns of the different forms of rh GM-CSF are shown as are the autoradiographs of the Western blots using patient's sera and a monoclonal anti GM-CSF antibody (figure 7.3)

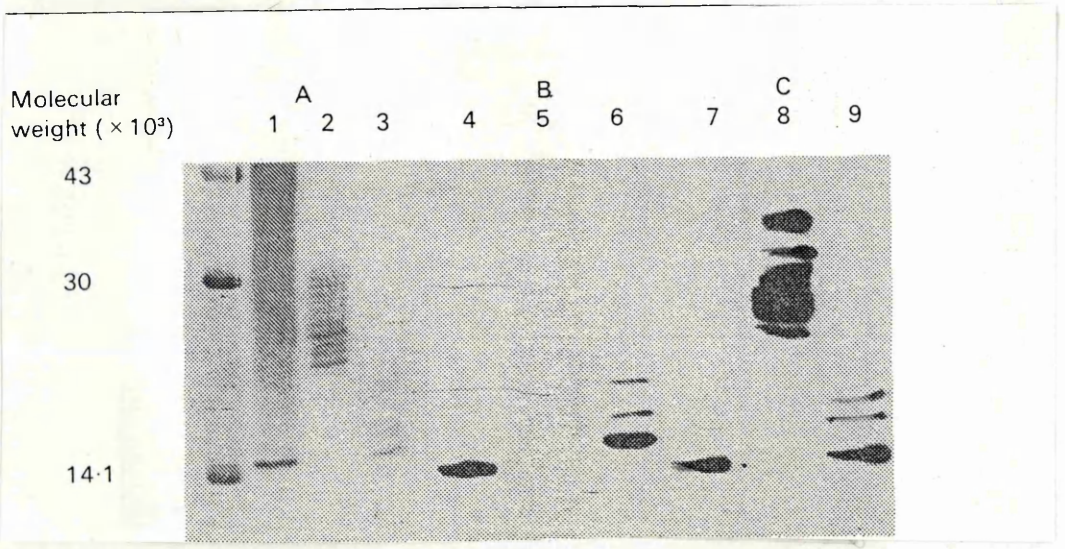


Figure 7.3.

The electrophoretic pattern of the recombinant forms of GM-CSF, unglycosylated E. coli, glycosylated CHO cell and glycosylated yeast derived recombinant GM-CSF is shown on the left (A) with molecular weight markers (Amersham, pre stained rainbow markers) of 14.1 and 30 kD. The autoradiographs obtained after incubation of the Western blots obtained with B) serum from patient 2 followed by [^{125}I] mouse anti human antibody and with C) a mouse monoclonal anti GM-CSF and [^{125}I] sheep anti mouse antibody are shown on the right.

The antibodies in all four patients did not react with the yeast N glycosidically linked carbohydrate side chains as there was full cross reactivity with the non glycosylated E coli product. The yeast material contains an amino acid substitution at position 23 (proline -> leucine) but again the reactivity with the E coli product which does not contain this substitution excludes this amino acid substitution as the antigenic site. It thus seemed probable that the antibodies were produced to a portion of the amino acid backbone protected by glycosylation in the native and Chinese hamster ovary (CHO) cell produced material. Whereas mammalian derived rh GM-CSF has two O-linked and two N-linked glycosylation sites, E coli derived material is not glycosylated and yeast derived material is only N-linked glycosylated. To test this hypothesis, N and O glycosidically linked sugars were removed from the mammalian derived rh GM-CSF. Removal of the O-linked sugars exposed antigenicity of the GM-CSF molecule to sera from the two patients studied (patients 1 and 4). (Table 2). Binding of antibody to the O-linked deglycosylated mammalian derived rh GM-CSF was inhibited by greater than 95% by addition of excess free E coli and yeast derived rh GM-CSF and binding of antibody to E coli derived rh GM-CSF was similarly inhibited by excess free O-linked deglycosylated mammalian rh GM-CSF (data not shown).

7.10. Biological activity of anti GM-CSF antibodies.

No evidence was found in the marrow culture assays to suggest that the antibodies detected had neutralizing activity. (Figure 7.4) but this may not be a sensitive assay of the neutralizing effects of the anti GM-CSF antibodies as described in detail below.

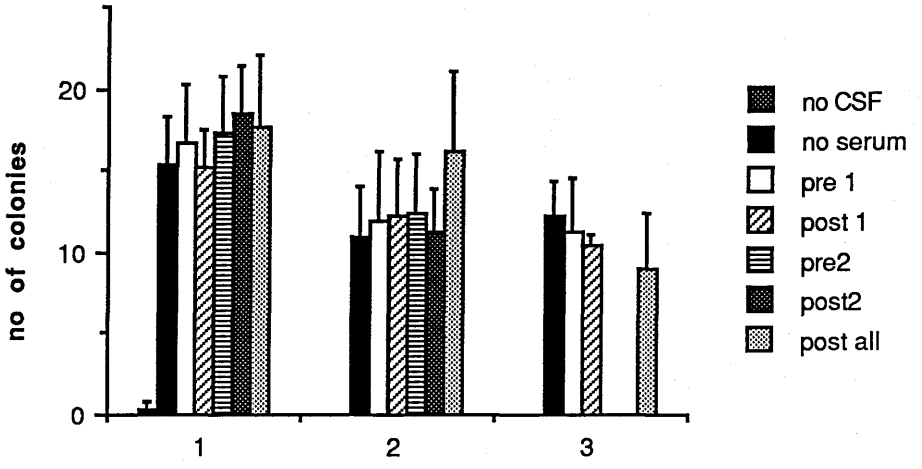


Figure 7.4.

This shows the number of colonies counted at day 12 from the non adherent cells cultured at a final concentration of 2 x 10^4/ml in 0.9% agar containing 25% foetal calf serum and 10% human serum with

1)rh GM-CSF at 12.5 ng/ml (optimal concentration)

2)1.25 ng/ml (suboptimal concentration)

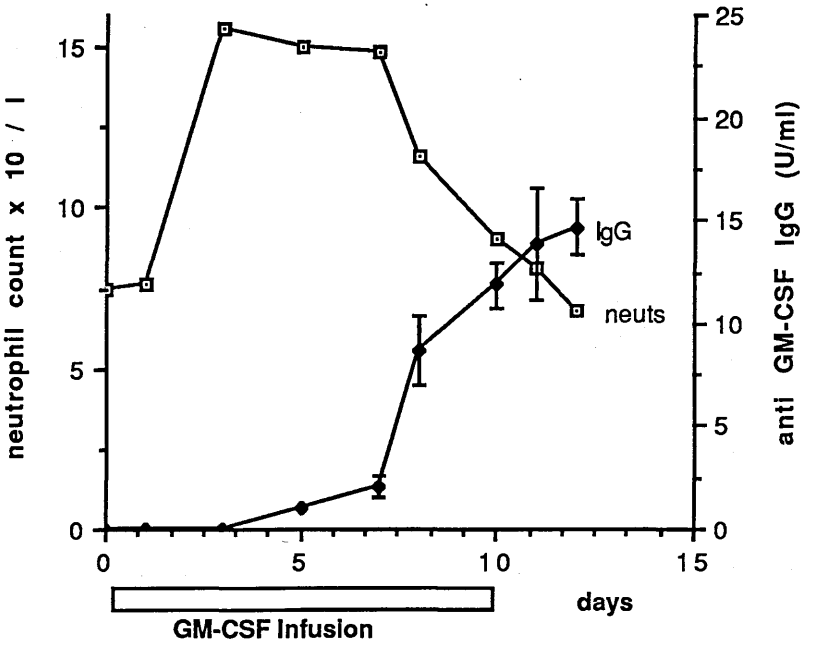
3) rh G-CSF at 2.5 x 10^3 U/ml

with or without sera obtained from patients 1 and 2 pre and post infusion of rh GM-CSF to assay for the presence of neutralizing antibodies and as a control from pre and post infusion of a combination of six patients who received rhGM-CSF but did not develop antibodies.

However, in the one patient who developed antibodies during the phase I study and who had not received prior chemotherapy there was a loss of the rise in neutrophil count which is normally seen during GM-CSF infusions and the plasma GM-CSF concentration fell to undetectable levels despite continuation of the rh GM-CSF infusion at the same dosage (Figure 7.5.). In two other patients in the Phase I study, who did not not develop anti GM-CSF antibodies, the levels of GM-CSF remained constant throughout the infusion. In the three ABMT recipients of rh GM-CSF who developed anti GM-CSF antibodies the levels of GM-CSF on day 7 or 8 of the infusions were 43%, 43% and 46% of the levels measured in the steady state on the first day of the infusion. In seven other ABMT recipients who did not develop detectable antibodies, there was a marked fall of the GM-CSF levels in one patient (26% on day 9) but in all other patients the level at days 7-9 were similar to those on the initial day of the infusion.

Evaluation of the clinical effects of antibody formation is difficult. The anti GM-CSF antibodies were detected retrospectively and were not suspected at the time of administration of growth factor. In the one patient who developed antibodies during the phase I study and who had not received prior chemotherapy there was a loss of the rise in neutrophil count normally seen during GM-CSF infusions and the plasma GM-CSF concentration fell to undetectable levels despite continuation of the rh GM-CSF infusion at the same dosage (figure 7.5). This may suggest that in this patient development of the antibodies resulted in more rapid plasma clearance of the rh GM-CSF. In the three recipients of ABMT it is not possible to tell whether the appearance of

anti GM-CSF antibodies influenced neutrophil regeneration as the time after ABMT to reach 0.5 x 10^9/l neutrophils was highly variable. One patient died of progressive disease before neutrophil recovery at 17 days after ABMT and the time to achieve a neutrophil count of 0.5 x 10^9/l in the other two patients was 14 days and 27 days respectively.



Serum GM CSF concentration(pg/ml)	**0**	**110**	**60**	**0**	**0**	**0**

Figure 7.5.

Neutrophil count, the level of anti GM-CSF IgG (U/ml) detected and serum GM-CSF concentration (pg/ml) in patient 1 during and after continuous infusion of rh GM-CSF (100 mg/m^2/day).

Although it is not possible to assess the biological response to GM-CSF in the ABMT patients in terms of their regeneration of neutrophils, by monitoring the serum level of GM-CSF it can be seen in figure 7.6 that despite continuing the infusion at a constant rate, GM-CSF levels fell in all patients who developed antibodies. In contrast, in those patients who did not develop antibodies, the serum level of GM-CSF fell in only one patient.

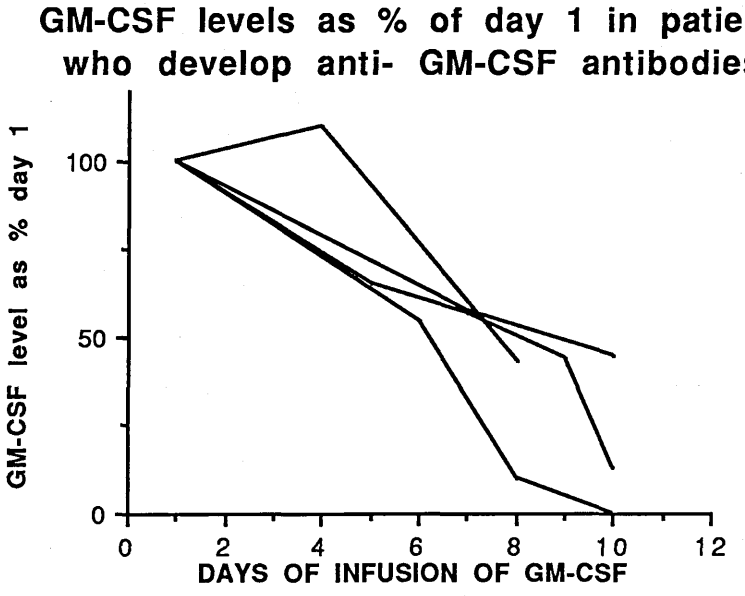


Figure 7.6.
Serum level of GM-CSF expressed as a percentage of the day 1 level in patients in whom antibodies were detected.

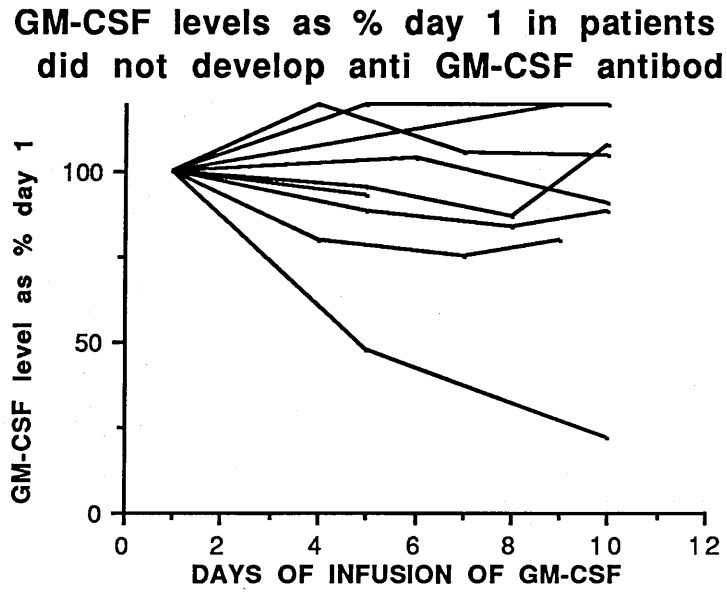


Figure 7.6.
Serum level of GM-CSF expressed as a percentage of the day 1 level in patients in whom antibodies were not detected.

7.11. Conclusions.

A high proportion of patients (four out of thirteen) who have received yeast derived rh GM-CSF have developed antibodies to this product . Although the yeast sugars do not contribute to the antigenicity it is possible that these sugars serve as haptens to augment the antigenicity of the unprotected O-linked glycosylation site(s). Only three patients received E coli derived material and although none developed antibodies, conclusions cannot be made about its

antigenicity. A large number of patients have received E. coli derived G-CSF and the development of antibodies to this protein has not been reported.

The antibody response against the rh GM-CSF was rapid, appearing in all cases by seven days after commencement of the infusion, despite the recent administration of myeloablative and immunosuppressive cytotoxic drugs in three of the patients. In these three cases the patients had previously received a test dose of rh GM-CSF immediately prior to the ablative chemotherapy and this may have initiated the antibody response. In all cases IgG antibody was produced suggesting that this is a secondary immune response, although in patient 1 the IgG response was equally rapid and no test dose was given. IgM responses were not ascertained as it proved impossible to eliminate non specific IgM binding under the ELISA conditions used. It is possible that the primary response had occurred previously to trace quantities of native non glycosylated protein, however, providing GM-CSF is produced in intra uterine life, exposure to native non glycosylated protein would be expected to induce tolerance It has previously been reported that GM-CSF may augment antibody responses by enhancing the function of antigen presenting cells (Morrisey et al. 1987). This itself may lead to an increase in the production of antibody against the antigenic sites on the recombinant derived protein.

Evaluation of the clinical effects of antibody formation is difficult. The anti GM-CSF antibodies were detected retrospectively and were not suspected at the time of administration of growth factor. No evidence was found to suggest that the antibodies detected had neutralizing activity. The marrow culture assays

were performed with 10% patients' sera and even with the highest titre of anti GM-CSF antibodies the specific antibody concentration would be only 3.6 ng/ml. With a GM-CSF concentration of 1.25 ng/ml there is still a molar excess of GM-CSF and this assay must therefore be considered an insensitive test of neutralizing activity. It is not possible to tell whether the appearance of anti GM-CSF antibodies influenced neutrophil regeneration as the time after ABMT to reach 0.5 x 10^9/l neutrophils was highly variable. One patient died of progressive disease before neutrophil recovery at 17 days after ABMT and the time to achieve a neutrophil count of 0.5 x 10^9/l in the other two patients was 14 days and 27 days respectively. The finding that the presence of anti GM-CSF antibodies was associated, in the one patient who had not received chemotherapy, with a decline in biological effect and a marked decrease in serum levels of GM-CSF despite continued infusion, suggests that development of this antibody resulted in more rapid plasma clearance of the rh GM-CSF. This is supported by the finding of a fall in circulating GM-CSF levels in the three ABMT recipients who developed antibodies compared to one out of seven who did not. This may indicate that when such antibodies are detected either the dose of administration should be increased or that there should be a change to an alternative source of recombinant product. Our findings may be particularly important where repeated administration of a growth factor is employed. Furthermore, it is clearly important that the sera of all patients receiving investigational cytokines and growth factors are analyzed for antibody formation using highly sensitive assays.

Chapter 8

Lymphocyte regeneration after ABMT

8.1. Introduction.

Consistent phenotypic abnormalities have been reported following both allogeneic and autologous BMT as discussed in chapter 2. Using available monoclonal antibodies the phenotype of the regenerating lymphocytes were assessed following ABMT. Lymphocyte phenotype characterization was performed in patients following ABMT by single antibody labelling in 93 cases In 34 of these cases dual antibody analyses were performed using commercially available antibodies directly labelled with fluorescein or phycoerythrin.

Blood was obtained from patients before the commencement of high dose therapy and at regular intervals following marrow regeneration in each of the patient groups studied. The mononuclear cell fraction was collected by separation over Ficoll as described in the method section. The cells were washed and incubated with monoclonal antibodies as described in the methods section. The cells were either stained fresh or after thawing of previously frozen cells. 2×10^5 cells were added to microtitre plates and predetermined optimal quantities of the appropriate antibodies were added to the cells and incubated on ice as described in the methods section.

Regeneration was studied in a total of 93 patients - 23 with AML (15 after double and 8 after single grafts), 8 with ALL, 45 with HD and 17 with NHL.

Results

The tabulated results obtained in individual patients with the mean and standrad deviation for each antigen for each patient group are shown in appendix 1. Before high dose therapy the total lymphocyte count, T cell count T cell subsets, B cells and CD56 cells were normal in patients with acute leukaemia and NHL. Compared to the patients with AML, patients with Hodgkin's disease had a significantly lower total lymphocyte count ($p<0.05$) and CD4/CD8 ratio ($p<0.005$). This reduced CD4/CD8 ratio was due to a decreased number of CD4 cells with a normal CD8 count.

8.2. T cell regeneration after ABMT.

The total peripheral blood lymphocyte count rapidly fell to near zero after high dose therapy and lymphocytes account for the earliest regenerating cells seen in the peripheral blood. The early cells have a distinct morphological appearance with increased size and a granular cytoplasm compared to the normal resting lymphocyte and are known as large granular lymphocytes. Figure 8.1 shows the cell numbers of regenerating lymphocytes, T lymphocytes (CD3+) and NK cells (CD56+) in all the patients after ABMT.

There was a significantly reduced total lymphocyte count after ABMT in the patients with ALL compared to the other patient groups for up to six months after ABMT ($p<0.05$).

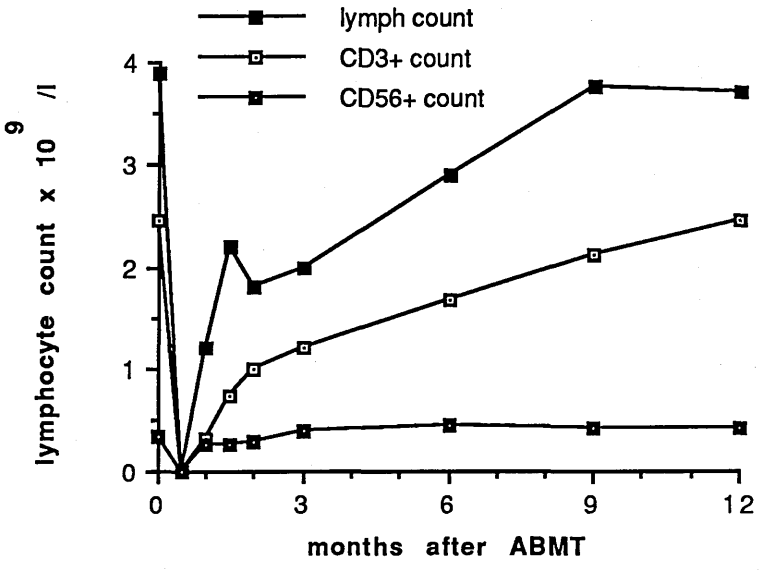


Figure 8.1. Regeneration of lymphocytes, T lymphocytes and NK cells after ABMT.

The percentage of cells staining positively with monoclonal antibodies against CD3 and CD56 are shown in figure 8.2.

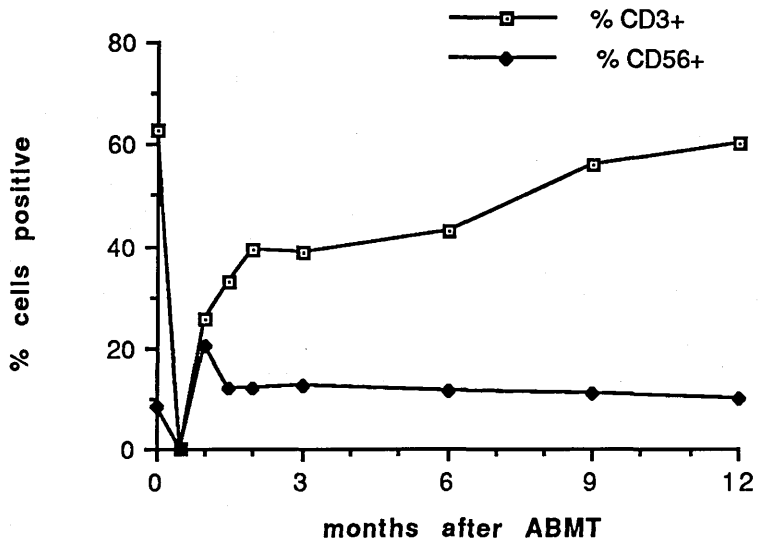


Figure 8.2. Regeneration of T cells and NK cells expressed as % positive cells expressing CD3 and CD56 after ABMT

As can be seen in figure 8.1. the total number of peripheral blood T lymphocytes (CD3+ cells) is markedly reduced for nine months after ABMT. There are differences, however, in the regeneration pattern of T cell subsets after ABMT. There is a rapid recovery of CD8+ cells which have reached normal numbers by two months after ABMT in most cases . The CD8+ cell count may be significantly elevated above the pre-transplant number until six to nine months after ABMT. In contrast the CD4+ cells are markedly reduced in

the early post transplant period and do not return to pre-transplant levels until one year after ABMT. The patients therefore have a reversal of the normal CD4+/CD8+ ratio. At 3-6 weeks after ABMT the mean CD4/CD8 ratio was 0.36 (±0.14), at six months 0.78 (± 0.25) and at one year 1.47 (±0.37). All results are expressed as mean ± standard deviation.

NK cells, as defined by positivity with the CD56 antibody NKH1, rapidly return to normal levels by six weeks after ABMT. Although these cells regenerated rapidly, there was no evidence of an actual increase in NK cells numbers during the early regenerative period. The CD8+/CD3- cells probably represent NK cells, but double labelling studies were not performed to confirm that these cells are the same population as those expressing CD56.

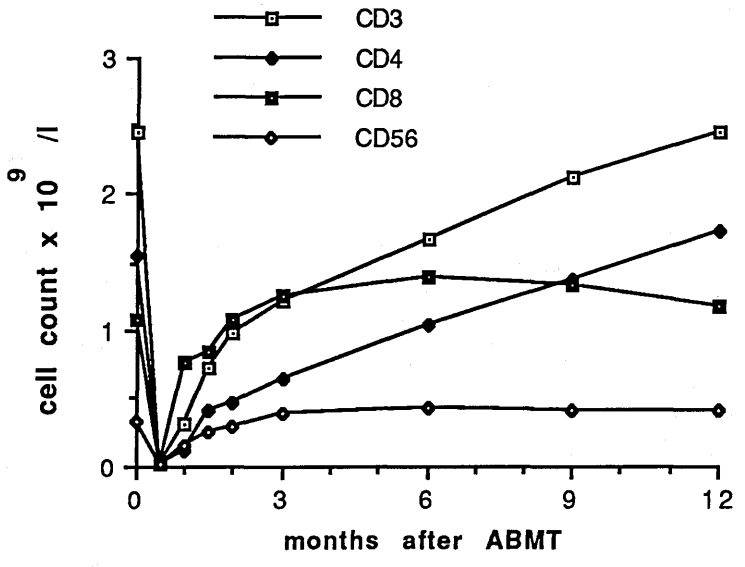


Figure 8.3.

Regeneration of T lymphocytes, T cell subsets and Natural Killer cells in patients after ABMT

Figure 8.4 shows the % CD3+ cells after ABMT in each of the patient groups. There is no statistical difference in regeneration of T cells at any time point after ABMT in any of the patient groups. The total T lymphocyte numbers remain below 50% of the pretransplant level for six months after ABMT and do not return to normal levels until nine months to one year after ABMT.

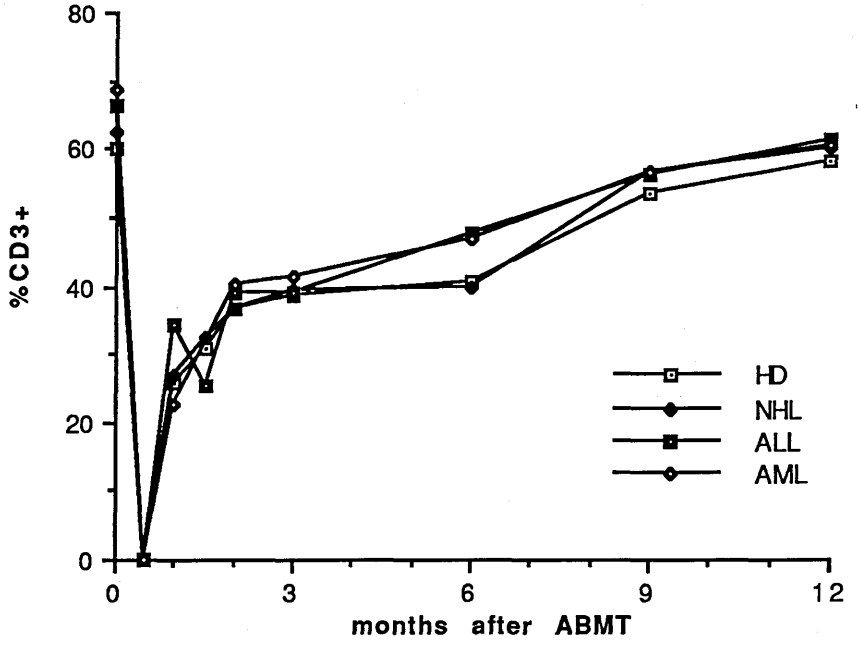


Figure 8.4.

Regeneration of CD3+ lymphocytes after ABMT in AML, ALL, HD and NHL patients. There is no statistical difference in T cell regeneration among any of the patient groups.

Regeneration of CD4+ cells after ABMT.

As can be seen in figure 8.5. CD4+ cells are markedly reduced in both % of lymphocytes and total number of cells nine months to one year after ABMT.

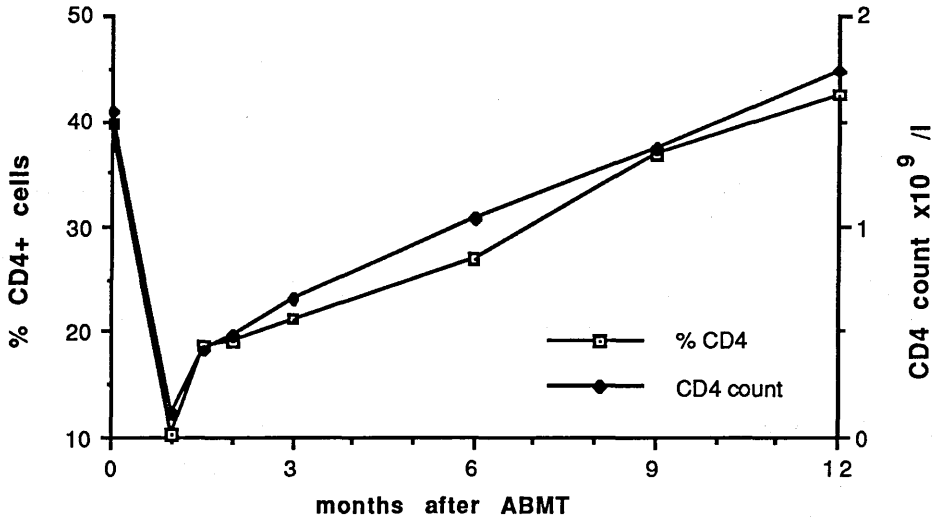


Figure 8.5.

Regeneration of CD4+ lymphocytes after ABMT . The results are expressed in terms of % CD4+ cells and also as total CD4+ cell count.

Regeneration of CD8+ cells after ABMT.

As can be seen in figure 8.6 there is a rapid regeneration of CD8+ cells after ABMT. These cells account for greater than 60% of the early regenerating cells after ABMT. Normal or above normal numbers of CD8+ cells are seen by two to

three months after ABMT. Clearly CD8+ cells exceed CD3+ cells and these cells do not therefore represent true T cells. CD3+/CD8+ cells classically represent suppressor function/cytotoxic T cells. CD3-/CD8+ cells most probably have NK function although double staining studies with CD56 were not performed to confirm this.

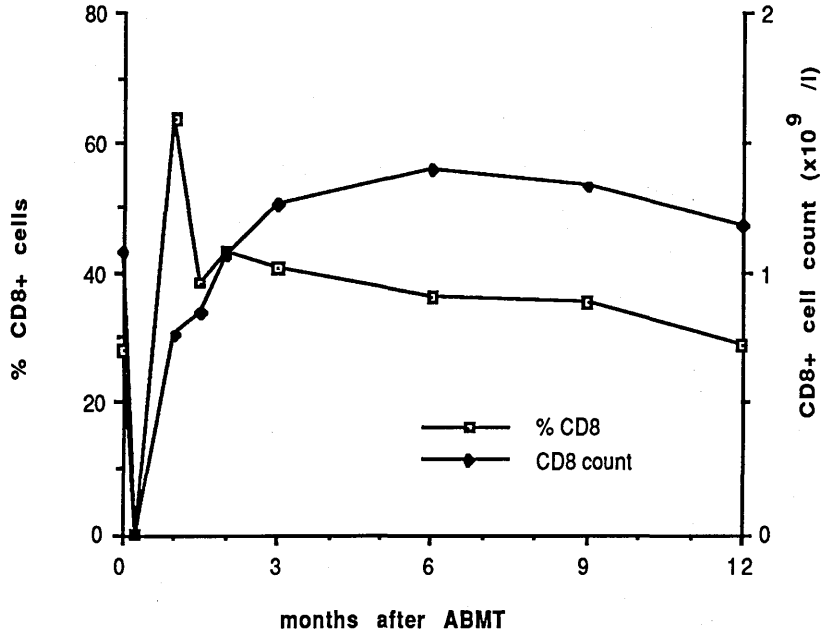


Figure 8.6.

Regeneration of CD8+ cells after ABMT. The results are expressed in terms of % CD8+ cells and also as total CD8+ cell count.

CD4/CD8 ratio after ABMT

Normally the CD4:CD8 ratio is approximately 2. Immediately prior to ABMT the ratio of the whole group was 1.68:1. The CD4:CD8 ratio was particularly depressed in the patients with HD (1.5 ± 0.46) before ABMT compared to the other patient groups.

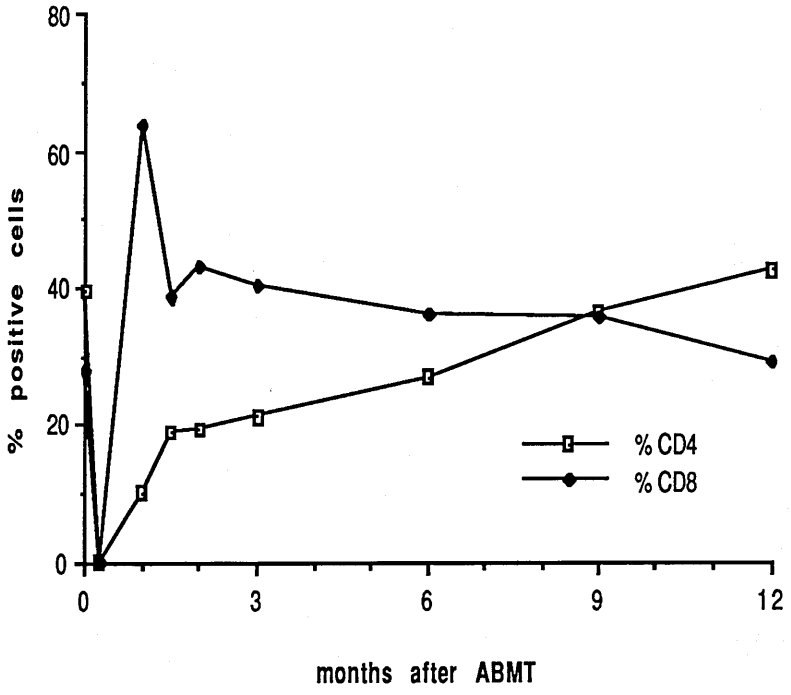


Figure 8.7.

CD4+ and CD8+ cells after ABMT expressed as % positive cells.

Figure 8.8 shows the absolute numbers of CD4+ and CD8+ cells after ABMT and clearly shows that this abnormal ratio is caused by the slower regeneration of CD4+ cells compared to the rapid regeneration of CD8+cells.

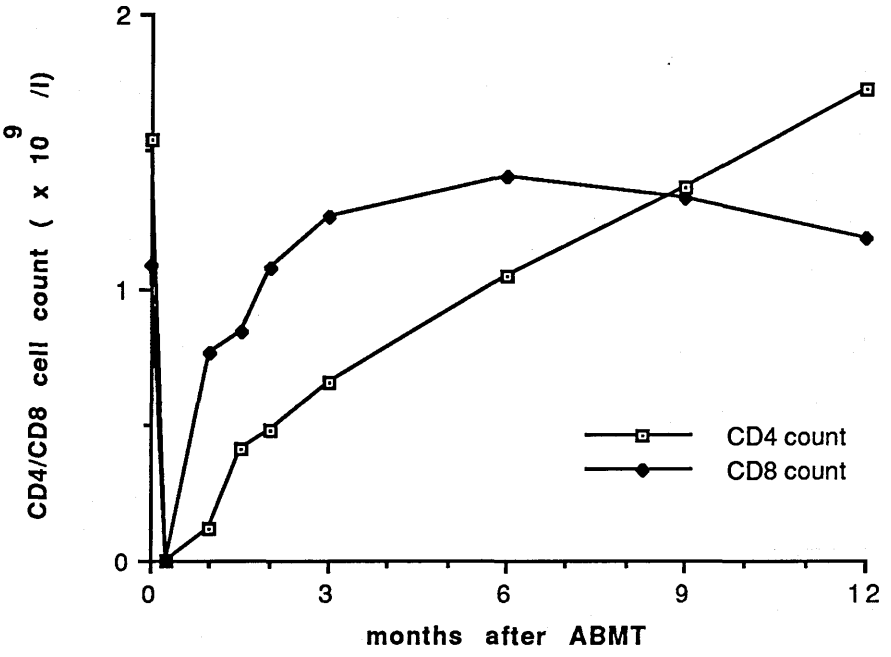


Figure 8.8.

CD4+ and CD8+ cells after ABMT expressed as absolute cell count.

As can be seen from figure 8.9 the normal CD4/CD8 ratio is markedly reversed during the early regenerative period. There is no significant difference in the CD4/CD8 ratio in any of the patient groups at any time after ABMT. At 3-6 weeks after ABMT the mean CD4/CD8 ratio was 0.36 (±0.14), at six months

0.78 (± 0.25), at nine months 1.01 (+0.21) and at one year 1.47 (±0.37). All results are expressed as mean ± standard deviation.

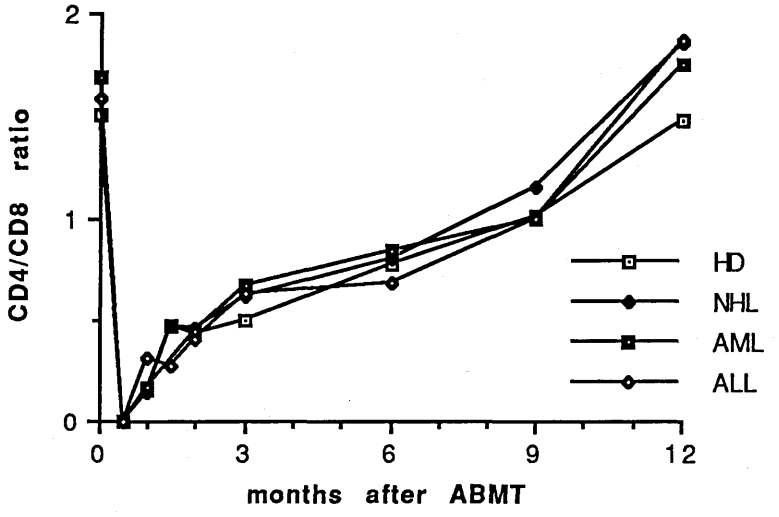


Figure 8.9. CD4/CD8 ratio after ABMT in patients with AML, ALL, NHL and HD.

There is no statistical difference in the CD4/CD8 ratio among the different patient groups for nine months after ABMT. At one year after ABMT the patients with Hodgkin's disease have a significantly reduced CD4/CD8 ratio compared to the other patients ($p < 0.05$). There is a reduced CD4/CD8 ratio in those patients with Hodgkin's disease who do not achieve CR after ABMT compared to those who do achieve CR ($p < 0.01$). There is no significant difference in CD4/CD8 ratio in those patients with Hodgkin's disease who achieve CR and patients with AML, ALL or NHL

8.3. B cell regeneration after ABMT.

There is a rapid regeneration of CD20+ cells after ABMT with a normal per centage of cells expressing CD20 cells by two months after ABMT in AML, HD and NHL patients. In no cases were there any cells which were CD10 +. There is a significant delay in CD20 regeneration in ALL patients compared to the other patient groups ($p<0.05$). Figure 8.10 shows the regeneration of CD20+ B cells after ABMT.

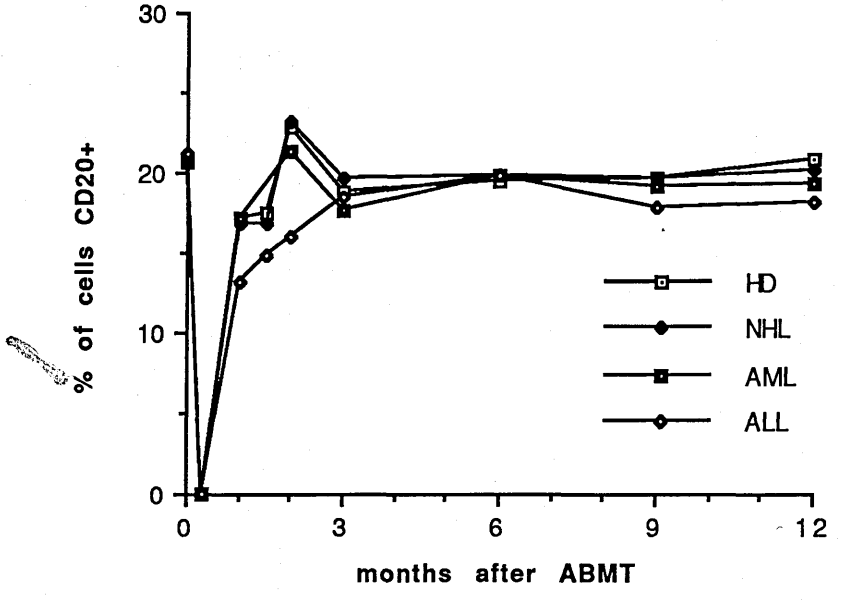


Figure 8.10.

Regeneration of B cells after ABMT expressed as a percentage of cells expressing CD20.

Figure 8.11 shows the regeneration of CD20+ cells expressed as total number of cells. Normal numbers of B cells are seen in AML, NHL and HD patients by three months after ABMT with no difference seen in regeneration of B cells among these groups. In contrast patients with ALL do not regenerate pre transplant numbers of B cells until six to nine months after ABMT.

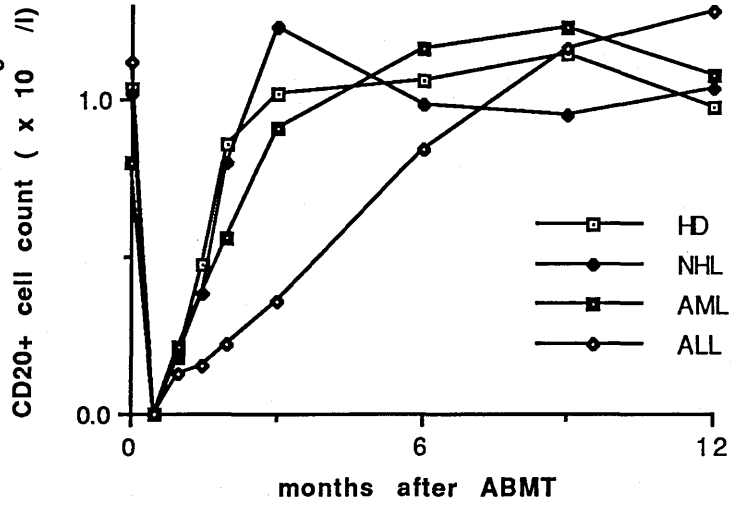


Figure 8.11.

Regeneration of CD20+ cells after ABMT expressed as absolute cell count.

8.4. T δ expression after ABMT

During the early regenerative period 13 of 31 patients examined had increased percentage of circulating T cells expressing the T δ chain suggesting that these cells expressed the Tγδ receptor. No patient had more than 5% of lymphocytes

expressing TCR δ before transplant but these cells accounted for up to 30% of peripheral blood lymphocytes in some cases in the first two months after ABMT. This increased expression was transient in all but one patient with Hodgkin's disease in whom the expression of T δ remained at 20 - 30% for one year after ABMT. Double labelling studies revealed that these cells do not co-express CD4, CD8 or CD56. The results obtained in individual patients are shown in figure 8.12.

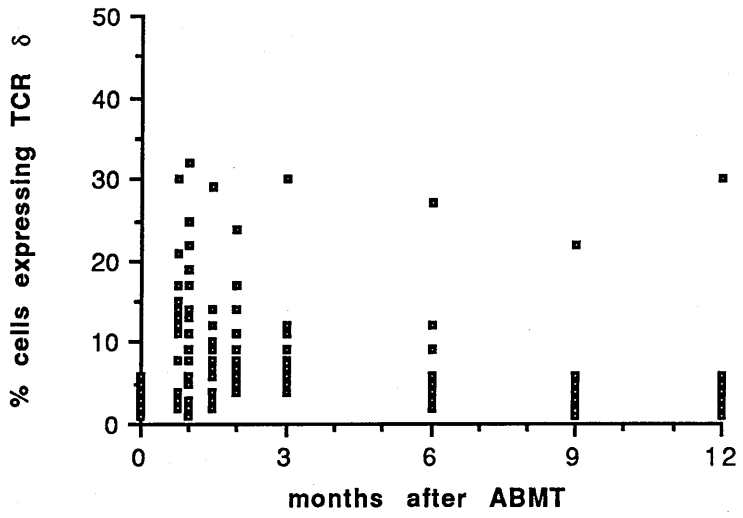


Figure 8.12.

% of cells expressing TCR δ before and after ABMT. There is an increased expression early after ABMT in 13 of 31 patients examined. This increase was transient in all but one patient.

Double labelling studies

Double labelling studies were performed using commercially available directly conjugated antibodies. These antibodies are extremely expensive and the studies were therefore directed at the determination of the cell phenotype in the earliest regenerating cells after ABMT. Obviously, the potential number of studies that can be performed with dual antibodies is large and the studies were further directed at the recovery of memory cells after ABMT. These studies were performed on 148 samples obtained during the recovery period in a total of 34 patients.

Although the CD4+ cells account for only a small percentage of the earliest regenerating cells, these CD4+ cells co-express $CD45R_0$ (84% $\pm$ 11%) and CD29 (89% $\pm$ 7%) but not CD45R (11% $\pm$ 5%). As discussed in chapter 3, this suggests that these cells are derived from the mature, memory T cell population. The CD8+ cells are largely $CD45R_0$ negative (12% $\pm$ 7%). No cells were found to co-express CD4 and CD8 during the very early regenerative stage.

CD56+ cells do not co-express CD4 or CD8. As discussed above, TCR $\gamma\delta$ expressing cells also do not co-express CD4, CD8 or CD56, although these cells may have NK function.

These results suggest that there is little evidence that during the early regenerative phase that the peripheral blood lymphocytes have an immature

phenotype. Although there is an increase in TCR δ expression, there are no cells which co-express CD4 and CD8. Although the CD4 cells are reduced in number they have almost entirely a memory cell phenotype. This suggests that the CD4 cells may be derived from either mature lymphocytes present in the re-infused bone marrow or from cells which survived the high dose chemotherapy and do not represent cells derived from the re-infused lymphoid progenitor cells.

Chapter 9.

In vitro T cell responses after ABMT.

9.1. Introduction.

As described in chapter 3, the CD3, the antigen specificity of individual T cells is determined by the TCR which recognises antigen complexed with self MHC. TCR $\alpha\beta$ are expressed on the cell surface in close association with CD3 which together form the TCR/CD3 complex. The CD3 complex is involved in signal transduction that occurs on antigen receptor binding. This antigen specific activation via the TCR/CD3 complex can be mimicked in a polyclonal fashion by antibodies with specificity on the TCR or on the closely associated CD3 structures. This leads to a hydrolysis of phosphoinositol bis phosphate to diacylglycerol (DAG) and inositol tris phosphate (IP_3). DAG induces activation of protein kinase C and IP_3 increases intracellular free Ca^{++}.

Human T cells produce a proliferative response when stimulated by antigen . This stimulation may be induced also by monoclonal antibodies against the CD3 complex which results in polyclonal activation of T cells. The lectins phytohaemagglutinin A (PHA) and conconavalin A (Con A) also mediate mitogenesis via CD3.

The CD2 antigen has also been implicated in T cell activation. Combinations of antibodies reactive with CD2 and CD2R, reactive with different epitopes on the

CD2 molecule are also mitogenic to mature T cells (Meuer et al 1984). This activation apparently involves the same intracellular second messengers (Alcover et al 1988). Peripheral blood T cells activated through either TCR/CD3 or CD2 pathways produce IL-2 and respond to it by proliferation (Weiss et al 1984).

PHA-P, but not anti CD3 activation of T cells can be blocked by anti CD2 antibodies, indicating that CD2 is involved in PHA-P induced stimulation (O'Flynn et al 1985). The exact mechanism of PHA-P activation of T cell remains unclear. However, since the mitogenic combination of anti CD2 antibodies was available in only very limited amounts, the lectin PHA-P was used to assess activation via this alternative pathway. It had been reported that this pathway was accessory cell independent, but it has recently been shown that this pathway is accessory cell dependent for maximal stimulation and that this dependence may be mediated by CD44 (Denning et al 1989).

9.2. Methods

Cell proliferation was assessed using the measurement of incorporation of radiolabelled thymidine as described in the methods section. Assays were plated out in quadruplicate at 2 x 10^5 cells in round bottomed microtitre plates in a final volume of 200 µl in RPMI supplemented with 10% FCS and the compound under assessment for growth stimulation. The lectins PHA-P and Con A and the monoclonal antibodies UCHT1 and 9.6/9.1 combinations were used at pretested

optimal concentrations. Assays were set up ± recombinant IL-2 (Boehringer Mannheim FRG). IL-2 was added at a final concentration of 10 U/ml.

The stimulation index (S.I.) represents the ratio of cpm of the test sample divided by the cpm obtained in the same patient sample set up in identical culture but with no added mitogen. The stimulation index was assessed in ten normal individuals to define a normal control value. Normal cells were set up in culture at the same time as the patient samples to confirm that optimal concentrations of mitogen were added.

T cell proliferative responses were investigated in 91 patients using PHA, Con A and IgG2a anti CD3 - 25 with AML, 7 with ALL, 12 with NHL and 47 with Hodgkin's disease. In 25 of these patients with Hodgkin's disease responses were also assessed using a combination of anti CD2 antibodies. The responses to the mitogens in individual patients with the mean and standard deviation for each group are shown in appendix 2.

9.3. Responses in patients with AML.

Samples from 25 patients with AML were studied before and at intervals after ABMT. The response to UCHT1 in 17 patients who received double grafts is shown in figure 9.1 and the responses of a further 8 patients who received only a single graft are shown in figure 9.2. Before ABMT all patients with AML in first CR had normal mitogenic responses. In all cases studied the response was markedly reduced for six months after ABMT, but returned to normal by one

year after the procedure. There was no difference in the pattern of response after a single procedure in those patients who did not have a second graft with the responses after the second graft..

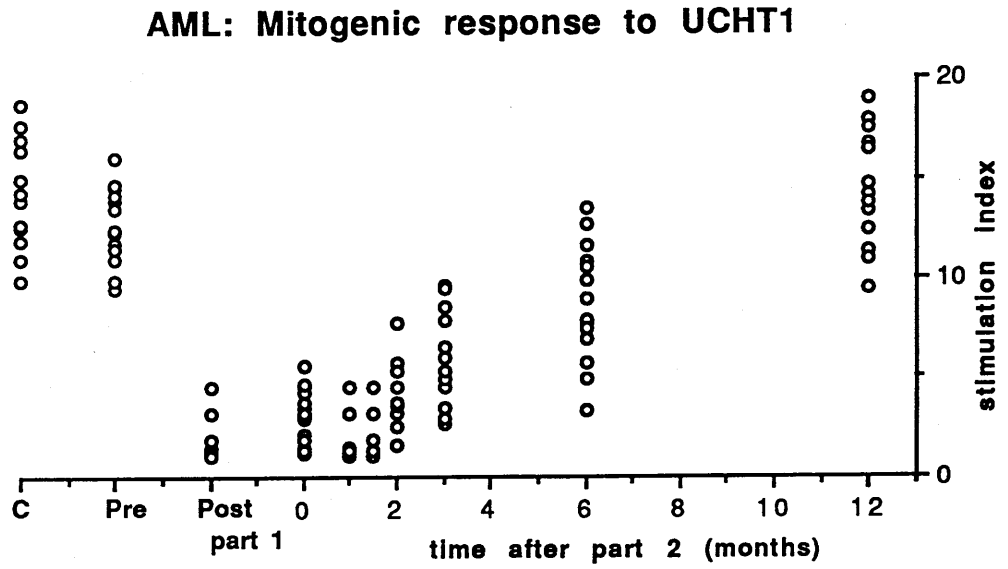


Figure 9.1

Mitogenic response to UCHT1 in AML patients who had double grafts. The stimulation index to UCHT1 in 10 normal individuals is shown as a control on the left. Time 0 is the day of re-infusion of marrow after the second ABMT.

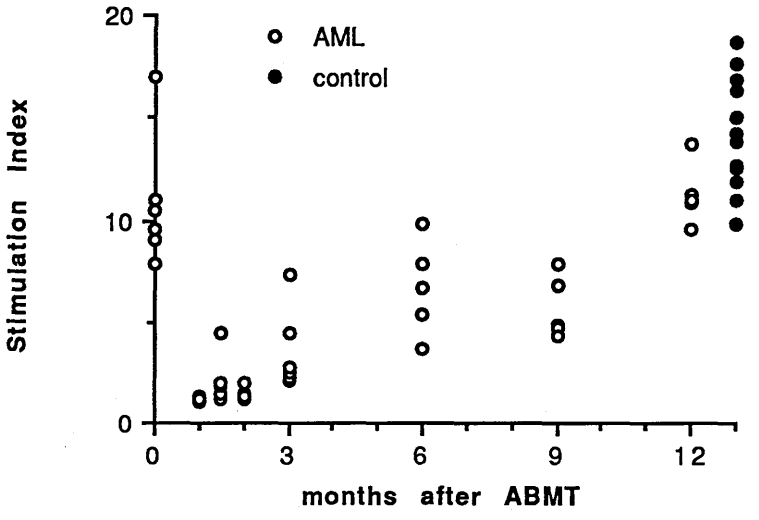


Figure 9.2.

Mitogenic response to UCHT1 in AML patients who had single grafts. The stimulation index to UCHT1 in 10 normal individuals is shown as a control on the right.

9.4. Responses in patients with ALL.

In the ALL patients tested the mitogenic responses to UCHT1 were significantly reduced prior to ABMT compared to normal controls ($p<0.05$). Responses were markedly decreased after ABMT in all cases until one year after ABMT.

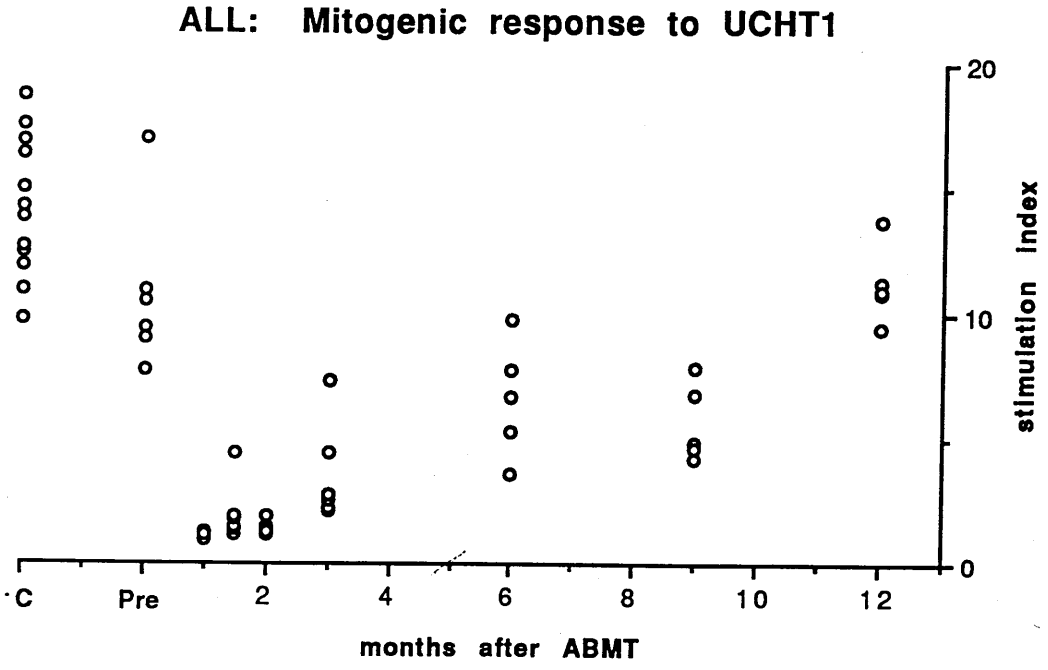


Figure 9.3

Mitogenic response to UCHT1 in ALL patients. The stimulation index to UCHT1 in 10 normal individuals is shown as a control on the left.

9.5. Responses in patients with NHL.

Before ABMT the response in patients with NHL was heterogeneous, with the majority of patients having responses below the normal limit of normal. The stimulation index after stimulation with UCHT1 in patients with NHL is shown in figure 9.4. Responses were markedly decreased for three months after ABMT but had returned to the pre transplant levels by six months after ABMT. By one year after ABMT the majority of patients had normal responses. Those patients who had not achieved normal responses by one year after ABMT were those patients who had not achieved CR with the transplant procedure, although it must be noted that a number of patients who still had active disease had normal responses by one year after ABMT.

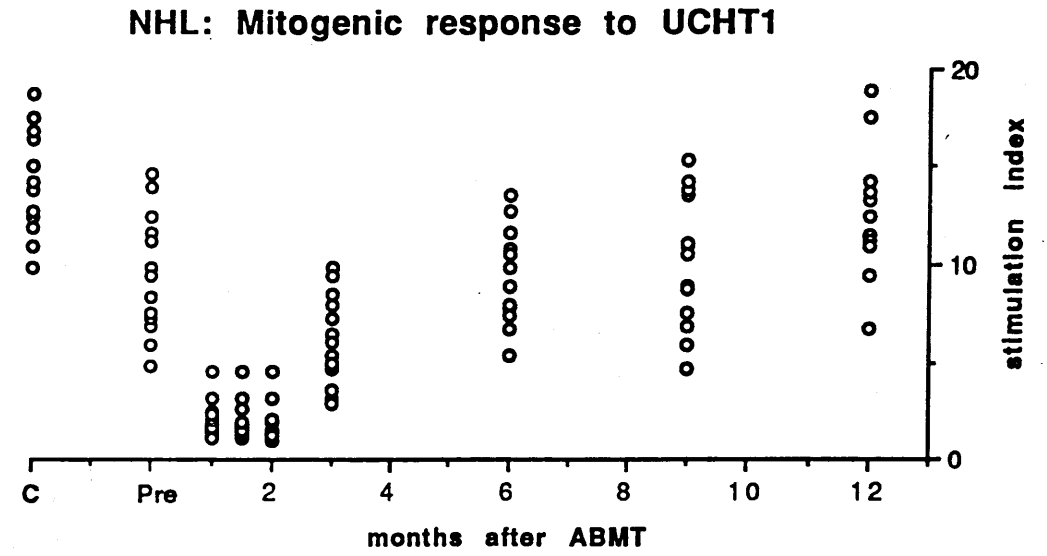


Figure 9.4

Mitogenic response to UCHT1 in NHL patients. The stimulation index to UCHT1 in 10 normal individuals is shown as a control on the left.

Similar patterns of responses were seen to each of the mitogens PHA-P and Con-A in each of the patient groups. The responses in all of the patients with AML, ALL and NHL to the mitogens UCHT1, PHA-P and Con A are shown as the mean response ± standard deviation in figure 9.5.

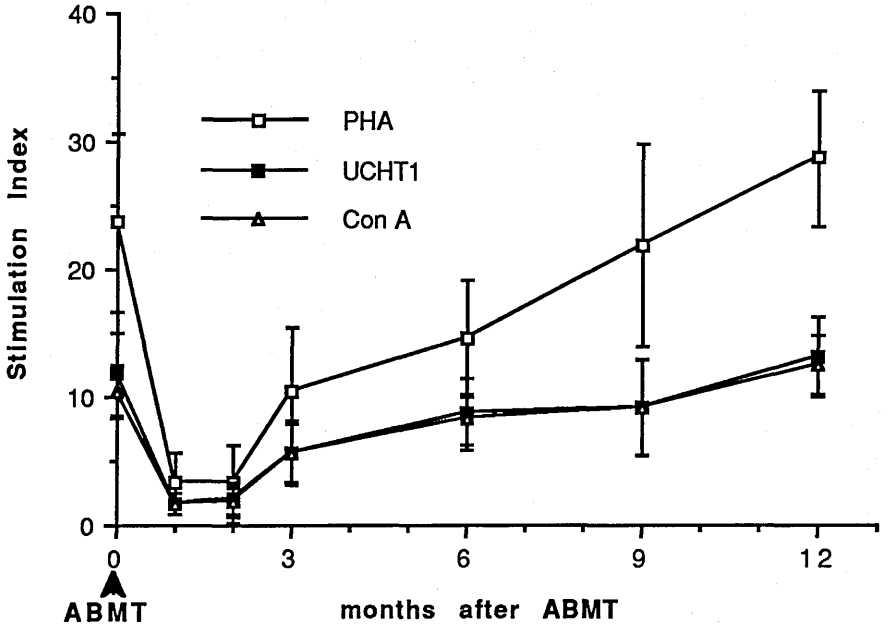


Figure 9.5.

Response to the mitogens UCHT1, Con A and PHA-P in patients with AML, ALL and NHL. The results are expressed as the mean ± standard deviation.

To each of the mitogens used there was a markedly decreased response seen after ABMT and this abnormal response was particularly marked in the early regenerative period. There was no evidence that the pattern of response after ABMT was different with the mitogens activating through the CD3 pathway, that is UCHT1 and Con A from the response with PHA-P acting through the CD2 pathway.

As discussed in chapter 2, there have been reports that there is defective production of IL-2 after bone marrow transplantation. The same assays were therefore set up with the addition of exogenous IL-2. The effect of the addition of this exogenous IL-2 at 10 U/ml final concentration is shown in figure 9.6. This illustrates the mean and standard deviation of the stimulation index to the mitogen UCHT1 in patients with AML, ALL and NHL $\pm$ IL-2.

There was no significant difference at any time point in the responses obtained to UCHT1 with exogenous IL-2 compared to those with no IL-2. The same results were obtained in responses to PHA-P and Con A. (data not shown).

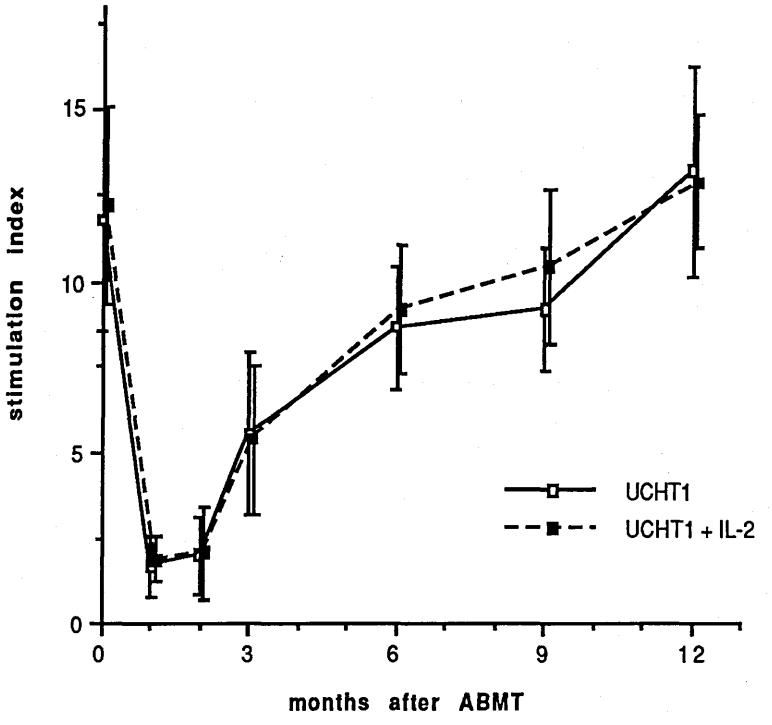


Figure 9.6.

The effect of the addition of exogenous IL-2 on the response to anti CD3 monoclonal antibodies

Although the total number of cells added to the cultures was constant, the percentage of cells expressing CD3 and therefore the total number of T cells was reduced in the cultures set up from the samples obtained from patients after ABMT. The mitogenic response to UCHT1 was therefore assessed in assays corrected to contain the same number CD3+ cells that were present in the controls. The results obtained are shown in figure 9.7.

Although this results in a more rapid return of the responses to the normal range, by six months after ABMT, there is no significant effect on the responses obtained during the first three months after ABMT when the responses are the most depressed.

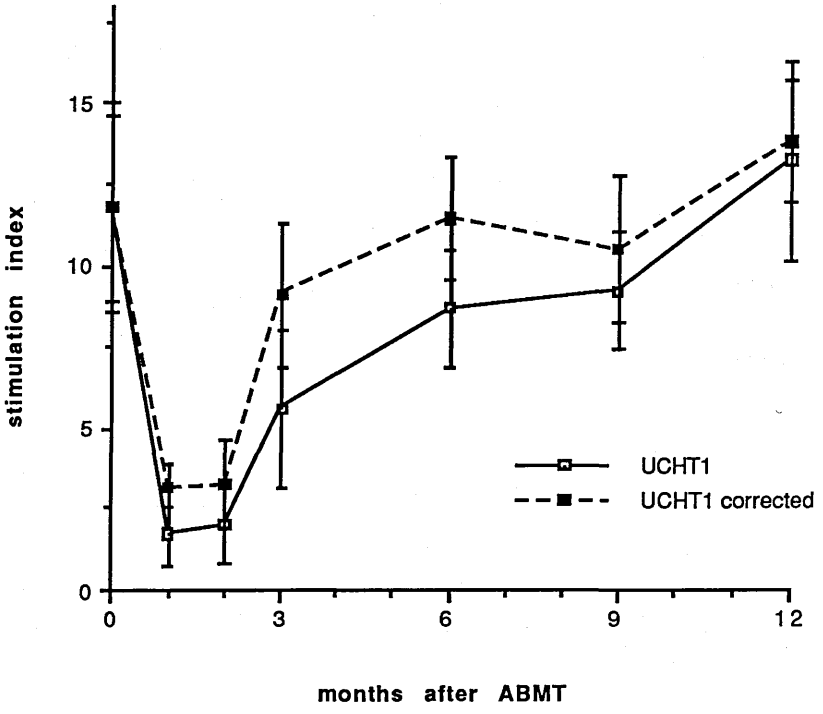


Figure 9.7.

Mitogenic responses to UCHT1 in cell cultures with numbers corrected for decreased numbers of CD3 cells present.

9.6. Responses in patients with Hodgkin's disease.

Samples from a total of 47 patients with Hodgkin's disease were available for analysis. In Hodgkin's disease patients the responses were markedly depressed in almost all cases immediately before ABMT. Figure 9.8 shows the mitogenic response response to UCHT1 in all patients with Hodgkin's disease. A similar pattern was obtained on testing with Con A (figure 9.9) and PHA-P (figure 9.10). In samples from 25 patients cells were set up in culture with the mitogenic combination of anti CD2 monoclonal antibodies. The results obtained are shown in figure 9.11.

Decreased responses were seen in all cases after ABMT. By six months after ABMT six patients had responses which were at the lower limits of normal, but many patients still had markedly abnormal responses by one year after ABMT.

In view of the known immune suppressive effect of Hodgkin's disease the mitogenic responses were assessed by the subsequent response the patient achieved after ABMT. The responses generated by each mitogen depended upon the response the patient achieved after ABMT. These responses are shown for the UCHT1 (figure 9.12), Con A (figure 9.13), PHA-P (figure 9.14) and the mitogenic anti CD2 combination 9.6/9.1 (figure 9.15)

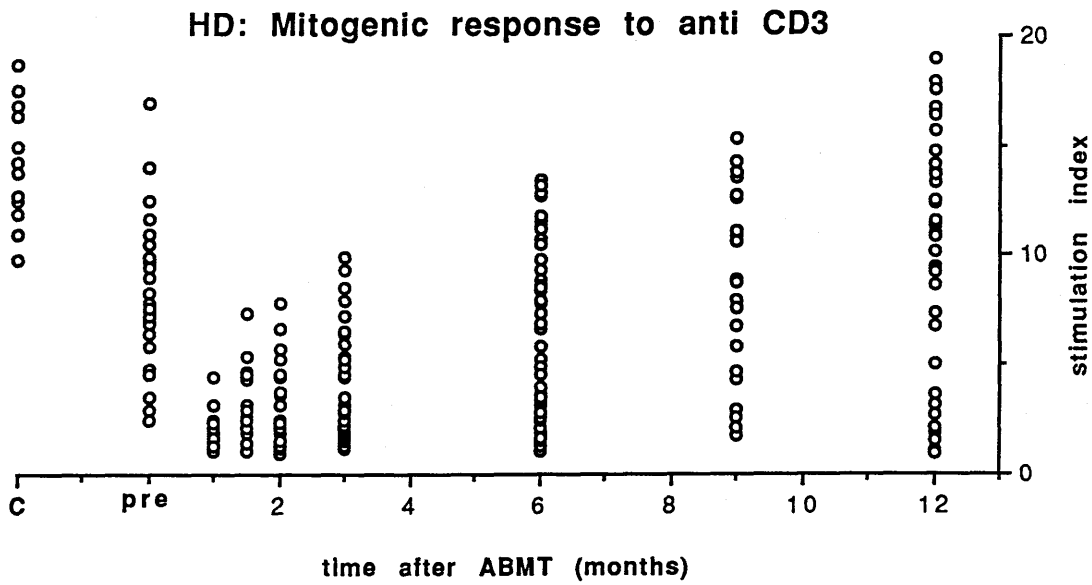


Figure 9.8. Mitogenic response to UCHT1 in patients with Hodgkin's disease after ABMT

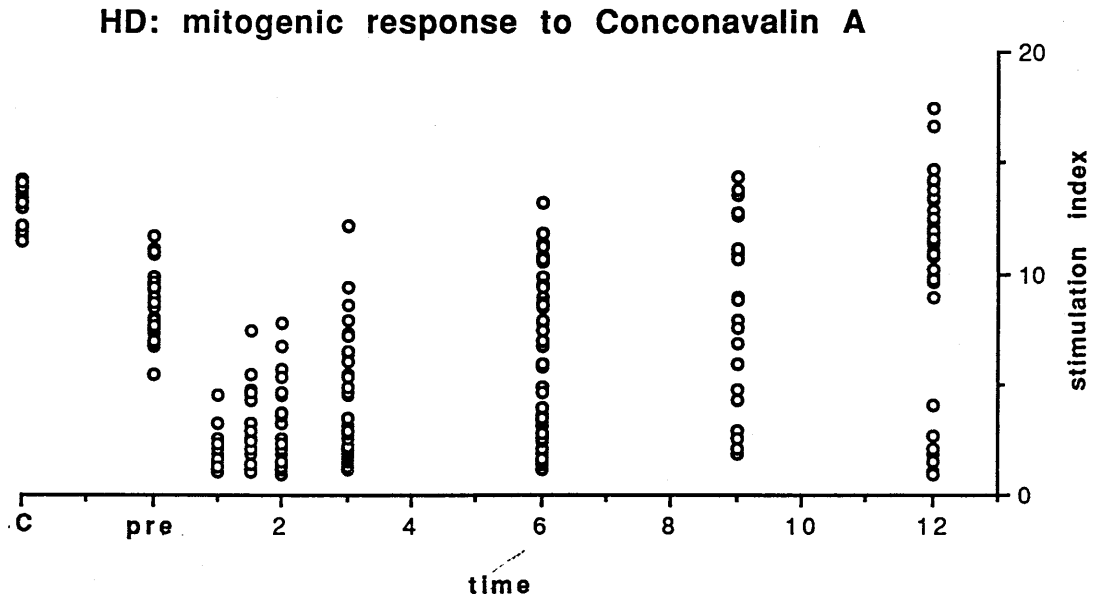


Figure 9.9. Mitogenic response to Con A in patients with Hodgkin's disease after ABMT.

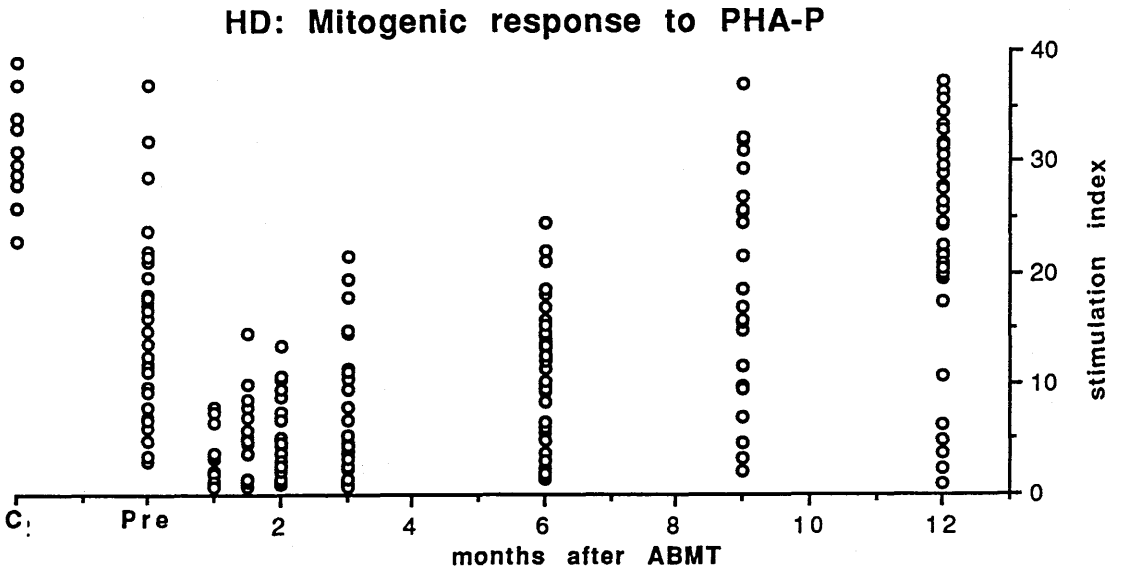


Figure 9.10. Mitogenic response to PHA-P in patients with Hodgkin's disease after ABMT.

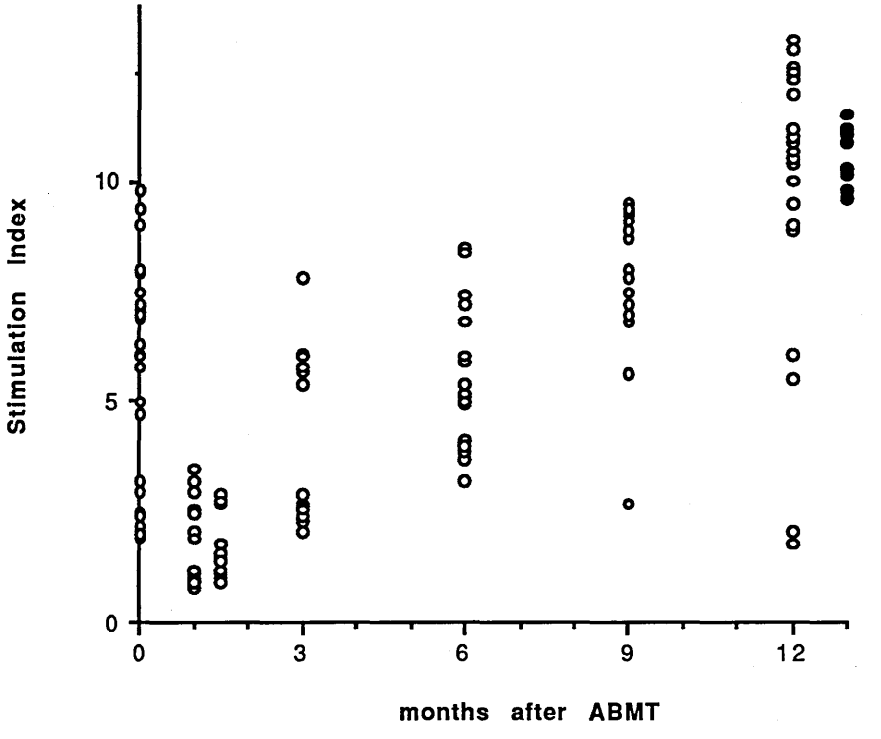


Figure 9.11. Mitogenic response to anti CD2 in 25 patients with Hodgkin's disease after ABMT.

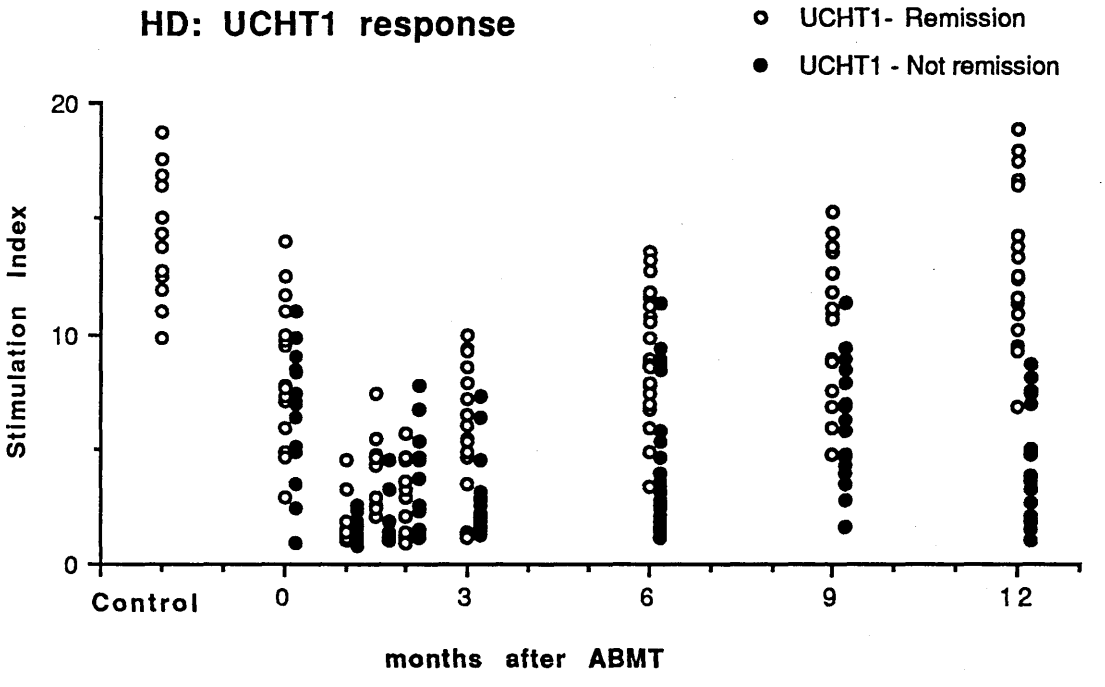


Figure 9.12.

Mitogenic response to UCHT1 in patients with Hodgkin's disease by the response the patient subsequently achieved after ABMT.

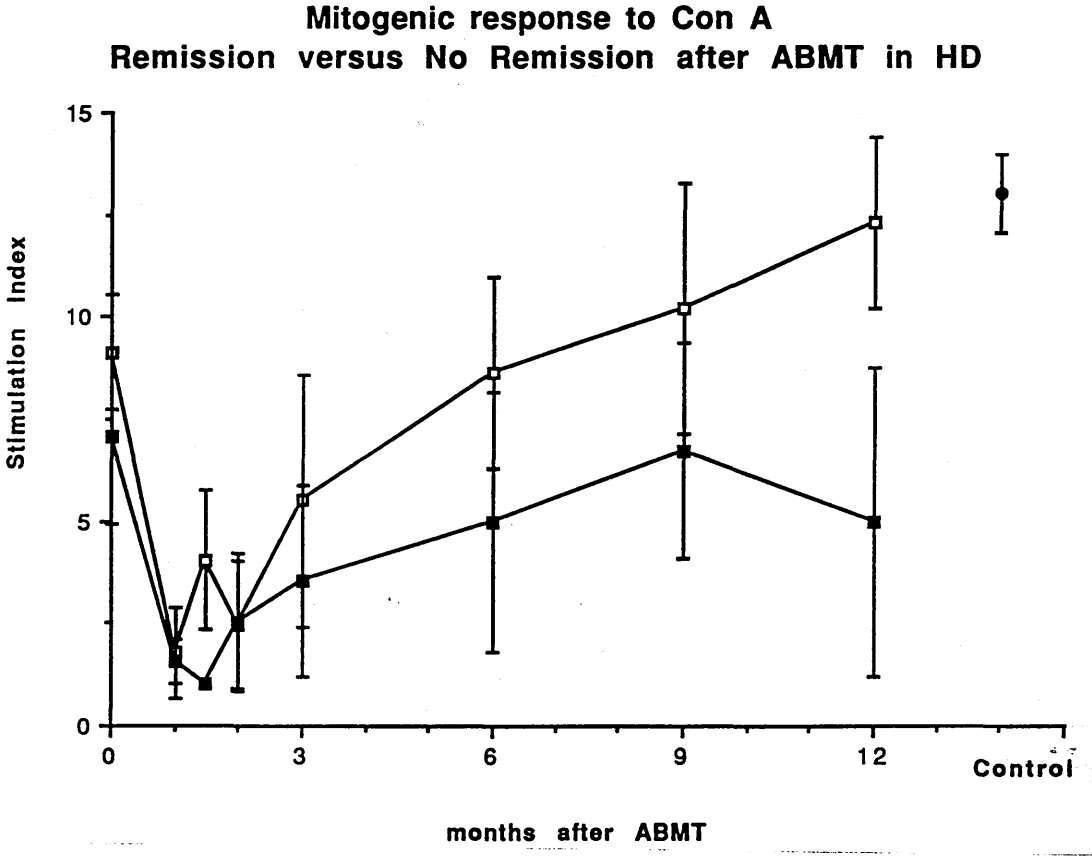


Figure 9.13.

Mitogenic response to Con A in patients with Hodgkin's disease by the response the patient subsequently achieved after ABMT.

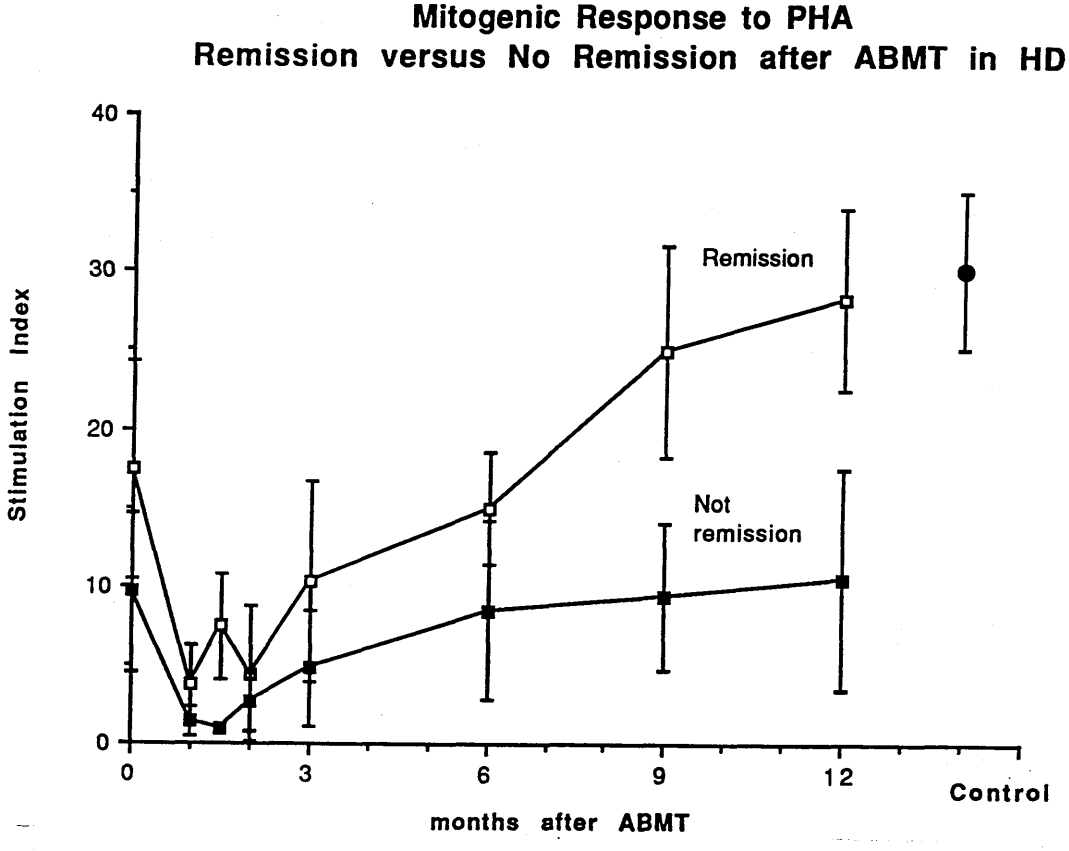


Figure 9.14.

Mitogenic response to PHA-P in patients with Hodgkin's disease by the response the patient subsequently achieved after ABMT.

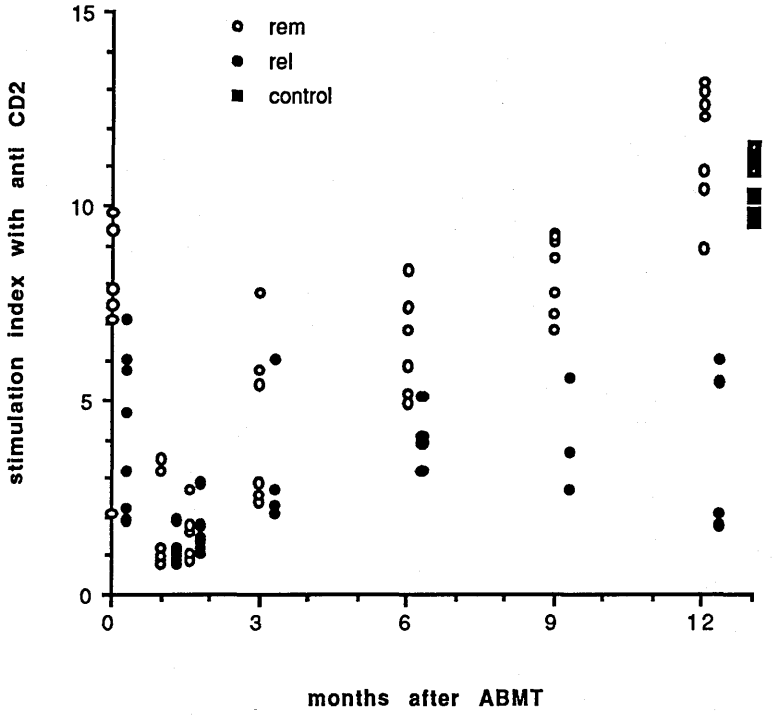


Figure 9.15.

Response to a mitogenic combination of anti CD2 monoclonal antibodies in 25 patients with Hodgkin's disease by the subsequent response those patients achieved to the ABMT. The responses in normal controls are shown on the right.

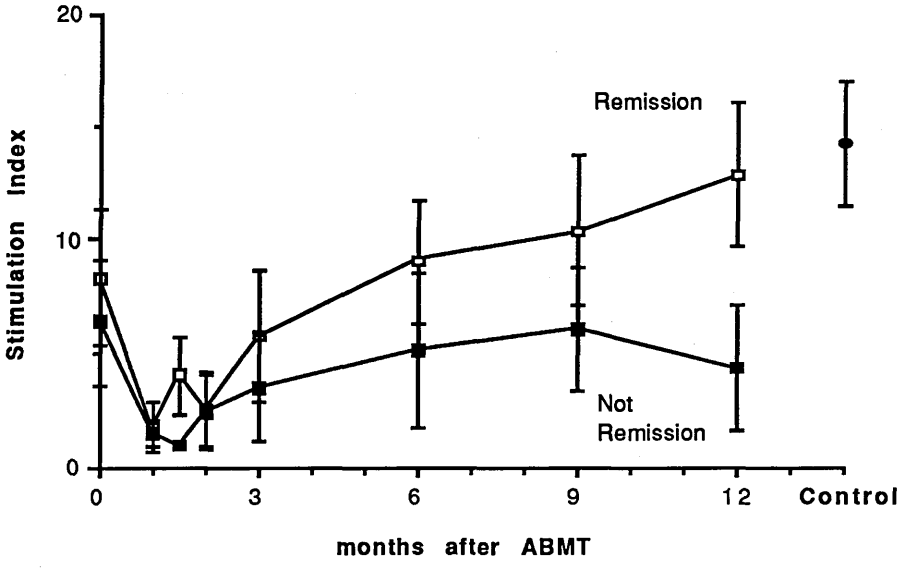


Figure 9.16.
The response to the mitogen UCHT1 expressed as mean ± standard deviation in those patients who achieved CR compared to those patients who did not achieve CR.

9.7. IL-2 Receptor expression after T cell activation.

IL-2 receptor (CD25) expression was assessed prior to culture by incubating cells with anti CD25 monoclonal antibody and subsequently goat anti mouse antibody couples with fluorescein as described in the methods section In no case did CD25 expression exceed 5% before culture. After three days of culture with each of the mitogens studied the CD25 expression was markedly increased, even in those samples obtained immediately after ABMT in which there was suboptimal response to the mitogens studied. Figure 9.17 shows the

mean responses in samples from patients at six weeks after ABMT after three days culture with UCHT1 and is typical of the pattern after culture with the other mitogens.

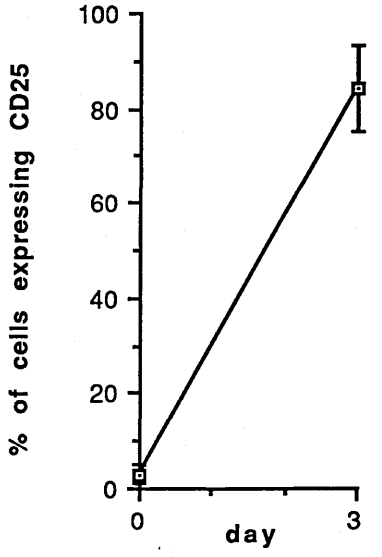


Figure 9.17.

CD25 expression before and after culture with UCHT1 in samples from 86 patients at six weeks after ABMT.

9.7. Conclusions.

T cell proliferative responses were investigated in 91 patients after ABMT. Before ABMT the responses achieved were normal in patients in AML, slightly reduced in patients with ALL and were heterogeneous in patients with NHL. Patients with active Hodgkin's disease had uniformly depressed responses before the administration of high dose therapy.

Abnormal proliferative responses were obtained after ABMT in all of the patients studied. This depressed response was most marked in the first three months after ABMT. The decreased response during this period was not due to the reduced numbers of T cells present, since the defect was not corrected by compensating for the decreased percentage of T cells present following ABMT. The abnormality was not corrected by the addition of exogenous recombinant IL-2, although each agonist resulted in an increased expression of the IL-2R (CD25).

These findings suggest that the defect present after ABMT may be caused by a decreased responsiveness of the cells to IL-2 or by a suppressive effect on cell proliferation by cells with suppressor phenotype which are increased in number during this period. Further studies will be necessary to address this question.

Chapter 10.

In vitro antibody responses by human B lymphocytes

10.1. Introduction.

The induction of an antibody response is a complex process involving the activation of resting B cells with subsequent proliferation and differentiation of some of these cells into mature immunoglobulin secreting cells. A number of model systems have been designed to examine the sequence of events involved in triggering this B cell activation, proliferation and differentiation.

Anti Immunoglobulin is a polyclonal B cell activator which stimulates resting B cells to enter the early G1 stage of cell cycle where they acquire responsiveness to the T cell derived factor B cell growth factor (BCGF). BCGF is necessary to drive the cells into late G1 where they now require the monocyte derived factor IL-1 to enter S phase (Muraguchi et al 1984)

In vitro antibody responses by human B lymphocytes can be studied by the use of these polyclonal activators of B cells. The response of B cells to the polyclonal activators pokeweed mitogen and anti Ig with BCGF were studied before and at intervals after ABMT in each of the patient groups. Specific antibody responses were studied using an in vitro assay for the production of anti influenza antibodies. I am grateful to Dr Robin Callard of the Institute of

Child Health in London for allowing me to spend time in his laboratory to learn these techniques.

10.2. Polyclonal activation of B cells

B cell actiavation responses were investigated in 91 patients - 20 with AML, 5 with ALL, 52 with Hodgkin's disease and 14 with NHL, before and after ABMT using the measurement of immunoglobulin secretion in vitro following activation with anti immunoglobulin or pokeweed mitogen.

Method.

Peripheral blood mononulclear cells were twice depleted of T cells by incubation with anti CD3 followed by incubation with goat anti mouse Ig coated magnetic beads as described in the methods section. The remaining fraction was further partially depleted of monocytes by plastic adherence for 45 minutes at 37^0C as described in the methods section. This B cell enriched fraction was washed three times with RPMI, 5% FCS and the cells set up in culture for ten days at 37^0C in round bottomed microtitre plates in quadruplicate at 2×10^5 cells/ml in a final volume of 200 µl with

1) anti Ig plus B cell derived growth factor (BCGF) - Gift from Dr. L Nadler, Dana Farber Cancer Institute, Boston.

2).Poke weed mitogen 10µl/ml final concentration plus BCGF.

BCGF was added at the concentration found to produce maximal immunoglobulin secretion in normal controls. There was considerable variation with different vials used.

The supernatants were assayed for IgG production by ELISA as described in the methods section. The Ig production was calculated by subtracting the levels detected in the control plates containing the cells from the same patient with the addition of no stimulatory factor from that produced in the test plate.

Results.

As can be seen from figure 10.1 there was a marked decrease in the in vitro IgG production with anti Ig and BCGF in the samples obtained after ABMT. The levels remained significantly decreased for six to nine months after ABMT in patients with AML, NHL and HD. The patients with ALL had decreased responses for a longer duration and responses were still not within the normal range at one year after ABMT.

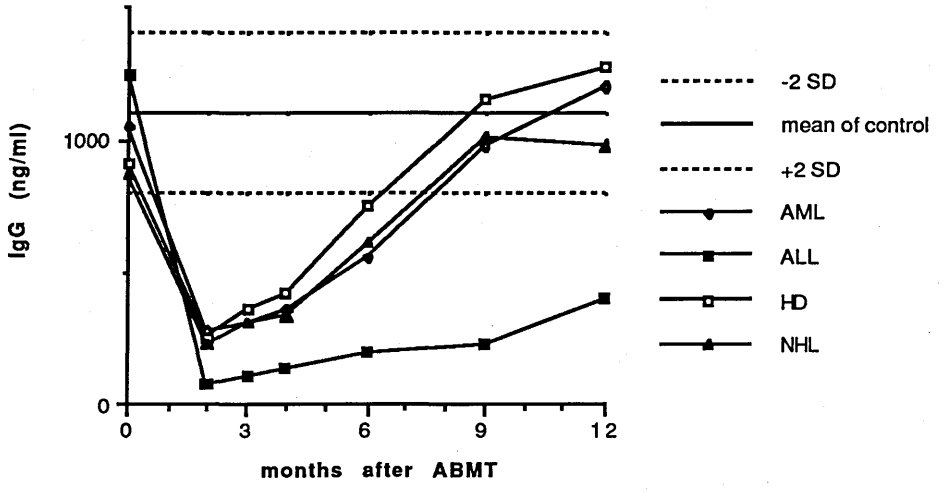


Figure 10.1.

IgG production (ng/ml) in vitro with anti Ig and BCGF in 20 patients with AML, 5 patients with ALL, 47 patients with HD and 14 patients with NHL before and following ABMT. The mean of normal control samples ± 2 SD are shown for comparison.

Figure 10.2 shows the response to the polyclonal activator pokeweed mitogen. Similar results were obtained, with decreased responses in all patients after ABMT. This was particularly marked in patients with ALL in whom responses reached the lower limits of normal only by one year after ABMT.

Unlike the T cell mitogen responses after ABMT, there was no difference in response in those Hodgkin's disease patients who achieved complete remission

after ABMT compared to those who did achieve complete remission (data not shown).

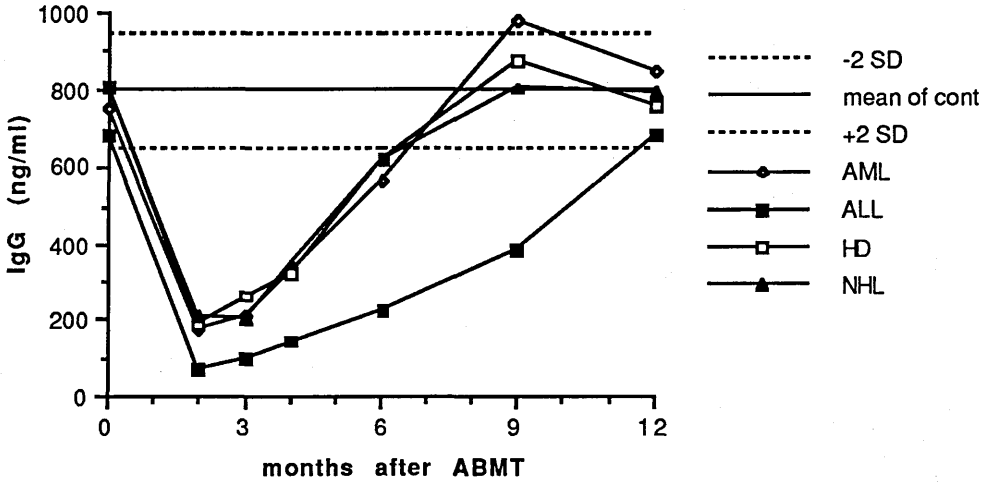


Figure 10.2.

IgG production (ng/ml) in vitro with Pokeweed mitogen (PWM) and BCGF in patients 20 patients with AML, 5 patients with ALL, 47 patients with HD and 14 patients with NHL before and following ABMT. The mean of normal control samples ± 2 SD are shown for comparison.

10.3. In vitro antibody production against influenza virus.

In this study patients undergoing ABMT for haematological malignancies were vaccinated with killed influenza vaccine on the day of marrow re-infusion and in vivo and in vitro assessments of Ig responses made at various times after ABMT. The in vivo responses were discussed in chapter 6.

Method.

(Modified from Zander et al 1981)

The strains of Influenza virus appropriate to that found in the vaccine were provided by Dr J Skehel (NIMR, Mill Hill). Peripheral blood lymphocytes were prepared blood samples obtained from normal volunteers and from patients pre and recovering from autologous bone marrow transplantation as described in the methods section. Separation of E- and E+ cell fractions were performed using AET treated sheep red blood cells as described in the methods section. Partial depletion of the monocyte/macrophage population was performed by adherence to plastic as described in the method section.

Cells were suspended at 2 x 10^6/ml in PRMI 1640 with 25 mM Hepes , 10% Horse serum and 10^{-5} M hydrocortisone and cultured in round bottom microtitre plates (Linbro) in quadruplicate in 200 µl aliquots. Influenza virus was added at 1 µg/ml final concentration. Optimal IgG production occurred when all three strains of virus were added together in the culture system.

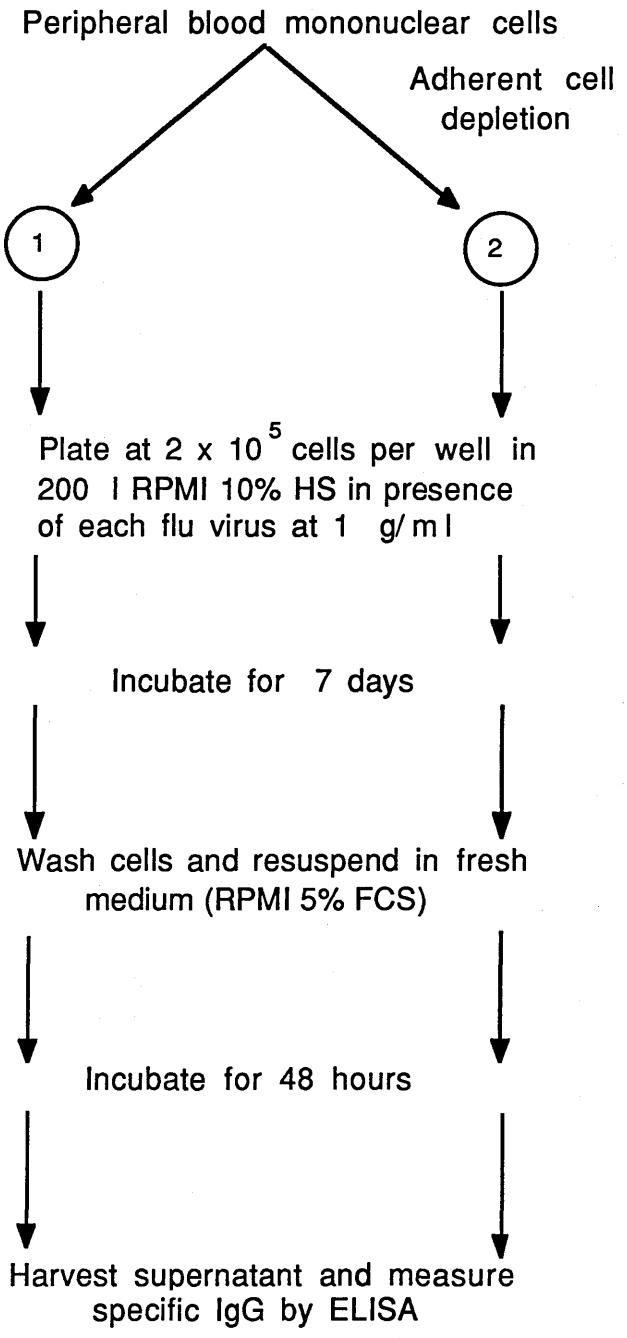


Figure 10.3.

Scheme for detection of specific antibody production in vitro.

Cells were incubated with antigen for seven days in an atmosphere of 10% CO_2 at 37^0C. After seven days the medium was removed by suction using a 21 guage sterile needle and the cells were washed twice in RPMI. The cells were re-suspended in 100 µl of RPMI containing 20 mM Hepes and 5% foetal calf serum for a further 48 hours. Supernatants were harvested and stored at - 20^0C in microtitre plates before assay.

Antibodies to influenza virus were assayed by ELISA as described in the methods section.

Results.

None of the normal controls or patients had detectable amounts of antibody against any of the viruses before vaccination. In the normal controls there was a rapid induction of in vitro response after vaccination which was detectable from peripheral blood samples obtained ass early as ten days after vaccination. Optimal responses in each case were obtained against the X/87 component of the vaccine. Response were increased after partial monocyte depletion, but the response is monocyte dependent as the responses were absent when the monocytes were totally depleted by immune rosetting with anti CD14 coated magnetic beads. As can be seen in figure 10.4, in the normal controls there was an early peak in response vaccination and that the response was maintained for more than one year after vaccination. The peak response was obtained from peripheral blood samples obtained from normal controls 10 - 20 days after vaccination.

In none of the patients studied after ABMT was there a detectable in vitro specific antibody response in samples obtained up to 100 days after ABMT. The response after vaccination in samples from normal controls and from patients in the first one hundred days after ABMT is shown in Table 10.1.

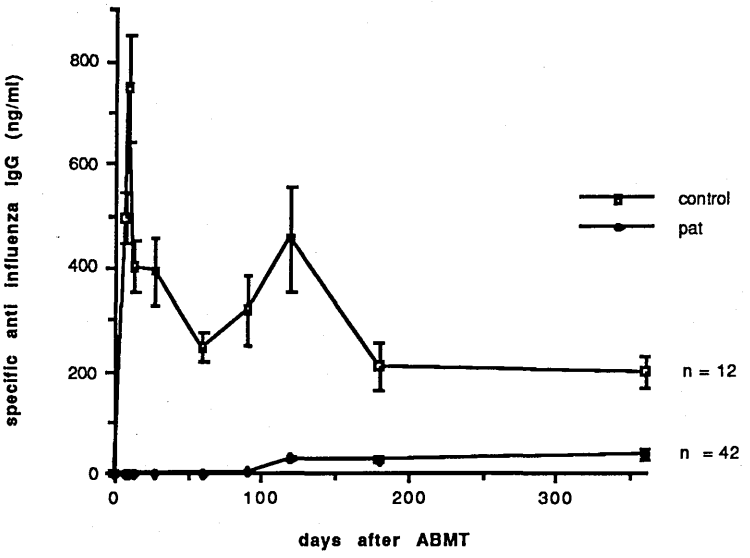


Figure 10.4.

In vitro specific anti-influenza IgG production against X/87 with partially monocyte depleted cell fraction.

In view of the fact that there are reports of decreased production of IL-2 after ABMT, exogenous IL-2 was added to the system at a final concentration of 10 U/ml. In an attempt to augment antigen presentation in the in vitro system, recombinant GM-CSF was also added to the culture system at a final concentration of 12.5 ng/ml.

Table 10.1.

		+IL-2	+GM-CSF
before vaccination			
1	0	0	0
2	5 ± 4	7 ± 3	5 ± 4
after vaccination			
Normal controls			
1	400 ± 163	460 ±123	375 ± 108
2	755 ± 120	780 ± 148	708 ± 95
Patients after ABMT			
three months after ABMT.			
1.	0	0	0
2.	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
Six months after ABMT			
6.	55 ± 14	62 ± 19	44 ± 22

Response in vitro in normal controls and patients ABMT.

1) Whole cell fraction

2) Partially monocyte depleted.

3) E+/E- 3:1 from sample post ABMT

4) E+ pre ABMT/E- post ABMT

5) E+ post ABMT/E- pre ABMT

6) Partially monocyte depleted.

The addition of IL-2 or GM-CSF had no effect on the peak response in normal controls. Presumably there is already optimal lymphokine production in the culture system in normal individuals. The addition of neither IL-2 nor GM-CSF had any effect on specific immunoglobulin production in vitro. Altering the ratio of E- to E+ fractions of cells present in the assay system similarly had no effect on immunoglobulin production during the first three months after ABMT.

10.4. Conclusions.

There was a marked decrease in B cell activation by polyclonal activators as measured by antibody production in vitro for periods up to one year after ABMT. These changes were particularly marked in patients with ALL who had received total body irradiation. Specific antibody production following vaccination with influenza virus occurred immediately in vivo but was absent in vitro for six months after ABMT and markedly reduced responses continued to be seen after this time.

The addition of IL-2 or GM-CSF had no effect on the failure by peripheral blood lymphocytes from patients after ABMT to respond to influenza virus in vitro. This non responsiveness was not due to a reduction in the number of B cells since the B cell numbers rapidly returned to normal in most of these patients while the responses were still absent. Optimal responses were reported to be optimal with mixing E+ and E- cell fractions in a ratio of 3:1. Experiments were performed altering the ratio of E+ and E- cells. This had no influence on non responsiveness after ABMT. Similarly, there was no effect by the addition

non responsiveness after ABMT. Similarly, there was no effect by the addition of T cells (E+) or B cells (E-) from cell samples frozen before ABMT, although it must be noted that these samples were obtained before vaccination and since none of the patients responded to the virus pre transplant, these presumably do not contain specific memory cells which are necesary for response.

In vitro Ig production with polyclonal activation and of specific anti influenza responses were detectable in most patients by 100 -120 days after ABMT, but these responses remained suboptimal and were not influenced by the addition of IL-2 or GM-CSF to the culture system. There was a particular delay in responses from patients with ALL in whom responses were absent in four out of five patients at six months after ABMT.

Chapter 11.

Discussion

Following allogeneic bone marrow transplantation there is a well documented period of immune suppression which is influenced by the presence of graft versus host disease. Following allogeneic bone marrow transplantation the regenerating immune system is derived from the donor and is thought to regenerate from the re-infused stem cell population. It has been postulated that the same pattern of regeneration would occur after autologous bone marrow transplantation, so that this setting would allow investigation of the re-capitulation of lymphocyte ontogeny without the effects of concomitent graft versus host disease.

Following autologous bone marrow transplantation, there are a number of analogous features to those found after allogeneic bone marrow transplantation. There was a rapid regeneration of NK cells, the earliest regenerating T cells expressed CD8 and there was slower regeneration of CD4, with a subsequent inversion of the normal CD4/CD8 ratio, all features which are seen after allogeneic bone marrow transplantation.

Although there is evidence of an increased TCR $\gamma\delta$ expression in some patients in the early regenerative period, there is little other evidence that the peripheral blood lymphocytes have an immature phenotype. The CD4 cells almost entirely co-express $CD45R_0$ and CD29 which are markers of cells

which have been previously activated and represent memory cells. No cells were found even in the earliest regenerating period which co-expressed CD4 and CD8. B cell regeneration is rapid after high dose chemotherapy as evidenced by the fact that CD20+ cells were among the earliest detected. Again there was no evidence that these cells had an immature phenotype, and no cells were found which were CD10+.

It may therefore be the case that the lymphocytes in the autologous setting are derived from an expansion of mature lymphocytes from the re-infused bone marrow. However, the fact that B cell regeneration may be more delayed after total body irradiation as evidenced by the slower regeneration of B cells in the small group of patients with ALL, may also be suggestive that there is a cellular fraction which is partly regenerated from cells which survived the high dose chemotherapy but did not survive the high dose irradiation therapy.

In vivo tests of T cell function showed a rapid recovery to normal despite continuing defects found in the peripheral blood phenotype and functional assays in vitro. Similarly, in vivo antibody production showed a remarkable resistance to the recent high dose therapy. This suggests that the antibody producing cells are derived from those re-infused with the cryopreserved marrow and are therefore not affected by the high dose chemotherapy. The fact that the patients who received TBI have a different response lends weight to the argument that antibody production is dependent on cells which are capable of functioning after very high dose therapy but not after radiotherapy. These cells may be not B cells, but either T cells or antigen presenting cells which

are necessary for in vivo cellular interactions with B cells. These interactions in vivo may not be apparent from investigation of peripheral blood lymphocytes.

In vitro tests of B cell function were markedly reduced after ABMT. These abnormalities were apparent at a time when the in vivo antibody responses were adequate. This was not due simply to a decreased number of B cells present since in vitro tests remained abnormally low at a time when the CD20+ cell numbers had returned to normal in those patients who had received chemotherapy alone and enrichment of the population with excess B cells did not increase the response. Similarly, the total number of T cells had no effect on antibody production post ABMT since altering the ratio of E+ to E- cells had no effect on the response. In the post ABMT patients it is possible that the lack of response is due to the presence of excess of T suppressor cells in the system since there is an excess of CD8+ cells in the patients during this period of non-responsiveness.

There are two possible explanations. Firstly, the peripheral blood lymphocytes may not be representative of the lymph node population which are responsible for in vivo antibody production, or the responses may be suppressed by the excess of suppressor function cells in vitro. This suppressor effect may perhaps be overcome in vivo by differences in cellular interactions which are possible within the lymph node microenviroment..The difference in response in patients who have received chemotherapy alone from the responses in the small number of patients who received TBI suggests that

these interactions would involve cells which have survived the chemotherapy but not the radiotherapy.

The patients with acute leukaemia were treated when in remission of disease. The patients with Hodgkin's disease all had active disease and one third had bulky disease. The differences in T cell function which occurred in the patients with Hodgkin's disease are largely explained by the immune suppressive effect of the disease

Defective cell mediated immunity with impaired responses to delayed hypersensitivity skin testing, impaired ability to reject allografts and depressed proliferative responses to T cell mitogens in vitro is a well recognized feature of patients with Hodgkin's disease. (Levy and Kaplan 1974). The reason for this remains unclear but intrinsic abnormalities of lymphocytes, the presence of immunosuppressive factors in serum or plasma and increased monocyte suppressor activity have all been implicated. Evidence for enhanced suppressor T cell activity in cancer patients has come from the demonstration of reduced lymphocyte proliferative responses to T cell mitogens. Severe impairment of lymphocyte function has been shown to correlate with poor outcome but this has been of limited clinical value. (Wedelin et al 1982)

In this study the immunological status of a total of fifty two patients with relapsed or refractory Hodgkin's disease was assessed prior to high dose chemotherapy and ABMT. Peripheral blood lymphocyte subset analysis of CD3,

CD4, CD8, CD14, CD20 and CD56 revealed no consistent abnormality although the CD4/CD8 ratio was reduced at 1.5 (± 0.46). The T cell proliferative response to mitogens was reduced compared to normal controls and to patients with acute leukaemia and non Hodgkin's lymphoma prior to ABMT. The stimulation index was 13.7 ± 4.2 in the patients with Hodgkin's disease compared to 30 ± 5 in the normal controls and similar data was obtained with Con A, anti CD3 and a combination of anti CD2 monoclonal; antibodies. T cell responses in vivo as measured by the skin response to recall antigens were measured in 32 individuals using a commercially available applicator and was found to be markedly suppressed - 8.5 ± 6.5 compared to 34 ± 3.9 in normal controls.

Following ABMT there was a return to normal CD8, CD20 and CD56 lymphocyte numbers within three months but complete recovery of CD4 lymphocytes took up to one year. Proliferative responses to T cell mitogens recovered progressively to normality at one year in those patients who achieved remission after ABMT (Stimulation index with PHA 28.2 ± 5.7), whereas in those patients with failing to achieve a CR the response remained low (Stimulation index with PHA 10.5 ± 7). The skin responses to recall antigens returned to normal levels by two to three months after ABMT in those patients who achieved remission whereas responses remained weak in those patients with persistent disease. This rapid in vivo recovery of T cell function was mirrored clinically by a lack of late infectious complications in this group of patients.

These studies have shown that there are consistent abnormalities of immune phenotype and in vitro function following autologous bone marrow transplantation. These studies have failed to show the precise nature of this defect, although there are suggestions that these effects may be caused by suppression of normal effector cells by the excess supprressor function cells which are present after ABMT. These putative suppressor effects are currently under investigation.

The in vitro antibody response to influenza virus has been shown to be T cell dependent (Callard and Smith 1981). Irradiation of the T cells has revealed a differential sensitivity of helper and suppressor cells. One possible way to examine the suppressive function of these cells is to use the differential effect of irradiation on these functional assays. The effect of using differential dosages of irradiation on the T cell fraction on the response to influenza vaccine is currently under investigation. It has further been shown that there is an HLA compatibility requirement for T cell help in this system as the combination of E+ and E - fractions with HLA non compatablilty resulted in no antibody formation. This nonresponsiveness was shown also to be mediated by suppressor activity which could be eradicated by the use of low dose irradiation. This failure does not appear to be due to genetic restriction since DR identical combinations also fail to respond (Callard and Smith 1981). This may be due to allo-activation of suppressor cells which suppress the specific antibody responses to influenza virus, but not the polyclonal production of antibody by poke weed mitogen. Irradiation of cells to 10 Gy completely abrogates the suppressor capacity of allogeneic T cells. Irradiation of

autologous T cells may enhance antibody formation whereas non irradiated cells tend to slightly inhibit. This may be explained by the existence of radiosensitive autologous suppressor T cells. Following irradiation there is an apparent loss of restriction between T and B cells in antibody response to influenza. This may be complicated by the fact that influenza virus may fuse with cell membranes and overcome the need for normal antigen processing. It is possible that by utilizing this radiation sensitivity of suppressor cell functions that remixing experiments could be performed using cell fractions from normal donors and patients after ABMT to more definitively establish the cause of the nonresponsiveness in these patients. These experiments are currently being performed but have proven to be technically difficult.

These studies have, however, shown that humoral immune responses remain intact in patients receiving high dose therapy and that this may have implications for the use of recombinant proteins which may be recognized as foreign to the body. Furthermore, the defect in cell mediated immunity seen in all patients persisted for only three months after ABMT and rapidly thereafter returned to normal ABMT. The defect in cell mediated immunity associated with active Hodgkin's disease rapidly corrected when remission was obtained even in heavily pre-treated patients. This early recovery of in vivo T cell function correlated with the low incidence of late opportunistic infections with ABMT and indicates that the persistent abnormalities seen in vitro with peripheral blood lymphocytes may have little physiological relevance.

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AML regeneration data

	PRE							3 - 4 weeks						
	CD 3	CD 4	CD 8	CD4/8 ratio	CD20	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
	72	49	32	1.53	19	9	15	25	7	61	0.11	11	13	7
	62	53	26	2.04	17	10	17	19	8	59	0.14	18	13	9
	69	36	23	1.57	21	14	19	18	9	73	0.12	25	15	11
	66	44	22	2.00	22	11	9	19	13	66	0.20	17	19	9
	68	45	25	1.80	21	11	18	16	8	61	0.13	25	10	14
	76	42	27	1.56	17	9	11	21	16	64	0.25	19	13	7
	64	39	24	1.63	21	8	10	22	15	66	0.23	14	14	9
	62	49	27	1.81	25	12	11	26	17	54	0.31	15	18	10
	77	47	31	1.52	27	14	14	34	13	53	0.25	11	12	9
	69	42	33	1.27	19	13	17	24	10	67	0.15	18	12	8
	59	38	31	1.23	11	8	14	25	7	63	0.11	14	17	9
	67	41	26	1.58	24	9	9	29	16	61	0.26	25	9	12
	74	42	25	1.68	25	13	11	24	7	58	0.12	18	10	11
	72	51	24	2.13	17	8	9	39	13	58	0.22	11	16	7
	75	47	23	2.04	23	9	8	19	7	61	0.11	17	17	9
Mean	68.8	44.3	26.6	1.69	20.6	10.5	12.8	24	11.1	61.7	0.18	17.2	13.9	9.4
SD	5.53	4.95	3.54	0.27	4.1	2.2	3.71	6.23	3.79	5.15	0.07	4.84	3.07	1.96

AML regeneration data

	Pre part 2							3 - 4 weeks						
	CD 3	CD 4	CD 8	CD4/8 ratio	CD20	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
	42	21	36	0.58	15	14	8	21	11	64	0.17	11	16	8
	29	19	39	0.49	25	13	11	18	7	63	0.11	17	18	10
	25	15	37	0.41	17	14	9	16	9	73	0.12	24	19	14
	31	24	31	0.77	17	16	7	16	12	68	0.18	19	19	9
	31	26	33	0.79	25	8	12	15	8	65	0.12	25	10	14
	25	19	38	0.50	19	13	10	21	14	64	0.22	19	15	7
	23	21	45	0.47	16	19	13	22	15	66	0.23	14	9	9
	29	17	44	0.39	18	17	9	26	14	59	0.24	15	18	10
	47	25	44	0.57	21	11	6	29	13	61	0.21	11	12	9
	38	25	39	0.64	15	13	15	27	16	67	0.24	18	12	8
	37	24	43	0.56	14	9	12	25	7	63	0.11	14	17	9
	31	15	33	0.45	25	8	8	22	11	61	0.18	25	9	12
	41	17	37	0.46	18	9	8	24	7	63	0.11	18	10	11
	46	21	34	0.62	11	7	9	36	13	58	0.22	11	16	7
	23	17	37	0.46	17	12	11	19	7	61	0.11	17	17	9
Mean	33.2	20.4	38	0.54	18.2	12.2	9.87	22.5	10.9	63.7	0.17	17.2	14.5	9.73
SD	8.12	3.76	4.39	0.12	4.2	3.55	2.45	5.63	3.22	3.79	0.05	4.75	3.74	2.19

AML regeneration data

	8 weeks							12 - 16 weeks						
	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
	40	19	37	0.51	25	16	10	50	25	31	0.81	19	9	12
	38	14	51	0.27	19	14	10	43	26	33	0.79	16	14	10
	44	16	47	0.34	22	9	15	44	25	41	0.61	15	15	11
	47	18	44	0.41	21	10	13	48	27	39	0.69	19	15	9
	42	22	42	0.52	25	8	14	41	27	35	0.77	19	12	15
	38	25	39	0.64	24	11	14	36	21	37	0.57	18	17	12
	46	17	44	0.39	15	9	9	39	21	39	0.54	17	15	16
	32	19	43	0.44	19	13	18	38	26	32	0.81	23	17	9
	38	21	40	0.53	18	8	9	41	19	31	0.61	19	15	13
	42	19	40	0.48	24	15	13	42	23	46	0.50	16	12	11
	37	16	48	0.33	19	17	11	37	29	26	1.12	15	11	8
	40	18	43	0.42	19	13	12	38	22	38	0.58	21	11	6
	40	23	39	0.59	29	18	11	42	21	41	0.51	15	13	17
	39	23	42	0.55	25	16	11	41	24	37	0.65	16	9	11
	42	15	42	0.36	16	13	17	38	19	37	0.51	18	9	14
Mean	40.3	19	42.7	0.45	21.3	12.7	12.5	41.2	23.7	36.2	0.67	17.7	12.9	11.6
SD	3.75	3.23	3.73	0.1	3.92	3.35	2.75	3.93	3.09	5	0.17	2.34	2.76	3.04

AML regeneration data

	24 - 30 weeks							36 - 42 weeks						
	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14	CD3	CD 4	CD 8	CD4/8 ratio	CD20	CD 56	CD 14
	46	38	37	1.03	20	12	11	59	35	38	0.92	17	10	21
	41	29	31	0.94	21	10	8	52	38	42	0.90	18	13	14
	48	25	26	0.96	16	9	9	59	38	51	0.75	23	15	11
	46	29	29	1.00	15	14	9	58	35	46	0.76	16	12	14
	48	24	39	0.62	26	12	12	54	33	44	0.75	16	10	17
	47	27	37	0.73	19	13	7	52	39	38	1.03	19	11	25
	51	27	26	1.04	21	12	15	62	46	39	1.18	21	12	15
	44	24	29	0.83	12	15	11	54	34	29	1.17	24	9	16
	47	31	34	0.91	15	12	16	57	37	34	1.09	23	13	13
	53	31	47	0.66	21	9	14	51	34	30	1.13	17	15	18
	41	27	39	0.69	26	13	8	70	51	37	1.38	13	12	13
	51	22	26	0.85	30	10	12	59	36	38	0.95	26	13	30
	52	21	32	0.66	23	7	17	59	35	31	1.13	13	11	32
	41	31	34	0.91	17	13	12	51	31	40	0.78	23	13	13
	44	31	36	0.86	15	14	13	54	38	36	1.06	17	10	8
Mean	46.7	27.8	33.5	0.84	19.8	11.7	11.6	56.7	37.3	38.2	1	19.1	11.9	17.3
SD	3.98	4.36	5.93	0.14	4.99	2.23	3.04	5.06	5.11	6	0.19	4.04	1.79	6.87

AML regeneration data

52 weeks

	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
	56	40	36	1.11	12	16	11
	54	44	28	1.57	18	18	14
	69	46	28	1.64	14	10	8
	55	41	25	1.64	27	13	12
	58	46	24	1.92	21	14	17
	60	48	22	2.18	19	12	13
	69	47	24	1.96	19	13	12
	63	44	22	2.00	17	8	17
	61	49	21	2.33	26	11	25
	54	37	22	1.68	29	12	11
	62	48	27	1.78	17	12	16
	59	39	27	1.44	23	13	13
	69	44	28	1.57	18	15	18
	57	46	23	2.00	12	12	13
	63	46	31	1.48	19	13	30
Mean	60.6	44.3	25.9	1.75	19.4	12.8	15.3
SD	5.26	3.58	4.05	0.31	5.1	2.4	5.69

ALL regeneration data

Time			Pre						3 weeks						
	CD3	CD4	CD8	CD4/8	CD20	CD56	CD14		CD3	CD4	CD8	CD4/8	CD20	CD56	CD14
	64	39	24	1.63	21	8	10		22	14	66	0.21	11	14	9
	62	49	27	1.81	25	12	11		26	15	54	0.28	10	18	10
	77	47	31	1.52	27	14	14		34	13	63	0.21	11	12	9
	69	48	31	1.55	19	13	17		24	10	67	0.15	14	12	8
	59	44	31	1.42	11	8	14		25	7	63	0.11	14	17	9
	67	41	26	1.58	24	9	9		29	14	61	0.23	15	9	12
Mean	**66.3**	**44.7**	**28.3**	**1.58**	**21.2**	**10.7**	**12.5**		**26.7**	**12.2**	**62.3**	**0.2**	**12.5**	**13.7**	**9.5**
SD	**6.31**	**4.03**	**3.08**	**0.13**	**5.74**	**2.66**	**3.02**		**4.27**	**3.06**	**4.63**	**0.06**	**2.07**	**3.39**	**1.38**

ALL regeneration data

	4 weeks							6 weeks						
	CD3	CD4	CD8	CD4/8	CD20	CD56	CD14	CD3	CD4	CD8	CD4/8	CD20	CD56	CD14
	23	19	48	0.40	16	19	13	22	20	66	0.30	14	9	9
	29	16	50	0.32	18	17	9	26	14	59	0.24	15	18	10
	47	15	61	0.25	16	11	6	29	18	61	0.30	11	12	9
	38	15	52	0.29	15	13	15	27	16	67	0.24	18	12	8
	37	17	62	0.27	14	9	12	25	19	63	0.30	14	17	9
	31	15	39	0.38	15	8	8	22	16	61	0.26	17	9	12
Mean	**34.2**	**16.2**	**52**	**0.32**	**15.7**	**12.8**	**10.5**	**25.2**	**17.2**	**62.8**	**0.27**	**14.8**	**12.8**	**9.5**
SD	**8.35**	**1.6**	**8.6**	**0.06**	**1.37**	**4.4**	**3.39**	**2.79**	**2.23**	**3.13**	**0.03**	**2.48**	**3.87**	**1.38**

ALL regeneration data

	8 weeks							12 weeks						
	CD3	CD4	CD8	CD4/8	CD20	CD56	CD14	CD3	CD4	CD8	CD4/8	CD20	CD56	CD14
	46	17	44	0.39	15	9	9	39	21	39	0.54	17	15	16
	32	19	43	0.44	19	13	18	38	22	32	0.69	23	17	9
	38	18	42	0.43	18	8	9	41	19	31	0.61	19	15	13
	42	17	44	0.39	24	15	13	42	23	46	0.50	16	12	11
	37	16	48	0.33	19	17	11	37	22	26	0.85	15	11	8
	40	18	43	0.42	19	13	12	38	22	38	0.58	21	11	6
Mean	**39.2**	**17.5**	**44**	**0.4**	**19**	**12.5**	**12**	**39.2**	**21.5**	**35.3**	**0.63**	**18.5**	**13.5**	**10.5**
SD	**4.75**	**1.05**	**2.1**	**0.04**	**2.9**	**3.45**	**3.35**	**1.94**	**1.38**	**7.09**	**0.13**	**3.08**	**2.51**	**3.62**

ALL regeneration data

	18 weeks							36 weeks						
	CD3	CD4	CD8	CD4/8	CD20	CD56	CD14	CD3	CD4	CD8	CD4/8	CD20	CD56	CD14
	51	22	31	0.71	21	12	15	62	41	39	1.05	21	12	15
	44	21	40	0.53	12	15	11	54	28	29	0.97	24	9	16
	47	27	34	0.79	15	12	16	57	37	34	1.09	23	13	13
	53	31	47	0.66	21	9	14	51	34	35	0.97	17	15	18
	41	24	39	0.62	26	13	8	54	37	40	0.93	13	12	13
	51	22	29	0.76	19	10	12	59	36	38	0.95	26	13	30
Mean	**47.8**	**24.5**	**36.7**	**0.68**	**19**	**11.8**	**12.7**	**56.2**	**35.5**	**35.8**	**0.99**	**20.7**	**12.3**	**17.5**
SD	**4.67**	**3.83**	**6.65**	**0.1**	**4.94**	**2.14**	**2.94**	**3.97**	**4.32**	**4.07**	**0.06**	**4.84**	**1.97**	**6.41**

ALL regeneration data

52 weeks

	CD3	CD4	CD8	CD4/8	CD20	CD56	CD14
	69	47	24	1.96	19	13	12
	63	44	22	2.00	17	8	17
	61	49	21	2.33	26	11	25
	54	37	22	1.68	29	12	11
	62	48	27	1.78	17	12	16
	59	39	27	1.44	23	13	13
Mean	**61.3**	**44**	**23.8**	**1.87**	**21.8**	**11.5**	**15.7**
SD	**4.93**	**4.98**	**2.64**	**0.3**	**5**	**1.87**	**5.13**

NHL regeneration data

Time	PRE							3 - 4 weeks						
	CD 3	CD 4	CD 8	CD4/8	CD20 ratio	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8	CD 20 ratio	CD 56	CD 14
	63	42	21	2.00	21	8	10	17	5	66	0.08	16	14	9
	62	45	27	1.67	25	10	11	26	7	59	0.12	18	17	10
	77	49	31	1.58	27	14	14	34	13	53	0.25	11	11	8
	69	37	33	1.12	19	10	17	24	9	67	0.13	15	13	8
	67	41	29	1.41	11	8	14	33	4	66	0.06	14	19	9
	46	33	26	1.27	24	9	9	29	12	61	0.20	25	9	11
	54	42	25	1.68	25	13	11	24	7	59	0.12	18	10	11
	54	39	21	1.86	17	8	9	37	13	58	0.22	11	16	7
	57	37	23	1.61	23	9	8	19	7	61	0.11	17	17	9
	63	43	27	1.59	17	7	7	24	12	63	0.19	22	15	7
	59	40	22	1.82	14	12	12	27	7	63	0.11	14	11	9
	73	49	30	1.63	24	10	9	22	14	63	0.22	17	13	9
	61	42	29	1.45	17	12	11	31	6	57	0.11	15	8	11
	56	41	29	1.41	26	11	26	29	17	69	0.25	15	9	9
	67	42	29	1.45	21	7	19	26	11	54	0.20	22	17	12
	63	48	24	2.00	19	14	15	20	8	73	0.11	21	15	8
	74	56	19	2.95	24	11	8	36	12	78	0.15	16	16	6
Mean	**62.6**	**42.7**	**26.2**	**1.68**	**20.8**	**10.2**	**12.4**	**26.9**	**9.65**	**62.9**	**0.15**	**16.9**	**13.5**	**9**
SD	**8.06**	**5.46**	**4**	**0.4**	**4.53**	**2.27**	**4.85**	**5.9**	**3.64**	**6.5**	**0.06**	**3.84**	**3.36**	**1.62**

NHL regeneration data

	6 weeks							8 weeks						
	CD 3	CD 4	CD 8	CD4/8	CD20 ratio	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8	CD 20 ratio	CD 56	CD14
	23	25	45	0.56	16	19	13	42	27	46	0.59	15	9	9
	29	18	44	0.41	18	17	9	29	17	43	0.40	27	13	18
	43	25	44	0.57	21	11	6	33	24	40	0.60	28	11	15
	31	22	39	0.56	15	13	15	40	19	42	0.45	28	15	13
	34	21	43	0.49	14	9	12	31	18	48	0.38	19	17	11
	29	15	39	0.38	25	8	8	33	17	43	0.40	19	13	12
	41	17	37	0.46	18	9	8	40	19	39	0.49	29	18	12
	46	21	41	0.51	11	7	9	39	23	34	0.68	25	16	11
	23	17	37	0.46	17	12	11	31	15	42	0.36	16	13	17
	31	21	39	0.54	22	10	10	41	16	37	0.43	28	14	8
	29	17	41	0.41	14	12	11	37	21	42	0.50	27	13	15
	34	21	48	0.44	7	11	7	44	21	43	0.49	24	14	18
	24	22	33	0.67	15	7	5	34	15	42	0.36	17	8	13
	34	19	39	0.49	15	14	9	37	21	48	0.44	26	11	8
	31	9	49	0.18	22	11	8	41	16	53	0.30	19	12	17
	37	14	31	0.45	21	13	9							
Mean	**32.4**	**19**	**40.6**	**0.47**	**16.9**	**11.4**	**9.38**	**36.8**	**19.3**	**42.8**	**0.46**	**23.1**	**13.1**	**13.1**
SD	**6.77**	**4.16**	**4.9**	**0.11**	**4.58**	**3.33**	**2.58**	**4.66**	**3.53**	**4.66**	**0.1**	**5.03**	**2.75**	**3.44**

NHL regeneration data

12 - 16 weeks							24 - 30 weeks						
CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
39	27	49	0.55	24	15	6	51	27	26	1.04	21	12	15
31	26	42	0.62	23	12	9	56	28	29	0.97	12	15	11
49	28	31	0.90	19	15	13	48	31	34	0.91	15	12	16
42	24	46	0.52	17	12	11	35	26	47	0.55	21	9	14
37	29	39	0.74	20	11	8	41	30	39	0.77	26	13	8
39	18	43	0.42	21	11	6	34	22	26	0.85	30	10	12
35	21	41	0.51	15	13	17	37	31	32	0.97	23	7	17
41	24	37	0.65	16	9	11	41	38	34	1.12	17	13	12
38	29	37	0.78	18	9	14	43	29	33	0.88	15	14	13
48	31	39	0.79	22	11	12	31	25	37	0.68	26	11	8
33	17	52	0.33	21	11	12	34	21	34	0.62	14	14	21
48	27	37	0.73	20	13	22	34	27	46	0.59	11	14	16
37	23	43	0.53	15	12	13	38	24	41	0.59	17	9	16
35	26	49	0.53	26	11	11	37	27	37	0.73	30	13	18
41	28	39	0.72	18	12	17							
Mean	**39.5**	**25.2**	**41.6**	**0.62**	**19.7**	**11.8**	**12.1**	**40**	**27.6**	**35.4**	**0.8**	**19.9**	**11.9**	**14.1**
SD	**5.45**	**4.06**	**5.59**	**0.16**	**3.27**	**1.74**	**4.27**	**7.27**	**4.27**	**6.43**	**0.18**	**6.4**	**2.35**	**3.69**

NHL regeneration data

	36 - 42 weeks							52 weeks						
	CD3	CD 4	CD 8	CD4/8 ratio	CD20	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
	57	46	31	1.48	21	12	11	74	58	25	2.32	19	13	12
	54	34	29	1.17	24	12	16	63	46	24	1.92	17	8	17
	57	38	34	1.12	23	13	13	61	44	21	2.10	26	11	25
	53	40	25	1.60	17	15	18	54	42	22	1.91	29	12	11
	70	51	37	1.38	13	12	13	62	51	31	1.65	17	12	16
	66	47	38	1.24	26	13	30	59	29	17	1.71	23	13	13
	59	29	31	0.94	13	11	32	69	50	31	1.61	18	15	18
	51	39	40	0.98	23	13	13	57	38	24	1.58	12	12	13
	57	40	37	1.08	17	10	8	63	46	31	1.48	19	13	30
	58	38	36	1.06	21	11	21	49	31	31	1.00	23	11	32
	56	37	31	1.19	22	14	26	53	49	24	2.04	18	13	13
	49	36	46	0.78	17	11	18	57	47	16	2.94	20	10	8
	49	31	34	0.91	18	13	28							
Mean	**56.6**	**38.9**	**34.5**	**1.15**	**19.6**	**12.3**	**19**	**60.1**	**44.3**	**24.8**	**1.85**	**20.1**	**11.9**	**17.3**
SD	**6.08**	**6.21**	**5.38**	**0.23**	**4.11**	**1.38**	**7.79**	**6.91**	**8.29**	**5.36**	**0.48**	**4.54**	**1.78**	**7.68**

HD regeneration data

	PRE							3 - 4 weeks						
	CD 3	CD 4	CD 8	CD4/8 ratio	CD20	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
	67	45	36	1.25	25	13	15	23	8	57	0.14	15	14	6
	62	35	29	1.21	19	12	17	17	7	61	0.11	25	13	14
	54	30	29	1.03	23	17	19	19	5	66	0.08	17	14	11
	69	41	27	1.52	22	16	9	24	12	77	0.16	17	16	9
	74	45	30	1.50	18	11	18	19	6	74	0.08	25	18	11
	67	41	29	1.41	22	9	11	21	14	64	0.22	19	13	7
	53	37	21	1.76	21	8	10	17	5	66	0.08	16	14	9
	62	45	27	1.67	25	12	11	26	7	59	0.12	18	17	10
	77	49	31	1.58	27	14	14	34	13	53	0.25	11	11	8
	69	37	33	1.12	19	10	17	24	9	67	0.13	15	13	8
	47	20	29	0.69	11	8	14	33	4	66	0.06	14	19	9
	46	31	26	1.19	24	9	9	29	16	61	0.26	25	9	11
	54	42	25	1.68	25	13	11	24	7	59	0.12	18	10	11
	54	39	21	1.86	17	8	9	37	13	58	0.22	11	16	7
	57	37	23	1.61	23	9	8	19	7	61	0.11	17	17	9
	63	43	27	1.59	17	7	7	24	12	63	0.19	22	15	7
	59	40	22	1.82	14	12	12	27	7	63	0.11	14	11	9
	73	46	30	1.53	24	10	9	22	14	63	0.22	17	13	9
	61	39	29	1.34	17	12	11	31	6	57	0.11	15	8	11
	56	41	29	1.41	26	11	26	29	17	69	0.25	15	9	9
	67	42	29	1.45	21	7	19	26	11	54	0.20	22	17	12
	63	48	24	2.00	19	14	15	20	8	73	0.11	21	15	8
	74	56	19	2.95	24	11	8	36	12	78	0.15	16	16	6
	51	38	26	1.46	24	13	25	31	19	58	0.33	14	11	8
	57	37	36	1.03	14	8	17	18	9	74	0.12	21	14	8
	46	30	39	0.77	25	7	11	25	7	56	0.13	15	9	14
	45	29	44	0.66	21	8	11	28	14	61	0.23	18	7	11
	47	37	30	1.23	19	9	12	24	5	73	0.07	19	16	7
	72	51	21	2.43	21	11	14	17	8	61	0.13	12	18	9
	62	41	19	2.16	23	13	7	23	11	61	0.18	21	16	7
	69	46	27	1.70	19	14	14	19	9	67	0.13	17	15	8
	56	39	24	1.63	21	11	7	26	12	61	0.20	16	14	9
	43	27	29	0.93	18	12	14	24	7	64	0.11	13	11	12
	49	39	27	1.44	19	13	20	37	11	65	0.17	12	8	11
	57	38	26	1.46	21	14	14	39	17	61	0.28	17	12	7
	59	41	24	1.71	19	9	18	27	14	63	0.22	16	10	9
	63	40	27	1.48	18	19	9	31	17	61	0.28	19	16	10
	68	43	22	1.95	21	27	12							
	74	55	29	1.90	23	11	10							
	79	52	24	2.17	22	13	11							
	47	28	41	0.68	17	8	14							
	48	29	28	1.04	19	11	19							
	61	39	27	1.44	17	12	12							
Mean	60	40	28	1.5	20.6	11.5	13.3	26	10	64	0.16	17.2	13.4	9.22
SD	9.8	7.5	5.4	0.46	3.48	3.65	4.55	6.1	4	6.2	0.07	3.7	3.2	2

HD regeneration data

6 weeks							8 weeks						
CD 3	CD 4	CD 8	CD4/8 ratio	CD20	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD14
39	14	39	0.36	15	14	8	39	17	37	0.46	26	11	12
19	19	39	0.49	25	13	11	31	12	51	0.24	17	13	11
24	11	37	0.30	17	14	9	42	14	47	0.30	25	8	15
33	12	31	0.39	17	16	7	42	15	44	0.34	28	9	13
31	19	30	0.63	25	8	12	39	19	42	0.45	25	8	16
24	19	33	0.58	19	13	10	37	25	39	0.64	35	11	14
23	25	45	0.56	16	19	13	42	17	44	0.39	15	9	9
29	16	44	0.36	18	17	9	29	17	43	0.40	27	13	18
43	25	44	0.57	21	11	6	33	21	40	0.53	28	11	15
31	25	39	0.64	15	13	15	40	19	40	0.48	28	15	13
34	23	43	0.53	14	9	12	31	15	48	0.31	19	17	11
29	15	33	0.45	25	8	8	33	17	43	0.40	19	13	12
41	17	37	0.46	18	9	8	40	19	39	0.49	29	18	12
46	21	32	0.66	11	7	9	39	13	34	0.38	25	16	11
23	17	37	0.46	17	12	11	31	15	42	0.36	16	13	17
31	21	39	0.54	22	10	10	41	16	37	0.43	28	14	8
29	17	41	0.41	14	12	11	37	21	42	0.50	27	13	15
34	21	48	0.44	7	11	7	44	21	43	0.49	24	14	18
24	22	33	0.67	15	7	5	34	15	42	0.36	17	8	13
34	19	39	0.49	15	14	9	37	21	48	0.44	26	11	8
31	9	49	0.18	22	11	8	41	16	53	0.30	19	12	17
37	14	31	0.45	21	13	9	36	15	58	0.26	25	10	12
19	13	45	0.29	16	8	17	42	17	54	0.31	16	11	15
28	20	44	0.45	24	17	10	38	20	51	0.39	28	13	17
41	21	47	0.45	21	9	12	34	19	43	0.44	17	9	11
40	22	39	0.56	15	8	10	43	18	39	0.46	26	12	13
24	19	43	0.44	18	11	8	30	17	39	0.44	19	14	18
27	14	33	0.42	19	13	8	34	19	36	0.53	24	13	9
20	13	32	0.41	12	14	14	37	19	41	0.46	16	13	16
31	20	38	0.53	21	11	11	31	22	45	0.49	16	11	14
28	15	36	0.42	17	12	12	30	23	38	0.61	13	10	10
26	22	35	0.63	16	13	14	42	21	39	0.54	25	11	13
24	16	41	0.39	13	12	12	37	19	41	0.46	26	12	12
34	27	31	0.87	12	15	11	35	24	47	0.51	28	10	14
45	16	38	0.42	17	13	9	43	21	40	0.53	24	11	8
29	19	45	0.42	21	14	9	29	19	43	0.44	19	8	13
38	25	36	0.69	17	12	8							
Mean	31	18	39	0.49	17.5	12	10.1	37	18	43	0.43	22.9	11.8	13.1
SD	7.2	4.4	5.3	0.13	4.13	2.89	2.55	4.6	3	5.4	0.09	5.26	2.48	2.91

HD regeneration data

12 - 16 weeks							24 - 30 weeks						
CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
34	27	34	0.79	25	14	8	43	27	37	0.73	20	12	11
37	16	39	0.41	15	13	10	32	24	31	0.77	21	10	8
40	19	41	0.46	16	17	11	40	25	26	0.96	16	9	9
43	24	39	0.62	18	14	9	39	29	29	1.00	15	14	9
41	25	38	0.66	19	13	5	38	27	39	0.69	26	12	12
36	19	43	0.44	18	17	8	35	21	37	0.57	19	13	7
39	21	49	0.43	24	15	6	51	27	26	1.04	21	12	15
31	26	42	0.62	23	12	9	34	24	29	0.83	12	15	11
29	19	31	0.61	19	15	13	48	31	34	0.91	15	12	16
42	18	46	0.39	17	12	11	35	22	47	0.47	21	9	14
37	29	39	0.74	20	11	8	41	21	39	0.54	26	13	8
39	18	43	0.42	21	11	6	34	22	26	0.85	30	10	12
35	21	41	0.51	15	13	17	37	31	32	0.97	23	7	17
41	24	37	0.65	16	9	11	41	38	34	1.12	17	13	12
38	19	37	0.51	18	9	14	43	29	33	0.88	15	14	13
41	19	39	0.49	22	11	12	31	17	37	0.46	26	11	8
33	17	41	0.41	21	11	12	34	21	34	0.62	14	14	21
48	18	37	0.49	20	13	22	34	27	46	0.59	11	14	16
37	18	43	0.42	15	12	13	38	24	41	0.59	17	9	16
35	26	49	0.53	26	11	11	37	27	37	0.73	30	13	18
41	17	39	0.44	18	12	17	42	26	31	0.84	20	13	14
30	13	37	0.35	21	14	6	43	21	30	0.70	17	12	9
35	16	37	0.43	22	14	9	35	31	46	0.67	23	13	16
43	22	39	0.56	17	14	8	36	32	37	0.86	13	12	8
32	19	56	0.34	25	14	10	42	29	36	0.81	21	14	12
47	17	34	0.50	21	12	10	38	32	37	0.86	25	12	11
41	19	39	0.49	15	13	12	43	28	44	0.64	15	12	12
37	16	37	0.43	16	11	13	41	32	31	1.03	21	8	11
39	22	42	0.52	13	13	6	59	26	29	0.90	19	9	9
36	19	41	0.46	18	13	5	54	29	37	0.78	22	11	9
42	21	47	0.45	19	9	14	47	27	37	0.73	26	12	14
58	17	37	0.46	16	15	17	42	30	53	0.57	17	9	8
36	19	44	0.43	15	14	12	51	29	44	0.66	15	15	21
40	22	42	0.52	16	15	13	37	26	38	0.68	20	9	9
							49	30	38	0.79	15	13	13
Mean	39	20	41	0.5	18.8	12.8	10.8	41	27	36	0.77	19.5	11.7	12.3
SD	5.56	3.62	4.86	0.10	3.35	1.99	3.85	6.5	4.2	6.4	0.17	4.92	2.08	3.72

HD regeneration data

36 - 42 weeks							52 weeks						
CD3	CD 4	CD 8	CD4/8 ratio	CD20	CD 56	CD 14	CD 3	CD 4	CD 8	CD4/8 ratio	CD 20	CD 56	CD 14
57	33	39	0.85	24	12	14	51	37	39	0.95	15	15	9
50	36	44	0.82	28	14	23	60	41	33	1.24	23	8	16
59	32	51	0.63	23	14	9	55	34	40	0.85	12	9	8
56	21	46	0.46	12	13	12	54	31	34	0.91	27	12	12
45	29	44	0.66	20	8	17	57	44	31	1.42	21	14	17
41	41	29	1.41	20	11	25	49	40	27	1.48	19	12	9
57	46	31	1.48	21	12	11	74	56	25	2.24	19	13	12
54	34	29	1.17	24	12	16	63	46	24	1.92	17	8	17
57	27	34	0.79	23	13	13	61	44	21	2.10	26	11	25
53	40	25	1.60	17	15	18	54	37	22	1.68	29	12	11
70	51	37	1.38	13	12	13	62	51	31	1.65	17	12	16
66	47	38	1.24	26	13	30	59	29	17	1.71	23	13	13
59	29	31	0.94	13	11	32	69	50	38	1.32	18	15	18
51	29	40	0.73	23	13	13	57	34	29	1.17	12	12	13
57	40	37	1.08	17	10	8	63	46	31	1.48	19	13	30
49	29	36	0.81	21	11	21	49	31	31	1.00	23	11	32
56	37	31	1.19	22	14	26	53	49	24	2.04	18	13	13
49	26	36	0.72	17	11	18	57	47	16	2.94	20	10	8
49	31	34	0.91	18	13	28	56	39	24	1.63	22	11	21
64	44	29	1.52	16	12	24	69	49	28	1.75	17	14	26
71	51	27	1.89	19	14	9	57	41	34	1.21	23	11	18
47	25	35	0.71	20	8	16	61	43	27	1.59	26	13	28
51	26	38	0.68	23	11	8	54	37	30	1.23	23	12	24
39	25	34	0.74	19	14	12	57	34	34	1.00	21	14	9
40	26	37	0.70	21	12	19	61	46	33	1.39	20	8	16
52	38	29	1.31	19	13	18	48	36	22	1.64	21	11	8
61	41	37	1.11	14	11	12	54	35	34	1.03	20	14	12
39	31	34	0.91	17	15	14	58	35	39	0.90	27	10	19
49	29	32	0.91	20	11	15	63	29	33	0.88	30	13	18
54	37	29	1.28	23	8	14	57	31	28	1.11	24	11	12
53	33	30	1.10	17	12	9	63	43	37	1.16	18	15	14
							49	46	21	2.19	23	13	15
							67	45	27	1.67	19	8	14
							65	38	26	1.46	17	10	9
							53	42	22	1.91	20	14	12
ean	53	34	35	1.02	19.7	12	16.7	58	40	29	1.48	20.8	11.9	###
D	8.2	8	5.9	0.34	3.86	1.85	6.61	6.2	6.9	6.3	0.47	4.23	2.06	6.46

AML - DOUBLE

	PHA	UCHT1	Con A
Pre	31	11	13.5
Pre	34	15	13.4
Pre	33	13.8	13.8
Pre	28	14.3	14.2
Pre	29	11.9	12.9
Pre	31	12.5	13.9
Pre	27	9.8	11.7
Pre	26	12.7	11.5
Pre	32	17.6	11.9
Pre	31.5	16.4	12.1
Pre	25	16.9	13.2
Pre	26	18.7	14.1
Pre	25	11	11.1
Pre	31.7	14.5	10.9
Mean	29.3	14	12.7
SD	3.1	2.7	1.16

	PHA	UCHT1	Con A
1	2.1	1.1	1.2
1	1.9	1.3	1.4
1	3.5	1.3	1.3
1	3.2	1	1
1	1.9	1	1
1	5.9	1.2	1.3
1	5.2	3.2	1.2
1	3.8	4.5	1.9
1	1	1.5	1.3
1	1	1.4	1.4
1	1	1.3	1.1
1	1	1.1	0.9
1	1	1	1.2
1	1	1.9	1.4
1	2.39	1.63	1.26
0	1.67	1	0.24

	PHA	UCHT1	Con A
Pre part II	3.9	2.1	1.3
Pre part II	4.3	2.9	1
Pre part II	3.5	3.1	1
Pre part II	3.2	3.5	1.2
Pre part II	4.6	5.6	3.2
Pre part II	5.9	1.2	4.5
Pre part II	6.5	3.2	1.5
Pre part II	7.8	4.5	1.4
Pre part II	1	1.5	1.3
Pre part II	3.8	1.4	1.15
Pre part II	5.4	4.2	1.9
Pre part II	1.9	4.7	1.9
Pre part II	4.6	3.7	2.1
Pre part II	3.8	1.9	4.1
	4.3	3.11	1.97
	1.76	1.36	1.15

AML - DOUBLE

	PHA	UCHT1	Con A
1	2.1	1.1	3.1
1	1.9	1.3	2.8
1	3.5	1	1.9
1	3.2	1	4
1	1.9	1.2	3.2
1	4.3	3.2	4.5
1	6.5	1.9	1.5
1	3.2	1.4	1.4
1	2.1	2.4	1.3
1	1.2	4.7	1.15
1	1.3	3.76	3.3
Mean	2.84	2.09	2.56
SD	1.55	1.27	1.17

	PHA	UCHT1	Con A
1.5	7.4	1.9	2.1
1.5	3.8	1.1	1.2
1.5	4.2	1.3	1.4
1.5	1.9	1.3	1.5
1.5	2.1	1	1.1
1.5	2	1	1.2
1.5	2.7	1.2	1.2
1.5	0.9	1	1.1
1.5	1.2	1.4	1.5
1.5	1.2	1.5	1.3
1.5	1.3	1.4	1.4
1.5	1.5	1.3	1.2
1.5	2.9	1.1	1.3
1.5	1.6	1	1.1
	2.48	1.25	1.33
	1.73	0.25	0.26

	PHA	UCHT1	Con A
2	7.4	1.3	1.8
2	10.5	1.2	1.7
2	10.7	2.1	1.2
2	9.1	1.9	1.2
2	4.6	1.2	1.8
2	7.9	1.8	1.5
2	8.9	2.2	1.3
2	9.4	1.9	1.4
2	5.2	1.4	2.1
2	4.7	1.9	3.1
2	6.7	3.2	2.9
2	2.1	3.2	3.2
2	2.9	4.5	4.5
2	3.1	3.2	3.2
	6.66	2.21	2.21
	2.9	0.97	1

AML - DOUBLE

	PHA	UCHT1	Con A
3	7.9	3.6	2.9
3	10.9	5.7	7.5
3	11.4	4.6	4.6
3	14.6	3.7	3.7
3	19.5	4.5	5.2
3	17.8	7.8	6.9
3	14.7	5.3	5.8
3	11.5	2.5	2.9
3	6.8	4.6	4.8
3	9.4	2.5	2.8
3	10.3	5.4	6.7
3	11.2	1.56	1.9
3	4.5	3.5	3.1
Mean	11.6	4.25	4.52
SD	4.22	1.64	1.81

	PHA	UCHT1	Con A
6	13.9	4.6	4.9
6	14.3	6	6.1
6	11.4	2.7	6.8
6	15.7	7.9	6.2
6	14.2	8.5	7.7
6	18	9.6	7
6	18.4	9.4	8.9
6	13.2	2.9	5.1
6	22	5.4	8.5
6	24.6	4.9	5.2
6	15.3	5.3	5.8
6	11.4	6.5	7.1
6	10.2	7.8	6.9
	12	5.8	
	15.3	6.24	6.63
	4.15	2.18	1.25

	PHA	UCHT1	Con A
12	22.5	14.7	9.8
12	29	17.9	16.7
12	31.7	16.7	10.8
12	34.5	17.5	17.5
12	37.2	18.9	9.6
12	32.9	13.3	13.3
12	35.7	12.4	12.4
12	31.2	16.4	13.4
12	30.5	14.2	14.2
12	29.7	13.7	13.7
12	26.4	11.3	11.3
12	25.7	10.9	10.9
12	24.3	9.5	10.9
	30.1	14.4	12.7
	4.46	2.93	2.47

AML - MITOGEN RESPONSES

AML - SINGLE

	PHA	UCHT1	Con A		PHA	UCHT1	Con A		PHA	UCHT1	Con A
Pre	28	14.2	12.7	1	1.1	1	1.1	1.5	2.1	3.2	3.2
Pre	30	13.2	13.2	1	3.2	1.4	1.4	1.5	1.8	1.5	1.4
Pre	27.5	11.4	11.5	1	1	1	1.3	1.5	1.4	1.5	1.5
Pre	29	11.7	11.5	1	1.2	1	1.15	1.5	1.3	1.4	1.4
Pre	33	17.4	11.9	1	1.3	1.4	3.3	1.5	1.5	1.3	1.3
Pre	31.5	15.3	12.5	1	1.9	1.5	1.6	1.5	2.4	1.1	1.1
Pre	26	14.5	14.2	1	1.9	1.3	1.4	1.5	1.7	1	1
Pre	22	14.2	13.9	1	3.5	1.3	1.3				
Mean	28.4	14	12.7		1.89	1.24	1.57		1.74	1.57	1.56
SD	3.41	1.94	1.03		0.97	0.21	0.72		0.4	0.74	0.75

AML - SINGLE

	PHA	UCHT1	Con A
2	6.7	2.9	3
2	5.4	2.4	2.9
2	3.9	1.9	1.7
2	2.7	1.9	2.4
2	2.9	2.2	2.7
2	3.1	2.1	2.5
2	4.1	2.6	2.2
2	6.6	3.1	3.4
Mean	4.43	2.39	2.6
SD	1.62	0.45	0.52

	PHA	UCHT1	Con A
3	11.2	4.1	4.4
3	17.1	3.5	3.7
3	14.3	4.2	4.9
3	12.9	4.7	6.7
3	15.1	5.3	5.8
3	11.8	3.9	2.9
3	7.4	3.7	4.8
3	9.4	2.5	2.8
	12.4	3.99	4.5
	3.14	0.83	1.36

	PHA	UCHT1	Con A
6	14.5	6.1	6.6
6	14.4	7.2	7.1
6	16.6	7.2	7.9
6	17.1	8.7	8.4
6	14.2	3.6	6.4
6	19.3	6.3	8.3
6	17.6	4.9	5.5
6	16.9	5.9	5.8
	16.3	6.24	7
	1.81	1.55	1.11

AML - SINGLE

	PHA	UCHT1	Con A
12	27.2	14.9	14
12	29.4	13.7	11.2
12	28.6	13.2	14.4
12	34.1	12.9	10.1
12	32.9	13.9	12.2
12	34.2	12.4	12.4
12	31.2	12.6	12.1
Mean	31.1	13.4	12.3
SD	2.78	0.87	1.49

ALL - MITOGEN RESPONSE

Time	PHA	UCHT1	Con A
0	22	11	11.1
0	17	9.5	10.9
0	15.9	7.8	8.6
0	18	9	9.2
0	37	17	13.2
0	21	10.5	9.8
Mean	21.8	10.8	10.5
SD	7.8	3.24	1.65

Time	PHA	UCHT1	Con A
1	2.1	1.1	1.2
1	1.9	1.3	1.4
1	3.5	1.3	1.3
1	3.2	1	1
1	1.9	1	1
1	7.9	1.2	1.2
	3.42	1.15	1.18
	2.3	0.14	0.16

Time	PHA	UCHT1	Con A
1.5	0.9	1.2	1.1
1.5	0.8	1.4	1.3
1.5	1.2	1.5	1.6
1.5	1.3	1.8	1.4
1.5	1.5	1.9	1.4
1.5	2.9	1.1	1.4
	1.43	1.48	1.37
	0.76	0.32	0.16

Time	PHA	UCHT1	Con A
2	2.2	1.2	1.5
2	1.9	1.1	1.4
2	1.6	1.2	1.2
2	1.9	1.3	1.4
2	4.5	1.3	1.7
2	3.8	1.6	1.9
	2.65	1.28	1.52
	1.2	0.17	0.25

Time	PHA	UCHT1	Con A
3	2.6	2.1	2.1
3	3.8	1.9	2.2
3	3.2	2.2	2.5
3	3.3	2.5	2.8
3	4.1	2.4	1.7
3	4.7	1.9	2.2
Mean	3.62	2.17	2.25
SD	0.74	0.25	0.37

Time	PHA	UCHT1	Con A
6	9.4	3.6	3.6
6	6.4	3.2	2.9
6	11.3	4.4	4
6	13.9	3.9	4.1
6	14.3	4	4.1
	11.1	3.82	3.74
	3.28	0.45	0.51

Time	PHA	UCHT1	Con A
9	8.7	4.9	3.9
9	9.7	6.8	6.8
9	11.6	7.9	6.4
9	14.9	4.7	5.2
9	15.6	4.3	4.8
	12.1	5.72	5.42
	3.07	1.55	1.18

Time	PHA	UCHT1	Con A
12	29.7	13.7	13.9
12	26.4	11.3	12.1
12	25.7	10.9	10.4
12	24.3	9.5	10.9
12	21.5	11	12.8
	25.5	11.3	12
	3	1.52	1.42

NHL - RESPONSE TO MITOGENS

Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A
0	11.6	5.9	12.6	1	7.9	1.2	1.4	1.5	1.1	1.1	1	2	1	1.37	1
0	12.6	11.7	7.94	1	6.5	3.2	3.1	1.5	1.2	1.2	1	2	0.9	0.9	1
0	9.2	6.9	11.4	1	7.8	4.5	2.7	1.5	1.15	1.3	1	2	1.1	1.5	1.1
0	13.4	11.2	8.87	1	1.1	1.5	1.1	1.5	1.18	1.4	1	2	1.2	1.4	1.2
0	16.8	8.3	9.8	1	1.25	1.4	1.2	1.5	1.4	1.5	1	2	1.3	1.2	1.4
0	14.7	9.5	9.4	1	1.3	1.3	1.3	1.5	0.9	1.6	1	2	1.4	1.4	1.2
0	28.6	4.8	11.7	1	1.5	1.15	1.1	1.5	0.8	1.7	1	2	4.5	1.3	1.3
0	23.7	9.9	1.1	1	1.7	1.04	1.2	1.5	1.25	1.8	1	2	3.8	1.9	1.6
0	21.6	12.5	8.7	1	1.45	1.9	1.5	1.5	3.1	1.45	1.4	2	2.2	2.1	1.6
0	14.7	14.6	7.9	1	3.5	1.8	2.1	1.5	1.5	1.9	1.9	2	6.9	3.2	2.1
0	17.9	7.3	7.5	1	3.8	1.6	1.7	1.5	5.8	4.5	4.5	2	7.4	4.5	2.9
0	16.7	7.6	9.6	1	7.4	2.1	2.1	1.5	3.7	3.2	3.2	2	10.5	3.2	2.6
0	32	14	7.6	1	0.9	2.5	2.6	1.5	4.7	3.2	3.2				
0	14.7	9.5	9.4	1	1.1	2.3	2.1	1.5	7.9	2.6	2.6				
Mean	17.7	9.55	8.82		3.37	1.96	1.8		2.55	2.03	1.77		3.52	2	1.58
SD	6.55	2.97	2.72		2.79	0.95	0.66		2.2	0.99	1.15		3.19	1.08	0.63

NHL - RESPONSE TO MITOGENS

Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A
3	5.3	3.1	3.4	6	6.4	5.3	8.7	9	21.6	13.5	13.5	12	16.5	6.7	7.6
3	6.7	6.4	3.7	6	11.3	6.7	6.7	9	26.9	10.9	10.9	12	34.5	17.5	17.5
3	7.9	3.5	3.8	6	13.9	7.8	7.8	9	31	13.8	13.8	12	37.2	18.9	9.6
3	10.9	4.6	4.1	6	14.3	9.8	9.8	9	21.6	10.6	10.6	12	32.9	13.3	13.3
3	11.4	6	4.9	6	11.4	8.9	8.9	9	16.8	4.7	4.7	12	35.7	12.4	12.4
3	14.6	7.2	7	6	15.7	10.8	10.8	9	15.7	5.9	5.9	12	19.8	11.5	13.4
3	19.5	7.9	6.5	6	14.2	7.9	7.9	9	18.6	14.3	14.3	12	30.5	14.2	14.2
3	17.8	8.5	9.2	6	18	10.5	10.5	9	37	15.3	13.7	12	29.7	13.7	13.7
3	14.7	9.9	11.2	6	18.4	11.6	9.5	9	32	11.1	11.1	12	26.4	11.3	11.3
3	21.5	9.4	8.2	6	13.2	12.7	10.6	9	32.1	8.9	8.9	12	25.7	10.9	10.9
3	6.8	2.9	3.1	6	22	13.5	8.9	9	25.7	6.8	6.8	12	24.3	9.5	10.9
3	9.4	5.4	6.2	6	24.6	7.4	7.4	9	24.6	7.56	7.56				
3	10.3	4.9	5					9	29.4	8.79	8.79				
Mean	12.1	6.13	5.87		15.3	9.41	8.96		25.6	10.2	10		28.5	12.7	12.3
SD	5.18	2.34	2.49		4.93	2.51	1.33		6.56	3.41	3.23		6.62	3.44	2.63

CMI score after ABMT

AML -single

Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp
0	26	0.75	4	1	14	1.5	15	3	25	6	26	12	26
0	34	0.75	6	1	19	1.5	21	3	22	6	31	12	30
0	41	0.75	0	1	20	1.5	24	3	28	6	26	12	41
0	32	0.75	6	1	12	1.5	17	3	34	6	34		
0	43	0.75	0	1	15	1.5	18	3	26				
0	28												
Mean	34		3.2		16		19		27		29.3		32.3
SD	6.84		3.03		3.39		3.54		4.47		3.95		7.77

AML - double

Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp
0	32	0.75	3	1	17	1.5	9	3	26	6	29	12	27
0	47	0.75	0	1	14	1.5	15	3	24	6	31	12	35
0	29	0.75	19	1	24	1.5	17	3	32	6	39	12	37
0	38	0.75	7	1	11	1.5	19	3	24	6	27	12	31
0	54	0.75	16	1	18	1.5	24	3	49	6	32	12	38
0	29	0.75	11	1	17	1.5	18	3	26	6	48	12	42
0	27	0.75	3	1	9	1.5	22	3	28	6	31	12	22
0	36	0.75	10	1	12	1.5	15	3	23	6	29	12	41
0	31	0.75	5	1	16	1.5	9	3	24	6	43	12	27
0	43	0.75	12	1	18	1.5	10	3	29	6	37	12	34
0	31	0.75	7	1	13	1.5	16					12	43
Mean	36.1		8.45		15.4		15.8		28.5		34.6		34.3
SD	8.62		5.8		4.15		5		7.72		6.9		6.86

HD - Response to Mitogens

RESPONDERS				NON - RESPONDERS				RESPONDERS				NON - RESPONDERS			
Time	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**
0	22	11	11.1	0	18	9	9.2	1	2.1	1.1	1.2	1	3.8	1.6	1.6
0	17	9.5	10.9	0	17	7.1	7.3	1	1.9	1.3	1.4	1	0.74	0.8	1.2
0	15.9	7.8	8.6	0	11	5.1	6.8	1	3.5	1.3	1.3	1	0.9	2.5	1.1
0	7.8	7	8.4	0	7	6.4	6.7	1	3.2	1	1.4	1	1.1	2.3	1.7
0	4.9	2.9	11	0	9.8	4.8	8.4	1	1.9	1	1	1	0.8	1.24	1.4
0	19.6	9.5	9.54	0	11.2	11	8.9	1	7.9	1.2	1.1	1	1	1.3	1.2
0	17.5	9.7	8.94	0	3.4	5.1	2.9	1	6.5	3.2	2.9	1	1.3	1.15	1.3
0	11.6	5.9	9.5	0	5.9	3.5	6.8	1	7.8	4.5	3.7	1	1.2	1.04	1.1
0	12.6	11.7	7.94	0	2.9	2.4	5.4	1	1	1.5	1.1	1	1.1	1.9	1.5
0	28.6	4.8	11.7	0	14.9	7.45	7.6	1	1	1.4	1.3				
0	23.7	9.9	9.6	0	11.7	9.8	8.7	1	3.5	1.8	2.1				
0	21.6	12.5	8.7	0	9.2	6.9	7.38								
0	14.7	4.6	7.9	0	13.4	8.4	8.87								
0	17.9	7.3	7.5	0	6.8	8.3	8.9								
0	16.7	7.6	6.9	0	1.7	0.95	2.1								
0	32	14	7.6												
0	11	5.9	9.4												
Mean	17.36	8.33	9.13		9.59	6.41	7.06		3.66	1.75	1.68		1.33	1.54	1.34
SD	6.97	3.03	1.41		5.01	2.79	2.15		2.57	1.10	0.87		0.95	0.58	0.22

HD - Response to Mitogens

RESPONDERS				NON - RESPONDERS				RESPONDERS				NON - RESPONDERS			
Time	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**
1.5	7.9	2.6	2.7	1.5	1	1.1	1	2	1	1.1	1.1	2	5.2	3.1	5.3
1.5	10	5.4	3.2	1.5	1	1	1	2	1	1.2	1.3	2	4.7	2.5	22.8
1.5	14.6	7.4	5.1	1.5	1	1	1	2	1	1.15	1.5	2	6.7	3.7	4.6
1.5	5	4.3	4.9	1.5	1	1	1	2	1	1.24	1.2	2	2.1	2.2	2.5
1.5	5.7	4.7	5.2	1.5	1	1	1	2	1	1.09	1	2	6.7	2.5	2.9
1.5	6.9	4.6	5.1	1.5	1	1	1	2	1	1.37	1.4	2	3.1	1.5	1.6
1.5	4.8	2.1	2.9	1.5	1	1	1	2	0.9	0.9	1	2	2.6	2.2	1.9
1.5	3.7	2.9	2.7	1.5	0.9	1	1	2	3.8	2.9	1.9	2	1.1	1.5	1.8
1.5	8.5	2.4	2.6	1.5	0.8	1	1.7	2	2.2	2.1	2.4	2	1.2	1.1	1.4
				1.5	1.2	1	1.5	2	6.9	3.2	3.2	2	1.3	1.2	1.2
				1.5	1.3	1.4	1.4	2	7.4	4.5	5.1	2	1.4	1.2	1.4
				1.5	1.5	1.9	1.7	2	10.5	3.2	3.3	2	4.5	1.3	1.6
				1.5	5.8	4.5	3.2	2	10.7	3.6	3.6	2	1.5	1.6	1.6
				1.5	3.7	3.2	3.2	2	13.5	5.7	4.9	2	0.9	1.7	1.4
				1.5	4.7	3.2	2.9	2	7.2	4.6	5.1	2	0.8	1	1.8
				1.5	4.9	3.7	2.5								
				1.5	8.9	4.5	4.8								
				1.5	9.4	5.2	7.8								
Mean	7.46	4.04	3.82		0.97	1.01	1.08		4.61	2.52	2.53		2.92	1.89	3.59
SD	3.34	1.72	1.20		0.07	0.03	0.23		4.38	1.56	1.55		2.11	0.79	5.45

HD - Response to Mitogens

RESPONDERS				NON - RESPONDERS				RESPONDERS				NON - RESPONDERS			
Time	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**	**Time**	**PHA**	**UCHT1**	**Con A**
3	1.4	1.1	1.4	3	1.3	1.9	1.6	6	11.3	6.7	7.6	6	4.8	2.76	2.8
3	1.2	1.3	1.3	3	2.3	2	1.9	6	13.9	7.8	8.1	6	11.7	7.8	8.9
3	1.2	1.21	1.1	3	2.6	2.1	2	6	14.3	9.8	10	6	14.2	6.2	8.4
3	7.9	2.9	3.5	3	3.8	2.2	2.1	6	11.4	8.9	8.9	6	15.7	8.1	9.4
3	10.9	4.6	4.4	3	3.9	2.5	2.4	6	15.7	10.8	11.1	6	3.6	3.5	3.5
3	11.4	6	5.7	3	3.3	2.8	2.4	6	14.2	7.6	7.9	6	13.7	11.3	9.1
3	14.6	7.2	7.7	3	4.6	4.5	4.2	6	18	9.8	10.5	6	14.6	3.3	3.9
3	19.5	7.9	8.1	3	1.3	1.3	1.2	6	18.4	8.7	9.5	6	2.9	4.2	4.6
3	17.8	7.4	8.5	3	1.4	1.5	1.5	6	13.2	12.7	11.2	6	17	8.1	8.7
3	14.7	9.9	12.1	3	6.8	7.3	5.7	6	22	13.5	8.9	6	1.7	1.3	1.34
3	21.5	9.4	9.4	3	5.4	2.9	3.1	6	24.6	7.4	7.4	6	2.4	1.5	1.56
3	6.8	2.9	5.3	3	5.3	3.1	3.1	6	15.3	4.89	5.3	6	2.9	1.7	1.89
3	9.4	5.1	5.4	3	6.7	6.4	5.1	6	11.4	6.93	7.2	6	5.6	2.1	2.2
3	10.3	4.4	4.9	3	12	4.9	5.8	6	10.2	3.4	3.4	6	5.9	2.2	2.4
3	11.2	4.9	5.3	3	13.4	8.9	7.3	6	12.6	8.7	7.2	6	12	4.9	5.8
3	6.5	4.5	6.5	3	1.9	1.6	1.8	6	16.8	8.5	8.2	6	13.4	7.8	8.9
								6	12.6	5.9	6.2	6	1.9	1.6	1.6
								6	13.5	11.1	13.2	6	9.2	2.9	3.1
								6	14.3	7.4	7.9	6	9.9	2.6	2.8
								6	14.6	11.2	12.1	6	9.4	3.6	3.7
								6	15.3	11.8	11.4	6	6.4	5.3	8.7
Mean	10.39	5.04	5.66		4.75	3.49	3.20		14.93	8.74	8.72		8.47	4.61	5.00
SD	6.24	2.78	3.05		3.59	2.28	1.85		3.52	2.55	2.37		5.65	3.05	3.18

HD - Response to Mitogens

RESPONDERS				NON - RESPONDERS				RESPONDERS				NON - RESPONDERS			
Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A	Time	PHA	UCHT1	Con A
9	14.6	11.8	9.8	9	4.8	2.76	2.9	12	33.2	17.9	14.1		6.3	3.6	9.7
9	25.4	12.7	13.1	9	8.5	8.94	8.4	12	36.4	13.8	13.8		2.2	1.5	1.5
9	21.6	13.5	13.8	9	14.2	8.1	8.4	12	31.2	16.5	9.65		1	1.1	1.08
9	26.9	10.9	11.2	9	15.7	8.6	9.4	12	31.7	16.7	17.1		21.6	3.8	4.5
9	31	13.2	13.8	9	3.6	3.3	3.5	12	34.5	17.5	17.5		10.6	5	7.6
9	21.6	10.6	10.8	9	13.7	2.3	5.9	12	37.2	18.9	9.6		17.2	8.1	11
9	16.8	4.7	4.9	9	11.2	33.6	3.96	12	32.9	13.9	13.3		15.2	8.7	12.8
9	15.7	5.7	5.9	9	2.9	4.1	4.6	12	35.7	12.4	13.1		19.4	7.4	4.1
9	18.6	14	14.3	9	3.9	6.1	8.7	12	31.2	16.4	13.4		8.7	2.67	2.67
9	37	15.3	13.7	9	12.1	6.9	8.7	12	30.5	14.2	15.6		12	7.5	5.9
9	32	11.1	11.6	9	11.6	6.8	8.6	12	29.7	13.1	13.7		12.5	4.7	4.9
9	32.1	8.9	9.2	9	9.7	7.9	7.9	12	26.4	11.3	14.7		19.6	6.9	4.8
9	25.7	6.8	8.4	9	14.9	4.7	7.9	12	25.7	9.8	10.9		1.3	1.1	1.1
9	24.6	7.56	7.7	9	11.1	4.3	9.4	12	24.3	9.5	10.9		1.8	1.3	1.4
9	29.4	8.79	8.9	9	2.4	1.9	1.9	12	31.5	11.9	12.4		8.3	1.5	1.67
				9	3.7	2	2.1	12	27.8	11.4	11.7				
				9	4.9	3.2	2.1	12	24.5	12.5	15.2				
								12	26.4	10.2	10.9				
								12	19.7	9.4	11.8				
								12	17.4	6.8	8.9				
								12	19.8	9.2	11.9				
								12	20.9	10.2	10.2				
								12	20.4	11.6	13				
								12	27.5	12.5	13.8				
Mean	24.87	10.37	10.47		9.35	7.35	6.68		28.19	12.82	12.80		10.51	4.32	4.98
SD	6.70	3.19	2.98		4.68	7.63	2.61		5.73	3.18	2.28		7.09	2.80	3.78

CMI score after ABMT

ALL

Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp
0	19	0.75	0	1	3	1.5	8	3	17	6	21	12	28
0	33	0.75	2	1	2	1.5	14	3	14	6	19	12	36
0	37	0.75	0	1	0	1.5	7	3	11	6	10	12	29
0	25	0.75	0	1	4	1.5	3	3	6	6	11	12	34
0	28	0.75	5	1	11	1.5	7	3	12	6	17	12	25
0	31	0.75	7	1	5	1.5	12	3	15				
Mean	28.83		2.33		4.17		8.50		12.50		15.60		30.40
SD	6.34		3.01		3.76		3.94		3.83		4.88		4.51

NHL

Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp
0	26	0.75	5	1	12	1.5	23	3	37	6	35	12	27
0	19	0.75	4	1	15	1.5	24	3	34	6	32	12	47
0	28	0.75	3	1	19	1.5	31	3	24	6	28	12	24
0	34	0.75	4	1	8	1.5	19	3	29	6	37	12	39
0	27	0.75	3	1	18	1.5	22	3	32				
0	25	0.75	6	1	7								
0	41	0.75	9	1	12								
0	36	0.75	4										
0	11	0.75	0										
0	14	0.75	0										
Mean	26.1		3.8		13		23.8		31.2		33		34.3
SD	9.5	0	2.66	0	4.62	0	4.44	0	4.97	0	3.92	0	10.7

HODGKIN'S DISEASE
CR after ABMT

Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp		
0	9	0.75	1	1	8	1.5	28	2	23	3	33	6	37	12	38
0	5	0.75	3	1	11	1.5	24	2	24	3	22	6	38	12	44
0	19	0.75	6	1	6	1.5	21	2	34	3	48	6	28	12	39
0	10	0.75	2	1	16	1.5	14	2	42	3	32	6	29	12	38
0	3	0.75	2	1	10	1.5	26	2	49	3	25	6	37	12	39
0	11	0.75	4	1	14	1.5	30	2	10	3	31	6	56	12	46
0	19			1	18	1.5	18	2	24			6	48	12	52
0	10			1	6	1.5	38					6	13	12	44
0	0			1	21	1.5	29							12	44
0	16			1	15	1.5	25								
0	6			1	14	1.5	29								
0	10			1	12	1.5	24								
0	7														
0	26														
Mean	10.8		3		12.6		25.5		29.4		31.8		35.8		42.7
SD	7.05		1.79		4.66		6.19		13.2		9.02		13		4.66

HODGKIN'S DISEASE
No CR after ABMT

Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp	Time	Resp		
0	6	0.75	0	1	5	1.5	13	2	18	3	19	6	27	12	20
0	4	0.75	2	1	7	1.5	8	2	17	3	31	6	14	12	27
0	10	0.75	4	1	8	1.5	14	2	26	3	24	6	22	12	14
0	8	0.75	3	1	14	1.5	12	2	9	3	12	6	25	12	19
0	16	0.75	5	1	10	1.5	6	2	33	3	18	6	14	12	17
0	6			1	12	1.5	16	2	21	3	15	6	14	12	23
0	4			1	3	1.5	17					6	11	12	33
0	7			1	16	1.5	21							12	19
0	4			1	9									12	22
0	3														
0	0														
0	5														
0	12														
0	0														
0	6														
0	4														
Mean	5.94		2.8		9.33		13.4		20.7		19.8		18.1		21.6
SD	4.12		1.92		4.18		4.84		8.21		6.79		6.36		5.66