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Use of "Real Time" CD34 Quantiation to Optimize Apheresis Harvests, Staffing As Well As Reduce Patient Charges. SJ Noga, C Grayson, L Rogers, LE Noffsinger, JT Ruskey, PV O'Donnell. The Johns Hopkins Oncology Center, Baltimore, Maryland, 21205, USA and Biometric Imaging, Inc, A Beckton Dickinson Company, Mountain View, CA, 94043, USA

High flow, extended time (6 hrs) apheresis reduces the total number of procedures and has low tumor cell contamination. However, the increased time commitment (10-15 litre procedures: 2 hrs) limits daily patient load and staff utilization. Also, many PBSC products far exceed the target CD34+ cell dose (5-10 x 10^6/kg) and can actually cause patient morbidity related to volume overload, DMSO toxicity and pulmonary leukostasis. We have found that the absolute peripheral blood (PB) CD34 measurement generated using IMAGN 2000® technology (50 µl sample size, 45 min turn-around) and the STELLer® CD34 assay tightly correlated with the CD34+/kg cell dose throughout the apheresis (r^2 = 0.94: 2 hrs, 0.95: 4 hrs, 0.93: 6 hrs) procedure. PB CD34 measurements were obtained daily on non-Hodgkin's lymphoma (NHL, n = 10) or multiple myeloma (MM, n = 5) patients undergoing mobilization with cyclophosphamide (2.5 g/m^2) and G-CSF (10 mcg/kg/day). An optimal (5x10^6/kg) CD34+ cell dose could have been collected in ≤ 2 hrs in patients with PB CD34 values >50/µl, 4 hrs: >20/µl, 6 hrs: >10/µl or never reached if <10/µl. Using this grouped data to generate an algorithm, the apheresis time was pre-set (2, 4, 6 hrs) based on the morning PB CD34 value for the next 13 patients (10 NHL, 3MM). Two hr collections were performed on 46% [PB CD34 57-164/µl, mean CD34 x10^6/kg =19 (range 9-31)] while 31% were pre-set at 4 hrs [PB CD34 21-45/µl, mean CD34 x10^6/kg =10 (range 5-14)]. Three patients had PB CD34/µl values <10 (9,6,6). One patient (9/µl) was collected (6 hrs) at physician request, yielding a final CD34+ product of 1.5x10^6/kg while the 2nd underwent marrow harvest (1x10^5/kg CD34 yield) and the third had no procedure performed. None proceeded to transplant. All 10 patients with optimal CD34+ collections engrafted with a median ANC>500/µl of day +9, platelets >50,000: day +12, and platelet transfusions: 3. Work and charge capture calculations based on relative value units (RVU's) showed a 20% reduction (18.7 to 14.8 RVU's) for procedures lasting 4 hrs and a 40% reduction (10.9 RVU's) for those of 2 hr duration. Therefore about 80% of the patients in this cohort would have apheresis charges reduced by 20 to 40%. The reduction in staff hours increases the flexibility of the apheresis team and along with pre-determined run times allows more efficient staff utilization and increased patient volumes. We conclude that "real time" PBCD34 measurement can be used to dictate the length of collection, even for high flow, large volume apheresis procedures, thus resulting in cost savings and improved staffing efficiency.

ALTERATIONS OF THE T CELL REPERTOIRE POST ALLOGENEIC STEM CELL TRANSPLANTATION

L. Barrow, M. Raitakari, R. Brown, J. Gibson, D.E.Joshua
Institute of Haematology, Royal Prince Alfred Hospital, Camperdown, NSW

GvHD is the major complication of allogeneic stem cell transplantation. Studies have focused on the identification of significant T cell subsets in the post transplant period in the hope of separating the GvH from the GvL effect. If a restricted T cell repertoire was found it could be positively selected, cloned and used to enhance the GvL effect. Alternatively, if cells were found that were associated with GvHD, these cells could be negatively selected from grafts or donor lymphocyte infusions.

We studied the PB T cell repertoire of 9 patients undergoing allogeneic stem cell transplantation, over an 18 month period, to determine if there was a clonal expansion of T cell subsets and whether these cells were associated with GvHD. Diagnoses: MDS n = 1; NHL n = 1; ALL n = 2; CML n= 3; AML n = 2. Six of the 9 grafts were T cell replete, the remainder underwent a 2-3.8 log reduction of T cells. Five of 9 patients received stem cells from HLA matched siblings; 1 was from a matched unrelated donor (BM) while the remaining 3 transplants were from siblings with a 1 or 2 loci mismatch. Six out of 9 patients had grade I or II aGvHD, one had grade IV disease and the remaining two had no evidence of aGvHD. Four of 8 patients progressed to chronic GvHD which was limited in 3.

The T cell repertoire was evaluated using a panel of 21 monoclonal antibodies directed at the TCR Vβ and which covered 62±9% of the T cell population in the peripheral blood of normal healthy donors. Increases in TCR Vβ expression > 3SD above the normal range was found in all 9 patients in the period up to 6 months post transplant. The majority of clones (in 8 out of 9 patients) were not present prior to transplant, indicating that such clones may have GvL potential. Three patients had marked Vβ subset expansions (> 20%) but these patients did not have severe (grade III or IV) aGvHD. No association could be found with the presence of expanded T cell clones post transplant and GvHD.

DELAYED T CELL DEPLETION OF MATCHED UNRELATED DONOR MARROW

D. Ford, J. Argyle, M. Vowels, R Lindeman.
Prince of Wales & Sydney Childrens Hospitals, Randwick. NSW

Twenty patients received a matched unrelated donor marrow (MUD) transplant for leukaemia, or other haematologic /oncologic diseases. Bone marrow was processed by T cell depletion for prevention of graft versus host disease (GVDH) combined with Cyclosporin (Csa). The T cell depletion was achieved by isolation of the mononuclear cell fraction of marrow before rosetting with sheep red blood cells. T cells were then removed by centrifuging the cell suspension through a ficoll gradient before infusion. The marrow manipulation described required a minimum processing interval of 7 hours.

The logistics of MUD bone marrow collections mean the majority of donations are transported from overseas and national centers. A policy for processing marrow was established so that collection in local and international centers were processed on arrival into the laboratory. National centre collections were stored overnight and processed the following day. The number of bone marrows received from local, national and international centers were 7,11 and 3 respectively. The median time interval between marrow collection and infusion was 28 hrs (7-36 hrs).

The patients were aged from 0.5 to 18.8 yrs (median 9) weighing from 8 to 62 Kg (median 31). The median nucleated cell, CD34, and CD3 cell dose was 0.4 x 10^8/Kg. (0.1-1.0), 1.2 x 10^6/Kg (0.1-6.2) and 3.1 x10^5/Kg (0.3-30.0) respectively. Engraftment occurred in 19 of 20 (95%) patients. The median time for neutrophils to 0.5 x10^9/l was 21 days and platelet count of 20-25 x10^9/l was 25 days.
The incidence of acute GVHD grade I/II was 40% with no chronic GVHD. Relapse occurred in 3 of 16 patients. Median follow-up of surviving patients 14/20 (70%) was 12 mths (1-48).
The bone marrow manipulation described provides an effective method for engraftment and together with Csa an effective GVDH prophylaxis.

EX VIVO PURGING WITH RITUXIMAB OF PERIPHERAL BLOOD STEM CELLS COLLECTED FROM PATIENTS WITH NON-HODGKIN'S LYMPHOMA

CJ Hutchins, KJ James, JV McAlonan, R Bird & STS Durrant
Bone Marrow Transplant Unit, Royal Brisbane Hospital, Herston, Qld & Cairns Base Hospital, Cairns, Qld

Rituximab (Mabthera^{TM}) is a chimeric mouse / human monoclonal antibody specific for the CD20 antigen expressed on the surface of normal and malignant B–cells. A method was developed for the *ex vivo* purging of $CD20^+$ B-cells from peripheral blood progenitor cells (PBPC) collected from patients with non-Hodgkin's lymphoma (NHL) using rituximab. PBPC (200ml) were collected on the COBE Spectra following mobilisation with cyclophosphamide (2g/m^2) and G-CSF (5ug/kg/d). On the day of purging, autologous serum (50ml) was prepared as a source of complement. Routine quality assurance procedures including nucleated cell count, FACS analysis of CD34, CD19 & CD20 expression and microbiological assessment were performed on PBPC prior to and following the purge procedure. Heparin (10U/ml), $MgSO_4$ (0.4mM) and $CaCl_2$ (0.01mM) were added to the PBPC, followed by incubation with rituximab (1 mg per 10^9 nucleated cells) at room temperature for 15min. Autologous serum was added and complement mediated lysis allowed to proceed at 37^{o}C for 45 min, followed by extensive washing of the cells in human serum albumin containing heparin (5U/ml) and cryopreservation. Preliminary results indicate that there is minimal loss of $CD34^+$ progenitor cells during the purging procedure (recovery range: 62.6 – 91.7%) and that the efficacy of purging of $CD20^+$ cells *ex vivo* correlates with the level of CD20 expression. Detection of the bcl 2-J_H gene rearrangement in PBPC post purge by PCR is in progress. One patient has undergone autologous transplantation with rituximab purged PBPC containing 2.9 x 10^6 $CD34^+$ cells per kg. Neutrophil recovery to 0.5 x 10^9/l occurred 8 days and platelet recovery to 20 x 10^9/l occurred 18 days post infusion. *Ex vivo* purging with rituximab provides a rapid method for the reduction of $CD20^+$ cells from peripheral blood progenitor cells prior to autologous transplantation or further *ex vivo* manipulation.

PERIPHERAL BLOOD STEM CELL TRANSPLANTATION – WHAT DO WE INFUSE?

K. Jones, D. Tucker, A. Fryga, S. Bol.
Dept.of Clinical Haematology/Oncology, Royal Children's Hospital, Melbourne.

Peripheral Blood Stem Cell (PBSC) transplantation is increasingly being used along with more intensive chemotherapy regimes in the treatment of children with malignant diseases. The reported cell doses to be infused for successful haemopoietic reconstitution are 3 x 10^8/kg white cells (WC) and 2 x 10^6/kg CD34+ cells. Traditionally, these cell numbers are calculated on the fresh PBSC product, and are not re-evaluated after the product has been cryopreserved and thawed prior to infusion. Thus the cell dose / kg is based on the assumption that cell numbers after freezing and thawing are not affected, or at least, that the cell recovery after thawing is constant and does not vary between individual transplant procedures.

It was investigated whether the analysis of cell numbers on the fresh product was a reliable indicator of the graft quality after thawing. Eighteen PBSC transplant harvests were evaluated for WC, CD34+ cell, and clonogenic cell recovery after thawing. The mean percentage recoveries were 75.1 $\pm$ 13.5, 76.5 $\pm$ 23.3, 64.6 $\pm$ 30.6 and 79.7 $\pm$ 27.4 for WC, CD34+ cells, BFU-E and CFU-GM, respectively. Although the mean values are similar, regression analysis showed no correlation between any of the parameters (for individual patients). Together with the observed high standard deviations in the cell recoveries, this shows that all parameters (with exception of BFU-E and CFU-GM) are independent. This means that to assess the quality of thawed products, individual parameters need to be determined separately and that one parameter alone cannot be used to predict the other.

In conclusion, the observed variation in post-thaw cell recoveries precludes the use of cell counts on the fresh PBSC product to determine the cell dose / kg. This indicates that cell dose parameters must be re-analysed post-thaw in order to determine the actual cell dose given to the patient. When correlated with engraftment kinetics, this data has implications on the target number of cells to be harvested, and ultimately, the number of apheresis cycles actually required by a patient. In addition, the re-evaluation of these cell parameters post-thaw represents good quality control practice, providing a full record of the entire procedure.

RT-PCR DETECTION OF TYROSINE HYDROXYLASE IN CD34 SELECTED STEM CELLS FROM PATIENTS UNDERGOING AUTOLOGOUS TRANSPLANTATION FOR NEUROBLASTOMA.

B. Kramer, G. McCowage.
Oncology Research Unit, The New Children's Hospital, Westmead, NSW, 2145.

Tyrosine Hydroxylase (TH) is the first enzyme in the biosynthesis of catecholamine, and its expression is confined to cells of neural crest origin. RT-PCR for the mRNA of TH has been shown to be a sensitive and specific marker for the presence of neuroblastoma cells in both peripheral blood and bone marrow.

A nested RT-PCR assay for TH has been established which can detect the presence of the neuroblastoma cell line IMR-32 at 1 in 10^5 peripheral blood mononuclear cells. This assay also amplifies a product of approximately 180 bp out of RNA extracted from primary neuroblastoma tumour samples and bone marrow from patients with Stage IV disease.

The aim of our study is to determine the utility of TH RT-PCR in measuring minimal residual disease in the CD34 positive cell populations selected out of Peripheral Blood Stem Cells (PBSC) harvested from patients undergoing autologous transplantation as part of their treatment.

CD34 selections are performed using the Isolex 300i Magentic Cell Separator (Baxter) using one or two pooled PBSC harvests to obtain at least 4 x 10^6 CD34+ cells /kg as an initial starting product. The mean yield of CD34 positive cells obtained to date is 47 % (n=5, range 25 – 83%) with a mean purity of the selected fraction (CD34%) of 73% (range 56 – 93%).

RNA is extracted from Pre-selected, CD34 selected and Negative (unselected) cell fractions, and used in the TH RT-PCR assay to test for the presence or absence of the TH specific product.

STEM CELL SELECTION AND CONCOMITANT T CELL DEPLETION USING CliniMACS FOR ALLOGENEIC TRANSPLANT

R Soares Mendes, PK Cannell and RP Herrmann
Department of Haematology, Royal Perth Hospital, Perth WA.

The depletion of mature T cells from HLA mis-matched related donor grafts is a prophylactic measure against graft-versus-host-disease. The associated risk of graft failure can be overcome by increasing the number of CD34+ stem cells infused. The CliniMACS device enables large numbers of CD34+ cells to be isolated with effective T cell depletion. Presented here are the results of three CliniMACS selections of mis-matched related donor stem cell harvests.

Two patients, one with lymphoblastic lymphoma and a 4/6 matched sibling and the other with secondary AML and a 5/6 maternal match, underwent transplant. Peripheral blood stem cells (PBSC) were harvested between days 4-6, following 300*u*g G-CSF twice-daily. One leucopheresis was required for the first donor, whereas the second donor required three to obtain adequate CD34+ cells. Two harvests were selected on the same day of collection, while one was left overnight at 4^{o}C and pooled with a fresh collection the next day. Cell labelling and washing was carried out on the Cobe 2991. Total nucleated cells (5.1, 3.5 and 5.4 $x10^{10}$) containing 1.2%, 0.5% and 0.4% CD34+ cells were selected. CD34 recovery post selection was 75%, 79% and 57% with purities of 97%, 95% and 93%. A log reduction in T cells (CD3+) of at least 4.6 was achieved. The absolute number of CD34+ cells recovered from each selection was 451, 136 and 135 $x10^{6}$. The lymphoma patient was infused with a final product containing $6.0x10^{6}$/kg CD34+ cells and $6.0x10^{3}$/kg CD3+ cells. Neutrophil recovery (ANC>$1.0x10^{9}$/L) was achieved by day 11. The infusion product available for the AML patient contains $4.2x10^{6}$/kg CD34+ cells and $4.1x10^{3}$/kg CD3+ cells. The selected PBSC retained colony forming ability.

The CliniMACS system resulted in highly effective T cell depletion. Combined with a high recovery of CD34+ cells, it is a useful advance in the preparation of grafts for mis-matched allografting.

PERIPHERAL BLOOD STEM CELL SELECTION USING THE ISOLEX 300*i*.

R Soares Mendes, PK Cannell, RP Herrmann
Department of Haematology, Royal Perth Hospital, Perth WA.

CD34 based immunomagnetic selection of autologous peripheral blood stem cells (PBSC) decreases the likelihood of reinfusing tumour cells which may contribute to relapse. For allogeneic harvests the concomitant reduction of T cells results in less graft-versus-host-disease. This study evaluates CD34+ stem cell selection using the Isolex 300*i* (Baxter).

Stem cell selection was performed on 51 autologous (non Hodgkin's lymphoma 24, multiple myeloma 27) and 5 fully matched allogeneic PBSC harvests. The mean percentage of CD34+ cells and number of total nucleated cells collected was 3.1±0.3%(SEM) and $3.0\pm0.3x10^{10}$ respectively for autologous and 0.9±0.1% and $5.5\pm1.6x10^{10}$ for allogeneic PBSC. Following selection the mean recovery of CD34+ cells was 54.6±0.02% for autologous and 48.3±0.07% for allogeneic with a mean CD34 purity of 90.6±1.3% and 74.8±4.0% respectively. The majority of autologous grafts remained positive for IgH rearrangement post selection. Selection resulted in a mean T cell log reduction of 3.2±0.1 for allogeneic PBSC. Patients received a mean of $2.7\pm0.2x10^{6}$/kg CD34+ cells for autologous and $3.4\pm0.5x10^{6}$/kg for allogeneic transplant. The number of days to neutrophil (ANC>$1.0x10^{9}$/L) and platelet (plt>$50x10^{9}$/L) recovery was 16 and 23 respectively. Of the autologous patients, 5 are deceased (3 relapsed, 1 sepsis/multi organ failure, 1 arrhythmia) and 3 have relapsed. Initial data shows no significant difference in patient survival at 500 days post transplant as compared to unselected transplants. Of the four patients allografted (NHL 2, AML 1, CML 1), 2 are deceased (regimen related toxicity, intracerebral haemorrhage) and 2 are in remission.

Selection of PBSC using the Isolex 300*i* provides a transplant product high in purity and capable of sustained engraftment. No survival advantage for positive selection is seen at 500 days but longer follow up is required.

CD8 DEPLETION USING T8 MURINE MONOCLONAL ANTIBODY- COATED DENSE NICKEL PARTICLES: EFFICACY OF DEPLETION AND SAFETY IN DONOR LYMPHOCYTE INFUSION (DLI).

K. Atkinson, E.P. Alyea, C. Canning, H. Houde, R.J. Soiffer, S. Giralt, A. Gee, R. Champlin. *Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA. Univ. Of Texas MD Anderson Cancer Center, Houston, TX, Eligix Inc., Medford, MA, USA.*

The development of graft-vs-host disease (GVHD) limits the effectiveness of DLI in patients (pts) who have relapsed after allogeneic BMT. Several studies suggest that the depletion of CD8+ cells prior to DLI may lead to a reduction in the incidence and severity of GVHD. However, current methods to deplete large number of cells from pheresis products are time consuming and difficult to perform. Recently, CD8 murine monoclonal antibody-coated dense nickel particles have been developed which allow for the rapid separation of cells by use of gravity. We conducted a pilot trial using CD8-High Density Microparticles (HDM) to determine the efficacy of CD8 depletion and the safety of infusing cells processed by this method. All pts received 3.0×10^7 CD4+ cell/kg, a dose that had been defined in previous studies. No other immune modulating therapy was used. Nine pts who relapsed after allogeneic BMT were enrolled. Three pts had CML, 3 multiple myeloma, 2 CLL and 1NHL. The median age was 40 (range 31-58) and the median time from transplant to DLI was 43 months (range 10-78). Two pts (1 multiple myeloma, 1 NHL) were withdrawn from the study due to rapidly progressive disease. Eight of 9 pts received the targeted cell number after 1 pheresis procedure. The time required for processing with CD8-HDM was approximately 1 hour. The median depletion of CD8+ cells from the pheresis product was 100% (range 97.8 to 100%): the mean percent yield of CD4+ cells was 105%. With depletion of CD8+ cells, the mean yield of CD3+ cells was 72.2%. No infusional toxicity related to the method of depletion was noted. One patient developed interstitial pneumonitis 2 weeks following cell infusion, which resolved. The overall incidence and severity of GVHD was low: 1 pt developed grade 2 acute GVHD and limited chronic GVHD. Three of 3 pts with CML developed a complete hematologic and cytogenetic response. Two of 2 on whom data are available achieved a complete molecular response. One pt with CLL had a good PR. The remaining patients had progressive disease. CD8-HDH appears to be highly selective and effective in depleting CD8+ cells from pheresis products. This method of depletion will allow for a multi-center trial to assess the impact of CD8 depletion on the incidence and severity of GVHD following DLI.

FROM SURVEY TO QAP OF $CD34^+$ STEM CELL ENUMERATION.

A Chang[1] and DDF Ma[2] for the BMT Scientists Study Group.
[1] *Dept. Haematology, PaLMS. Royal North Shore Hosp, Sydney.*
[2] *Dept. Haematology, St. Vincent's Hospital, Sydney.*

Enumeration of $CD34^+$ haemopoietic stem cells (HSC) by flow cytometry is used routinely for clinical decisions and management of HSC transplant patients. In Australasia, standardisation of the method for $CD34^+$ cell estimation has progressed through several stages, and shows a strong trend to adopt the ISHAGE protocol as the consensus method.

As methods are still evolving, modifications need to be evaluated. Modifications to the ISHAGE protocol include (1) incorporation of fluorobeads for absolute counting using a single platform to eliminate variations introduced by the white cell count, (a major variable affecting the absolute CD34 counts identified in our study) and (2) addition of a viability stain to estimate viable stem cells rather than total $CD34^+$ cells, so that the true graft potential can be determined for harvested cells stored either overnight or after cryopreservation.

Comparisons are made of the reagents used, method of analysis and results from the most recent sample to that at the start of this study. Whereas the first sample (analysed in November 1994 by 20 centres), showed a 4 fold difference in the $\%CD34^+$ cell results with a CV of 29%, the results from the latest sample (analysed in February 1999 by 27 centres) showed a 2 fold difference with a CV of 13%.

The Australasian centres have thus achieved a major reduction in the site-to-site variation of $CD34^+$ counts, with most centres adopting a consensus method. With support from participants (38 centres), it now appears appropriate to initiate a formal quality assurance programme for $CD34^+$ cell enumeration.

Hematopoietic stem cell transplantation for the treatment of systemic lupus erythematosus

J. Ouyang, L.Y.Sun, Y.G.Yang, X.M. Sun
Affiliated Gulou Hospital of Nanjing University, Nanjing, P.R.China

A 29 year old female was diagnosed with systemic lupus erythematosus (SLE) 8 years previously. Conventional dose corticosteroids and cyclophosphamide, plasma exchange and traditional Chinese medicine had no therapeutic effect. In an attempt to achieve effective long-term immunosuppression for this patient's active disease, high dose myeloablative chemotherapy followed by autologous bone marrow transplantation was performed. At the time of transplantation, the patient complained of arthralgia, fever and haemoptysis. She had vasculitic ulcers on fingers and toes, facial erythematosus, extensive livedo reticularis on her back and lateral chest, and radiological appearances of interstitial pneumonia. Anti-nuclear antibody (ANA), anti-ds DNA and anti-U_1RNP C_4 antibody titres were elevated. Renal function was normal.

Haemopoietic stem cells were harvested from bone marrow. Mononucleic cells (approx. $2.0x10^8$/kg) were re-infused after administration of the immunosuppressive regimen cyclophosphamide (120mg/kg) and mephalan (140mg/kg). Leukocyte engraftment (absolute neutrophil count >500/ l) and platelet engraftment (non-transfused platelet count >20,000/ l) occurred on 10 and 14 day respectively. By 2 months after the transplant, the ulcers, erythematosus and the livedo reticularis had completely resolved and ANA, anti-ds DNA and anti-U_1RNP C_4 antibody titres had become undetectable; 7 months later she remains well and in complete remission.

High dose chemotherapy with autologous hematopoietic stem cell transplantation (without T cell depletion) successfully induced a remission in this patient's active autoimmune disease for the first time in 8 years. We suggest it be considered a therapeutic option in similar circumstances.

VALIDATION OF A STEM CELL WASHING PROCEDURE PRIOR TO AUTOLOGOUS TRANSPLANTATION

Nicole Wiggins, Scott Ragg and Ray Lowenthal
Stem Cell Transplant Laboratory, Royal Hobart Hospital, Hobart, Tasmania.

Cryogenic storage of stem cells necessarily utilises the cryopreservative dimethyl sulphoxide (DMSO). Whilst the toxic side effects of resultant DMSO infusion are well known, there has been little motivation for removing this compound prior to transplant due to concerns of potential contamination and cell loss. The publication of the New York Cord Blood Bank's washing protocol, along with improvements in closed system processing techniques, has reignited interest in applying such methodology to other stem cell products.

We have performed a validation study on a modified NYCBB washing protocol for use with cryopreserved bone marrow and peripheral blood stem cell harvests. Parameters investigated included cell loss, cell clumping, overall viability, viable CD34+ stem cell enumeration, clonogenic potential and reduction in free haemoglobin content. Overall cell viability was improved markedly by DMSO removal, a factor which correlated with the decreased incidence of cell clumping in washed samples. Enumeration of viable CD34+ cells demonstrated that washing did not reduce the number of these crucial cells. Washing also removed >90% of free haemoglobin (from lysed red cells) from harvests. The results demonstrate that washing of cryopreserved stem cells does not have an appreciable negative impact upon these parameters (compared to unwashed controls) and actually improves the outcome of several key parameters.

Our study demonstrates that a modified NYCBB stem cell washing protocol is a feasible clinical practice that should reduce the morbidity associated with the reinfusion of cryopreserved stem cells.

RETROSPECTIVE ANALYSIS OF OUTCOME FOLLOWING AUTOLOGOUS HAEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT) AFTER LONG-TERM (≥12) VERSUS SHORT-TERM (<12 MONTHS) CRYOPRESERVATION.

RA Harrup, E Tegg, RM Lowenthal.
Clinical Haematology/Medical Oncology Unit, Royal Hobart Hospital , TAS.

Autologous HSCT, using bone marrow, peripheral blood, or more recently umbilical cord blood as a source of stem cells is now a commonplace medical procedure. In certain circumstances cells may need to be cryopreserved for long periods. Nineteen patients reinfused following cryopreservation for a median of 21 (range 12-72) months were compared to controls matched for cell source, harvest era and priming protocol who were transplanted following cryopreservation for a median of 4 (range 1-11.9) months.

Results:	Patients	Controls	p value
Neuts ≥ 0.5 x 10^9/L	14 days	13 days	0.42
Plat ≥20 x 10^9/L	26 days	16 days	0.20
Plat ≥50 x 10^9/L	41.5 days	22 days	<0.006
Survival day +30	95%	84%	0.3071
day +100	79%	59%	0.2645

The difference in time to platelet recovery to ≥ 50 x 10^9/L did not lead to significant differences in blood product requirements. A significant continuous relationship was identified between length of cryopreservation and time to neutrophil and platelet recovery, p= 0.0196 and p= 0.0099 respectively, following long-term cryopreservation. CD34+ cells and/or GM-CFU doses were comparable between groups. HSCT can be performed safely using stem cells cryopreserved for ≥12 months. The relationship between length of cryopreservation and increasing time to haematopoietic recovery warrants further study.

ZINC FINGER PROTEINS IN THE CONTROL OF ERYTHROID GENE EXPRESSION

Merlin Crossley
Department of Biochemistry, G08, University of Sydney, NSW, Australia, 2006

The expression of erythroid genes is known to be regulated by a complex set of DNA-binding proteins but the precise mechanisms by which these transcription factors operate is poorly understood. We have focussed on two zinc finger proteins that are thought to play significant roles in the regulation of globin gene expression. The first, GATA-1, is a strong activator of transcription. It binds to GATA sequences in DNA through its C-terminal zinc finger and to the cofactor protein FOG (Friend of GATA) through its N-terminal finger[1]. The second, BKLF, is a repressor of transcription that binds to CACCC-box elements in DNA through its three zinc fingers and associates with a transcriptional co-repressor protein CtBP through its non-finger domain[2,3]. Interestingly, FOG also makes physical contacts with the co-repressor CtBP[4]. Certain CACCC-boxes within the promoters and enhancers of the globin genes are closely linked to GATA sequences, it is thus possible that protein combinations such as BKLF/CtBP/FOG are involved in repressing the activity of GATA-1 operating through adjacent elements. We will discuss experiments to test whether these proteins are involved in repressing specific globin genes during development.

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Globin gene switching.

W. G. Wood, MRC Molecular Haematology Unit, University of Oxford, UK.

It is widely accepted that continued expression or reactivation of fetal haemoglobin (HbF) in adult life would significantly ameliorate the severe genetic disorders of haemoglobin such as ß thalassaemia and sickle cell disease. A thorough understanding of the normal switch from fetal to adult haemoglobin should increase the potential for therapeutic intervention. Although there have been considerable advances in our understanding of the molecular basis of this process over the last few years, many of the details remain elusive.

The ß globin gene cluster on chromosome 11 is arranged in the developmental order of expression of the genes (5' ε-^{G}γ-^{A}γδ-β 3') and spans a region of ~100kb. Switching from γ to β gene expression occurs in the perinatal period and is unaffected by birth. Developmental regulation of globin gene expression lies at the transcription level and is determined at least as early as the BFU-E stage of erythroid differentiation.

High level expression of any of the globin genes is dependent on upstream regulatory sequences, the locus control region (LCR) that comprises four erythroid-specific DNase 1 hypersensitive sites (HSs 1 - 4). Each alone is capable of enhancing globin expression although all four are required for maximum levels of transcription. It is currently believed (but yet to be directly demonstrated) that the HSs form a holocomplex that interacts with the promoters of individual genes to drive transcription. Switching, therefore, will be determined by factors that differentially affect the access of the γ and β gene promoters to the LCR or the stability of the interaction once established. These factors include both trans-acting factors and cis-active epigenetic modifications of chromatin structure.

The search for developmentally restricted transcription factors that might be important for switching has revealed few candidates. The best characterised is EKLF which binds to the β globin gene CACC box and not to the similar element in the γ globin gene. EKLF is essential for β gene expression and mice in which the EKLF gene is knocked out die in utero of β thalassaemia. However, EKLF is also found at high levels in embryonic erythroblasts where β is inactive, suggesting that either the protein gets post-translationally modified later in development or that the β gene is not accessible to the factor in embryonic cells. More recently, a related protein (FKLF) that binds to the γ gene CACC box has been found in fetal but not adult erythroid cells. The importance of this factor will be established when its gene has been knocked out.

The analysis of the human β globin cluster in transgenic mice has been a crucial method for increasing our understanding of the switching process. The pattern of switching is variable and dependent on the size and structure of the construct. Developmental specificity partially resides in sequences in and around the γ and β genes themselves but this can be overcome by altering the order of genes in the fragment. If the β gene is moved closer to the LCR, it can be inappropriately expressed in embryonic life. Experiments in transgenic mice need to be interpreted with caution, however, since even large integrated fragments may be subject to position effects and it must be borne in mind that the mouse does not have a fetal haemoglobin so regulation may differ somewhat between the two species. Nevertheless, the fact that developmental expression of the γ and β genes can alter depending on the construct and epigenetic modifications demonstrates that trans-acting factors are not the sole determinants of which gene will be expressed.

The complexity of the processes involved in the normal switch from fetal to adult haemoglobin suggests that sustained reactivation of the fetal genes in adult life may not be simple to achieve.

AN OVERVIEW OF THE CYTOKINE NETWORK

A Kelso

The Queensland Institute of Medical Research and the Joint Transplantation Biology Program, University of Queensland, Brisbane

Cytokines participate in the induction and effector phases of all immune and inflammatory responses, and can promote or exacerbate disease in different circumstances. They are therefore obvious candidates for exploitation as drugs or drug targets to stimulate, limit or alter these responses in infection, allergy, autoimmunity and other disease states. Although some cytokines and related molecules are already in clinical use, the full therapeutic potential of this class of hormones has yet to be realized and this will depend in part on understanding their normal functions and regulation in health and disease. This overview will summarize recent advances in our knowledge of the cytokines and their receptors, their genetic and structural relationships, and the regulation of their activities by naturally-occurring antagonists and other physiological mechanisms. Some of the ways in which new therapeutic strategies are being developed from knowledge of the structure, function and regulation of these various components of the cytokine network will be outlined.

INDUCTION OF FETAL HEMOGLOBIN AS A THERAPY FOR THE HEMOGLOBINOPATHIES

Wen-lai Zhou, David R. Clouston, Melissa Holmes, John M. Cunningham and Stephen M. Jane

Bone Marrow Research Laboratory, Royal Melbourne Hospital Research Foundation, Parkville VIC 3050; Division of Experimental Hematology, St Jude Children's Research Hospital, Memphis TN USA 38101.

Our laboratory has focused on defining the molecular mechanisms governing the expression of the human fetal ( ) globin genes. The importance of these studies is emphasised by the observation that elevated levels of fetal hemoglobin are protective in patients with the devastating genetic disorders, sickle cell disease and -thalassemia. Thus, identification of the transcription factors that control -gene expression may lead to new genetic therapy approaches for these diseases. We have adopted two strategies in our search for these factors. The first is centred on biochemical and molecular approaches to define the stage selector protein (SSP), a key regulator of fetal gene expression. We have previously demonstrated that the ubiquitous transcription factor CP2 is a major component of this complex. We have now utilised the yeast two hybrid assay to clone the developmental and tissue restricted component of the SSP, a novel gene we have named NF-E4. Antiserum to NF-E4 ablates the binding of the SSP to the proximal -promoter. Preliminary functional analysis suggests that enforced expression of NF-E4 in fetal erythroid cells leads to a marked induction of fetal globin expression. Further characterisation of the molecular role of NF-E4 in the regulation of fetal globin gene expression in primary human erythroid cells and in animal models is in progress.

Our second strategy to define new fetal globin regulatory genes utilises novel pharmacological compounds capable of inducing -globin expression. This approach has led to the identification of the HLH factor Id2 as a new globin regulatory factor.

DIAGNOSIS, CLASSIFICATION AND THERAPY OF VON WILLEBRAND DISEASE

Evan Sadler

Von Willebrand factor (VWF) is a large, multimeric blood protein that performs two major roles in hemostasis; it mediates the adhesion of platelets to sites of vascular injury, and it is a carrier protein for blood coagulation factor VIII. Inherited defects in VWF therefore may cause bleeding by impairing either platelet adhesion or blood clotting, resulting in von Willebrand disease (VWD). The characterization of mutations in VWD has provided useful insight into the synthesis, structure, and function of VWF. This growing body of information has prompted a reclassification of VWD types. The three major categories are partial quantitative deficiency (type 1), qualitative deficiency (type 2), and total deficiency (type 3).

VWD type 1 typically is an autosomal dominant disorder and patients usually have mild bleeding symptoms. VWD type 3 is an autosomal recessive disorder that is characterized by severe bleeding throughout life. Although the mechanism of VWD type 3 does not differ fundamentally from that of VWD type 1, “type 3” receives a separate label because it is distinguished by exceptional clinical severity and distinct therapeutic requirements. Qualitative defects in VWF (VWD type 2) are divided into four subtypes: 2A, 2B, 2M, and 2N. VWD type 2A is characterized by decreased platelet-dependent function that is associated with the absence of high molecular weight multimers. VWD type 2B refers to variants with increased affinity for platelets; such patients often have thrombocytopenia. VWD type 2M (“M” for “multimer”) refers to qualitative variants with decreased platelet-dependent function despite the presence of high molecular weight multimers. This phenotype may be caused by mutations that specifically inactivate the major binding site for platelet glycoprotein Ib. VWD type 2N refers to qualitative variants with markedly decreased affinity for factor VIII. The “N” is for “Normandy”, the province in France where one of the first patients identified with this variant was born. Such patients have isolated factor VIII deficiency and have been misdiagnosed with hemophilia A. These VWD categories correlate with the efficacy of standard therapeutic interventions, such as administration of desmopressin or clotting factor concentrates.

The cell biology of VWF provides a useful framework for understanding the pathophysiology of VWD. The biosynthesis of VWF is unusual because it involves the intracellular assembly and storage of enormous multimers, and it requires the formation of disulfide bonds in the acidic environment of the Golgi apparatus. Some of the structural requirements for targeting and disulfide assembly have been characterized. Depending on which step in this complex process is affected, VWF mutations can have a dominant effect and produce several distinct VWD phenotypes. Retention of mutant subunits in the ER appears able to cause relatively severe quantitative deficiency (VWD type 1), whereas mutant subunits that make it as far as the Golgi can cause qualitative defects. For example, mutations that interfere with multimer assembly or stability cause VWD type 2A. Mutations that inactivate specific ligand binding sites, or disrupt the regulation of binding to platelets, cause distinct VWD phenotypes that clearly illustrate the biological importance of VWF-dependent platelet adhesion and factor VIII stabilization.

Although our understanding of its pathophysiology has advanced, VWD remains a diagnostic problem because the bleeding symptoms are not specific, and heterozygous transmitters of VWD may be phenotypically normal. Many aspects of the biological function of VWF are poorly understood. These include the mechanism by which binding of VWF to platelets is induced at sites of vascular injury, and the factors that influence the likelihood of bleeding symptoms in patients with VWD type 1.

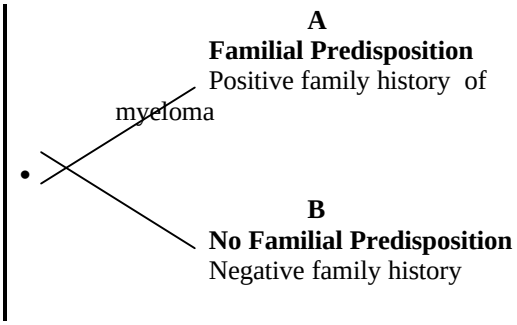
The aetiology of myeloma

Brian G. M. Durie, Director of Research and Myeloma Programs Cedars Sinai Comprehensive Cancer Center, Los Angeles, CA 90048

The development of myeloma is multifactorial and multistep. At least three types of processes are involved: 1. **DNA damage** to the idiotypic clone of origin; 2. **Antigen triggering** of the damaged clone; 3. **Abnormal regulation** of the expanded idiotypic clone. Specific factors involved range from carcinogenic chemicals, ionizing radiation, oncogenic and/or triggering viruses or other infectious agents to the complex interplay between cytokines, hormones, adhesion molecules, angiogenic factors and bone cells to name a few. Genetic susceptibility is influenced by several polymorphic genes such as P450 and GST (involved with activation and catabolism of toxic chemicals), HLA/MHC and related immune response genes, plus racially variable ALU sequences. Despite the complexity some correlations with chemicals e.g. herbicides/pesticides/dioxins and radiation exposure have nonetheless emerged. However, up to now, the multifactorial, multistep nature of myeloma has not been explicitly considered in epidemiologic studies often making negative associates invalid. This combined with the inherent biases, confounding variables and measurement errors makes traditional epidemiology of a rare disease such as myeloma potentially futile. New strategies include "cluster" analyses combined with tissue analyses and molecular/susceptibility studies and a search for the organ or tissue of origin of myeloma in individual cases. Recent successful cluster analyses include dioxin exposure linked to 5 clusters and radiation exposure linked to Pacific atomic tests. Tissues of origin of myeloma include at the site of implants (e.g. breast, pacemaker), appliances (e.g. denture adhesive) and focal tissue injury (e.g. infection, electrical burn). Coexistence of numerous infections is linked to myeloma including HIV (odds ratio 4.5), hepatitis B and C, herpes zoster and pneumococcal infection. Whether or not any specific infection such as HHV-8 or some new mutant virus (e.g. "stealth") is involved remains to be seen. Nonetheless it should prove helpful to classify myeloma by potential causal factors e.g.

Risk Factors

1. **Chemical** e.g. dioxins, solvents, heavy metals, others
2. **Radiation** e.g. ionizing, other
3. **Virus** e.g. HIV, Hepatitis C, HHV-8, others
4. **Other** e.g. EMF, other



In the future such classification will guide not just prognostic assessment and treatment selection, but potential preventive strategies.

HIGH DOSE THERAPY AND STEM CELL TRANSPLANTATION IN MYELOMA

D Joshua and J Gibson. *Institute of Haematology, Royal Prince Alfred Hospital, Sydney Australia.*

Multiple myeloma remains an incurable disease with little improvement in survival being achieved despite over three decades of trials with conventional chemotherapy protocols. Multi-agent protocols have not been definitively demonstrated to be superior to melphalan alone and alpha interferon is of relatively minor importance. There appear to be very few "new drugs" on the therapeutic horizon with the exception of the anti-angiogeneic agents such as thalidomide. The failure of conventional dose therapy to prolong survival has spurred interest in high dose therapy. The rationale for the use of high dose therapy relates to the demonstration of a drug dose-response relationship in myeloma such that increased dose will lead to cure or prolonged remission. In addition, in patients undergoing allogeneic transplantion a graft-versus-myeloma effect is now well recognised and believed to be a contributor to the response. Currently the ratio of patients undergoing autologous as opposed to allogeneic transplantation for myeloma is approximately 3:1 (IBMTR data). The high toxicity of allogeneic transplantation in this setting has to date limited its use. Data from the EBMTR long term follow up study of allogeneic transplantation in myeloma suggests that at nine years less than 20% of patients are alive and many of these still have residual disease. A recent matched pair analysis performed by the EBMTR also demonstrated a superior survival for patients undergoing autologous transplants compared to those undergoing allogeneic transplants. Pivotal to autologous transplantation development in myeloma was the Attal study which demonstrated that a single (bone marrow) transplant was superior to conventional multi agent chemotherapy both in terms of overall survival and disease-free survival. Subsequent randomised studies have looked at the issues of one versus two transplants and early versus late transplantion. As yet both studies show no difference in overall survival although follow up is still relatively short.

NETCORD EXPERIENCE IN CORD BLOOD BANKING E. Gluckman for Netcord-Eurocord organisation. Hôpital saint Louis. Paris France.

As more than 1,000 unrelated cord blood transplants have been performed world-wide, the number of cord blood banks has been increasing with more than 25,000 units collected and available for unrelated donor hematopoietic stem cell transplant searches. The main advantages of cord blood transplants are the large donor pool, the low incidence of viral infection at birth, the low incidence of graft versus host disease due to the immune immaturity of the new-born and the increased speed of search as the units are readily available, having been previously tested and cryopreserved. Compared to unrelated bone marrow transplants where a complete HLA identity for class I and class II antigens are required, most of the cord blood transplants have been performed with donors having 1, 2 or 3 HLA antigens mismatches. Clinical analysis has shown that in fact the most important factor predicting the outcome of the transplant is the number of nucleated cells infused which must be superior to $3X10^7$/kg. Placental/umbilical cord blood banking for unrelated transplantation, being a relatively new field, it is very important to set up minimum standards and reach an international agreement on aspects essential for the safety of the donor and the mother and the best possible chance for the recipient. For this purpose, Netcord was founded in 1998, it currently includes experienced cord blood banks (Barcelona Spain, Denver USA, Duesseldorf Germany, Leiden Netherlands, London UK, Milano Italy, New-York City USA, Paris France, St Louis USA and Tokyo Japan). The joint inventory available on a single research file currently contains 28,650 validated units, 1,017 have been transplanted to paediatric and 272 to adult patients. The Board of directors of Netcord has developed a detailed set of standards for cord blood banking. These include respective national and international regulatory aspects and meanwhile have been submitted to FACHT (USA) for accreditation. Furthermore a joint allocation system employing most recent internet technology has been implemented to facilitate rapid allocation of cord blood units according to histocompatibility and number of nucleated cells within an average time of 48 hours. In conclusion, Netcord has achieved to provide a world wide and rapid service of equally high quality standard to the international transplant centers.

Results of cord blood transplantation from the Eurocord Netcord Registry. E. Gluckman Head of bone marrow transplant program Hopital Saint Louis .Paris France.

E. Gluckman for Eurocord Hopital saint Louis Paris France

Cord blood is increasingly used as a new source of hematopoietic stem cell transplant, from 10/88 to 07/99, 485 cord blood transplants (CBT) have been reported to the Eurocord Registry from 110 transplant centers from 30 countries in and outside Europe. The donor was related in 126 cases and unrelated in 349 cases. Most of the patients were children (n=376) and 99 adults only were reported. In related cord blood transplants, the overall 2-year survival was 64%, it was 55% in children with malignancies, 67% in children with aplastic anaemia and 71% in inborn errors. In acute leukemia survival was 55% in children transplanted in first or second complete remission. Factors influencing survival were age, weight, number of cell infused, sex match and number of nucleated cells infused. The degree of HLA disparity did not influence survival. Engraftment depended on the number of cells infused. The incidence of GVH was decreased when compared to bone marrow transplants, it was influenced by HLA disparities. In unrelated cord blood transplants in children, the 2 year survival was 35%. Factors associated with survival were diagnosis, CMV serology and ABO match. Neutrophil and platelet engraftment were delayed. The speed of engraftment was related to the number of cells infused and the degree of HLA disparity. The incidence of acute GVHD was reduced, it was not associated with the number of HLA disparities. In adults survival was 20%. The prognosis was related to the status of the disease and to the number of cells infused. None of the patient who received less than $1x10^7$ nucleated cells /kg survived. Preliminary studies show that immune reconstitution was faster than after a mismatched bone marrow transplantation. The follow-up is too short to evaluate the incidence of relapse in malignant diseases. We therefore focus our analysis by comparing cord blood transplants with other source of stem cells. The first analysis compared the outcome of HLA identical related bone marrow transplant to cord blood transplants in children in order to test the hypothesis that the risk of GVHD is lower after CBT compared to BMT because of the immaturity of umbilical cord blood cells. We studied in children 113 related HLA identical sibling CBT reported to Eurocord and the International bone marrow Registry (IBMTR) compared to 2,052 HLA identical sibling BMT performed during the same time period. Several pretransplant variables were different and were adjusted in the Cox analysis. Multivariate analysis demonstrated lower risks of grade II-IV acute GVHD (RR 0.40 p=0.001 and chronic GVHD (RR 0.35 p=0.02) in CBT recipients. Neutrophil recovery was significantly delayed after CBT (RR 0.40 p=0.0001) as was platelet recovery (RR 0.20 p=0.0001). Survival was similar in the two groups(RR 1.15 p=0.43) These clinical results are consistent with the hypothesis that immaturity of the neonatal immune system leads to decreased alloreactivity in the transplant setting.
We then compared the outcome of unrelated CBT (n=99) to unrelated BMT (n=416) in children with acute leukemia transplanted from 01/94 to 05/98 in 51 centers. The donor was HLA mismatched in 91% CBT and 29% BMT. T cell depletion was performed in 43% BMT. After adjustment relative risks of outcomes showed no differences between both groups for overall survival (RR 1.27 P=0.16), event free survival (RR 1.27 p=0.15), acute GVHD$\geq$II (RR 0.98 p=0.92). The incidence of grade III-IV GVHD was 9% after TD-BMT, 21% after CBT and 29% after unmanipulated BMT (p=0.0001).There was no difference on the incidence of relapse (RR 0.79 p=0.37). Neutrophil recovery was significantly delayed after CBT RR 0.42 p=0.0001 and this could explain an increased 100 days transplanted mortality (.RR 2.32 p=0.0015) In conclusion, this study shows that cord blood from related or unrelated, matched or mismatched donors can be used to treat children with various haematological diseases with results analogous to other sources of hematopoietic stem cells. Engraftment is delayed, the number of cells required to obtain a successful engraftment is superior to $3x10^7$ nucleated cells/kg. Ex vivo progenitor cells expansion is currently Investigated. The incidence of GVH is reduced even in mismatched transplants. This outcome was predicted by the properties of the lympho hematopoietic system at birth characterised by naivete, immaturity and enrichment with immature hematopoietic stem cells.

Design of GMP compliant cell therapy facilities.

DM Wall, HM Prince. *Division of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, East Melbourne, Victoria 3002*

The Peter MacCallum Cancer Institute has recently built a facility suitable for production of gene therapeutics as well as to allow other forms of cell manipulation, such as blood cell expansion and high-speed therapeutic cell sorting by flow cytometry. Whilst low level manipulation in the context of closed processing may only require generic laboratory facilities, we shall describe here designs for GMP compliant facilities intended for higher orders of cell manipulation such as gene therapy.

We have applied a hybrid regulatory approach:

- Production of a safe therapeutic. Designs incorporated the requirements of AS1386:1989 to produce Class 350 areas associated with Class 3.5 workzones within Class II biological safety cabinets.
- Protection of staff, the public and the environment. Designs also followed the provisions of PC3 physical containment as described in the Guidelines for Small Scale Genetic Manipulation (1998) so that this facility would be suitable for Category A procedures.

Further requirements were that these facilities could be flexible, so that the five processing areas could change function from being a clean area to being a clean and contained area, and vice-versa. One of the primary complications was how to address the incompatible differences between Class 350 and PC3 facilities. The hybrid approach of using Class 350 and PC3 designs is relevant to any centre intending to produce gene therapeutics, especially with the developing interest of the TGA in regulating the facilities producing such therapeutics. We will discuss further the specific issues related to combining the requirements for clean areas with containment, with an obvious focus upon the conflicting requirements.

REGULATION OF THE AUSTRALIAN BLOOD SYSTEM – CURRENT AND FUTURE PERSPECTIVES

Albert Farrugia
Senior Advisor on Blood Products, Therapeutic Goods Administration, P.O Box 100, Woden ,A.C.T,2606, Australia

Australia's federal system of Government allocates different responsibilities to the Commonwealth and the States. In the regulation of the blood system, the Commonwealth has historically assumed the responsibility for the safety and efficacy of pooled plasma products while the States have been responsible for the cellular and small pool components of blood. As a result of intense consultation between Governments, stakeholders and the TGA, the Australian Health Ministers Advisory Council agreed in April 1999 that the regulation of all components should be subject to the *Therapeutic Goods Act 1989*. This presentation will discuss the process whereby the TGA has formulated its regulatory strategy for components and the principles which will be used to assure standards in this part of the blood system.
Another aspect of Australia's blood system involves Australia's commitment to the principle of national self-sufficiency in blood products, in conformance to international obligations. The integration of this principle with policies ensuring blood safety and supply will also be discussed. An integrated approach to the management of the blood supply and its regulation will be proposed.
[The views of the author are not necessarily those of the Therapeutic Goods Administration]

WHAT IS VON WILLEBRAND DISEASE TYPE 1?

Evan Sadler

An enduring mystery regarding VWD is to explain how the mutant alleles in VWD type 1 cause a dominantly inherited bleeding disorder without seeming to affect the structure or function of VWF. For most hemostatic proteins, one functional allele is sufficient to confer a normal phenotype. In contrast, VWD type 1 seems to be caused by heterozygous, partial, quantitative deficiency of VWF. Symptomatic VWD type 1 is the most common form of VWD, affecting approximately 125 per million population at most. This immediately poses a problem, because the typical figures for the prevalence of recessive VWD type 3 (0.5 per million) require the prevalence of obligate heterozygous individuals to be „1400 per million. Type 3 VWD usually is caused by deletion, frameshift, and nonsense mutations; therefore, inheritance of a single null VWF allele cannot cause symptoms in most persons. In fact, population screening indicates that heterozygous persons with few or no symptoms are extremely common. What, then, is the cause of bleeding in VWD type 1? Several explanations have been proposed, including differences among normal VWF alleles, the influence of genotype at other loci and nongenetic factors on VWF levels, unsuspected compound heterozygosity for VWD mutations, and dominant negative VWD type 1 alleles.

A more fundamental problem is that VWD type 1 can be difficult to identify consistently, so that misdiagnosis is a constant hazard. The bleeding symptoms of VWD are not specific and may occur with any cause of platelet dysfunction. Some symptoms, such as bruising and menorrhagia, occur frequently in apparently normal persons. Low VWF levels in the blood can be hard to evaluate because the normal range of VWF concentration is very broad. Therefore, bleeding symptoms and low VWF levels are individually common and may occur together by chance. The case for VWD is strengthened by evidence that bleeding symptoms and VWF deficiency are coinherited, but in practice the diagnosis often is uncertain. Assigning a diagnosis of VWD is not necessarily harmless to patients because it may delay necessary surgery, lead to needless laboratory testing, raise difficult questions about the transmission of a genetic disease, and cause difficulties in obtaining medical insurance. We need a better approach to patients with mild mucocutaneous bleeding symptoms and borderline low VWF levels.

DOSE INTENSITY IN NON-HODGKIN'S LYMPHOMA (NHL)

M Wolf, Division of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, Melbourne

The CHOP regimen remains the generally accepted standard treatment for NHL having been shown in a prospective, randomised Phase III trial conducted by SWOG and ECOG to be equivalent to the more intensive third generation chemotherapy regimens. However in the ANZLG trial comparing CHOP to MACOP-B, long-term follow-up indicates a significant advantage for MACOP-B in terms of failure-free and overall survival.

In several retrospective analyses, dose intensity was found to be an important determinant of outcome in NHL. One prospective randomized study specifically addressing the importance of dose intensity randomized patients to BACOP therapy given either in standard dosages or a regimen with an increased dose of doxorubicin. No differences were seen in the response rate, overall survival or disease-free survival. The ANZLG has recently completed a Phase III study in 250 patients comparing standard dose CEOP to high-dose CEOP with filgrastim. Preliminary results of this study will be presented.

Another strategy for improving the results of lymphoma therapy involves the incorporation of stem cell transplantation into the treatment protocol. Patients with chemosensitive first or subsequent relapse have been shown in a randomized study to benefit from this approach compared to conventional chemotherapy. In newly diagnosed 'poor-prognosis' patients a number of recent randomized prospective trials have compared upfront high dose therapy to conventional induction therapy. The studies report conflicting results and additional definitive trials are in progress around the world. The current status of high-dose therapy in NHL will be discussed.

Globin gene regulation during erythropoiesis.

W. G. Wood, MRC Molecular Haematology Unit, University of Oxford, UK.

Globin gene transcription is a late event in the differentiation pathway from the haemopoietic stem cell to the erythroblast yet it is now becoming clear that many of the important regulatory steps that lead to globin gene expression take place in the early stages of this pathway. These steps include cis-active, epigenetic modifications to the chromatin structures of the genes and the sequential activation or repression of critical transcription factors. A complete description of the events involved in this process, and hence a better understanding, will require the development of new experimental systems.

It has become clear that the closely related and globin gene clusters show surprising differences in their structures and regulation. The cluster is telomeric, GC-rich, full of CpG islands and interspersed among several housekeeping genes that are themselves widely expressed in many tissues. It is in a generally open chromatin conformation in all tissues although DNase 1 hypersensitive sites at the regulatory element (HS -40) and the gene promoters are erythroid specific. The cluster, in contrast, lies in AT-rich DNA, has no CpG islands and is in an inaccessible chromatin conformation in all non-erythroid cells. It is not closely flanked by other genes and appears to occupy it own domain which has to be opened up before the genes can be expressed. These differences between the clusters are also apparent when they are expressed in transgenic mice. The intact cluster (~100kb), including its more complex regulatory region (LCR) is expressed at the same level as the endogenous mouse genes and is unaffected by the position of integration whereas even large fragments (>150 kb) containing the cluster show suboptimal expression levels and are subject to position effects.

The regulatory regions and the genes of both clusters share binding sites for many ubiquitous and erythroid restricted transcription factors that are necessary for their activation. Analysis of the concentration of these factors during the later stages of differentiation allows one to monitor changes in these factors at the time when the globin genes, as well as other erythroid specific genes, are activated. In addition, both the - and -like genes require at least one factor that is gene specific. Loss of the EKLF transcription factor severely reduces globin expression without affecting the genes while mutations in the ATRX protein have the opposite effect.

The complex cis- and trans-acting processes that are necessary for the coordinated expression of the genes are initiated much earlier in the developmental and differentiation history of the cell than the time at which the globin genes are actively transcribed. When DNA fragments containing the whole clusters are transferred into erythroid cell types, gene expression is dysregulated relative to when intact chromosomes are transferred into the same cells. Only in embryonic stem cells are the two equivalent, implying that epigenetic modifications to the DNA are required for proper regulation and that these modifications are acquired early in the differentiation history of the cell.

A more complete analysis of these early events has been hampered by the limited availability of adequate numbers of haemopoietic progenitor cells. Transformed cell lines are, at best, a poor substitute. The mouse MPLV virus produces a wide range of multi- and uni-lineage progenitor cell lines, all immortalised in the same way. These lines confirm that structural modifications of the globin genes occur at the earliest stages of differentiation that can be examined and demonstrate alterations in the levels of various transcription factors as lineage potentials become progressively restricted. Such results need to be confirmed in primary progenitor cell populations and experimental systems capable of producing inducible proliferation of these cells without differentiation and without immortalisation are currently being developed.

DETECTION OF HEPARIN-DEPENDENT ANTIBODY BY ENZYME-LINKED IMMUNOFILTRATION ASSAY

CM Vun. *Haematology Department, SEALS, Barker St, Randwick, New South Wales 2031, Australia.*

Heparin-dependent thrombocytopenia (HIT) is a potentially fatal disorder if not recognised promptly. As heparins and heparinoids are widely used clinically, it is important to confirm or exclude HIT by rapid and accurate laboratory test. Functional assays like serotonin release assay, platelet aggregation and flow cytometric detection of platelet activation, depend on the availability of donor platelets which are highly responsive to HIT antibody so as to minimise false-negative results. In the past five years, detection of anti-heparin-PF4 antibodies using solid phase microtitre plates has been advocated as superior to other assays. However, the main problem with ELISA detection of anti-heparin-PF4 antibody is its poor specificity and sensitivity. This is likely due to the altered conformation of PF4 when bound onto microtitre plate. An immunofiltration assay based on heparin-PF4 complex coated onto positively charged nylon membrane is developed to overcome this shortcoming. It also has an added advantage of short performance time. The assay is evaluated by comparison with serotonin release assay. Of 60 HIT patients confirmed by the serotonin release assay, anti-heparin-PF4 antibody is detected in all. The majority of these HIT antibodies are IgG, whilst those few with barely detectable HIT IgG have higher levels of HIT IgA. Detection of IgM antibody is not diagnostic of HIT as this is often detected in healthy and non-thrombocytopenic individuals. Of 120 patients with negative serotonin release assay, less than 5 % have just detectable levels of anti-heparin-PF4 antibody. ELIFA is preferable to ELISA as the membrane-bound PF4 maintained the native conformation crucial for the tri-molecular interaction with glycosaminoglycan and HIT antibody. This assay may serve as a substitute for the serotonin release assay until a suitable peptide mimotope is found for the HIT antibody.

IMPROVED DIAGNOSIS OF HEPARIN INDUCED THROMBOCYTOPENIA BY MODULATION OF PLATELET FcγRII REACTIVITY.

Ross Baker, James Thom and Mark Hogarth.
Coagulation Unit, Haematology Department, Royal Perth Hospital, University of Western Australia, Perth, and Austin Research Institute, Melbourne, Australia.

Binding of $F(ab)_2$ fragments of a non IgG blocking, anti FcγRII antibody 8.2, enhances FcγRII dependent platelet activation. In the two point heparin induced platelet aggregation assay (0.5 and 100U/ml heparin), $8.2(ab)_2$ pre-incubation in PRP increases the sensitivity of the variable platelet response without loss of specificity. We prospectively studied 101 samples referred with a possible diagnosis of HIT. Comparison between the standard HIT platelet aggregation, the 8.2 modification and a heparin- PF4 (HPF4) ELISA assay was performed.

HPF4	HEPARIN DEPENDENT PLATELET AGGREGATION			
	positive	***8.2 only***	***negative***	***total***
positive	19 (70%)	6 (46%)	15 (24.6%)	40
negative	8 (30%)	7 (54%)	46 (75.4%)	61
total	27	13	61	101

We found $8.2(ab)_2$ pre-incubation substantially increased the positive platelet aggregation result from 27 to 40 out of 101. There was an increased concordance between aggregation and HPF4 results after $8.2(ab)_2$ pre-incubation. 15 samples were positive with HPF4 only. It is likely that this group represents IgA or IgM antibodies that do not cause FcγRII dependent platelet activation. Conversely another 15 samples were only detected by aggregation and may represent FcγRII dependent antibodies directed against alternative but similar antigens such as IL-8 or NAP-2. In the 13 samples uniquely diagnosed with 8.2, only 46% had heparin PF4 antibodies. These results suggest the 8.2 modification improves sensitivity of the platelet aggregation test and detects additional antibodies not detected by the HPF4 assay. Performing both tests provide useful and complementary diagnostic information for immediate decisions in patients with suspected HIT.

HYPERCOAGULABLE MARKERS IN VENOUS THROMBOEMBOLIC DISEASE

M. Kalev, S. Bhattachatyya, T. Day, N. Van de Water, A. Bennett, P. Ockelford

Venous Thromboembolism Unit, Haematology Department, Auckland Hospital, Auckland, New Zealand

Patients referred to Auckland Hospital with suspected venous thromboembolism (VTE) are all managed through the Thrombosis Unit following objective confirmation of the diagnosis by compression ultrasound, or V/Q scan/ angiography as appropriate. The management protocol is primarily based on outpatient treatment using the LMWH – Enoxaparin followed by six months of secondary warfarin prophylaxis. Whenever possible patients completing warfarin therapy prospectively undergo a full series of thrombophilia studies. The objective of this study was to determine the usefulness of thrombophilia screening in a routine outpatient setting. Patients were subdivided into those having an acute risk factor, namely a "provoked" or secondary thrombosis, or an idiopathic event. There were 223 patients (123female/ 99male), median age approximately 55 years, tested to December 1998. Of these 164 had DVT+/- PE and 59 PE alone. The events were idiopathic (primary) in 39%(N=86) and secondary in 61%(N=137) of the patients. The commonest secondary events were recent surgery 20%(45), malignancy 19%(43), trauma 15%(33), air travel 11%(24) and recent hospitalisation 4%(9). 19(15%) of the females were OC users. Thrombophilia studies were completed in up to two thirds of the patients. The most frequent abnormality was an elevated factor VIII>150% (52.3%); significant reduction in any of the inhibitors (ATIII/PrC/PrS) was observed in 4.7% of those tested. Activated protein C resistance/ FVLeiden was common (23%) with the prothrombin variant seen in 12% of cases. Features consistent with lupus-like anticoagulant were observed in 10.7% and a potential defect in fibrinolysis in almost one third of those studied.

These results indicate that prothrombotic abnormalities are common in an unselected group of patients presenting to an acute hospital. The relevance of many of these abnormalities for long term management remains uncertain.

HOMOCYSTEINE IN HEALTH AND DISEASE: A REASSESSMENT

NPB Dudman

Centre for Thrombosis and Vascular Research, Prince Henry Hospital, University of New South Wales, Sydney, NSW.

Moderate hyperhomocysteinemia is a familiar finding in occlusive vascular disease. Homocysteine has been thought to cause vascular disease, while increased homocysteine levels in these patients have been ascribed to various genetic abnormalities and vitamin deficiencies that decrease homocysteine catabolism, as well as to renal disease. However there are several observations that do not fit with these conclusions. In the present study vascular occlusion is proposed to *result in* hyperhomocysteinemia, when post-occlusion processes upregulate homocysteine-producing methylation reactions. Experimentally, moderate increases in homocysteinemia induce leukocytes to adhere to and migrate through the vascular wall in anaesthetised rats, and stimulate cultured neutrophils and monocytes to become cytolytic (Dudman et al, Circulation Research;84;409;1999). These findings suggest that homocysteine released from ischaemic tissues and from trapped blood may be involved in recruiting the leukocytes that infiltrate and remodel tissues following an occlusive event. In this way, moderate hyperhomo- cysteinemia is hypothesised to be a natural part of the healing process in occlusive vascular disease, and as such may be beneficial. The possibility that homocysteine priming of neutrophils and monocytes in ischaemic tissues could set the stage for reperfusion injury is also considered. In the absence of reperfusion, it is proposed that moderate hyperhomocysteinemia is not necessarily pathogenic to the vasculature.

THROMBOPHILIC GENOTYPES IN LUPUS ANTICOAGULANT PATIENTS. J. Moore*, M.Aboud, K.Cheong*, L.Coyle and D.D.F. Ma*. *Haematology Department, St.Vincents Hospital* and Royal North Shore Hospital, NSW*.

The lupus anticoagulant (LA) is a common acquired thrombotic risk factor present in 2% of the population and up to 14% of patients presenting with their first DVT. However, not all LA patients actually develop thrombotic disease. We hypothesised that recently recognised thrombophilic genotypes may account for this discrepancy. We retrospectively assessed 67 LA patients in our hospital between 1991-1996 and performed PCR for FV Leiden, the prothrombin G20210A and MTHFR mutations in both LA and control patients (n=64). We then correlated this with various clinical parameters. 42 patients (23F:19M, 21.4% SLE) had a "persistent LA" (>2 positive LA, 3 months apart) and 25 no longer had a LA present ("transient LA" – 17F:8M, 16% SLE). Persistent LA patients were significantly older compared to transient patients (52.9 ± 17.8 yrs vs. 40.2 ± 15.9yrs, respectively, p = 0.005). The rate of thrombosis was similar in both groups (52.3% in persistent LA and 48% in transient LA). The prevalence of thrombophilic genotypes is outlined in the table below:

	Persistent LA (n = 42)	**Transient LA (n = 25)**	**Control (n = 64)**
FV Leiden (%)	4.8	16#	3.1
G20210A (%)	4.8	4	9.4
MTHFR TT (%)	19*	12	3.1

** p= 0.01 for MTHFR homozygote compared to controls*
 # p= 0.05 for FV Leiden compared to controls(Fisher's exact test).

The increase of MTHFR homozygotes in patients with a persistent LA did not correlate with an increased rate of thrombosis in these patients (only 2/8 patients). In addition, the increased rate of FV Leiden in the transient group did not correlate with increased thrombosis. From this data it would appear that these common thrombophilic genotypes are not an additional risk factor for thrombosis in LA patients.

RANDOMISED BLINDED COMPARISON OF BUFFY COAT PLASMA OR T-SOL SUPPORTED PLATELET TRANSFUSIONS.
RA Harrup, JT Kennedy*, J Kiss*, B Daniels.
*Department of Haematology, Royal Hobart Hospital (RHH), TAS; *Australian Red Cross Blood Service-Tas, Hobart, TAS; Department of Psychological Medicine, RHH, TAS.*

T sol is a platelet additive solution produced by Baxter and currently licensed in Australia. It is used for approximately 100 000 platelet transfusions annually in Finland and some other Scandinavian countries. Abundant laboratory data is available to suggest that T Sol^R is an equal or better platelet support medium than plasma however little clinical data, and no randomised data, comparing the clinical efficacy of plasma supported versus T Sol^R supported transfusions has been published. All patients requiring platelet transfusion, as determined by the treating physician, will be randomised via an envelope to either plasma or T Sol^R supported group specific platelets which are otherwise visually indistinguishable and have been produced using identical methods and personnel. Each transfusion will be treated as a separate event and be administered according to existing hospital guidelines which do not include routine pre-medication. Equivalence will be assessed with respect to non-haemolytic febrile transfusion reactions, immediate platelet increment, 12-24 hour platelet increment and episodes of clinically significant bleeding. We hypothesize that the true treatment difference, using two sided confidence intervals, lies between a lower and upper equivalence margin of the clinically acceptable difference in efficacy of 10%. If this is confirmed then platelet production in Tasmania will switch to T Sol^R supported, permitting increased plasma supplies for fractionation.

THROMBOPOIETIN MOBILISED AUTOLOGOUS PLATELET TRANSFUSION

M. Bentley, K. Taylor, S Wright, R Rodwell, C. Kelly, D Taylor and A Kerwick., *Mater Hospital, Brisbane.*

Availability of thrombopoietin has renewed interest in autologous platelet support for intensively treated malignancy. We pursued this approach in a 38 year old female patient with AML who received Thrombopoietin - Megakaryocyte Growth and Development Factor with induction chemotherapy. Her course was complicated by severe haemorrhage and platelet refractoriness. The patient's platelet type was HPA-1a positive and platelet alloantibodies to HLA Class I were detected. Autologous platelet apheresis was performed on days 28 and 29 (venous platelet counts $856x10^9$/L and $558x10^9$L). The collections were divided into 16 aliquots (median platelet dose of $2.3x10^{11}$/unit [range, 2.1-2.44]), irradiated, cryopreserved with saline and 10% DMSO, controlled rate frozen to -100 ^oC and stored in vapour phase of liquid nitrogen. During first consolidation, autologous platelets were administered on day 15 (platelet count $6x10^9$/L) following failure to increment to fresh single-donor platelets. Post-thaw recovery was 52% which resulted in a reinfused platelet dose of $2.3x10^{11}$. Reinfusion was well tolerated with 1 hour count being $21x10^9$L (CCI=11.6m^2/μL); at 24 hours $29x10^9$L. No other transfusions were required. During second consolidation, autologous platelets were administered for vaginal bleeding following inadequate boost to random-donor platelets (CCI=5m^2/μL) with excellent increment and clinical response. No further platelet transfusions were required. Platelet aggregation studies were normal prior to freezing and post thaw. Transfusion of thrombopoietin mobilised autologous platelets may overcome alloimmunisation, negate leucocyte depletion/filtration, avoid transfusion-associated GVH, in addition to providing effective, sustained increments. We are now proceeding on a phase II feasibility autograft study using this approach.

REPETITIVE HIGH-DOSE THERAPY IS ASSOCIATED WITH SUBSTANTIAL REQUIREMENTS FOR SINGLE DONOR AND HLA-MATCHED PLATELETS.

HM Prince, D Westerman, P Gates, S Juneja, JF Seymour, M Wolf, H Januszewicz, M Brettell, M Quinn, D Wall, R Maisano, P Gates, M Brettell. *Blood and Marrow Transplant Service, Div. Haematology and Medical Oncology, Peter MacCallum Cancer Institute, Melbourne, Victoria.*

High-dose therapy (HDT) with peripheral blood progenitor cell (PBPC) support for the treatment of solid tumours is being actively investigated. However, an important complication of this therapy is the induction of severe thrombocytopenia, requiring aggressive platelet (plt) support. Furthermore, with the use of repeated cycles of HDT, there is the potential to develop platelet antibodies with subsequent refractoriness to random donor platelets (RDP). We sought to determine the plt support requirements of 69 consecutive pts (68 female) with metastatic breast cancer (MBC) treated with a planned 3 cycles of HDT (total of 177 cycles). Bedside blood product filtration was not routinely used. All cellular blood products were irradiated. There was a median number of 3 plt transfusion episodes [1 episode ≈ 5U RDP ≈ 1U single donor platelets (SDP)] per cycle (range:1-37). SDP were required during 26 cycles (14.6%) in 19 pts (27%). Eight-eight separate donor aphereses were performed to supply these SDP, with a median of 2 separate donor aphereses per SDP-requiring cycle (range:1-11). The requirement for SDP increased substantially during the 2nd and 3rd cycles: cycle 1=2/69 cycles (2.9%), cycle 2=15/63 cycles (24%) and cycle 3 =10/45 cycles (22%). HLA-matched platelets were required during 15 cycles (9%) in 14 pts (20%). In an attempt to predict the requirement for SDP/HLA-matched plts, we routinely examined for the presence of HLA (Class I) Abs prior to HDT (n=64pts tested). HLA Abs were detected in 10/69 (14.5%) (range of HLA Abs = 6% to 96%) pts prior to any HDT. Of these 10 pts, 5 ultimately required HLA-matched platelet transfusions (50%). In addition to these 5 pts, a further 9 pts (with no HLA Abs pre-HDT) ultimately required HLA-matched plts. Bleeding episodes occurred during 42 cycles, however all but 4 were of grade 1 toxicity (grade 2=3, grade 4=1) and all 4 of these bleeding episodes were in the setting of RDP support.
We conclude that; 1) there is a substantial incidence of HLA Abs in women with MBC (≈ 15%), 2) the use of repeated cycles of HDT is associated with an 8-fold increase in the requirement for SDP and 3) the routine determination of HLA Abs in women with MBC prior to HDT, can predict the need for HLA-matched plt support.

OPTIMISATION OF PLATELET COLLECTION VIA THE COBE SPECTRA (V-6) TO FACILITATE AUTOLOGOUS PLATELET CRYOPRESERVATION

C.Kelly, S.Wright, K.Taylor, K.Williams, R.Rodwell, D.Taylor, M.Bentley. *MaterHospital Apheresis/Transplant Unit, Brisbane.*

Platelet refractoriness and alloimunisation can lead to life threatening complications and necessitate dose reduction and premature truncation of treatment regimens. A 55yo female experienced severe thrombocytopenia during induction chemotherapy for acute myeloid leukaemia. There was a total of 14 transfusion days, 11 units of random donor and 2 units of single donor apheresis platelets administered. Platelet recovery occurred on day 22 and the patient entered complete remission. On day 28 (platelet count 556 x 10^9/L) autologous platelets (APLTS) were collected on the Cobe Spectra V-6. The collection procedure was extended to 120 mins. Inlet flow at 33mL. Target predicted platelet volume was 843mLs, which posed considerable problems for cryopreservation including cost, storage space limitations and long reinfusion preparation time. To reduce the collected volume the concentration of APLTS in the collection bag was slowly increased from 1,400,000 to 2,700,000/microlitre. At this time the collection line and interface was very concentrated with APLTS. The ratio was increased by 0.5 every 10 mins until clumping was observed and therafter reduced and maintained at 9.5. These changes reduced the target collect volume to 402mL. The total platelet yield was 18.73 x 10^{11}. The APLTS were irradiated to 25Gy and cryopreserved into 3 alloquots of 6 x10^{11} platelets. Platelet count the next morning was 417 x 10^9/L and the procedure was repeated with a platelet yield of 9.57 x 10^{11}. This allowed a total 5 potential APLTS transfusions to be cryopreserved. We conclude that modification of collect parameters in this fashion enables the collection of large numbers of platelets. Further evaluation of this apporach is planned following cytokine mobilisation of APLTS for autologous transplant patients.

THE PRIMITIVE PROGENITOR CELL CONTENT OF THE GRAFT PREDICTS PLATELET RECOVERY IN TRANSPLANTED MICE

Marilyn Pike[1], Kally Yuen[2], Ralph Green[1], Ivan Bertoncello[3].
[1]Department of Medical Laboratory Science, RMIT University; [2]Statistical Centre and [3]Trescowthick Research Laboratories, Peter MacCallum Cancer Institute, Melbourne, Victoria.

Hierarchical models of hematopoietic organization propose that the early regeneration of circulating blood cells following transplantation is due to the engraftment and proliferation of progenitor cells, whereas long-term blood cell regeneration is restored by the proliferation and differentiation of primitive hematopoietic stem cells (PHSC). This model has been used to explain the more rapid recovery of neutrophil and platelet counts in humans following autografting of mobilized peripheral blood stem cells (PBSC) when compared to transplantation of bone marrow (BM). In this study, we have tested this assumption by analysing and comparing the patterns of peripheral blood cell recovery in mice transplanted with unmanipulated cells from normal bone marrow, and the mobilized PB and bone marrow from cyclophosphamide mobilized mice. The recovery of PB platelet counts following transplantation was transplant cell dose dependent. Stepwise multiple linear regression analysis of platelet counts at 9 and 14 days post-transplantation identified the high proliferative potential colony-forming cell (HPP-CFC) content of the graft as the only significant variable responsible for abrogation of the nadir in platelet counts at day 9 post-transplantation ($P<0.001$). Multiple linear regression analysis also showed that HPP-CFC content ($P<0.001$) was a significant determinant of the rate of platelet regeneration: HPP-CFC content predicted platelet recovery, whereas the committed progenitor cell content of the graft failed to do so. Cell source was also a significant factor in platelet recovery, but contrary to expectations, platelet counts at 14 days post-transplantation of mobilized PB were inferior to those observed following transplantation of either normal BM ($P<0.017$) or the BM of mobilized mice ($P<0.001$). The finding that hematopoietic stem cells and not committed progenitor cells more closely predict early engraftment and rapid recovery of platelet counts in transplanted mice has significant implications for the use of ex vivo expanded progenitor cell populations in transplantation.

A REVISED MODEL OF PLATELET AGGREGATION

S Kulkarni, SM Dopheide, CL Yap, C Ravanat, M Freund, KA Heel, A Street, IS Harper, F Lanza, SP Jackson
Australian Centre for Blood Diseases, Department of Medicine, Monash Medical School,Victoria, Australia,Etablissement de Transfusion Sanguine, Institut National de la Santé et de la Recherche Médicale Unité 311, Strasbourg, France, Department of Pathology, Alfred Hospital, Victoria, Australia.

The formation of stable adhesion contacts between platelets (platelet aggregation) at sites of vascular injury is essential for the arrest of bleeding and for subsequent vascular repair. However, an exaggerated platelet aggregation response at sites of atherosclerotic plaque rupture can lead to the development of vaso-occlusive thrombi, resulting in a heart attack, stroke or sudden death. To examine the mechanism of platelet aggregation under physiological flow conditions we have established an *in vitro* flow-based platelet aggregation assay and an *in vivo* rat thrombosis model. These studies demonstrated an unexpected complexity to the platelet aggregation process in which platelets in flowing blood continuously tether, translocate and/or detach from the luminal surface of a growing platelet thrombus at both arterial or venous shear rates. Studies of platelets congenitally deficient in von Willebrand factor (vWf) or integrin $_{IIb\ 3}$ demonstrated a key role for platelet vWf in initiating platelet tethering and translocation while integrin $_{IIb\ 3}$ mediated cell arrest. Platelet aggregation under flow appears to be a multi-step process involving: i) exposure of vWf on the surface of immobilized platelets; ii) a reversible phase of platelet aggregation mediated by the binding of GPIb on the surface of free-flowing platelets to immobilized vWf; and iii) an irreversible phase of aggregation dependent on integrin $_{IIb\ 3}$. Studies of platelet thrombus formation *in vivo* demonstrate that this multi-step adhesion mechanism is indispensable for platelet aggregation in arterioles and also appears to promote platelet-platelet adhesive interactions in venules. Taken together, our studies demonstrate an important role for platelet vWf in initiating the platelet aggregation process under flow and challenge the currently accepted dogma that the vWf-GPIb interaction is exclusively involved in promoting platelet adhesion processes at elevated shear rates.

THE ROLE OF CHOLESTEROL AND CHOLESTEROL-ENRICHED MEMBRANE DOMAINS IN EGF RECEPTOR-MEDIATED CELL SIGNALING

Linda J, Pike,
Washington University School of Medicine, Dept. of Biochemistry and Molecular Biophysics

Caveolae are small, plasma membrane invaginations that contain high levels of cholesterol and glycosphingolipids. A variety of proteins involved in signal transduction have been shown to be localized to these low density membrane domains. This includes proteins such as the EGF receptor, the PDGF receptor and heterotrimeric G proteins. We have shown that ~50% of the signaling lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) is also localized in these cholesterol-enriched membrane domains and that it is this pool of PIP_2 that is subject to turnover in response to the hormones EGF and bradykinin.

If localization of the PIP_2 to cholesterol-enriched membrane domains is important for proper function of PI turnover, then disruption of these domains should impair signaling via this pathway. Indeed, we have shown that depletion of cellular cholesterol by treatment methyl -cyclodextrin, inhibits EGF- and bradykinin-stimulated PI. This is associated with the loss of PIP_2 as well as signaling proteins such as the EGF receptor and G_q from the low density, membrane fraction. Repletion of cellular cholesterol led to the reorganization of signaling proteins and lipids into low density domains and the reconstitution of hormone-stimulated PI turnover. Addition of excess cholesterol to naive cells leads to an increase in PI turnover. Thus, cholesterol contributes positively to the function of this signal transduction pathway.

Several oxysterol analogs of cholesterol were also able to reconstitute hormone-stimulated PI turnover. Those compounds that were able to restore signaling were also able to restore domain integrity as judged by the re-localization of signaling molecules to the low density membrane fraction. Conversely, those oxysterols that were ineffective in reconstituting PI turnover were also unable to reform the low density membrane. The correlation between functional signaling and domain integrity suggests that the role of cholesterol may be to promote the localization of PIP_2 and/or signaling-related proteins to low density membrane domains.

In contrast to what was found for EGF-stimulated PI turnover, we have shown that modest cholesterol depletion leads to an increase in EGF-stimulated MAP kinase activity. We have traced this effect to increased receptor binding and kinase activity following removal of cholesterol. Conversely, increasing cholesterol levels led to decreased EGF binding

and receptor autophosphorylation and a concomitant inhibition of EGF-stimulated MAP kinase activity. The correlation between receptor binding, autophosphorylation and MAP kinase activation suggests that in this pathway, cholesterol negatively impacts EGF receptor function leading to the inhibition of down stream signaling. These findings demonstrate that cholesterol can have reciprocal effects on different signaling pathways and may modulate signal transduction via a number of mechanisms

Dissection of activation, signalling and biological effects of gm-csf and il-3 receptors using constitutive mutants of the common β subunit

Thomas J Gonda, Timothy Blake, Brendan Jenkins, Matthew McCormack, Carolyn Butcher and Richard D'Andrea

Hanson Centre for Cancer Research and Institute of Medical and Veterinary Science, Frome Road, Adelaide South Australia 5000

Saturation point mutagenesis of the common β subunit ($h\beta_c$) of the human GM-CSF/IL-3/IL-5 receptors has identified twelve sites at which activating amino acid substitutions can occur. These result in constitutive activity and factor-independent growth of certain factor-dependent haemopoietic cell lines. Our current studies aim to use these mutants to help understand the basis of GMR/IL3R/IL5R activation and dissect some of the signals generated by this receptor complex. This has been aided by the observation that the activating mutations fall broadly into classes defined by their locations: extracellular, transmembrane or cytoplasmic. Representatives of the first two classes have been characterised with respect to cell type-specificity, intracellular signalling and their effects on the proliferation and differentiation of haemopoietic cells *in vitro* and *in vivo*.

We have found that the *in vitro* specificity of extracellular mutants for myeloid cells reflects, in part, a requirement for a GMR α subunit. *In vivo*, however, these mutants can induce a trilineage myeloproliferative disorder which includes a profound erythrocytosis; we believe this reflects crosstalk between the activated h $_c$ and the erythropoietin receptor. A further difference between these classes of mutants is that only transmembrane mutants exhibit constitutive β subunit tyrosine phosphorylation. These observations lead us to propose a model for the mechanism by which the mutants are activated; this model also bears on the function of the normal GMR/IL3R/IL5R complexes.

PHOSPHOINOSITIDES AND 5-PHOSPHATASES: THE ON AND OFF SWITCHES THAT REGULATE CELL GROWTH

CA Mitchell
Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168.

Following growth factor or cytokine stimulation, phosphoinositides undergo a complex series of phosphorylation and dephosphorylation events that generate a large number of second messenger molecules. Phosphatidylinositol can be phosphorylated by specific 3, 4 and 5 -kinases to form 3-position phosphoinositides. These lipid signaling molecules regulate actin cytoskeletal rearrangement, protein and vesicular trafficking, cell growth and inhibition of apoptosis.

The inositol polyphosphate 5-phosphatases (5-phosphatases) remove the 5-position phosphate from phosphoinositide and inositol phosphates and thereby terminate growth factor and cytokine-mediated signaling events. Nine mammalian 5-phosphatases have been identified and are all characterized by a highly conserved 300 amino acid “5-phosphatase domain”. 5-phosphatases regulate both B and T-cell activation. Recent studies have shown targeted deletion in mice of the 5-phosphatase SHIP, results in death from myeloid infiltration of the lung and a "myeloproliferative-like" syndrome.
We have identified, cloned and characterized several novel 5-phosphatases in mammalian cells and *Saccharomyces cerevisiae.* Studies of the yeast 5-phosphatases indicate the enzymes may localize with actin patches during agonist cell stimulation and thereby regulate cell division. In addition, we have determined the catalytic mechanism of action by which the 5-phosphatases regulate second messenger molecules. Collectively these studies demonstrate that the 5-phosphatases are a widely expressed family of enzymes that terminate and thereby regulate growth factor mediated signals.

STRUCTURE AND FUNCTION OF THE PLATELET GP IB-IX-V COMPLEX

MC Berndt.
Baker Medical Research Institute, Melbourne

Thrombosis can result in unstable angina, acute myocardial infarction or stroke, all major causes of death in the western world. Circulating platelets become adherent and form an occlusive thrombus either by exposure to sclerotic lesions following plaque rupture or in response to pathological shear stress in stenosed arteries. At high shear, thrombus formation is initiated by a specific platelet membrane adhesion receptor, the glycoprotein (GP) Ib-IX-V complex, which binds the adhesive glycoprotein, von Willebrand factor, in the vessel wall or plasma. The vWF binding site on GP Ib-IX-V is within the N-terminal 282 amino acid residues of GP Ibα, which consists of an N-terminal flanking sequence (His1-Ile-35), seven leucine-rich repeats (Leu-36 to Ala-200), a C-terminal flank (Phe-201-Gly-268), and a sulfated tyrosine sequence (Asp-269-Glu-282). We have used mammalian cell expression of canine/human chimaeras of GP Ibα, corresponding to precise structural boundaries, to demonstrate the first specific requirement for individual leucine-rich repeats for binding of vWF either induced by the modulator, ristocetin, or under hydrodynamic flow. Implicit in this approach was that the GP Ibα chimaeras retained a functional conformation, a supposition confirmed by modeling canine/human Ibα leucine-rich repeats, analyzing restoration of function to reversed human/canine chimaeras, and demonstrating that all chimaeras bound vWF activated by botrocetin, a modulator that is indiscriminate between species. Leucine-rich repeats 2, 3 and 4 of Ibα were identified as being critical for vWF adhesion to GP Ib-IX-V .

ENDOTHELIAL CELL CONTROL OF vWF MULTIMER SIZE

L Xie, CN Chesterman, PJ Hogg.
The Centre for Thrombosis and Vascular Research, School of Pathology, University of New South Wales and Department of Haematology, Prince of Wales Hospital, Sydney

The haemostatic activity of plasma von Willebrand factor (vWF) is a function of multimer size. Only the large vWF multimers are effective in promoting platelet adhesion to a site of vascular injury. We observed that the conditioned medium of cultured human umbilical vein, human microvascular and bovine aortic endothelial cells contained an activity which reduced the average multimer size of plasma or purified vWF and its affinity for collagen. The average multimer size of vWF produced endogenously by human umbilical vein endothelial cells was similarly reduced following secretion. The reducing activity was ablated by pre-treatment with heat or the thiol blocking agents, iodoacetamide, N-ethylmaleimide or E-64, but not by a range of specific serine-, cysteine-, aspartic-, or metallo-proteinase inhibitors. Reduction in vWF multimer size was associated with formation of new thiols in vWF and there was no evidence for additional proteolytic processing of vWF. The reducing activity was associated with a protein with an anionic pI that binds heparin and contains reactive thiol(s). These results suggested that the interchain disulfide bonds that link the vWF homodimers near the N-termini were being reduced by a vWF reductase secreted by endothelial cells. In support of this hypothesis, incubation of vWF with the protein reductants, protein disulfide isomerase and thioredoxin, resulted in formation of new thiols in vWF and reduction in the average multimer size of vWF. In summary, we have evidence that vWF multimer size is regulated by a vWF reductase secreted by endothelial cells. This result may have consequences for normal control of vWF haemostatic activity and perturbation of this control may contribute to the abnormally large vWF multimers associated with thrombotic thrombocytopenic purpura and haemolytic ureamic syndrome.

A ZEBRAFISH HOMOLOGUE OF CYTOKINE-LIKE FACTOR-1, A NOVEL SOLUBLE MEMBER OF THE HAEMOPOIETIN RECEPTOR SUPERFAMILY

Graham J. Lieschke and Judith E. Layton
Cytokine Biology Laboratory, Ludwig Institute for Cancer Research, The Royal Melbourne Hospital, Victoria 3050.

The zebrafish (*Danio rerio*) has emerged as the pre-eminent vertebrate for developmental genetic studies, and offers several advantages as an animal model system for functional genomic studies, particularly of developmentally important genes. As part of our efforts to study cytokine and myeloid biology using the unique advantages of this animal model, we searched for zebrafish members of the haemopoietin family of receptors. One zebrafish cDNA isolated encodes a homologue of mammalian cytokine-like factor-1 (Elson et al, J Immunol 1998;161:1371), a soluble family member most closely related to the extracellular domains of the CNTFR, IL-6R and gp130. The conceptual translation of the zebrafish *clf1* cDNA is a 389 amino acid protein with 64.1% amino acid identity to human CLF-1. Two less abundant cDNA species encode apparent splicing variants with different carboxyl termini. Whole mount in situ hybridization analysis of zebrafish embryos evidences a developmentally regulated and restricted expression pattern of *clf1* expression confined to newly forming somites from 13 hours-post-fertilization (hpf), and becoming restricted by 24-26 hpf to the most dorsal paramedial region of the most caudal body segments. The function of mammalian CLF-1 is unknown. Given the early embryological expression of zebrafish *clf1*, we have embarked on a study of its role in development through overexpression by intra-zygotic injection of in vitro-transcribed *clf1* mRNA, a particularly versatile technique for the study of early gene function in zebrafish. This gene, along with other zebrafish haemopoietin, JAK and STAT family members cloned in our laboratory, confirm that cytokine signaling pathways are conserved in this teleost and support the prospect of using zebrafish for a genetically-based molecular dissection of cytokine signaling and myeloid development.

RETROVIRAL TRANSDUCTION OF HAEMOPOIETIC CELLS TO CONFER CHEMOPROTECTION FROM ALKYLATING AGENTS.

B. Kramer*, P. Gunning*, I. Alexander[#], G. McCowage*.
*[#]Gene Therapy Research Unit, *Oncology Research Unit, The New Children's Hospital, Westmead, NSW 2145*

The use of the chemotherapeutic agent BCNU in the treatment of brain tumours results in significant bone marrow toxicity. This may be due to low levels of expression of the DNA repair enzyme Methyl-Guanine–Methyl-Transferase (MGMT) in haemopoietic stem cells in contrast to high levels of expression in the tumour cells being targeted. Depletion of MGMT within tumour cells by administration of the substrate O^6-Benzylguanine (O^6-BG) prior to BCNU can increase its chemotherapeutic activity but also results in increased toxicity to the bone marrow.

Mutant forms of MGMT differ in their ability to bind to O^6-BG. In mouse haemopoietic transplant models, marrow stem cells that have been retrovirally transduced with cDNA for mutant MGMT show resistance to the depleting effects of O^6-BG and show enhanced survival after subsequent exposure to BCNU.

Our aim is to develop a gene therapy protocol in which $CD34^+$ stem cells will be retrovirally transduced with cDNA for mutant MGMT and used in an autologous transplant to provide chemoprotection against the side effects of subsequent BCNU therapy in paediatric patients being treated for brain tumours.

Retroviral vector plasmids based on the MML-V based MFG vector, carrying the cDNA for the wild type (wt)MGMT or a mutant form (*D*140 or *D*156) have been constructed. Two retroviral packaging cell lines for wtMGMT which pseudotype virus with either GAL-V or VSV-G proteins have been produced. Transduction of K562 cells with these vector stocks have resulted in expression of MGMT in up to 52 % of cells and resistance to subsequent BCNU exposure.

Optimisation of vector stocks capable of transducing CD34 selected bone marrow cells will proceed using both the GAL-V and VSV-G pseudotyped retroviral vectors.

FUNCTIONAL CD44 EXISTS ON THE CELL SURFACE IN A LARGE PROTEIN COMPLEX CONSISTING OF A DISULFIDE LINKED HOMODIMER AND OTHER NON-COVALENTLY ASSOCIATED PROTEINS

LJ Bendall, A James, A Zannettino*, PJ Simmons*, DJ Gottlieb, KF Bradstock.
*Departments of Haematology and Medicine, Westmead Hospital, Westmead and *Hanson Centre, Adelaide*

CD44 is an adhesion molecule which has been implicated in the interaction of haematopoietic cells with the bone marrow stroma. Malignant cell migration, a function necessary for metastasis formation and dissemination of leukeamia, can also be influenced by the expression of CD44. However considerable discrepancy exists between the expression of CD44 and the ability of cells to recognise hyaluronic acid. We have identified a large molecular weight complex which contains CD44 on the cell surface using a novel monoclonal antibody 7H9D6. This antibody only recognises CD44 incorporated into a complex containing disulfide linked CD44 homodimers as well three other non-covalently associated proteins. The complex is partially resistant to solubilization in Triton X-100 but dissociates in the presence of SDS. Partitioning into a Triton insoluble fraction could be due to cytoskeletal association, although cytochalasin B has no effect on 7H9D6 epitope expression. Alternatively partitioning could result from association with Triton insoluble lipid rafts. Despite recognizing only a subset of CD44 molecules on the cell surface 7H9D6 is capable of completely inhibiting cell adhesion to hyaluronic acid suggesting that complex formation is necessary for its function as an adhesion molecule. 7H9D6 may be a useful marker for hyaluronic acid binding capacity of cells and a better predictor of the metastatic potential of tumor cells.

MOLECULAR CLONING OF cDNA FOR A SUBUNIT OF PHOSPHATIDYLINOSITOL 3-PHOSPHATASE

H Nandurkar, K Caldwell and C Mitchell

Department of Biochemistry and Molecular Biology, Monash University; University of New Mexico, USA

Phosphoinositides phosphorylated in the D3 position by phosphoinositide 3-kinase (PI3K) modify downstream targets such as protein kinase B (or Akt), a mediator of anti-apoptotic signaling. Activating mutations of PI3K and Akt exhibit oncogenic potential. Effects of kinases are regulated by specific protein and lipid phosphatases, such as the tumour suppressor protein PTEN, a tyrosine/serine phosphatase also exhibiting a critical lipid 3-phosphatase activity.

A phosphatidylinositol 3-phosphatase protein heterodimer has been purified and consists of 65 kilo Dalton (kDa) and 78 kDa subunits. Relative contribution of the two subunits to enzyme activity has not yet been determined. We have recently isolated cDNAs encoding the 78 kDa protein from human leukaemia cell line (K562) and rat lung. The human cDNA encodes a putative 747 amino acid (aa) protein. Northern analysis revealed expression in rat and mouse lung, brain and testis, and in several haemopoietic cell lines.

Alignment of the cDNA and predicted amino acid sequences with molecular databases have revealed a striking homology (aa homology 44%) with myotubularin, a tyrosine/serine protein phosphatase and the product of a recently described gene mutated in human X-linked myotubular myopathy (MTM1). Interestingly, the 78 kDa protein lacks the protein phosphatase catalytic motif, and resembles SET domain-binding factor-1 (Sbf1), a dominant negative homolog of myotubularin. In addition, alignment is noted with human cisplatin resistance-associated protein. We are currently expressing the protein, identifying interacting partners and intracellular localisation. We propose that this novel protein will function to regulate PI3K derived signals and thereby modulate cell growth and differentiation.

GUIDELINES FOR APHERESIS EDUCATION AND COMPETENCY

R McKenna, M Brettell, M Armstrong, P Chase, L Steele, S Burtt, K Atkinson, D Thorp.

Peter MacCallum Cancer Institute, Cell Separator Unit, East Melbourne, Victoria, Australia.

The International Council for Nurses (ICN) places the mandate for setting standards in education and practice as the responsibility of the profession. The Royal College of Nursing Australia already has a brief to investigate credentialling, therefore as a demonstration of professional accountability, the Australian and New Zealand Apheresis Association (ANZAA) initiated a working party to investigate the educational components and criteria for apheresis operators to work at a professional level.

In order to incorporate the range of disciplines involved in apheresis and their different educational issues, it was decided that detailed guidelines linking the educational, technical, teaching and learning requirements of specialists in this profession, with theories of adult education, learning characteristics and assessment were required. Furthermore consideration must be given to the economic context in which ANZAA operates.

Newble & Cannon (1995), say much curriculum development must inevitably be a matter of revising and adapting existing courses and materials. Therefore building on content developed by the American Society For Apheresis (ASFA) and the training guidelines and assessment tools developed by the Ucommonly available, commercial Cell Separator companies, Cobe, Baxter and Haemonetics; the outline for the guidelines will be presented.

THE PERCENTAGE OF CD34^{bright} or CD34^{dim} CELLS IN THE PRE-SELECTION APHERESIS PRODUCT IS A RELIABLE PREDICTOR OF CD34+ CELL YIELD FOLLOWING ISOLEX 300i CD34-SELECTION.

HM Prince, L Barber, P Chapple, DM Wall, J Bashford, M Quinn, M Brettell, M Wolf, H Januszewicz, J Seymour, S Juneja, P Gates, R Maisano. Autologous Blood and Marrow Transplant Service, Division of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, East Melbourne, Victoria and Clinical Haematology and Medical Oncology, The Wesley Clinic, Brisbane, Queensland

Selection of CD34$^+$ cells with the Isolex 300i usually leads to a highly pure CD34+ product (>85% CD34+ cells). However, we have previously demonstrated that two-thirds of the CD34+ cells are 'lost' in the negative-fraction during the processing with a range of 1%-60% [Exp Hematol 1998, 26:68]. To date, there has been no reliable predictor of CD34+ cell yield following Isolex processing. In flow cytometry studies, we analysed the CD34 histogram and divided the CD34+ population into CD34^{dim} and CD34^{bright} populations. In pre-clinical studies using the MiniMACS CD34+cell-selection system, we have demonstrated that the expression of the CD34 antigen correlates with the yield of CD34$^+$ cells post-selection with an increased percentage of CD34^{dim} cells leading to lower CD34$^+$ cell recovery. In this study, we sought to determine if this finding applied to the clinical Isolex 300i CD34-selection system.

The CD34$^+$ cells from apheresis products from 10 pts were purified using the Isolex 300i and the pre- and post-selection CD34$^+$ cells were analysed using the ISHAGE guidelines. The initial apheresis product contained 1.47% (range: 0.23%-3.63%; ± 0.36% SEM) CD34$^+$ cells. Following Isolex 300i CD34 selection, the purity of CD34+ cells was 73.8% (range: 16.3% to 92.9%; ± 9.1% SEM) with a CD34$^+$ cell recovery (yield) of 31.6% (2.3% to 60.0%; ± 5.1%SEM).

The median %CD34^{dim} cells in the pre-selection apheresis product was 20.8% (range: 5.9% to 31.7%; ± 2.8% SEM) with CD34^{bright} contributing 78.4% (range: 68.1% to 91.8%; ± 2.8% SEM) of CD34+ cells. Although there was no correlation between CD34$^+$ cell yield and apheresis product white cell count, platelet count, % total CD34$^+$ cells nor absolute CD34$^+$ cell count, there was a correlation between the %CD34^{bright} cells and CD34$^+$ cell yield following selection (r= 0.648, p= 0.0377). Furthermore, there was inverse correlation between %CD34^{dim} cells and CD34$^+$ cell yield (r= -0.673, p= 0.029). There was no influence of the CD34 antigen expression on post-selection CD34+ cell purity (p= 0.327, r= -0.333).

We conclude that the intensity of CD34 antigen expression affects the CD34$^+$ yield but not CD34+ purity following Isolex 300i selection. Indeed, the percentage of CD34^{bright} or CD34^{dim} cells in the apheresis product is a valuable predictor of CD34$^+$ yield following CD34-selection.

Automatic closed bag processing of autologous blood stem cell collections

DM Wall, G Moore[1], M Quinn, P Chapple, HM Prince. *Division o Haematology and Medical Oncology, Peter MacCallum Cancer Institute, Eas Melbourne, Victoria 3002 and [1]Baxter Healthcare Pty Ltd*

Apheresis collections for autologous blood stem cell transplantatio are routinely processed to reduce the overall volume of the collection prior t addition of cryopreservative and freezing. This is currently performed using a entirely open manual approach using 50ml tubes and centrifugation. A close procedure was developed in conjunction with Baxter Healthcare. The metho had to be suitable to manage the various apheresis collection machines as we as sub-optimally centrifuged product. The approach chosen was an adaptatio of the methodology of the Baxter Optipress II automated blood componer extractor and a Terumo TSCD sterile tubing welder.

Apheresis collections were centrifuged (200*g*, 15 minutes) and wer then transferred to the Optipress II, where a program originally designed t automatically isolate platelet rich plasma from pooled buffy coats was usec Initial development was applied upon discarded blood components altered t resemble autologous blood cell apheresis collections. Volume was reduce from a mean of 104ml down to 45ml, whilst a white blood cell recovery o 89% (SD 10%) was achieved with 55% (SD 18%) of platelets being removec Further work then proceeded using autologous blood cell apheresis product obtained with the Baxter CS3000. In subsequent processing with the Baxte Optipress II, volume was reduced from a mean of 185ml to 30ml, with 99.8% (SD 0.1%) of white cells being retained, and 58% (SD 5.3%) of platelets bein removed.

We conclude that the use of a sterile tube welder and an automated blood component extractor has allowed us to develop a wholly operator-independent and closed processing system to volume-reduce apheresis collections for autologous blood cell transplantation.

PERIPHERAL BLOOD STEM CELL (PBSC) SELECTION USING THE ISOLEX 300*i*.

R Soares Mendes, PK Cannell, RP Herrmann
Department of Haematology, Royal Perth Hospital, Perth WA.

CD34 based immunomagnetic selection of autologous PBSC decreases the likelihood of reinfusing tumour cells which may contribute to relapse. For allogeneic harvests the concomitant reduction of T cells results in less graft-versus-host-disease. This study evaluates CD34+ stem cell selection using the Isolex 300*i* (Baxter).

Stem cell selection was performed on 51 autologous (NHL 24, MM 27) and 5 fully matched allogeneic PBSC harvests. The mean percentage of CD34+ cells and number of total nucleated cells collected was 3.1±0.3%(SEM) and 3.0±0.3x10^{10} respectively for autologous and 0.9±0.1% and 5.5±1.6 x10^{10} for allogeneic PBSC. Following selection the mean recovery of CD34+ cells was 54.6±0.02% for autologous and 48.3±0.07% for allogeneic with a mean CD34 purity of 90.6±1.3% and 74.8±4.0% respectively. The majority of autologous grafts remained positive for IgH rearrangement post selection. Selection resulted in a mean T cell log reduction of 3.2±0.1 for allogeneic PBSC. Patients received a mean of 2.7±0.2x10^{6}/kg CD34+ cells for autologous and 3.4±0.5 x10^{6}/kg for allogeneic transplant. The number of days to neutrophil (ANC>1.0x10^{6}/L) and platelet (plt>50x10^{9}/L) recovery was 16 and 23 respectively. Of the autologous patients, 5 are deceased (3 relapsed, 1 sepsis/multi organ failure, 1 arrhythmia) and 3 have relapsed. Initial data shows no significant difference in patient survival at 500 days post transplant as compared to unselected transplants. Of the four patients allografted (NHL 2, AML 1, CML 1), 2 are deceased (regimen related toxicity, intracerebral haemorrhage) and 2 are in remission.

Selection of PBSC using the Isolex 300*i* provides a transplant product high in purity and capable of sustained engraftment. No survival advantage for positive selection is seen at 500 days but longer follow up is required.

THE CD34+ HOECHST/RHODAMINE DULL/DULL SUBSET OF CML – A UNIQUE SOURCE OF NORMAL PROGENITORS.

D White, M Forte, L McDiarmid, & T Hughes. *Hanson Centre for Cancer Research, IMVS Adelaide.*

To investigate biological differences between normal and CML cells we have analysed the cell cycle status of primitive cells collected from 10 CML patients at presentation and 3 in late chronic phase. Low retention of Hoechst 33342 (Hst) and Rhodamine 123 (Rh) defines a population in normal individuals which are quiescent and capable of long term repopulation in the murine transplant model. In all CML cases studied we have demonstrated the majority of cells within the Hst/Rh dull/dull subset are bcr-abl negative (Median 33% bcr-abl+ R 10-46%) in contrast to the Hst/Rh bright/bright subset which are bcr-abl positive (Median 85% bcr-abl+ R 56-88%). Using the monoclonal antibody Ki67 and propidium iodide we have confirmed that greater than 99% of CML CD34+ dull/dull cells reside in either the G0 or G1. Using a stroma free culture system and the 6 haemopoietic growth factor (6 HGF) combination IL-3, IL-6, SCF, G-CSF, Flt 3 ligand and TPO we have demonstrated preferential expansion of these bcr-abl neg dull/dull cells over a 14 day period. In CFU-GM assays, dull/dull cells are capable of giving rise to approximately double the number of colonies as CD34+ cells at day 0, and of demonstrating maximal expansion of CFU-GM over a 21 day period. In 3 patients examined to date minimal or no expansion is observed when dull/dull cells are exposed to SCF alone, which is consistent with our observations in dull/dull cells collected from normal individuals. Further evidence for the normality of these dull/dull cells comes from the observation that exposure to p-selectin inhibits their proliferative response to HGF. This is in keeping with previous observations in normal cells, but in contrast to CML CD34+ cells which are able to overcome the inhibitory effects of p-selectin. The CD34+ dull/dull subset of CML provides a source of functionally and genetically normal cells.

RATIONALISATION OF STEM CELL HARVEST USING CIRCULATING CD34+ CELLS.

D Thorp, P Dyson, T Hughes.

Haematology Day Centre and Department of Clinical Haematology, Royal Adelaide Hospital and Hanson Centre, Adelaide, South Australia.

In our institution Peripheral Blood Stem Cell Collections [PBSC] are commenced when the patient's/donor's CD34 count has reached a predetermined value. Our aim is to collect sufficient cells for transplantation in the least number of apheresis procedures.

Our standard protocol requires $3x10^6$/kg CD34 cells for transplantation. However, when the stem cells are to be selected a greater starting number is required because there is an approximate 50% loss of cells during the selection process, one research protocol requires $4x10^6$/kg CD34 cells and paediatric hospital requires $5x10^6$/kg CD34 cells.

We examined data from 279 consecutive collections (240 adults and 39 children). Data from five collections was discarded because of missing numbers.

Using the patient's circulating CD34 count and the known collection efficiency of our machines, we can predict the amount of blood to be processed to obtain the desired PBSC for each patient.

By commencing the PBSC when the patients/donor CD34 is >20µl we collected $1x10^6$/kg CD34 cells in 95% of procedures and $3x10^6$/kg CD34 in 56% of procedures in adult patients using the standard two blood volume blood processed procedure.

HAIRY CELL LEUKAEMIA 25 YEARS ON.

RA Harrup, RI Kimber, RM Lowenthal.

Clinical Haematology/Medical Oncology Unit, Royal Hobart Hospital TAS

Hairy cell leukaemia (HCL) is an uncommon lympho- proliferative disorder which was first recognised as a separate entity in 1958, but not widely known until the 1970's. Recent therapeutic advances, including the development of purine analogues such as 2-chloroxyadenosine, have led to long-term remissions, making recognition of this relatively rare entity worthwhile.

Case History

An otherwise well 78 year old woman was seen in outpatients in October 1998 regarding worsening thrombocytopenia (platelets 58 x 10^9/L). Examination revealed a tippable spleen. Further questioning revealed that she had previously attended the haematology clinic in 1973 for investigation of bone pain and a fourteen year history of 'anaemia'. A bone marrow biopsy was performed in February 1974 resulting in a diagnosis of 'myelofibrosis'. The patient was lost to follow-up but remained well in the intervening 24 years. A repeat full blood count at our hospital showed atypical lymphocytes with features suggestive of hairy cells. The previous trephine from 1974 was located and a retrospective diagnosis of HCL was made. The availability of archive material saved this patient the necessity of an invasive procedure and also provides a new insight into the natural history of untreated HCL, highlighting the need to reverse the current trend to destroy such historical records.

CASE STUDY: PENICILLIN AND CEPHALOSPORIN-ASSOCIATED IMMUNE NEUTROPENIA

BT Leach, B Dawkins
Platelet and Granulocyte Immunobiology Laboratory, Australian Red Cross Blood Service - Qld

Background: Samples were referred from a 67 year old male patient with suspected drug-associated immune neutropenia. The patient experienced a profound neutropenia, following treatment with penicillin. Cephalosporin was then administered, with no improvement to the neutrophil count. Laboratory investigations were performed using both of the above drugs to confirm or exclude the presence of drug-associated neutrophil antibodies.

Method: The patient's serum was investigated by Granulocyte Immunofluorescence Test methodology and analysed by flow cytometry. Briefly, serum and neutrophils (both autologous and random donor) were incubated with and without the presence of the suspected drug(s) over a range of drug concentrations, and then labelled with fluorescein-conjugated anti-human globulin. Optimal test sensitivity was achieved experimentally, with alterations to the assay configuration required for each drug under investigation.

Results: Neutrophil specific penicillin-, and cephalosporin-dependent antibodies of class IgM (only), as well as non drug-dependent (auto)antibodies (class IgG and IgM) were detected in the patient's serum over the weeks immediately following the episode of neutropenia. A normal neutrophil count was eventually attained with G-CSF and non-penicillin antibiotics. This patient's complex clinical history of autoimmune neutropenia, with drug involvement typifies a polyclonal immune response to multiple drugs, supporting the "unified hypothesis" for drug-induced immune cytopenias proposed by C.Mueller-Eckhardt and A.Salama (Transfusion Medicine Reviews, **IV**, 1990:69-77).

MUTATION ANALYSIS OF PATIENTS WITH NON-DELETIONAL FORMS OF α-THALASSAEMIA REVEALS A LOW INCIDENCE OF NON-DELETIONAL MUTATIONS

DM Barbulescu[1], G Casey[2], J Hall[2], DM Quinn[1], Z Rudzki[2]
[1]School of Pharmacy and Biomedical Sciences, University of South Australia and [2]Department of Molecular Pathology, Institute of Medical and Veterinary Science, Adelaide, SA.

Alpha-thalassaemia (α-thal) is an inherited disorder of haemoglobin characterised by reduced synthesis of α-globin chains. The α-globin chains are the expression products of two genes (α_2- and α_1-) located in a gene cluster at the tip of chromosome 16. Deletions of one or both of the α-globin genes from one chromosome are the main cause of α-thal (95%). The remaining defects consist of point mutations or small deletions/insertions in the α-globin genes. This study aimed to determine the molecular defects in the α-globin genes of 24 patients diagnosed with non-deletional α-thal. The differential diagnosis of these patients included the detection of microcytosis, normal Hb A_2/Hb F levels and Hb H inclusion bodies. The samples were also analysed for gross gene deletions by Southern blotting and gap polymerase chain reaction (PCR). Samples that were negative for gene deletions were then further analysed using specific PCR amplification of α_2- and α_1- globin genes and automated sequencing of the PCR products. Sequence analysis showed that 4/24 patients had point mutations/small deletions in their α-globin genes, the remainder (22/24) displaying normal sequences. The mutations detected were α^{TSAUDI} (polyadenylation site; 3 patients) and α^{Hph} (5 base pairs deletion in the first intervening sequence; 1 patient). This low frequency (17%) of non-deletional mutations in patients predicted to have this type of molecular lesion suggests that other defects (*e.g.* mutations at locus control region) are the cause of the non-deletional α-thal syndrome.

FUNCTIONAL SIGNIFICANCE OF CLONALLY EXPANDED CD8 T CELLS IN MULTIPLE MYELOMA

MYD Sze, RD Brown, J Gibson, D Joshua
Institute of Haematology, Royal Prince Alfred Hospital, Sydney

Myeloma tumour cells produce unique idiotypic structures which can be regarded as tumour-specific antigens. Many *in vitro* studies have demonstrated the presence of such idiotype-specific T cells. We have recently found T cell expansions with phenotype of $CD8^+$, $CD28^-$, $CD57^+$ and $perforin^+$ in the majority of patients tested. We further examined whether these expanded T cells are activated *in vivo* and produce appropriate cytokines. Peripheral blood mononuclear cells (PBMC) freshly obtained from myeloma patients were firstly surface-stained, fixed and permeabilised prior to the intra-cytoplasmic cytokine staining. We found that neither the expanded nor the non-expanded T cells from five myeloma patients secreted γ-IFN or IL-4. Cells treated with PMA and ionomycin were used as a positive control.

We then investigated the antigen-specificity of the T cell clones in a myeloma patient who had 2 expanded Vβ populations (Vβ1=20.8% and Vβ3=8.8% of total CD3 cells). We tested if these expanded T cell clones were specific for the autologous idiotype. PBMC were cultured with autologous myeloma F(ab')2 protein in the presence of rhIL-2 (2U/ml) for 2 weeks. After culturing, antigen-specific CD4 T cells and CD8 T cells of expanded TCRVβ were obtained by MACS separation. Standard 6-hour ^{51}Cr release assays were performed using γ-irradiated autologous or allogenic $F(ab')_2$-primed, CD3-depleted PBMC as targets. The cultured TCRVβ1 and Vβ3 T cells caused little specific lysis. However in the presence of autologous cultured CD4 cells, the cultured TCRVβ1 and Vβ3 T cells caused 58% and 33% specific lysis of autologous F(ab')2-primed target cells but not the allogenic-primed counterparts. CD4 T cells alone did not cause any lysis to either the autologous or allogenic-primed target cells. Freshly obtained CD8 T cells did not show specific lysis in the presence of either fresh or cultured CD4 T cells. The results suggest that antigen-specific CD4 T cell may have an important role in upregulating CD8 effector function. The lytic effect of these cultured CD8 T cells on the actual myeloma tumour cells remains to be tested.

CHARACTERISATION OF T-CELL CLONES IN MYELOMA

M Raitakari, W Esdale, R. Brown, J. Gibson and D. Joshua.
Institute of Haematology, Royal Prince Alfred Hospital, Sydney,

Patients with multiple myeloma (MM) who have clonal T-cell populations in blood have previously shown to have a significantly better prognosis, suggesting that T-cell clones may be involved in anti-tumour activity. Using flow cytometry and a panel of 21 monoclonal antibodies to the variable region of the T-cell receptor β chain (TCRVβ), we studied the TCRVβ repertoire on peripheral blood lymphocytes of 38 patients with MM. The phenotype of the T-cell expansions and the expression of the functional antigens bcl-2, Fas (CD95) and perforin were also investigated. The TCRVβ panel covered 62∀10% (mean∀SD) of the $CD3^+$ cells. Oligoclonal (1-4 clones) TCRVβ expression was found in 79% of patients. During an 18 month follow-up of 26 patients, the TCRVβ repertoire remained relatively stable. When compared to the non-expanded T-cells, the expanded Vβ clones had a higher expression of CD8 (84∀19% vs. 58∀24%, $p<0.007$), CD57 (70∀21% vs. 43∀23%, $p<0.001$) and CD45RA (71∀23% vs. 52∀18%, $p<0.03$), and a lower expression of CD28 (26∀15% vs. 54∀22%, $p<0.001$). The expression of bcl-2 and Fas were similar in the expanded vs. non-expanded T-cells, whereas the expression of perforin was higher in the expanded vs. non-expanded Vβ populations (49∀15% vs 26∀16%, $p<0.05$). The expression of perforin was associated with the expression of CD57 ($r=0.80$, $p<0.001$). The average number of TCRVβ expansions was lowest in Stage I (Durie-Salmon) patients (1.0), who had never received any chemotherapy, and highest in Stage I patients (1.6) who had received therapy. In Stages II and III, the numbers of Vβ expansions were 1.3 and 1.5, respectively. Furthermore, in Stage I patients without therapy, none of the patients had TCRVβ expansions >15% of CD3 cells, whereas in the other groups, expansions of up to 25% of CD3 cells were observed. We conclude that the majority of patients with MM have persistent clonal expansions of $CD8^+$ $CD57^+$ $CD28^!$ and $perforin^+$ T-cells, a phenotype characteristic for cytotoxic T-cells. Since such T-cells were less common in patients with mild disease it is suggested that the T-cell expansions emerge following disease progression and may potentially take part in the immune regulation in myeloma.

MINIMAL RESIDUAL DISEASE ANALYSIS USING PATIENT SPECIFIC PCR AFTER ALLOGRAFTING IN CHRONIC LYMPHOCYTIC LEUKAEMIA

M. Higgins, P. Cannell and R. Herrmann
Department of Haematology, Royal Perth Hospital, Perth, WA.

Chronic lymphocytic leukaemia (CLL) represents 25% of all leukaemias but although diagnosis and staging is straightforward predicting prognosis and measuring minimal residual disease (MRD) remains a challenge. New therapeutic agents and the use of transplantation for treatment of CLL has increased the proportion of patients achieving clinical complete remission (CR). Methods are needed to monitor MRD to predict relapse and define molecular CR, although the significance of these results is still unknown. Clonal rearrangements of the immunoglobulin heavy chain locus (IgH) that occur in CLL provide a useful marker for MRD detection. Molecular techniques to measure this have evolved into highly sensitive PCR systems. We report a single patient diagnosed with CLL whose transient responses to chemotherapy and young age made him a suitable candidate for allogeneic bone marrow transplantation (BMT). MRD post BMT was monitored by PCR using concensus primers to the FR1, FR3 and J regions of the IgH gene. PCR product was sequenced and patient specific primers designed to unique regions of CDR1 and CDR3. These were used in a nested PCR specifically amplifying patient clonal DNA, increasing the sensitivity of detecting clonal cells in normal DNA from $1:10^2$ (concensus PCR) to $1:10^6$. This is the most sensitive tool available for MRD detection in CLL. Both PCR techniques were performed on peripheral blood and bone marrow samples post BMT and show transition from PCR positive to negative after 30 days. The patient is considered in molecular and clinical CR. To our knowledge this is the first report of patient specific PCR being used in MRD detection in CLL. It has the potential to provide the earliest indication of relapse but further monitoring is needed to ascertain the relevance of the results in predicting disease outcome.

ANALYSIS OF TEL/AML1 GENE FUSION BY FISH IN PAEDIATRIC ACUTE LYMPHOBLASTIC LEUKAEMIA

M. Kalev, P. Oei, L. Teague, P.J. Browett
Haematology and Cytogenetics Laboratories, Auckland Hospital, Private Bag 92024, Auckland, New Zealand

TEL/AML1 fusion is the most frequent gene rearrangement in childhood ALL and is considered a good prognostic indicator.
The aim of this study was to assess the incidence of the TEL/AML1 gene fusion by FISH using the Vysis dual colour translocation probe in 57 consecutive samples from children with ALL. 41 were tested at presentation, and 16 at the time of relapse. 34 patients had c-ALL, 14 pre-B, 4 T-ALL, 1 null ALL and 3 biphenotypic leukaemias. 15/57(26%) children were positive - 11/41(27%) at diagnosis and 4/16(25%) at relapse. TEL deletion was present in 7 patients and a double fusion in 5 patients. TEL gene deletion with no associated fusion was observed in a further 6 patients. Cytogenetic analysis revealed a normal karyotype in 7 of 15 positive patients, numerical abnormalities in 5 and structural changes in 5. Hyperdiploidy (8/42) and structural abnormalities (24/42) were more frequent in the group without evidence of the TEL/AML gene fusion. The t(12;21) was not observed by conventional G banding in any case. All positive patients had a B cell precursor phenotype (TdT+, CD10+, CD19+) but only 2/15(13%) were positive for CD20 compared with 24/32(75%) in the TEL/AML1 negative group. 11/15(73%) expressed myeloid antigens, 10(67%) CD13 and 6(40%) CD33, whereas in the negative group CD13 was expressed in 11/32(34%) and CD33 in 4/32(13%). Patients were treated on ANZCCSG protocols VI and VII, with a median time to relapse of 3.7 years in the TEL/AML positive group versus 1.7 years in the negative group.
This study has found a comparable incidence of the TEL/AML1 fusion gene in paediatric ALL cases to that previously reported (26%). A normal karyotype appears more frequent in this group, but numerical changes do not preclude the presence of the fusion gene. We were not able to identify a characteristic immunophenotype as suggested by some authors, although increased myeloid antigen expression and loss of CD20 were observed.

OPTIMIZED CORD BLOOD STEM CELL RECOVERY AND VOLUME REDUCTION WITH THE OPTIPRESS II.

Rodwell RL, Taylor D, Ambrosoli P, Bentley M, Taylor K.
Queensland Cord Blood Bank At The Mater, Brisbane, Qld.

Volume reduction of cord blood units (CBUs) by red blood cell (RBC) depletion, mononuclear separation or buffy coat extraction (BCE) is a prerequisite to cost-effective cord blood banking. The Optipress II (Baxter Healthcare, Australia) employs BCE within a closed system. The aim of our study was to optimize this process to provide maximum stem cell (SC) recovery. We examined factors that may influence SC recovery including CBU volume, storage conditions and duration, centrifugation conditions and adjustable Optipress variables. Initial experiments were performed with the latter variables fixed at force 30 and volume 25mL. These showed the potential for cell recoveries >90%. Results however were inconsistent, with cell loss being greatest when processing large volume (>120mL) CBUs. We found that CBUs could be stored overnight at 4^0C provided adequate mixing was performed prior to processing. Centrifugation conditions were also important. Significant SC loss occurred in the Optipress RBC output line and a final volume of 25 mL was at the instrument's limit of operation. The variables were modified (force 15, volume 30mL) and with CBUs of ≥100mL, 20-30 mL RBC was removed (either by attached syringe port or preferential opening of the RBC cannula), prior to processing. With these optimized conditions, data (mean ± SEM) for the first 70 CBU banked were nucleated cell and $CD34^+$ recoveries of 80.3 ± 1.0% and 88.3 ± 2.86% respectively; corresponding cell yields were 13.0 ± 0.5 x 10^8 and 5.41 ± 0.51x 10^6 respectively with RBC reduction of 67.2 ± 1.6%. Samples were processed in a median time of 5.5 hours from collection. The Optipress II provides a simple and rapid method of volume reduction for CBUs within a closed system. Optimization ensures consistently high SC recoveries even with large volume CBUs.

COMPARATIVE STUDY OF POSITIVE AND NEGATIVE SELECTION TO REDUCE TUMOUR LOAD IN AUTOGRAFTS FROM MYELOMA PATIENTS

M J Sturm, R Soares Mendes, CDS Mamotte, WN Erber*, RP Herrmann
Department of Haematology Royal Perth Hospital and Pathcentre, Western Australia*

In this study, plasma cells in peripheral blood stem cell (PBSC) harvests from myeloma patients were quantitated and the effect of stem cell selection assessed. Since plasma cells have been previously shown to bind the lectin peanut agglutinin (PNA), PNA purging of autografts was evaluated experimentally. Plasma cells were identified in PBSC products from 23 myeloma patients by flow cytometry on the basis of CD38 and CD45 expression. Molecular detection of the malignant clone was assessed in all samples by PCR, using primers specific for each VH family and a JH consensus primer; CD34 stem cells were immunomagnetically selected using the Isolex 300I (Baxter). Six PBSC samples were incubated with biotinylated PNA and PNA bound cells were removed with MACS strept-avidin magnetic beads.
Contaminating plasma cells were identified in all PBSC harvests. Stem cell selection resulted in a substantial reduction (1-3 logs), however, immature and primitive plasma cells were still detectable in 52% of cases and most grafts remained positive for IgH gene rearrangement. A similar result was achieved with PNA treatment. The recovery of CD34 stem cells following PNA treatment was 57.2 ± 5.2% (SE) as compared to Isolex selection of 47.6 ± 2.7%. The preliminary results of PNA purging suggest that this technique may be as effective as positive stem cell selection in reducing tumour load, with the potential of improving stem cell yields.

COMPARISON OF SINGLE PLATFORM, DUAL PLATFORM, AND MICROVOLUME FLUORIMETRY FOR THE QUANTITATION OF CD34 POSITIVE PROGENITORS IN PERIPHERAL BLOOD

P. Chapple[1,2], HM Prince[1], DM. Wall, M. Quinn[1], M. Brettell[1] and D.Venter[2]. [1]*Blood and Marrow Transplant Service and* [2]*Department of Pathology, Division of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia*

Quantitation of peripheral blood progenitor cells is now an established method for timing of peripheral blood stem cell harvesting for transplantation. Furthermore our group, and others, have shown the utility of peripheral blood CD34 counts for predicting success in harvest procedures. Recent refinements to the ISHAGE gating strategy for progenitor enumeration have seen the introduction of microbeads to enable absolute counting of cells on a single instrument platform. This eliminates the need for total white cell counts performed on an automated haematology analyzer and potentially increases the analytical precision of the methodology. At the same time alternative methods for CD34 positive cell enumeration have started to emerge, notably by microvolume fluorimetry, which forms the basis of fully the automated STELLer CD34 ® method using the Imagin 2000 instrument by Biometric Imaging®

We have sought to perform a three-way evaluation of these methods to establish the most appropriate protocol in our institution. Sixty-eight samples of peripheral blood from patients undergoing peripheral stem cell mobilisation and collection were analysed by all three methods and corrections between all three calculated. The two-platform ISAHGE method was used as the reference method. Table 1 shows the correlation matrix.

Table 1. Correlation matrix (R values)

	2 platform ISHAGE	Single platform ISHAGE	STELLer® CD34 assay
2 platform ISHAGE		0.9325	0.8215
Single platform ISHAGE	0.9325		0.8865
STELLer CD34 ® assay	0.8215	0.8865	

Both the single platform ISHAGE and the STELLer CD34 ® methods gave acceptable correlation with the reference method. Results were slightly higher on the single platform ISHAGE method and slightly lower on the STELLer CD34 ® method but the differences were not statistically significant ($p > 0.05$). The STELLer CD34 ® methodology is technically simpler than the ISHAGE single platform method and this may confer benefits in the context of near patient testing prior to harvesting.

FROM SURVEY TO QAP OF CD34$^+$ STEM CELL ENUMERATION.

A Chang[1] and DDF Ma[2] for the BMT Scientists Study Group.
[1] *Dept. Haematology, PaLMS. Royal North Shore Hosp, Sydney.*
[2] *Dept. Haematology, St. Vincent's Hospital, Sydney.*

Enumeration of CD34$^+$ haemopoietic stem cells (HSC) by flow cytometry is used routinely for clinical decisions and management of HSC transplant patients. In Australasia, standardisation of the method for CD34$^+$ cell estimation has progressed through several stages, and shows a strong trend to adopt the ISHAGE protocol as the consensus method.

As methods are still evolving, modifications need to be evaluated. Modifications to the ISHAGE protocol include (1) incorporation of fluorobeads for absolute counting using a single platform to eliminate variations introduced by the white cell count, (a major variable affecting the absolute CD34 counts identified in our study) and (2) addition of a viability stain to estimate viable stem cells rather than total CD34$^+$ cells, so that the true graft potential can be determined for harvested cells stored either overnight or after cryopreservation.

Comparisons are made of the reagents used, method of analysis and results from the most recent sample to that at the start of this study. Whereas the first sample (analysed in November 1994 by 20 centres), showed a 4 fold difference in the %CD34$^+$ cell results with a CV of 29%, the results from the latest sample (analysed in February 1999 by 27 centres) showed a 2 fold difference with a CV of 13%.

The Australasian centres have thus achieved a major reduction in the site-to-site variation of CD34$^+$ counts, with most centres adopting a consensus method. With support from participants (38 centres), it now appears appropriate to initiate a formal quality assurance programme for CD34$^+$ cell enumeration.

GRAFT ENGINEERING USING THE ISOLEX® 300i RUNNING SOFTWARE VERSION 2.0b1; CLINICAL RESULTS FROM A SYSTEM WHICH OFFERS CD34 SELECTION CONCURRENT WITH THE DEPLETION OF OTHER CELL TYPES. **J. T. Kemshead, M. Schilling, L. Hami, C.van de Ven and N. Wyman on behalf of the Isolex users group.** ***Nexell Therapeutics, Irvine, California, USA.***

The Isolex® 300i offers an automated approach to the purification of CD34 cells from either peripheral blood stem cells (PBSC) or bone marrow (BM), in a functionally closed system compliant with GMP requirements. Recent improvements to the system include a newly designed disposable and enhanced on line washing. Version 2.0(b1) of the software controlling the Isolex® 300i allows for a CD34 selection step concurrent with the further depletion of another cell population such as tumour cells and T cells. Sufficient data (n>200) has been accrued from multiple users to now report on the functionality of the system. Analysis of data from commercial sites in Europe and test sites in the USA indicates that the median CD34+ve cell purity has been increased to 94% and the median yield to 58%. This compares to a median purity of 89% and yield of 48% of CD34 +ve cells using the older version of software (v1.12). Using a variety of anti-T cell monoclonal antibody (MoAb) combinations, including CD4/CD8, CD2/CD6 and CD3 a median 4.9 log T cell depletion was achieved. No significant difference was observed in the level of T cell depletion recorded with any of these MoAb combinations. Mathematical models indicate that this figure is at least one log greater than that achievable with CD34 cell selection alone, a point independent of the system used for the selection procedure. Furthermore, in a small cohort of patient samples, high levels of B (4.5 log) and T cell (4.9 log) reduction have been achieved through the concurrent use of CD4/8 and CD19/20 MoAbs in the depletion step. No impairment in the regraftment times of patients receiving either autologous or allogeneic CD34 selected cells isolated using the Isolex running V2.0b1 software has been observed. We conclude that the Isolex® 300i offers a safe and highly efficient approach to the isolation of CD34 cells free of capture reagents and the versatility of the machine is currently being further expanded so that a multitude of cell selection procedures can be undertaken with one instrument.

HAEMATOLOGY ADVANCED NURSE PRACTITIONER: DEVELOPMENT OF THIS ROLE IN THE TRANSPLANT TEAM.

S Eerhard, L Clark, HM Prince, P Gates, D Spencer, J Gale, A Pollard.
Blood and Marrow Transplant Service, Division of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, East Melbourne.

Peter MacCallum Cancer Institute (PMCI) has been successful in an application to the Victorian Government Department of Health Services for one of eight Nurse Practitioner Project Grants selected from 108 submissions. One million dollars of State health funding has been allocated to the development of nurse practitioners within Victoria. The project plans to demonstrate sustainable models for nurses who have been educated for advanced practice in clinical roles.

At PMCI the Haematology Nurse Practitioner (HNP) will perform as a core member of the Haematology Autologous Blood and Marrow Transplant Service with a specific focus on the transplant program.

In addition to a requisite post registration qualification in oncology and extensive clinical experience in haematology nursing, the HNP will receive further context-based clinical knowledge and develop skills and attitudes relevant to the specialist area of autologous transplantation. An educational program has been designed specifically for this purpose, and will be provided by a range of professionals across all disciplines.

The highly educated HNP will work independently (with the assistance of written policies, as there are selected procedures, investigations and management decisions that can be well defined by appropriate and well documented best practice guidelines, and the support of the Haematology Consultant) and interdependently with all members of the haematology interdisciplinary team.

In addition to advanced knowledge and skills in haematology/autologous transplantation, the HNP will develop and achieve a level of proficiency in physical and psychological assessment and will have the authority to initiate selected pathology and radiological investigations, interpret results, obtain informed consent for selected procedures, initiate standing pharmaceutical orders and order blood products. The performance of some medical procedures may constitute substitution, however advanced practice nursing will offer insights into the clinical area from experiential, theoretical, and philosophical nursing perspectives. The HNP practice field spans the entire care continuum. The HNP will value and work purposefully to achieve a collaborative and interdisciplinary model of care with an emphasis on consultation and appropriate referral.

HAEMOPOIETIC CELL TRANSPLANTATION IN AUSTRALASIA: AN INTERNATIONAL PERSPECTIVE.

I Nivison-Smith. *Australasian Bone Marrow Transplant Recipient Registry, St Vincents Hospital, Darlinghurst NSW.*

Bone marrow and stem cell transplantation activity has been recorded by the Australasian Bone Marrow Transplant Recipient Registry (ABMTRR) since 1992 for Australia, and for New Zealand since 1998. Haemopoietic cell transplants in Australia increased in number from 478 in 1992 to 1,003 in 1998. There were 91 haemopoietic cell transplants in New Zealand in 1998.

Between 1997 and 1998 the annual number of autologous transplants in Australia increased from 619 to 683. The number of allogeneic related transplants increased from 212 to 238, while the number of allogeneic unrelated procedures increased from 74 to 82. In 1998 in New Zealand, there were 52 autologous transplants, 31 allogeneic related transplants and 8 allogeneic unrelated transplants.

In Australia in 1997, the main indications for allogeneic transplant were AML (27%), CML (22%) and ALL (18%). This compares closely with international figures compiled by the International Blood and Marrow Transplant Registry (IBMTR) for 1997 where the main indications were CML (24%), AML (23%) and ALL (16%).

The 5-year survival probability for allogeneic transplant recipients with HLA-identical sibling donors is currently 66% for CML patients in first chronic phase from Australian data, compared to 58% from IBMTR data. Mortality in the first 100 days post transplant after allogeneic transplant with HLA-identical sibling donors for CML patients in first chronic phase is currently 15% from Australian data compared with 14% from IBMTR data.

Current patterns of haemopoietic cell transplantation in Australia and New Zealand show an increasing use of these procedures as treatment for a widening range of indications, with outcomes that compare favourably with international data. The ABMTRR is a valuable national resource providing timely and detailed information on transplant activity and outcome.

FACTOR V, MTHFR, PAI-1 AND PROTHROMBIN POLYMORPHISMS IN PATIENTS WITH VENOUS THROMBOEMBOLISM.

K.F. Cheong*, K. Wong, K. Sheather, L. Coyle and D.D.F. Ma*. *Haematology Department, St. Vincent's Hospital* and Royal North Shore Hospital, NSW.*

Genetic variance of factor V (1691G→A), thermolabile MTHFR (C677C→T), PAI-1 (-675 4G/5G insertion/deletion polymorphism) and the prothrombin 3'-untranslated region (20210G→A) have been implicated as risk factors for venous thromboembolism (VTE) in studies reported in Europe and the United States. We commenced a prospective study to investigate the incidence of these genetic polymorphisms in patients referred to a single institution for management of VTE. The diagnosis of VTE was confirmed in all patients by independent reviewers. During a period of 2 years, 250 individuals were recruited to this study including 141 patients being confirmed to VTE disease and 64 individuals without any evidence of VTE. The age and sex of the two groups were closely matched. Genetic polymorphism of these four genes were performed by standard PCR and restriction analysis methods. Our results showed there was no significant difference between the disease and control groups in the distribution in genetic variance of PAI-1 and prothrombin genes. As reported previously, a significant difference in factor V Leiden (FVL) was found between these two groups. The patients with VTE had a high incidence of FVL (15.8% versus 2.9%, P=0.005) with a risk ratio of 5.5. For the MTHFR mutation, the incidence of the thermolabile variant (TT) was 12.8% compared to the control of 3.1% (P=0.031). The estimated risk ratio was 4. Further analysis showed that patients with FVL and also carrying the homozygous or heterozygous genetic variant of MTHFR have a slight increased incidence of VTE (18.4% versus 5.3%, P=0.057).

INCIDENCE OF THE PROTHROMBIN G20210A MUTATION IN AN ADELAIDE-BASED STUDY

JE Hardingham, LT Gooley, MB Abbott, WM Hague, RE Sage, NW Wickham.
Haematology-Oncology Department, The Queen Elizabeth Hospital, Woodville Road, Woodville, South Australia.

The 20210 G to A variation in the prothrombin gene was first described by *Poort et al. (1996) and was found to be associated with increased risk for venous thrombosis (18% of patients compared to 1.2% of case controls). The aim of this study was to determine the prevalence of this mutation in patients referred to us with venous thrombosis or a family history, or because of past or present complications of pregnancy.

The G20210A mutation was determined by PCR amplification of the 3' untranslated region of the prothrombin gene. The reverse primer was that of Poort et al. (1996) which contains a mismatch to create a *Hind*111 digestion site if the G20210A mutation is present. We designed the forward primer such that a second naturally occurring *Hind*111 site is included in the PCR product, as a control digestion site. Amplification results in a product of 486 bp; digestion with *Hind*111 produces a fragment of 407 bp if both alleles are normal (GAG), a fragment of 384 bp if both alleles are mutated (AAG homozygous), or both fragments if only one allele is mutated (GAG/AAG heterozygous).

One hundred and forty one patients were analysed. There were 26 males and 115 females. Overall 6/141 (4%) patients were positive for the mutation; 2 males were heterozygous for the G-A mutation (one was also heterozygous for the factor V Leiden mutation), and 4 females, one of which was homozygous for the prothrombin gene mutation.

*SR Poort, FR Rosendaal, Reitsma PH and RM Bertina. *Blood* 88:3698-3703, 1996

POSITIVE LUPUS ANTICOAGULANT TESTS AND THROMBOTIC RISK

Murphy B, Wheeler M, Butler J, Gan T
Haematology Department, Monash Medical Centre, Victoria.

Lupus anticoagulants (LA's) are antibodies which interfere with the phospholipid dependent coagulation in vitro and in vivo resulting in increased risk of thromboembolic events. It has been suggested that some tests are more predictive of thrombophilia. We performed a retrospective analysis of 466 patients who were screened for the LA. The aim of this study was to investigate any correlation between a positive LA screening test and thrombotic risk. In our laboratory we perform the following LA screen; Activated Partial Thromboplastin Time (APTT), Dilute Russell Viper Venom Time (dRVVT), Kaolin Clotting Time (KCT), Dilute Thromboplastin Time (dTT). Mixing studies with normal pooled plasma (NPP) and phospholipid reagent. Of the 466 patients 65 had one or more abnormal results which did not show correction with NPP. Of these 65 patients 31 (48%) had a positive thrombotic history; DVT (n=12), PE (n=8), Thrombotic stroke (n=7), Spontaneous abortion (n=1), retinal thrombosis (n=3). The results of our findings are as follows;

TEST	Abnormal test		Positive thrombotic Hx	
APTT	(25/65)	38 %	(3/25)	12 %
DRVVT	(22/65)	34 %	(11/22)	50 %
KCT	(6/65)	9 %	(3/6)	50 %
DTT(1/100)	(23/65)	35 %	(9/23)	39 %
DTT 1/200)	(36/65)	55 %	(19/36)	53 %

Two types of LA have been described and it has been suggested that the anti-B2 glycoprotein type have a higher thrombotic risk, the pathogenesis of which is not fully understood. Our studies support this and show that a battery of tests is required for the detection of LA patients with increased thrombotic risk.

DETECTION OF PROTEIN S DEFICIENCY: SOME NEW AND OLD METHODS COMPARED.

Emmanuel J Favaloro, Anthony Henniker, Rennie Coombs.
Haematology, ICPMR, Westmead Hospital NSW 2145 Australia.

Protein S (PS) deficiency (PSD) is a well characterised disorder, associated with thrombosis in affected individuals. In plasma, PS exists in a C4b BP-bound form and an unbound (ie 'free') form; together, these comprise the 'total' plasma PS. It is the unbound (free) form that has functional activity. Type I PSD occurs when free PS levels and PS activity are low; Type IIa PSD when free PS levels, total PS levels and PS activity are low; Type III PSD when PS activity only is low. Accordingly, whilst assessment of free PS, total PS, ***and*** PS activity is optimal, in practice, most laboratories perform only a single assay. Moreover, laboratory assessment of PSD is possible using a variety of alternate methodologies and procedures. Currently, assessment of free PS is typically by one of two separate approaches: (i) ELISA procedure using a monoclonal antibody (MAB) against the C4b BP binding site on PS; (ii) initial removal of the C4b BP (including the 'bound' PS material) from plasma using a PEG-precipitation step, followed by measurement of residual (free) PS. The first assay is known to work well, and is available commercially; however, its high cost prohibits its use in most laboratories. The second assay is technically more demanding, very time consuming, and suffers considerable assay to assay and inter-laboratory variation. Accordingly, we undertook to produce various MAB against PS in order to develop alternate assay systems. Some 12 MAB were produced, including one which appears to detect free PS. We are currently assessing the utility of these MAB in ELISA based PS assay systems. Results from these are being compared to alternate ELISA systems, involving the novel approach of detecting total PS vs C4b BP-bound PS. Although results are preliminary, the MAB based systems so far developed are capable of detecting plasma PS. In addition, total PS and C4b BP-bound PS can also be detected by similar ELISA steps, leading the way to a more comprehensive PS testing approach.

EXPERIENCE WITH A DNA-BASED TEST FOR THE DETECTION OF GENETIC RISK FACTORS ASSOCIATED WITH THROMBOPHILIA

N A Marsh, N M Pecheniuk, T P Walsh
Co-operative Research Centre for Diagnostic Technologies, School of Life Sciences, Queensland University of Technology, Brisbane, Queensland

Currently, reliable functional tests are available for the detection of the factor V Leiden variant, the molecular cause of 90% of all cases of activated protein C resistance and the main genetic basis for familial thrombophilia. However, there are no functional tests for the detection of the prothrombin 20210 and methylene tetrahydrofolate reductase 677 variants which may also be significant risk factors for thrombotic conditions. We have previously described a combined test based on the first nucleotide change (FNC) technology: a simple, direct minisequencing protocol which is easily adaptable for examining multiple targets and/or samples. The test is based on a single nucleotide extension of a primer hybridised next to the mutation site which has the power to discriminate the genotype of the variant. The assay endpoint is a ratio of colourimetric optical densities generated from the incorporation of the two bases: adenine (mutant) and guanine (normal). We have established numerical cutoffs for the technique so that homozygous wild-type samples can be clearly distinguished from heterozygote and homozygote variants for two of the mutations. However, we could not delineate cutoffs successfully for the homozygote MTHFR variant. Using this technique, we tested 500 samples from a population of normal blood donors and the results were: factor V Leiden, 3.6%, prothrombin 20210, 2.8% and MTHFR, 11.0%. The advantages of an objective numerical endpoint over examination of gel electrophoresis patterns, include computer assisted interpretation, direct importation of data into statistical packages and facilitation of data storage.

A randomised comparison of G-CSF Stimulated Bone Marrow (G-BM) and G-CSF Mobilised Peripheral Blood (G-PBSC) as sources of stem cells for allogeneic transplantation.

James Morton, Natasha Woodward, Cheryl Hutchins, Judy Cummins and Simon Durrant.
Bone Marrow Transplant Unit, Royal Brisbane Hospital.

The use of G-PBSC instead of bone marrow for allogeneic transplantation is increasing due to accelerated engraftment, however the higher T-cell load is likely to result in an increase in the occurrence of clinical extensive chronic GVHD. Small studies using G-BM for autologous transplantation have reported rapid engraftment with smaller T-cell loads. The aim of this study was to compare G-PBSC with G-BM for allogeneic transplantation. The study was designed to detect a difference of 1 day in neutrophil engraftment with a power of 80%, α=0.05 and planned to enrol 128 patients. This report represents an interim analysis of the first 47 patients.

Donors all received Neupogen 10μg/kg for 5 days and were randomised to either bone marrow harvest (15-20ml/kg) or 1-2x 12L peripheral blood collections (minimum $2.5x10^6$/kg $CD34^+$). Randomisation was stratified by disease risk (high or standard) in blocks of 4 patients. All patients received GVHD prophylaxis with CSP and day +1, 3, and 6 MTX with leucovoron the following day.

At this time 47 consecutive patients have been enrolled (35 standard, 12 high risk) with 24 receiving G-BM (6 high risk). Median follow-up is 486 days (range 70,703). Median times to neutrophil and platelet recovery have been significantly faster in the G-PBSC group (table 1).

	BM	PBSC	p
ANC>500/ul	16.5 (14.5,18)	14 (13,16)	0.007
PLT>20/ul	14 (12,17)	12 (11,14)	0.05

The Cumulative incidence of grade II-IV, and grades III-IV acute GVHD are not significantly different. Overall and relapse free survival are similar. Of patients alive and in remission beyond 100 days, 10 of 27 developed clinical extensive chronic GVHD, including 2/13 G-BM and 8/14 G-PBSC (ρ=0.03).

From this interim analysis engraftment with G-BM is slightly slower than with G-PBSC, however it appears that the incidence of clinical extensive chronic GVHD is reduced. Patient accrual in this study is ongoing.

PERIPHERAL BLOOD STEM CELL TRANSPLANTATION FOR RHEUMATOID ARTHRITIS – AN AUSTRALIAN MULTICENTRE TRIAL. **J. Moore,** ***St.Vincents Hospital, Sydney, on behalf of the Australian Cooperative Transplant Group.***

Rheumatoid arthritis (RA) has recently been suggested as a good candidate for PBSCT based on animal studies and favourable remissions obtained in patients with coexistent malignancy. Phase I dose escalation trials, in Australia, have demonstrated significant remissions in 8 RA patients undergoing PBSCT with unmanipulated grafts. This lead to our current randomised multicentre trial comparing CD34 selection (as a form of T cell depletion) with unmanipulated PBSCT in an attempt to ascertain the role of infused T cells in RA. 30 severe resistant RA patients aged 18-65 years who have failed at least 2 disease-modifying agents, are eligible. Stem cell collection is with GCSF and conditioning with Cyclo 200mg/kg. CD34 selection is performed with the Isolex device. 12 patients have now been randomised in 3 centres (7 selected and 5 unmanipulated). 8 patients have been transplanted with acceptable toxicity levels, 1 patient is off study for failing to collect adequate stem cells and 3 patients are currently having cells collected for PBSCT. Stem cell mobilisation has been difficult with 4/12 not achieving $>2 \times 10^6$ CD34/kg with the first attempt of GCSF 10mcg/kg. Patients have required on average 2 aphereses (range 1-4) with 5.1×10^6/kg CD34 cells collected (range 2.4 - 9.03). Subsequent patients will be mobilised with 12mcg/kg GCSF bd. Engraftment was 12 days for neut >0.5 and 12 days for plat. $>20 \times 10^9$/L. Thus far 7/8 patients have had significant improvements in swollen and tender joint scores along with ESR and CRP correlating with complete remission or major responses (based on American College of Arthritis criteria). Follow up is short, but extensive immunological and clinical data is being collated to ascertain the mechanisms for these initial encouraging findings in patients who have severe resistant disease.

NON-MYELOABLATIVE ALLOGENEIC BLOOD STEM CELL TRANSPLANTATION FOR METASTATIC MELANOMA: A PILOT STUDY

KF Bradstock[1], DJ Gottlieb[1], RF Kefford[2], R Childs[3], AJ Barrett[3].

[1]BMT Service, and [2]Dept of Medicine, Westmead Hospital, NSW, Australia, and [3]BMT Unit, Hematology Branch, National Institutes of Health, Bethesda, Maryland, USA

Melanoma is an immunogenic tumour which responds to immunotherapy-based protocols in approximately 30% of patients with metastatic disease. We have explored the effects of allogeneic T lymphocytes from HLA-matched siblings in 7 patients with advanced melanoma who had failed conventional treatment. Patients were prepared for transplant with non-myeloablative chemotherapy (Fludarabine 25mg/M^2/day x 5, plus Cyclophosphamide 60mg/kg/day x 2 (5 patients) or Cytarabine 2g/M^2/day x 5 (2 patients). Unmanipulated G-CSF-mobilized blood stem cells from their HLA-identical sibling donors were infused on day 0. Cyclosporin given as GVHD prophylaxis was tapered from day +42, and donor leucocytes were given in some patients as additional anti-melanoma therapy, or to boost donor chimerism. Of seven patients transplanted, 2 died before day +28 of rapidly progressive melanoma. One patient died of transplant-related complications (pneumonia, encephalopathy) at day 50, after engrafting fully with donor lymphoid and myeloid cells. Four other patients also engrafted and showed evidence of donor chimerism. Two subsequently rejected their grafts; one of these underwent a second transplant at 6 months, and survives at 9 months. Of 4 patients evaluable for graft-versus-tumour activity of the transplant, 3 have shown evidence of melanoma regression (cerebral metastases in 1, lymph node metastases in 2), while 1 died of progressive disease at 9 months after graft rejection. This pilot study shows that allogeneic anti-melanoma effects may occur even in patients with advanced disease, and suggests that further research is warranted.

A COLLABORATIVE TRIAL OF OUTPATIENT CARBOPLATIN-SUSTITUTED ESHAP FOR PBPC MOBILISATION AND TREATMENT IN RELAPSED AND REFRACTORY LYMPHOMA

A Spencer[1], I Kerridge[2], S Deveridge[2], M Hertzberg[3], M Seldon[2], A Schwarer[1], A Enno[2]

[1]BMT Programme, The Alfred Hospital, Prahran; [2]Hunter Haematology Unit, Mater Misericordiae Hospital, Newcastle; [3]Department of Haematology, Westmead Hospital, Westmead.

We have treated 50 patients (age 15 – 80 years, median 54 years, 10 patients >65 years) with relapsed (n = 39; previous CR = 20 or PR = 19) or primary refractory (n = 11) lymphoma (NHL = 45, Hodgkins Disease = 5) on an outpatient basis with a 5 day schedule of etoposide, methylprednisolone, Ara-C and carboplatin (ESHAC). All patients received G-CSF 5µg/kg daily commencing on day +10. At diagnosis disease was stage III or IV in 64%, B-symptoms were present in 42%, bone marrow involvement was present in 26% and the LDH was elevated in 45% of cases. The median number of prior treatments was 1.5 (range 1 – 4). To date 125 cycles of therapy have been delivered with a subsequent 17% hospital admission rate and a 12.5 % incidence of febrile neutropenia. Grade IV neutropenia and Grade IV thrombocytopenia have occurred following 21% and 33% of evaluable cycles respectively. There have been no treatment related deaths. Total response rate is 62% (CR + PR). Twenty four patients have undergone PBPC collection commencing on days 14 – 16 following ESHAC therapy (1 – 4 leucaphereses, median 2). Median CD34+ cell dose collected was 5.0 x 10^6/kg (range 0.12 - 32.0 x 10^6/kg) with 3 patients achieving < 2 0 x 10^6/kg and 28 patients have proceeded to ASCT at a median of 3.3 months from post-salvage therapy. At 2 years post-salvage therapy OS is 42% with PFS in those undergoing ASCT of 66%. We conclude that ESHAC is an effective salvage therapy for relapsed/refractory lymphoma which also facilitates effective disease-specific PBPC mobilisation to enable early ASCT.

CD8 DEPLETION USING T8 MURINE MONOCLONAL ANTIBODY- COATED DENSE NICKEL PARTICLES: EFFICACY OF DEPLETION AND SAFETY IN DONOR LYMPHOCYTE INFUSION (DLI).

K. Atkinson, E.P. Alyea, C. Canning, H. Houde, R.J. Soiffer, S. Giralt, A. Gee, R. Champlin.

Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA. Univ. Of Texas MD Anderson Cancer Center, Houston, TX, Eligix Inc., Medford, MA, USA.

The development of graft-vs-host disease (GVHD) limits the effectiveness of DLI in patients (pts) who have relapsed after allogeneic BMT. Several studies suggest that the depletion of CD8+ cells prior to DLI may lead to a reduction in the incidence and severity of GVHD. However, current methods to deplete large number of cells from pheresis products are time consuming and difficult to perform. Recently, CD8 murine monoclonal antibody-coated dense nickel particles have been developed which allow for the rapid separation of cells by use of gravity. We conducted a pilot trial using CD8-High Density Microparticles (HDM) to determine the efficacy of CD8 depletion and the safety of infusing cells processed by this method. All pts received 3.0 x 10^7 CD4+ cell/kg, a dose that had been defined in previous studies. No other immune modulating therapy was used. Nine pts who relapsed after allogeneic BMT were enrolled. Three pts had CML, 3 multiple myeloma, 2 CLL and 1NHL. The median age was 40 (range 31-58) and the median time from transplant to DLI was 43 months (range 10-78). Two pts (1 multiple myeloma, 1 NHL) were withdrawn from the study due to rapidly progressive disease. Eight of 9 pts received the targeted cell number after 1 pheresis procedure. The time required for processing with CD8-HDM was approximately 1 hour. The median depletion of CD8+ cells from the pheresis product was 100% (range 97.8 to 100%): the mean percent yield of CD4+ cells was 105%. With depletion of CD8+ cells, the mean yield of CD3+ cells was 72.2%. No infusional toxicity related to the method of depletion was noted. One patient developed interstitial pneumonitis 2 weeks following cell infusion, which resolved. The overall incidence and severity of GVHD was low: 1 pt developed grade 2 acute GVHD and limited chronic GVHD. Three of 3 pts with CML developed a complete hematologic and cytogenetic response. Two of 2 on whom data are available achieved a complete molecular response. One pt with CLL had a good PR. The remaining patients had progressive disease. CD8-HDH appears to be highly selective and effective in depleting CD8+ cells from pheresis products. This method of depletion will allow for a multi-center trial to assess the impact of CD8 depletion on the incidence and severity of GVHD following DLI.

ANTIPROLIFERATIVE EFFECTS OF AG957 AND AG490 (TYRPHOSTIN INHIBITORS OF *BCR-ABL* AND *JAK2* RESPECTIVELY) ON MYELOID CELL LINES

X.M. Sun[1], J.E. Layton[1], A.G. Elefanty[2], A. Levitski[3] and G.J. Lieschke[1]

[1]Ludwig Institute for Cancer Research, and [2]Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria 3050; [3]Hebrew University, Jerusalem, Israel.

The *BCR-ABL* fusion gene protein product P210 with enhanced protein tyrosine kinase (PTK) activity is a hallmark of chronic myeloid leukaemia. Also, recent reports implicate constitutive *JAK2* PTK activity in acute lymphoblastic leukaemia. Hence specific PTK inhibitors directly acting on P210 or *JAK2* PTK could be useful in the treatment of leukaemia. AG957 and AG490 are small molecule tyrphostin PTK inhibitors: P210 kinase activity is 7-fold more sensitive to AG957 than c-*ABL*; AG490 selectively blocked leukaemic cell growth with *JAK2* PTK inhibition. Cell lines used in ^{3}H-thymidine incorporation proliferation assays were: FDCP1(wild-type & IL-3-independent P210 expressing clones), 32D (wild-type & an EGR-responsive clone expressing a EGFR-muJAK2 chimera), K562 (*BCR-ABL*- expressing human leukaemic cell line) and U266 (human myeloma cell line). Exposure of K562 cells and P210-FDCP1 cells to AG957 for 30 min-18 h inhibited proliferation (IC_{50}0.7-6 M); only minimal restoration of IL-3 dependence of the P210-FDCP1 cells was demonstrated (IC_{50} in IL-3 above 90% of those determined without IL-3). However, AG957 also inhibited proliferation of wild-type (i.e. non-P210-expressing) FDCP1 cells and all the other cell lines (IC_{50}1.2-15 M). AG490 inhibited all cell lines with 18 h exposure (IC_{50}1-30 M; for U266 IC_{50}>40 M), but 30 min exposure to AG490 was non-inhibitory. Synergistic inhibition occurred when 30 min AG957 exposure was preceded by low-dose AG490 exposure. We conclude that AG957 is a more potent antiproliferative agent than AG490 for K562 and P210-FDCP1 cells, however, its action was not specific for P210-stimulated proliferation. AG490 is a less potent inhibitor, but could synergize with AG957.

THE DISULPHIDE BOND BETWEEN CYS-4 AND CYS-17, IN THE PLATELET GPIb-alpha SUBUNIT, IS CRUCIAL FOR vWF BINDING

AS Tait, IW Dawes and BH Chong

Haematology Department, Prince of Wales Hospital, Randwick, NSW, 2031

The amino acid cysteine is important in the conformational structure of proteins. In the platelet GPIb-alpha subunit, of the nine cysteine residues, six are involved in intra-disulphide bond formation. This includes Cys-4 and 17, Cys-209 and 248 and Cys-211 and 264. Previous studies have shown that Cys-209/248 and Cys-211/264 are crucial for the interaction between GPIb and vWF. The cysteine residues at 484 and 485 are thought to be involved in inter-disulphide bonds with GPIbβ. To date, there is only limited knowledge regarding the remaining three residues and their involvement in the interaction with vWF. These include the Cys-4 to 17 disulphide bond and the Cys-65 residue, thought to have a free thiol group.

To examine the function of these residues, wild-type GPIb-alpha, Cys4Ser, Cys17Ser and Cys65Ser GPIb-alpha mutants were expressed in CHO cell lines previously transfected with GPIb-beta and GPIX. When the disulphide bond between Cys-4 and Cys-17 is disrupted, the interaction with vWF is abolished. When the free thiol group is removed from GPIb-alpha at position 65, the binding to vWF reaches saturation at lower levels of ligand. From 0.5 – 2 μg/ml vWF, there is no difference in binding between the mutant and wild-type cell lines. At 4 – 8 μg/ml, there is 50% less vWF bound to the mutant than to the wild-type cell line.

These results indicate that the disulphide bond between Cys-4 and 17 is crucial in the interaction between GPIb-alpha and vWF. The free thiol at position 65 seems to play a role in regulating vWF binding.

USEFULNESS OF FACTOR VIII BINDING ASSAY AND TWO-STAGE FACTOR VIII ASSAY IN THE DIAGNOSIS OF HAEMOPHILIA A, CARRIERS AND VON WILLEBRAND'S DISORDER TYPE 2N.

SE Rodgers[1], EM Duncan[1], NV Lerda[2], DM Quinn[2], RLP Flower[2], JA Rowell[3], RI Baker[4] and JV Lloyd[1]. *[1]Haematology Division, IMVS, Adelaide, [2]University of South Australia, [3]Royal Brisbane Hospital, [4]Royal Perth Hospital.*

In von Willebrand's disorder type 2N (vWD 2N), haemophilia A and carriers, there are low levels of factor VIII coagulant activity (FVIII:C) and normal von Willebrand antigen. We have used the factor VIII binding assay to differentiate between these disorders in 20 families. As controls, we also tested 18 normal subjects, 10 patients with haemophilia A and 8 with von Willebrand's disorder. We have also assessed the usefulness of the two-stage factor VIII assay (FVIII:C-2), compared to the one-stage assay (FVIII:C-1).

Of the 37 patients tested, 20 were male and 17 female; 24 were from SA, 7 from WA and 6 from Qld. Severely reduced factor VIII binding (≤25%) was found in 7 patients (from 4 families). Four of these were homozygous for a vWD 2N mutation, and 3 were heterozygous. One homozygous vWD 2N patient had previously been diagnosed with mild haemophilia A. A binding defect was excluded in 28 patients (15 families). In the remaining family, there was a mildly reduced binding defect and a heterozygous mutation.

In the 7 patients diagnosed with vWD 2N, FVIII:C-2 (0.04-0.37 IU/ml) was substantially less than FVIII:C-1 (0.20-0.69 IU/ml). FVIII:C-2 was less than half FVIII:C-1 in another 18 patients, consistent with variant mild haemophilia known to show this discrepancy. In one of these patients, FVIII:C-1 was normal (0.70 IU/ml) while FVIII:C-2 was low (0.12 IU/ml) and diagnostic for haemophilia.

We have found the factor VIII binding assay to be useful for diagnosis of vWD 2N, and also for its exclusion. The two-stage factor VIII assay shows better discrimination from the normal range in some patients with a bleeding disorder.

CLASSIFICATION OF FACTOR VIII INHIBITORS IN HAEMOPHILIA A BASED ON KINETIC STUDIES

M Ling[1,2], EM Duncan[1], SE Rodgers[1], JV Lloyd[1], and A Street[3].
[1]Haematology Division, IMVS, Adelaide, SA; [2]Pharmacology Department, University of Adelaide, Adelaide, SA; [3]The Alfred Hospital, Melbourne, VIC

The classic reference for classification of factor VIII (FVIII:C) inhibitors, published nearly 30 years ago (Biggs 1972), described type I inhibitors as causing second-order inactivation kinetics while type II inhibitors follow a more complex pattern. We re-examined this classification using antibody dilution and time course studies. Our aim was to develop a routine laboratory approach to classify these inhibitors.

Ten haemophilia patients with inhibitors were studied, and included 5 severe, 3 mild and 2 acquired cases. In antibody dilution studies normal plasma was incubated for two hours with a series of dilutions of patient plasma and residual FVIII:C measured as in the Bethesda method. For plasma samples with inhibitor levels >10 BU/ml, FVIII:C plotted against antibody dilution showed two different patterns. A steep slope and complete FVIII:C inactivation at high antibody concentrations is typical for type I, and was seen with 2 severe and 1 mild case. A curvilinear pattern with a FVIII:C plateau at high antibody concentrations characterises type II, which was seen with 1 mild and 1 acquired case. This indicated incomplete inactivation of FVIII:C even when antibody was in excess. In plasma with inhibitors <10 BU/ml, no clear classification could be made.

In a 2-hour time course study, all cases showed rapid initial loss of FVIII:C to near maximal inactivation followed by a slight continuous decline. Extended time course studies with addition of extra AHF after 4 hours gave complex results. Type I and II inhibitors could only be differentiated when a critical ratio of antibody to factor VIII was used.

In summary, when the level of inhibitor in plasma was >10 BU/ml, the antibody dilution study could distinguish type I from type II inhibitors, whilst other approaches were less successful.

RH(D) IMMUNOGLOBULIN POTENCY ASSAY - A COMPARATIVE STUDY BETWEEN FLOW CYTOMETRY AND COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY

Kim Elliott, Albert Farrugia, Em Tran, Alison Charnock, Mirek Nemec

Blood Products Group, Molecular Biology Section,, Therapeutic Goods Administration, P.O Box 100, Woden , A.C.T, 2606, Australia

Anti-D immunoglobulin is administered to rhesus negative women to decrease the incidence of Haemolytic Disease of the Newborn (HDN) in subsequent pregnancies. Accurate quantification of the anti-D content is essential in order to ensure the quality, efficacy and safety of Rh (D) Immunoglobulin (Ig) administered. The current pharmacopoeial method for quantitation of Anti-D is based on red cell agglutination detected on the Auto-Analyser. New techniques to determine Rh(D) Ig potency are currently being validated. Our laboratory has developed a Flow Cytometric (FC) assay using R_2R_2 RBC (CSL Bioplasma) incubated with Anti-D, stained with FITC conjugated IgG (gamma chains), then analysed on a Coulter Epics-XL Flow Cytometer. Results obtained over the past 12 months indicate the intra-laboratory %CV for this method is 11.22% (n=32).

In collaboration with the National Institute for Biological Standards and Control (NIBSC), we are currently trialing a competitive ELISA method using R_2R_2 coated plates, incubated with Anti-D and biotinylated human IgG1 Anti-D antibody (Bio-Brad-5; NIBSC code 99/578). Binding of Brad-5 is then detected with alkaline phosphatase labelled Extravidin and absorbance read on the Spectramax™ plate reader. This method is more labour intensive than the FC assay, but the %CV reported by NIBSC is stated to be 3%. We are currently analysing the same sample of Rh(D) Ig by both methods. Intra-laboratory %CV is closer to 7.3% (n=4) by ELISA and 12.97% (n=10) by FC. This study will continue to help establishment of a rapid and reproducible method to replace the current European Pharmacopoeial Auto-Analyser assay.

MONITORING RESPONSE TO TREATMENT IN CHRONIC MYELOID LEUKAEMIA: REAL-TIME QUANTITATIVE PCR OF BCR-ABL IN BLOOD IS A RAPID AND RELIABLE SUBSTITUTE FOR BONE MARROW CYTOGENETICS.

S Branford, TP Hughes, Z Rudzki.

Division of Molecular Pathology, Institute of Medical and Veterinary Science; Leukaemia Research Unit, Hanson Centre for Cancer Research, South Australia.

We have developed a rapid real-time quantitative PCR method for measuring BCR-ABL mRNA levels in peripheral blood in chronic myeloid leukaemia. The technique was used for the early detection of relapse after allogeneic transplant and as an assessment of treatment response. The method was sensitive, reproducible and readily detected a change in BCR-ABL transcript levels in serial blood samples. Normal BCR mRNA was quantitated to control for RNA degradation and the results reported as a percentage of BCR-ABL/BCR. Every patient measured at diagnosis (n = 23), had increased expression of BCR-ABL of up to five fold above the normal BCR levels. With effective treatment the BCR-ABL levels decreased. The molecular data was correlated with Philadelphia chromosome levels in bone marrow and a good correlation was found when treatment induced a cytogenetic response (Spearman correlation = 0.94, $p < 0.0001$, n = 70 samples). In patients receiving interferon-α therapy, we found a significant difference in the BCR-ABL levels between cytogenetic response groups. In all patients with transformed disease (n = 7), the BCR-ABL levels were raised, similar to the diagnosis levels. Serial monitoring was performed on five of these patients and showed that high levels preceded cytogenetic or haematological signs of disease progression. We conclude that real-time quantitative PCR monitoring of peripheral blood can be used to reliably monitor disease response in CML.

DISCRIMINATION OF VWD SUBTYPES BY ELISA: VWF:CBA VERSUS MONOCLONAL ANTIBODIES.

Emmanuel J Favaloro, Anthony Henniker, David Facey.

Haematology, ICPMR, Westmead Hospital NSW 2145 Australia.

Discrimination of von Willebrand's Disease (VWD) subtypes is important as it will influence management. Qualitative [ie Type 2] defects exhibit von Willebrand factor (VWF) discordance and give high VWF:Ag to VWF:'activity' ratios. Classically, VWF:'activity' is assessed using the VWF:RCof assay. The VWF:CBA is a relatively new, ELISA-based VWF-functional 'adhesive' assay which has consistently proved to be superior to VWF:RCof (1). A commercially available monoclonal antibody (MAB) based ELISA assay system claimed to mimic a VWF:RCof-like activity has also been recently described ('SE'), as has the production and characterisation of a large number [n=10] of anti-VWF MAB as part of a previous collaborative project involving our laboratory. In the current study, we have compared the ability of various assay systems to discriminate VWD subtypes. Thus, the VWF:CBA, VWF:RCof by agglutination, and SE assays have been directly compared for their ability to discriminate Type 1 VWD samples [n=9] from Type 2 [n=11]. We have also utilised the in-house produced anti-VWF MAB in recent in-house developed ELISA assays to assess whether these could also be utilised in VWD diagnosis and subtype discrimination. In summary, MAB-based systems, when optimised, could be shown to have some VWD-discriminatory capabilities. Thus, VWF assays using MAB (both in-house and SE systems) could be developed to yield results showing a pattern of VWF discordance when using Type 2 VWD plasma, and when compared to VWF assessed by the classical VWF:Ag procedure. However, better evidence of VWF-discordance was usually achieved using the VWF:RCof by agglutination assay, and greatest VWF-discordance was always observed using the VWF:CBA assay. In conclusion, the VWF:CBA assay proved to offer the best 'diagnostic' predictive tool for a Type 2 VWD defect, and use of MAB-based systems appear the least effective in this regard.

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RETROVIRAL TRANSDUCTIONOF PRIMARY HUMAN CHRONIC MYELOID LEUKAEMIA CELLS WITH BCR-ABL RIBOZYMES

Presgrave PJ, Wright LA, Kearney PK, Biggs JC, Ma DDF.
Department of Haematology, St. Vincent's Hospital, Sydney, Australia.

Chronic Myeloid Leukaemia (CML) is characterised by the presence of the chimaeric *bcr-abl* gene, which is strongly implicated in the pathogenesis of the disease. The *bcr-abl* mRNA is consequently an ideal target for ribozyme-medicated inactivation and it has been hypothesised that down regulation of *bcr-abl* expression may lead to an alteration of the malignant phenotype. Our laboratory has previously designed a series of hammerhead ribozymes (Rz6-10) targeted to the b3a2 type *bcr-abl* mRNA. In vitro experiments with synthetic *bcr-abl* and *bcr* substrates have shoed differing degrees of cleavage and specificity exhibited by these ribozyme sequences (Wright *et al*., 1993; Kearney *et al*., 1995).

To assess the activity of these molecules in primary human CML cells, ribozyme cDNAs were cloned into the retroviral vector pLGL1, containing the GFP reporter gene, and high titre clones for each construct isolated. A pure population of peripheral blood CD34+Ph+ CML progenitors were obtained by MACS purification. Cells were transduced with supernatant containing the control vector or Rz. Two rounds of infection were performed at 24 hour intervals. All cultures were performed in the presence of IL-3 and IL-6. Following 48 hour expression culture, the transduced cells were FACS sorted based on GFP expression, yielding a highly purified population of CD34+ cells expressing the transgene. The cells were seeded into LTCIC assays, which were demipopulated at weekly intervals, and the non-adherent cells seeded into methylcellulose cultures. Initial results from single colony RT-PCR in one patient demonstrates transgene expression out to day 42 in both vector and Rz transduced cells (representing > 50 days *ex-vivo* culture). RT-PCR of day 17 colonies showed that 90% of colonies expressing the control vector continued to express *bcr-abl*. In contrast, 100% of Rz expressing colonies were *bcr-abl* negative, suggesting an effect of Rz on *bcr-abl* mRNA expression. This change in *bcr-abl* expression was accompanied by effects on cell proliferation and clonogenicity. Further primary human Ph+ cells (b3a2/b2a2) are being analysed for Rz specific alternations in the malignant phenotype.

CYTOLYTIC P2X7 PURINOCEPTORS ARE EXPRESSED IN LYMPHOCYTES OF B-CHRONIC LYMPHOCYTIC LEUKAEMIA BUT MAY BE NON-FUNCTIONAL

JS Wiley, B Gu, P Dao, W Zhang, L Bendall, I Chessell
Sydney University Department of Medicine, Nepean Hospital, Penrith, NSW, Australia

Chronic lymphocytic leukemia (B-CLL) is a disease in which the lymphocytes accumulate in blood and bone marrow with little evidence of apoptotic death in vivo. However brief exposure of B-CLL cells to extracellular ATP triggers subsequent apoptotic cell death in vitro. We have measured the surface expression of P2X7 receptors with a P2X7 monoclonal antibody against an extracellular epitope as well as measuring the unique functional response to ATP in which the P2X7 ionic channel dilates to a cytolytic pore allowing entry of ethidium dye. Six out of eight patients with B-CLL showed strong surface expression of P2X7 although all eight showed immunoreactivity within the cytosol. The two patients without surface P2X7 as well as another two patients with immunoreactive P2X7 on the cell surface showed absence of functional responses to ATP. In all eight patients mRNA for P2X7 was present on RT-PCR analysis. Normal peripheral blood B lymphocytes expressed P2X7 receptors and showed the normal permeability responses to extracellular ATP. Thus B-CLL lymphocytes show two separate defects in the cytolytic P2X7 receptor. First, some patients show impairment of movement of P2X7 protein to the cell surface while other patients have immunoreactive P2X7 receptors on the cell surface which is non functional. Since P2X7 activation causes apoptotic death of B-CLL lymphocytes, it is possible that the above defects may contribute to the long survival of B-CLL lymphocytes in vivo.

SERIAL SECTIONING OF THE TREPHINE BIOPSY INCREASES THE DIAGNOSTIC ACCURACY OF BONE MARROW INVOLVEMENT IN DIFFUSE LARGE CELL LYMPHOMA.

J.K. Campbell, S.K. Juneja and M. Wolf on behalf of the Australian Leukaemia and Lymphoma Group.

Department of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, East Melbourne 3002.

Bone marrow involvement occurs in 5-34% of patients with diffuse large cell lymphoma (DLCL). In 50-70% of patients histologic discordance is noted between the lymph node and the involved bone marrow. There are few guidelines regarding the length of the trephine and the number of sections necessary for accurate diagnosis. As part of a wider study of the prognostic significance of discordant histology in DLCL we determined the incidence, proportion and pattern of bone marrow involvement in 131 patients with DLCL enrolled in the ANZLG NHL05 and NHL07 trials. The effect of serial sectioning of the trephine biopsy on the sensitivity of the diagnosis was also examined.

The H and E stained original trephine biopsy and 3 deeper levels cut 0.1 mm apart were examined. Bone marrow involvement was present in 41 of 131 cases (31%). Minimal infiltration was common, with 46% of patients having <5% and 27% having 6-24% involvement. Discordant histology (<50% large cells) was present in 54% of patients. Nodular and paratrabecular aggregates alone or in combination were the most common pattern of marrow infiltration (68%). In 7 of 39 patients (18%) with both the original trephine and deeper levels available for comparison, lymphomatous aggregates were present in the deeper levels only. Examination of deeper levels also enabled a positive diagnosis in 3 patients and a negative diagnosis in 4 patients where the original trephine histology was equivocal.

Bone marrow involvement was detected in 31% of patients with DLCL. Examination of serial sections of the trephine biopsy increases the rate of diagnosis of marrow involvement and may provide a useful means to determine the significance of otherwise equivocal lymphoid aggregates.

TARGRETIN™ FOR PREVIOUSLY TREATED PATIENTS WITH CUTANEOUS T CELL LYMPHOMA: THE PETER MACCALLUM CANCER INSTITUTE/ST VINCENTS HOSPITAL EXPERIENCE.

HM Prince, G Ryan, JF Seymour, C McCormack, C Baker, H Rotstein, M Wolf, H Januszewicz, J Davison, S Juneja. *Division of Haematology and Medical Oncology and Division of Radiation Oncology, Peter MacCallum Cancer Institute and Dermatology Unit, St Vincents Hospital, Melbourne, Victoria*

Targretin™ (LGD1069) is a novel synthetic retinoid anologoue which binds to RXR receptors. Results of Phase I studies suggest activity in mycosis fungoides (MF)/Sezary syndrome (SS). Three Phase II studies have recently completed accrual. Eight patients at PMCI have entered these studies and received either the oral formulation (n=7) or the topical gel (n=1). Patients receiving the oral preparation (see table), must have failed either topical therapy and phototherapy (PT)/radiotherapy (RT) or systemic chemotherapy. The majority of pts had received topical nitrogen mustard (NM). Others had received chrorambucil (C), methotrexate, chrorodeoxyadenosine (CDA) or CVP.

#	Stage	chemoRx	PT	RT	Max response		TOX
1	IIB	NM	Y		PR	16	↑TG
2	IVA	NM,C			PR	16	Itch
3	IIB	NM,M	Y	Y	PRO	4	
4	IIB	NM	Y		PR	16	↑TG
5	IIB	C		Y	ST	12	
6	IVA	C,CVP	Y		PR	12	
7	IIB	NM,CDA	Y	Y	VGPR	16+	↑TG,h'ache

In the 5 responding patients, maximum response was observed at 4w. One patient has achieved a VGPR. All but one patient progressed at 16w [time to progression (TTP); 4-16+ weeks]. The major toxicity has been hyper-triglyceridemia (TG) requiring therapy. One pt discontinued treatment because of severe headache (Pt #7). The single pt receiving the topical preparation (Stage IB) remains in VGPR at 44w. We conclude that Targretin is an active agent in MF/SS and studies examining its role in previously untreated patients are warranted.

PILOT STUDY OF HIGH DOSE THERAPY WITH PURGED PERIPHERAL BLOOD STEM CELL TRANSPLANT (PBSCT) IN POOR PROGNOSIS FOLLICULAR NON-HODGKIN'S LYMPHOMA (NHL).

K Gelly, A Cook, S. Carnoutsos, A Moore, M Carpenter, D Fearnley, GR Hill,[1] RL Spearing, DC Heaton, S Gibbons, DNJ Hart[1], WN Patton, on behalf of the South Island Bone Marrow Transplant Unit, Christchurch Hospital, New Zealand and Mater Medical Research Institute,[1] Brisbane, Australia.

Innovative therapies for patients with poor risk follicular NHL include new combination chemotherapy regimens ± IFN, autologous high dose therapy (HDT) ± purging, allo BMT, anti-B cell antibodies, anti-idiotype vaccinations and bcl-2 anti-sense therapy. Patients with successfully purged autologous grafts and those whose bone marrow remains negative for molecular markers post HDT have shown good disease free survival post HDT. We report our initial experience with 14 patients in a phase II study in poor risk follicular (n=13) and mantle (n=1) NHL of initial chemotherapy, CD 34^+ selected (Ceprate SC) PBSCT and IFN post HDT. Age ranged from 30 to 54 yrs, 7 had relapsed disease, 7 de novo disease with poor risk features and 13/14 patients had a molecular marker (IgH/bcl2 = 11; IgH = 2). Initial study therapy included CHOP (n=12) or ESHAP (n=2) and cyclo $2g/m^2$ + G-CSF was used for mobilisation. 1/14 failed to respond to initial therapy and 4/13 failed mobilisation, but 3 of these proceeded to HDT with unpurged autologous marrow which in one case supplemented a poor yield $CD34^+$ PBSCH (0.1×10^6 $CD34^+$/kg). The remaining 9/13 underwent satisfactory $CD34^+$ selection with median yield of $CD34^+$ cells x 10^6/kg = 1.9 (range 0.8 - 4.0) with a median recovery of 28% (8-51) and median purity of 69% (27 - 85). 6/9 patients had PCR detectable contamination of stem cell harvests with 2/3 evaluable remaining positive post $CD34^+$ selection. 1/9 patients could not proceed to HDT due to cardiac toxicity but 8/9 patients proceeded to $CD34^+$ PBSCT (Cy/TBI n=6; CBV n =2). One patient (previously splenectomised) died from sepsis at d +11 and one patient died from progressive NHL at 3 months post PBSCT. The remainder engrafted successfully (median $n>0.5 \times 10^9$/L = d+13; median pl > 20×10^9/L = d +16) with 4/5 evaluable in CR (3 molecular) and 1/5 in stable PR. These data indicate that $CD34^+$ PBSCT is feasible in poor risk follicular NHL and that this approach might be better considered with a B cell negative selection procedure and considered early in the course of the disease.

USE OF LOW DOSE G-CSF POST AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION (PBSCT) IN LYMPHOPROLIFERATIVE DISORDERS

IE Llewellyn, FR MacLean, J Sanders, DC Heaton, S Gibbons, RL Spearing, WN Patton for the South Island Bone Marrow Transplant Unit, Department of Haematology, Christchurch Hospital, New Zealand.

Aims:

To determine the effect of low dose G-CSF on haematological recovery and supportive care requirements for patients receiving high dose therapy with PBSCT for lymphoproliferative disorders.

Methods:

Retrospective open label non-randomised study of 2 consecutive cohorts of patients treated at one institution. Group 1 (n=23; NHL=18; myeloma=2; HD=3) received no G-CSF post PBSCT and Group 2 (n=18; NHL=8, myeloma=5; HD=5) received G-CSF ($50\mu g/m^2$) from day +1 post PBSCT until neutrophils $>0.5 \times 10^9$/L. Conditioning regimens included CY/TBI (n=10), CBV (n=15), BEAM (n=7), HDM (n=6), MEL/TBI (n=1), CY/BCNU/VP (n=1) and ETOP/TBI (n=1). Excluding G-CSF, supportive care policies were identical for each patient group and CD34 cell dose was not significantly different between the groups. (median {range} $CD34^+$ cells Group 1 = 3.2×10^6/kg {1.1-14.5}; Group 2 = 2.5×10^6/kg {0.7-5.4}).

Results:

There were no transplant related deaths and no failures to engraft. Neutrophil recovery was more rapid in the G-CSF treated group (median days to $n>0.5 \times 10^9$/L = 11 v 13; p=0.02) and this was associated with a significant reduction in days on IV antibiotics (median = 7 v 9; p=0.03) and a trend for a decrease in days until discharge (median = 15 v 17; p=0.32). Platelet support requirements however, were significantly increased in the G-CSF treated group (median = 24 v 12 units; p=0.03) where there was also a trend for delayed platelet recovery (median = days to $pl>20 \times 10^9$/L = 14 v 12; p=0.22).

Conclusions:

Low dose G-CSF post PBSCT enhances neutrophil recovery with some clinical benefits but can result in increased requirements for platelet suppport. It is uncertain whether higher threshold values for $CD34^+$ cells might influence these observations.

MOBILIZATION OF TUMOUR CELLS IN PATIENTS WITH MYELOMA ACCEPTED FOR HIGH DOSE THERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION.

D Thuraisundaram, NS Van de Water, J Nelson, E Theakston, PJ Browett, *Department of Molecular Medicine, University of Auckland and Molecular Haematology Laboratory, Auckland Hospital, Auckland, New Zealand.*

High dose therapy followed by autologous peripheral blood stem cell transplantation (APBSCT) is now part of standard therapy for patients with multiple myeloma. Recent studies however have raised the possibility of tumour cell mobilization during the stem cell collection procedure, with subsequent contamination of the pheresis product. The aim of this study was to assess the potential for tumour cell mobilization in myeloma patients in remission following C-VAMP induction therapy. Between June 1997 and June 1998 14 patients with stage II/III myeloma were considered potential candidates for APBSCT. The CDR3 region of the immunoglobulin heavy chain gene was successfully amplified from the diagnostic bone marrow in 10/14 (71%) cases. Following sequencing of the PCR fragment, a patient specific primer was designed for each case and conditions optimized to produce a level of sensitivity of 1 in 10^4. Seven of the 10 informative patients were subsequently mobilized with cyclophosphamide 2 gm/m2 and G-CSF 5 µg/kg/day. Prior to mobilization 6/7 bone marrow and 1/7 peripheral blood samples were CDR3 positive. Following mobilization sequential peripheral blood samples became positive in 5 of 7 cases. The recruitment of tumour specific B cells coincided with the mobilization of CD34 positive cells, resulting in tumour contamination of 9 of 14 leucopheresis products in the seven patients. Although the clinical significance of tumour cell contamination of leucopheresis products in myeloma remains to be determined, this preliminary study indicates a potential for tumour cell mobilization, even in patients in remission following intensive induction therapy. There does not appear to be a "window of opportunity" for collecting tumour free stem cells with the mobilization of tumour cells coinciding with the rise in CD34 positive cells in the peripheral blood.

B7-2 POSITIVE MYELOMA IS A POOR PROGNOSIS DISEASE : IMPLICATIONS FOR IMMUNOTHERAPY

RD Brown, B Pope, E Yuen, J Gibson, D Joshua.
Institute of Haematology, Royal Prince Alfred Hospital, Sydney.

Deficiencies in the B7:CD28 costimulatory pathway are considered to be a major cause of the failure to generate a tumour specific immune response. Upregulating the expression of the B7 family of molecules on malignant B cells by CD40 ligand has been shown to generate the proliferation of cytotoxic T cells. Plasma cells of patients with myeloma express a tumour-specific idiotype but lack CD80 (B7-1) and have a variable expression of CD86 (B7-2). Whether endogenously high levels of CD86 expression are clinically significant has not yet been determined. We have identified the incidence and clinical significance of high CD86 expression on plasma cells at diagnosis and studied the ability of trimeric human CD40 ligand (huCD40LT- Immunex) to upregulate the expression of the B7 family on malignant plasma cells. High CD86 was expressed on the plasma cells of 51% of the patients studied at diagnosis (n=37) and was associated with a significantly shorter survival (median 28 vs 57 months; χ^2=4.6; p=0.03) and a high tumour load (patients with bone marrow plasma cell >50% was 47% vs 6%; χ^2=8.2; p=0.05). CD86 expression was highest on immature plasma cells (CD38hi, CD45+) and was associated with a CD40+, CD20+, CD19-, CD138+ phenotype. There was no correlation between high CD86 and other known prognostic markers (B2M, STK, LI) nor the presence of expanded T cell clones as detected using a 0.4kb TCR CTβ probe and 3 restriction enzymes. The addition of huCD40LT to short term cultures upregulated both CD80 and CD86 expression on B cells (CD19+) and CD80 on plasma cells (CD38++) but did not upregulate CD86 expression on plasma cells. These studies demonstrate that B7-2 positive myeloma consists of a subgroup of patients with a relatively poor prognosis. The upregulation of costimulatory molecules after huCD40LT therapy increased CD80 expression on malignant plasma cells without any significant change in CD86 expression. It is uncertain why B7-2 positive myeloma has a worse prognosis but as huCD40LT therapy does not upregulate CD86 on plasma cells this agent may be useful in immunotherapy protocols.

ILLEGITIMATE RECOMBINATIONS AT THE IMMUNOGLOBULIN HEAVY CHAIN (IgH) LOCUS IN PRIMARY MYELOMA TUMOUR.

PJ Ho, RD Brown, A Basten, J Gibson, DE Joshua.
Institute of Haematology, Royal Prince Alfred Hospital; Centenary Institute of Cancer Medicine & Cell Biology, Sydney.

Chromosomal translocations at the IgH locus at 14q32 have been found in myeloma. Many involve the switch regions, 5' of the constant region genes, where recombination for isotype switching occurs. Analysis of myeloma cell lines have elucidated some of the breakpoints, partner chromosomes & candidate oncogenes. In contrast, relatively few primary tumours have been similarly studied, for which recent examination by fluorescent in situ hybridisation (FISH) analysis have found IgH translocations in up to 80%. In this study, 35 primary myeloma marrow samples (plasma cells 2-95%) were studied by Southern Blot, using probes 5' and 3' of the switch regions (Bergsagel et al, 1996). Mononuclear cells were isolated by density gradient centrifugation. To identify samples in which clonality and thus a legitimate switch is detectable by Southern blot, a JH region probe was used to detect non-germline bands. Clonality was found in 17/35 samples. 12/17 samples (2 plateau, 6 active, 4 progressive) were analysed by the 6 pairs of switch probes. Eight of the 12 had IgG isotype, 2 IgA, one IgM and one κ light chain. Germline controls were derived from non-lymphocyte DNA. Illegitimate recombinations were detected in 6/12 cases (2 progressive, 3 active, 1 plateau), while no illegitimate recombinations were detectable in 2/12 cases (1 active, 1 progressive). In 4/12 cases recombinant bands did not demonstrate a productive switch. Of the 6 cases with illegitimate recombinations, 3/6 (2 IgG and one IgM) were detected by a probe upstream of the μ switch region (Sμ), indicating a possible translocation breakpoint in this region. In the other 3 cases, illegitimate recombinant bands were detected by multiple probes, implying more than one illegitimate recombination - at Sα & Sγ in one (IgG), and Sμ & Sγ in 2 cases (IgG & κ light chain). To elucidate the partner chromosome & breakpoint, long range-vectorette PCR has been applied to 2 cases so far. Using this "one-sided" PCR method, primers for the known end were designed according to the translocation region indicated by the switch probes, & the unknown translocated DNA amplified & sequenced; results are pending. The combination of Southern Blot and long range PCR allows efficient identification of the translocation region, then elucidated by PCR and sequencing. Unlike myeloma cell lines with abundant DNA for examination, this approach should be most useful in the analysis of IgH translocations in primary myeloma tumour. Ref: PNAS 93:13931.

FREQUENT EXPRESSION OF RANKL IN HUMAN MULTIPLE MYELOMA CELLS.

H-G Schneider, M-H Cheng, M. Gillespie, M. Cole-Sinclair, V. Grill.
Clinical Biochemistry Unit, Alfred Hospital and St. Vincent's Institute of Medical Research, Melbourne.

Severe bone disease of myeloma patients is mediated by osteoclasts. Myeloma cells have been presumed to produce an osteoclast activating factor. A stimulator of osteoclast activity and formation that is produced in osteoblasts has recently been identified as receptor activator of nuclear factor kappa B ligand (RANKL). We have investigated the expression of RANKL and the soluble decoy receptor for RANKL osteoprotegerin (OPG, OCIF, TR1) in bone marrow biopsies of patients with multiple myeloma. In situ hybridisation with a mouse antisense RANKL riboprobe that cross-reacts with human mRNA was used. Detection is using digoxigenin labeling of the probe and anti-digoxigenin antibodies coupled to alkaline phosphatase. We found high expression of RANKL in myeloma cells of 14 of 17 non-selected biopsies of patients with multiple myeloma. Little expression of RANKL was detected in non-myeloma cells of the bone marrow specimens. Three normal control patients without haematological disease showed no expression of RANKL. Two biopsies of patients with plasmacytoma showed no expression of RANKL. Osteoprotegerin was detected in the cytoplasm of myeloma cells, as in many other cells and the level of expression was not different from that seen in control patients. High levels of mRNA for RANKL in myeloma cells make it likely that this stimulator of osteoclast formation and activation is involved in the bone complications of multiple myeloma. We hypothesise that it is the disregulated production of RANKL that leads to stimulation of bone resorption while there is also constitutive expression of OPG.

FRESHLY ISOLATED MYELOMA CELLS SHOW VARIABLE LEVELS OF APOPTOSIS INDUCED BY RECOMBINANT HUMAN TNF-RELATED APOPTOSIS INDUCING LIGAND (TRAIL)

LF Lincz[1] and A Spencer[1,2,3,4]

[1]Hunter Haematology Research Group, Mater Misericordiae Hospital, NSW; [2]Hunter Area Pathology Service, NSW; [3]Hunter Haematology Unit, Mater Misericordiae Hospital, NSW; ***[4]Alfred Hospital BMT Program, Melbourne, VIC.***

TRAIL is a member of the TNF family and is known to induce apoptosis in cancerous but not normal cells. We investigated the possibility of using TRAIL to selectively remove tumour cells from haemopoietic progenitor cell harvests from multiple myeloma (MM) patients. Preliminary dose response curves on four human MM cell lines revealed that RPMI 8226 and NCI H929 underwent a maximum of 55-65% relative apoptosis after 24 hours of culture with 2 μg/ml TRAIL (Immunex Corp. Seattle, WA), while U266 and MCCAR displayed less than 15% apoptotic cells under similar conditions. When susceptible cells were mixed with normal BM, TRAIL selectively killed the malignant cells as evidenced by a 77% decrease in the percentage of BB4 $(CD138)^+$ cells detected by flow cytometry and plasma cells identified in cytocentrifuged preparations. The ability of TRAIL to induce apoptosis in ex-vivo MM cells was tested using fresh BM and PBPC samples from 10 MM patients. Of these, only 2 samples showed decreases (22% and 59% respectively) in the number of $BB4^+$ cells following TRAIL treatment. Overall there was no statistically significant difference in the percentage of $BB4^+$ cells, morphologically identified plasma cells or ASO-PCR detectable DNA clones in treated versus untreated samples. As in previous studies, TRAIL did not effect on the number of $CD34^+$ cells or the growth of CFU-GM colonies. We conclude that freshly isolated myeloma cells are more resistant than their cell line counterparts to apoptosis induced by TRAIL, suggesting that other strategies may have to be employed before TRAIL can be used as an effective purging tool.

THALIDOMIDE FOR TREATMENT OF RELAPSED AND RESISTANT MYELOMA.

CK Arthur, L Coyle, H Wheeler. *Haematology and Oncology Departments of Royal North Shore Hospital, St. Leonards 2065.*

Thalidomide was recently reported to be capable of producing partial remission in patients with myeloma including patients who relapsed following autologous transplantation (ASH meeting 1998). We have entered 6 myeloma patients into a trial of thalidomide if they had failed or progressed on therapy. Thalidomide up to 500 mg daily was given. Patients were monitored with monthly blood tests and bone marrow at 3 monthly intervals. Three patients (aged 71, 77, 79) have reached the 3 month assessment and so can be analysed for response. Prior treatment variously included melphalan/prednisolone, autografting with high dose melphalan, interferon, high dose dexamethasone, radiotherapy and intermittent pulse cyclophosphamide. Thalidomide dose was 400mg daily in 2 pts and 500mg daily in the other. All 3 patients have shown a response. Paraprotein has fallen from 23 → 8g/l and 6.2 → 3.8g/l in two patients and IgA 14 → 4 g/l in the 3rd patient (paraprotein not estimable). The blood count has remained stable in all three except in one who had a rise in platelets from <15 to >100 x 10^9/L. Bone marrow changes have been difficult to assess because of patchy involvement and hypocellular fragments. However bone marrow has probably also improved with plasma cells changing from 10% → 2% in one patient, and in another a heavily infiltrated marrow with 85% plasma cells to a hypocellular marrow with a few clusters of plasma cells and in the final patient marrow fragments have become hypoplastic with few cells and was previously infiltrated with 7-30% plasma cells. The main toxicity has been moderate drowsiness in all patients but this improved with continuation of the drug. Two patients had moderate constipation. In conclusion, although very preliminary, these results support previous studies indicating thalidomide is an active agent in myeloma even in previously treated patients. Its place in myeloma therapy however awaits randomised studies.

DEVELOPMENT OF NOVEL ANTITHROMBOTICS

SP Bottomley, JC Whisstock and RN Pike

Department of Biochemistry and Molecular Biology, Monash University, Clayton, 3168 Victoria Australia.

The control of coagulation is vital in diseases such as thrombosis. The search for new anticoagulant therapies to supplement the use of warfarin and heparin, which both have a number of limitations is therefore the focus of intense research efforts. Most emphasis has been placed on thrombin, however its ability to catalyse many contrasting processes in the body makes it a complex and challenging target. An alternative target is factor Xa which when bound to factor Va is responsible for the generation of thrombin. The generation of low molecular weight factor Xa inhibitors has been restricted due to the lack of high-resolution structures of factor Xa-inhibitor complexes. An alternative approach is to design factor Xa specific inhibitors based upon the structure and function of existing plasma proteinase inhibitors. Antithrombin is a candidate but it inhibits both thrombin and factor Xa, however antitrypsin a structurally similar molecule does not inhibit either. Using a protein engineering approach we have attempted to modify antitrypsin by incorporating fragments of antithrombin such that it becomes an effective inhibitor of factor Xa while not significantly inhibiting thrombin. This presentation will demonstrate the potential use and pitfalls of engineered plasma serpins.

PURIFICATION OF PROTEINS USING THE GRADIFLOW – PURE AND SIMPLE.

Hari Nair, Andrew Gilbert, Tracey Edgell, May Lazar, Elizabeth Seabrook, Thomas Turton, Indira Wathugala, Gradipore Ltd, ,Riverside Corporate Park, North Ryde, North Ryde, NSW, AUSTRALIA

Coagulation research has long relied on the availability of clotting proteins and their use in diagnostic tests. There are times when the purity of a blood protein determines the extent to which this protein can be characterised. Furthermore, future and potential protein based therapeutics need to be purified and treated to remove pathogens to be acceptable to regulatory authorities. The major limiting factor in the biotechnology industry is the processing of complex biological solutions. Whilst the resolution of proteins with 2D gel electrophoresis is revolutionizing proteomics and protein identification, this technology is rarely suitable for functional studies due to both the denaturing conditions used for the separation and small amounts recovered. There is, therefore, a major demand for cost-effective preparative technologies for the purification of naturally occurring and recombinant proteins.

The Gradiflow is a simple device, which utilizes the established resolution of electrophoretic techniques to separate proteins in their native state by both size and charge on a preparative scale. This technology employs a disposable cartridge comprising three thin 0.1mm polyacrylamide membranes, a cooling system that also acts as an electrophoresis buffer and separate streams for the addition of sample and collection of the product. The top and bottom (restriction) membranes in the cartridge are heavily cross-linked limiting the passage of molecules to a size smaller than 3 kDa. The degree of cross-linking in the middle separating membrane varies according to the specific application and is useful for separating molecules with native molecular weight ranging from 10-1000kDa. The use of standard pH and ionic strength conditions, efficient cooling and excellent resolution have meant that individual proteins can be rapidly purified to near homogeneity from complex mixtures with yields and recovery of biological activity often greater than 90%.

Using the Gradiflow a number of proteins have been separated including monoclonal antibodies from murine ascitic fluid, fibrinogen, albumin, thrombin and immunoglobulins from human plasma and a variety of other proteins
The distinct advantage of this new system over other conventional preparative systems is the ability of the instrument to be configured to simultaneously concentrate and/or desalt the protein samples at the same time. The Gradiflow technology, thus, has the potential to be a rapid, cost effective alternative to conventional chromatography for the preparative purification of many proteins. Furthermore, the system has the potential to concurrently remove viruses, bacteria and endotoxins. Collectively these results point to a powerful new tool in the search for new drugs and technology to replace Cohn Fractionation in the blood product industry

THE RELATIONSHIP BETWEEN THE PROTHROMBIN G20210A POLYMORPHISM AND PROTHROMBIN mRNA STABILITY

Angela M. Carter, Fabienne Maurer and Robert L. Medcalf, *Monash University Department of Medicine, Box Hill Hospital, Box Hill 3128 Victoria, Australia*

The prothrombin (PT) G20210A polymorphism is associated with increased plasma PT levels and implicated in the pathogenesis of venous thrombosis (VT). The mechanism(s) whereby G20210A leads to elevated PT is unknown but its location in the 3' untranslated region (3'-UTR) immediately preceding the poly (A) tail of PT mRNA suggests it may influence PT mRNA stability or protein translation. To determine whether the mutation could influence prothrombin protein production, we inserted the wild-type (20210G) or mutant (20210A) PT cDNAs into a CMV-promoter driven expression vector. These constructs were stably transfected into NIH3T3 cells and secreted PT protein was assessed in cell supernatants by Western blotting. 2- to 3-fold more PT protein was produced by cells transfected with the mutant PT cDNA compared to the cells transfected with the wild-type PT cDNA indicating that the 20210A polymorphism indeed results in an increase in PT protein. To determine whether this was due to an increase in PT mRNA stability, a mRNA decay system was established using plasmid pfosHGH which contains the human growth hormone gene (HGH) under the control of the transiently serum responsive c-fos promoter. Both variants of the 97 nt 3'-UTR of PT were inserted into the 3'-UTR of pfosHGH and stably transfected into NIH3T3 cells. Transfected cells were treated with 15% serum for up to 8 hours and chimeric HGH-PT mRNA levels assessed by Northern blotting. Preliminary results indicate that both the wild-type and mutant HGH-PT chimeric mRNAs were surprisingly stable with no clear evidence of a difference in the mRNA decay rate of the two variants. Finally, RNA electrophoretic mobility shift assays indicate that the 3'-UTR of PT provides a binding site for HuR, a RNA binding protein that is associated with RNA stability. In summary, the level of PT protein production is enhanced by the G20210A prothrombin gene polymorphism but it remains to be determined if this is due to changes in PT mRNA stability or translation. Finally, our observation that HuR interacts with the 3'-UTR of PT mRNA may provide an explanation for the surprising stability of the PT transcript.

MODIFICATION OF DENDRITIC CELL FUNCTION - IMPLICATIONS FOR AUTOIMMUNITY AND CANCER

GM Woods[1], YP Chen[1], KV Doherty, AL Dewar, BH Toh[2] and HK Muller[1]
[1]*Discipline of Pathology, University of Tasmania, Hobart, Australia*; [2]*Department of Pathology and Immunology, Monash University, Prahran, Australia.*

Throughout the epidermis is a network of Langerhans cells (LC), which must be maintained at an appropriate density of mature and functional cells to generate an effective immune response. If adult epidermis is depleted of mature LC and new antigen applied through this LC depleted skin an immune response fails to generate, but instead, antigen specific tolerance is induced. If antigen is applied through neonatal skin, antigen specific suppression is also induced. We propose that immature LC provide a signal which can down regulate the immune response. We hypothesised that immature LC could therefore be useful in preventing the development of immunological diseases, such as autoimmunity. To test this hypothesis we used the day 3 thymectomy model (3dnTx) of autoimmune gastritis. With this model, BALB/c mice are thymectomised at day 3 after birth and these mice are prone to developing autoimmune gastritis. The autoantigen in this disease has been identified as the β- subunit of the H^+/K^+ ATP-ase gastric proton pump and an autoreactive peptide from this chain has been produced. When immature LC of 3dnTx BALB/c mice were exposed *in vivo* to this autoreactive peptide, the mice were prevented from developing autoimmune gastritis. This provides encouragement that skin immunisation via immature LC may provide a useful strategy for immunisation against autoimmune disease

ZEBRAFISH: A NEW MODEL FOR STUDYING BLOOD DEVELOPMENT

KE Crosier

Department of Molecular Medicine, School of Medicine, University of Auckland, Auckland, New Zealand

The tropical freshwater fish *Danio rerio* (zebrafish) has emerged as an excellent experimental system for the analysis of vertebrate development. Its advantage over other vertebrate models is the powerful combination of experimental embryology and classical genetics to which the fish are amenable. Saturation mutagenesis screens in the zebrafish have provided blood mutants, and positional cloning of these genes is beginning to establish zebrafish models of human haematopoietic disorders. The conservation of genomic structure between zebrafish and human is remarkable and enables researchers to move between databases for each in search of candidate genes in the region near mutations. This type of approach is illustrated by work from our laboratory that seeks to identify the function of several recently cloned human TGF-β superfamily molecules. One of these, GDF6, is a candidate regulator of vascular and haematopoietic development. In other work, we have used signal sequence trap (SST) technology to clone secreted proteins in zebrafish and developed a rapid expression screening strategy using whole-mount *in situ* hybridisation. In addition, we are commencing a genetic screen to identify regulators of haematopoietic stem cell development. The zebrafish as a vertebrate model system is now poised at the forefront of genetic research, and promises substantial rewards in terms of our understanding of developmental mechanisms and disease processes.

IMMUNOTHERAPY FOR ADVANCED STAGE LOW GRADE NON-HODGKINS LYMPHOMA USING ANTIGEN PULSED AUTOLOGOUS DENDRITIC CELLS.

D.S.Ritchie[1], J.Yang[1], P.J. Dady[2], M.Findlay[2], J. Carter[2], C.A. Johnson[2], F.Ronchese [1]

[1]Malaghan Institute of Medical Research, Wellington;
[2]Wellington Cancer Centre, Wellington.

Low grade Non-Hodgkins Lymphoma (NHL) of advanced stage currently remains incurable by conventional chemotherapeutic and radiotherapeutic approaches. Accordingly, much attention has been given to the role of immunotherapeutic modalities in the treatment of this disease.

Here we report on the production of clinical grade dendritic cells for use in a clinical trial of immunotherapy for the treatment of patients with relapsed or chemoresistant low grade NHL.

Tumour antigen preparations were obtained by the acid elution of a lymphoma cell suspension produced from the surgical excision of an involved lymph node. Acid elution results in the dissociation of the MHC Class-I heavy chain from β_2 -microglobulin and the subsequent release of bound antigens, which may be collected from the supernatant.

Clinical grade autologous dendritic cells (DC) were obtained by culturing peripheral blood monocytes in a combination of GM-CSF and IL-4 for 7 days. Dendritic cell yield was determined by flow cytometric analysis of the combined expression of MHC Class-II, CD11c, and CD86 in the absence of the expression of the lineage markers CD19, CD3, and CD14. Dendritic cell function was confirmed by the stimulation of T cells in an allogeneic MLR.

Each patient has received an infusion of antigen pulsed DC fortnightly for 8 weeks. Infused DC numbers ranged from 2 million cells to 30 million cells per infusion.

To date, 10 patients have been enrolled with 5 patients completing the full therapeutic protocol. Minimal acute toxicity has been observed. Objective reduction in lymphadenopathy and splenomegaly has been observed in one patient along with a fall in LDH and evidence of T cell infiltration of residual lymphoma. A further patient has shown evidence of an immune response to DC vaccination.

THE ESTIMATION OF NEUTROPHIL ALKALINE PHOSPHATASE BY FLOW CYTOMETRY.

JG Ivey
Cell Biology Unit, Department of Haematology, Royal Perth Hospital

The use of an antibody (clone 1B12.1) directed against alkaline phosphatase has previously been used to study neutrophil alkaline phosphatase (NAP) in normal and pathological leucocytes. The original flow cytometric gating strategy and the use of an unconjugated antibody showed poor discrimination between patients with low NAP levels and normal controls. We have developed a flow cytometry gating strategy which together with the use of the commercially available antibody clone 1B12.1 directly labelled with phycoerthyrin has resulted in a flow cytometric NAP score which displays better discrimination between patients with low NAP levels compared to normal. The gating strategy used assigns an arbitrary score depending on the linear fluorescence intensity to the cells falling within arbitrarily defined gates. The aggregate of these scores is reported as a FLOW NAP score. This strategy is similar but not identical to the current cytochemical based NAP score. The advantages of a flow based assessment are that samples up to 24 hours old may analysed, the use of EDTA anticoagulated blood is acceptable, neutropenic samples may be analysed and large numbers of granulocytes can be assessed which improves the standardisation of the assay. All together the flowcytometic analysis of NAP is well suited to replace the cytochemical NAP score which is tedious to perform and subject to great interlaboratory variability.

GINSENOSIDES ENHANCE CYTOKINES-INDUCED EXPANSION OF HAEMATOPOIETIC CELLS EX VIVO.

H Tao, JM Jin, BH Chong. *Haematology Department, Prince of Wales Hospital, NSW 2031, Australia.*

We investigated the effects of ginsenosides, extracted from Panax ginseng, on ex vivo lineage-specific expansion of $CD34^+$ haematopoietic stem cells derived from human cord blood. Expansion was performed using both semi-solid and liquid culture in serum-free conditions. Erythroid expansion was induced using EPO, SCF, IL3 and IL6, myeloid expansion by G-CSF, SCF, IL3 and IL6, and megakaryocytic expansion by TPO. The concentrations of ginsenosides studied were up to 200 μg/ml. In the liquid culture, the cell lineage-specificity and maturation were analysed by flow cytometry using monoclonal antibodies: glycophorin A and CD71 for erythroid progenitors, CD33 and CD15 for myeloid progenitors, CD41 and CD61 for megakaryocytic progenitors. Cellular proliferation was measured by DNA synthesis using BrdU labelling and metabolic activity based on bioreduction of tetrazolium compound. Ginsenosides showed a dose dependent growth enhancing effect in all three lineages peaking at 10 to 25 μg/ml. As the concentration of ginsenosides was further increased, the enhancing effect tapered off and became inhibitory at high concentration. Similar dose-dependent effects were found in semi-solid culture, as measured by CFU-GM, CFU-E, BFU-E, CFU-MK and CFU-GEMM. Ginsenosides contain multiple components, some of which may have enhancing effect on haematopoiesis, whilst others inhibitory effect. Our results are consistent with the presence of one or more growth enhancing components with saturable effect, as well as inhibitory components present at lower specific activity in the extract. Further studies with single ginsenoside component at the molecular level will help to elucidate the mechanism of action and the therapeutic potential of extract from ginseng.

INHIBITORY EFFECT OF PF4 ON MEGAKARYOPOIESIS IS DEPENDENT ON VERY FEW AMINO ACID RESIDUES.

CM Vun, H Tao. *Haematology Department, SEALS, POWH, Sydney, New South Wales, 2031, Australia.*

PF4 is produced by megakaryocytes and stored abundantly in the α-granules of platelets. As PF4 is known to inhibit proliferation and differentiation of megakaryocytes *in vitro*, it is suggested that it may exert negative feedback control on megakaryopoiesis. However, the receptor for PF4 remains unknown. The PF4 binding sites which mediate its inhibitory effect have been investigated mostly by peptides based on its C-terminal portion. In physiological condition PF4 exists as a tetramer whose interaction with other biomolecules will be difficult to mimick by peptides derived from fragments of PF4. Therefore, it is important to investigate the binding sites of PF4 using mutant PF4s which still fold to the native conformation. To select the amino acid residues for mutagenesis, the 3D structure of PF4 is examined to identify those residues which are exposed on its surface. Selected residues are mutated using PCR spliced overlap extension and mutagenic mismatch primers. More than 40 PF4 mutants are generated. Using *ex vivo* suspension culture of cord blood $CD34^+$ cells induced to differentiate into megakaryocytes by TPO, the proliferation of megakaryocytes is assessed by BrdU-based DNA synthesis and flow cytometric phenotyping using CD41 monoclonal antibody. Only a few residues on PF4 are essential for the inhibitory action on megakaryopoiesis. Interestingly, some mutants show stronger inhibitory effect than wild-type metPF4. This is probably due to reduced steric hindrance as the implicated residues are mutated to either alanine or glycine. PF4 binds avidly to heparin and heparan sulfate, the later of which is present on megakaryocytes. Although the heparin-binding sites of PF4 are still not absolutely determined, the finding of residues on PF4 essential for megakaryopoietic inhibition different from known heparin-binding sites suggests that a receptor is involved.

REGULATION OF MATRIX METALLOPROTEINASE PRODUCTION BY CO-CULTURE OF BREAST CANCER CELLS AND BONE MARROW FIBROBLASTS

S Saad, LJ Bendall, A James, DJ Gottlieb, KF Bradstock.
Departments of Haematology and Medicine, Westmead Hospital, Westmead 2145

In women with poor prognosis breast cancer encouraging results have been obtained using autologous stem cell transplantation to facilitate dose intensification. However, breast cancer cells frequently metastasise to the bone marrow resulting in malignant cell contamination of both bone marrow and peripheral blood stem cells. An understanding of the mechanisms involved in the interaction of breast cancer cells with the bone marrow may make it possible to inhibit breast cancer cell invasion of the bone marrow and decrease the risk of malignant contamination of harvested stem cells.

We have previously shown that metalloproteinases are involved in the invasion of bone marrow stromal layers by the more aggressive breast cancer cell lines. Co-culture of the invasive breast cancer cell line, MDA-MB-231 with monolayers of bone marrow fibroblasts (BMF) results in an increase in matrix metalloproteinase (MMP) 1 and 2 in culture supernatants. MMP-1 is produced by the MDA-MB-231 cells in response to a soluble factor produced by the BMFs. In contrast regulation of MMP-2 is contact dependent. No increase in MMP-2 mRNA could be detected suggesting that increased synthesis of MMP-2 was not occurring. It appears that increased MMP-2 levels in co-culture supernatants results from the release of MMP-2 from the cell surface and from cytoplasmic stores of BMFs.

UNIQUE BCR-ABL TRANSCRIPT (E8A2) WITH AN INSERTION OF AN INVERTED SEQUENCE OF ABL INTRON Ib IN A PATIENT WITH CHRONIC MYELOID LEUKAEMIA

S Branford, TP Hughes, Z Rudzki.

Division of Molecular Pathology, Institute of Medical and Veterinary Science; Leukaemia Research Unit, Hanson Centre for Cancer Research, South Australia.

The BCR-ABL hybrid gene is the main product of the t(9;22)(q34;q11) translocation and is present in 95% of patients with chronic myeloid leukaemia (CML). The BCR-ABL mRNA varies in size, depending on the breakpoint in the BCR gene. Three breakpoint cluster regions have been identified, major (M-bcr), minor (m-bcr) and micro (mμ-bcr). In 99% of patients, the break occurs within M-bcr and either exon b2 (13) or exon b3 (14) is fused to ABL exon a2.

We report an unusual BCR-ABL transcript in a patient with Philadelphia chromosome positive CML who showed no amplification of the usual BCR-ABL transcripts. The atypical mRNA was amplified using a long polymerase chain reaction technique. Sequence analysis of the fusion region of the amplified cDNA fragment revealed a joining of BCR exon e8 and ABL exon a2, with a 55 base pair insert between them, giving rise to an in-frame e8a2 BCR-ABL transcript. The inserted sequence was 100% homologous to a reverse complement sequence of ABL intron 1b. The probable cause of insertion was the introduction of a cryptic splice site caused by an inversion event involving the region of intron 1b. The patient progressed to the acute phase of the disease within two years of diagnosis and was refractory to treatment. To our knowledge this is the first report of a patient with a BCR-ABL transcript including an inverted sequence of ABL intron 1b.

INDUCED MUTATIONS IN LYMPHOBLASTIC CELL LINES USING LOW DOSE γ-IRRADIATION.

Neil Granter[1] and Andrew Spencer[1, 2, 3]

[1]Hunter Leukaemia & Lymphoma Research Group [2]Hunter Area Pathology Service, Mater Misericordiae Hospital, Newcastle and [3]BMT Programme, Alfred Hospital, Prahran.

We have previously demonstrated differential induction of BCR-ABL transcription using high dose gamma-irradiation in lymphoblastic cell lines (LCL) derived from leukaemic and non-leukaemic individuals. In this present investigation we exposed similar LCL to low dose gamma-irradiation and measured the mutation frequency of the marker gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). Eleven LCL (4 normal, 3 CML, 4 AML) and 3 Ataxia Telangiectasia mutant (ATM) LCL were studied. Duplicate sets of 1 million cells were exposed to 0.5, 1.0, 1.5 or 2.0 Gy. Following overnight incubation 6-thioguanate was added and the cells plated into 96 well plates at a concentration of 10,000 cells/well. After 14 days incubation the surviving mutant colonies were counted and the mutation frequency calculated. Between 4 and 8 replicate experiments were performed for each LCL at each radiation dose. The normal LCL showed a negative correlation between mutation frequency and increasing radiation dose ($p = .05$, Spearman Rank correlation). In contrast the mutation frequency in 1 AML and 1 CML derived LCL showed a significant increase after radiation compared to the unirradiated equivalents (0Gy vs 2Gy, $p = .05$ and 0Gy vs 1Gy, $p = .025$, respectively). These preliminary results add further to the hypothesis that certain individuals may harbour a 'leukaemic' predisposition preceding the acquirement of more typical 'leukaemia-associated' genetic lesions.

GENERATION OF DENDRITIC CELLS (DCs) FROM HUMAN PERIPHERAL BLOOD USING CYTOKINE CONDITIONING

Hammett M, Badami K, Ball G, Mak R, Schwarer AP.
Bone Marrow Tranplant Programme, Alfred Hospital, Melbourne, VIC.

DCs are potent initiators of immune responses that have been widely used in vitro to induce T cell responses to human antigens in cancer. Three combinations of cytokines (GM-CSF (G) 100ng/ml, IL-4 (I) 1000 U/ml, and TNF-α (T) 10 ng/ml), were used to derived DCs from the plastic-adherent fraction of PBMCs from healthy donor and chronic myeloid leukaemia patients. The 3 cytokines conditions were GIT, GI and GT. The cells were harvested on day 7, then compared with untreated PBMCs phenotypically (CD1a, 2, 4, 8, 14, 20, 33, 80 and HLA-DR), and functionally (MLR). The GIT conditioned cells were the most potent stimulators in 6/10 experiments at the highest (0.1×10^6 cells/well) and lowest (0.001×10^6 cells/well) stimulator concentration.

IL-2-MLR assays using GIT conditioned DCs were set up to specifically test T cell responses due to stimulation by PBMCs and DC. The results support those observed from the proliferative MLR assays. The DCs appear to be the more potent stimulator of T cell responses compared to unmanipulated PBMC.

DCs were also generated using extra purification steps to remove cells likely to inhibit DC development. The addition of TNF-α was added after a 5 day incubation with GM-CSF + IL-4, to facilitate DC maturation. Small cell yields were obtained however a greater proportion of the cells were CD1a+/CD80+ indicating that the majority of the cells were of a DC phenotype. Initial results demonstrated that this method did not produce DCs which were significantly more potent than the GIT conditioned cells. The advantage of the second method tested is the ability to pulse the DCs with a peptide of interest, before maturation with the addition of TNF-α, shutting down the antigen uptake and processing machinery of the DC

MATURATION OF ANTIGEN PRESENTING CELL FUNCTION IN NEONATAL LANGERHANS CELLS

AL Dewar, KV Doherty, GM Woods, HK Muller
Discipline of Pathology, University of Tasmania, Hobart, Australia.

Langerhans cells (LC) drive the induction of immune responses following cutaneous exposure to antigen. As little is known of their ontogeny, the aim of this study was to examine the development of the LC network within the neonatal epidermis and to correlate this with acquisition of function.

To examine the morphology and phenotype of LC in neonates, epidermal sheets were prepared from mice aged 3, 7, 14 days and 6 weeks. Striking differences in the structure and morphology of the LC network were observed with age. While MHC Class II was expressed at day 3, the dendritic cell marker DEC-205 was absent. By day 7, some MHC II+ cells expressed DEC-205 and by day 14, all LC coexpressed MHC II and DEC-205.

To assess the ability of neonatal LC to take up and transport antigen, the fluorescent antigen, FITC, was applied to the dorsal skin of neonatal mice. 24 hours later lymph nodes were examined for fluorescent cells by flow cytometry. The number of cells carrying antigen and the amount correlated with age. The LC were further characterised by *in vitro* FITC-Dextran uptake. Maximal uptake was significantly lower in day 3 LC when compared to adult LC.

To determine whether neonatal LC were able to initiate a contact sensitivity response, TNCB was applied to the dorsal skin of mice aged 3, 7, 14 days and 6 weeks. The mice were resensitised after 6 weeks and challenged 5 days later. The response of mice sensitised at 3 or 7 days of age was significantly less than mice sensitised at 14 days or 6 weeks.

These results demonstrate that the LC network undergoes a gradual period of maturation before it is able to effectively stimulate an immune response.

CHRONIC RENAL FAILURE: HAEMODIALYSIS ACCELERATES METHYLATION OF ERYTHRO-CYTE MEMBRANE

K. Yang, W.Y. Fu and N.P.B. Dudman

Centre for Thrombosis and Vascular Research, Prince Henry Hospital, University of New South Wales, Sydney, NSW.

Patients with chronic renal failure (CRF) often have erythrocytes with a severely shortened half-life. Here we hypothesised that erythrocyte plasma membranes deteriorate more rapidly than normal in CRF patients, and that this would lead to enhanced methylation-dependent repair. Methylation was assessed by incubating red cells in whole blood or medium for 1 hr at 37^{o}C, with [methyl-^{3}H]methionine. We then measured the amount of [methyl-^{3}H]CH_3OH released during incubation, and the extent of residual labeling of esterified erythrocyte membrane following incubation. We studied 10 adult CRF patients (mean age ± SD = 51.5 ± 11.0 y; 5 males) before and after 5 hr haemodialysis, and 11 healthy adult controls (52.0 ± 10.2 y; 5 males). Compared with healthy controls, CRF patient membrane methylation was 32.6 ± 10.2% higher (mean ± SEM; not significant) before haemodialysis, and 104.9 ± 22.2% after dialysis ($p<0.01$). Similarly, release of [methyl-^{3}H]CH_3OH was increased in CRF patients before dialysis by 50.5 ± 13.9% ($p<0.05$) and after dialysis by 123.6 ± 31.3% ($p<0.01$). The increased rate of methylation caused by haemodialysis was found by *in vitro* dialysis studies to reflect loss of a dialysable inhibitor from plasma. Present results do not show whether this inhibitor acts against the methylation step, or the preceding protein isomerisation step. In conclusion, we have found evidence that the erythrocyte membranes of CRF patients in a haemodialysis program are being more actively methylated than those of healthy controls. The methylation rate is enhanced following dialysis.

IFOSPHAMIDE, VP-16 AND ARA-C (IVAC) FOR DISEASE CONTROL AND PBPC MOBILISATION IN REFRACTORY AGGRESSIVE NHL

A Spencer[1], S Deveridge[2], M Seldon[2], A Schwarer[1], A Enno[2]

[1]BMT Programme, The Alfred Hospital, Prahran; [2]Hunter Haematology Unit, Mater Misericordiae Hospital, Newcastle

We have treated 8 patients (ages 29 – 63 years, median 50.5 years) with primary refractory (n = 4) or refractory relapsed (n = 4) non-Hodgkin's lymphoma with ifosphamide, VP-16 and Ara-C (IVAC). Diagnoses were B cell - DLC, n = 4; T cell - DLC, n = 2; FCC with Burkitt's transformation, n = 1 and T cell lymphoblastic, n = 1. LDH was elevated at diagnosis or relapse in 6 patients (range 149 – 6048 IU/l, median 484 IU/l, NR <250 IU/l). Median number of prior therapies was 1.5 (range 1 – 3). All patients received G-CSF 5µg/kg commencing at a median of 2.5 days (range 1 – 24 days) following completion of IVAC. Treatment was well tolerated with no treatment related deaths. Responses following 1 cycle of therapy were – resistant disease, n = 1; stable disease, n = 2; partial remission, n= 2 and complete remission, n = 3. All patients underwent PBPC collection during recovery from IVAC at a median of day +16 (range 13 – 28) from commencing therapy. Median CD34+ cell yield was 13.3 x 10^{6}/kg (range 0.12 – 36.1 x 10^{6}/kg) achieved with a median of 1.5 leucaphereses (range 1 – 4). Only the patient with FCC with Burkitt's transformation and extensive prior alkylator therapy failed to mobilise sufficient PBPC to enable stem cell transplantation (ASCT). The remaining 7 patients have undergone ASCT with LACE conditioning and 3 remain alive 2 months (undergoing ASCT), 10 months (CR) and 28 months (CR) post-IVAC. A further patient died from an unrelated cause whilst in CR 7 months post-IVAC. We conclude that IVAC should be considered in selected patients with refractory NHL for disease control and PBPC mobilisation.

PHASE I STUDY OF REPETITIVE HIGH-DOSE TOPOTECAN (T), CARBOPLATIN (C) AND PACLITAXEL (P) IN PREVIOUSLY UNTREATED OVARIAN CANCER.

HM. Prince, D. Rischin, M. Quinn, D. Allen, R. Planner, J. Davison, P. Gates. *Division of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, Melbourne, Victoria*.

Purpose: In view of the significant activity of T in ovarian cancer with dose limiting toxicity of myelosuppression, we evaluated the addition of T to C and P with peripheral blood progenitor cell support.
Methods: Patients received 2 cycles of C AUC 5 and P 175mg/m^2 with collection of progenitor cells after the second cycle. They then received 3 cycles of intensive therapy with T on a daily x 5 schedule, P 250 mg/m^2 (24 hr) on day 3 and C AUC 12 on day 4. Eligibility included previously untreated stage 3 or 4 ovarian cancer with either macroscopic residual disease following primary debulking surgery or clear cell histology.
Results: 14 patients, median age - 49 (range 21-63). T was escalated in 4 patient cohorts up to a dose of 3.5 mg/m^2/d. This dose level met the criteria for defining the dose limiting dose level with 2/4 patients experiencing grade 4 mucositis. The preceding dose level with T 2.5 mg/m^2/d is the recommended dose. Toxicities in the 3 patients treated at this dose level were grade 3 mucositis in 1/9 high dose cycles and febrile neutropenia in 2/9. Responses in 14 patients who have had second look laparoscopy or laparotomy: pathologic CR - 7, microscopic residual - 1, PR – 5, PD - 1.
Conclusion: When combined with C (AUC 12) and P (250 mg/m^2) the recommended topotecan dose is 2.5 mg/m^2/day. This outpatient high dose regimen combines 3 of the most active drugs in ovarian cancer with acceptable toxicity and promising activity.

INFECTIOUS COMPLICATIONS IN AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTS.

Broady R, T E Hawkins, S J Palmer, J Norcott, P J Browett
Haematology Department, Auckland Healthcare, Auckland, NZ

Autologous peripheral blood stem cell transplants (AuPBSCT) engraft rapidly with a shorter duration of neutropenia than autologous bone marrow transplants. Consequently a lower rate of infectious complications are expected. The aim of this study was to evaluate infectious complications following AuPBSCT by a retrospective analysis of 55 consecutive patients treated between May 1995 and March 1999. 19 female and 36 male patients with a median age of 42 years (range 19–63) who were treated for Myeloma (18), non-Hodgkin's lymphoma (17) Hodgkins Disease (9), AML (3), ALL (2), amyloidosis (2) and germ cell tumour (4) were analyzed. Myeloablative chemotherapy regimes were as follows: MM/AL= HDM; NHL = BEAM; AML = Bu/Cy; ALL= Cy/TBI; HD=CBV; and GCT = ICE. Patients received antimicrobial prophylaxis consisting of Fluconazole 200mg daily or itraconazole 2.5mg/kg/bd and acyclovir 200mg bd from day 0. 14 patients treated on an outpatient protocol received ciprofloxacin 500mg bd from day 0. Patients received a median of 4.53 x 10^6 CD 34-positive cells (range 1.12 – 110.1) for stem cell rescue. The median time to ANC >0.5 x 10^9/l was 15 days (range 10-35). All patients experienced at least one neutropenic febrile episode and received empirical antibiotic therapy with Gentamicin 4mg/kg od and Cefpirome 2g q12h. 36 pathogens were isolated in 29 patients (53%); gram positive bacteraemia occurred in 21 patients; St.epidermidis (n=10), α haemolytic Strep (n=3), other (n=8) and gram negative bacteraemia in 15. Five patients developed oral HSV 1 infection. No invasive fungal infections were observed. There were no infection-related deaths. Patients received a median of 11 days (range 3-26) parenteral antibiotic therapy. The median length of inpatient treatment was 13 days (range 10-35). We conclude that although AuPBSCT is associated with a high incidence of bacterial infection, life threatening, fungal and viral infections are rare.

CYTOMEGALOVIRUS SEROLOGY AT TRANSPLANT INFLUENCES SURVIVAL OF HAEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENTS WHO REQUIRE MECHANICAL VENTILATION.

Peter H Scott, Simon Durrant, Thomas J Morgan, Rob Boots.
Royal Brisbane Hospital, Brisbane, Queensland, Australia.

Survival of bone marrow transplant (BMT) recipients requiring mechanical ventilation is poor. Identifying predictors of outcome specific to these patients may guide therapy and improve resource utilisation. A review was conducted of all BMT and peripheral blood stem cell transplant (PBSCT) recipients at our institution from January 1988 to December 1998. Records of those receiving mechanical ventilation in intensive care (ICU) were studied, with survivors ventilated for <24 hours excluded. Of 717 transplant recipients, a higher incidence of mechanical ventilation was seen in BMT patients (BMT 9.6% vs PBSCT 2.8% $p<0.001$) and in allogeneic transplants (allogeneic 10.8% vs autologous 1.3% $p<0.0001$). The BMT ventilation rate was less than in other published series ($p<0.005$). 50 patients met the study criteria comprising 42 BMT and 8 PBSCT. The indication for ventilation was respiratory failure in 90% of these patients. Median transplant-ventilation interval was 58 days (range 5-1356). Median ventilation period was 8 days (range 0.25-75). Survival to ICU discharge was 30%, falling to 12% at 6 months post-ventilation. No patient survived to 6 months post-ventilation in any of the following categories: 1) CMV negative recipient (n=17) or CMV negative donor (n=20); 2) receiving haemodialysis while ventilated (n=13); 3) serum bilirubin >68mmol/L during the first episode of ventilation (n=29); 4) APACHE II >23 in first 24 hours of ICU (n=27). By logistic regression, survival to 6 months was adversely influenced by a higher APACHE II score, a shorter transplant-ventilation interval, the need for vasopressors, and a matched sibling donor.

HAEMORRHAGIC CYSTITIS IS ASSOCIATED WITH ACUTE GRAFT VERSUS HOST DISEASE AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

J Crawford, R P Herrmann, P K Cannell and M Trent
Haematology Department, Royal Perth Hospital, Perth Western Australia

Haemorrhagic cystitis (HC) is a potentially dangerous complication after bone marrow transplantation (BMT) and attributable to viral infection on occasion but usually ascribed to urothelial damage from cyclophosphamide. We report a retrospective single institution experience of 50 patients, 29 receiving conditioning with busulfan 16mg/kg and melphalan 140-200mg/m^2 (Bu/Mel), 5 receiving TBI 1320cGy/melphalan 140mg/m^2 (TBI/Mel) for allogeneic BMT and 16 receiving Bu/Mel for autologous BMT for malignant disease. We compared this group with 15 receiving cyclophosphamide-containing regimens concurrently. No early onset HC was seen. Late onset HC occurred in 10 of 29 (34.5%) allotransplants receiving Bu/Mel, none of 16 autotransplants receiving Bu/Mel and 3 of 22 (13.6%) receiving cyclophosphamide-containing regimens ($p=0.09$). The incidence of HC increased with increasing severity of acute graft versus host disease (AGVHD). No HC was observed in grade 0-2 AGVHD but 4 of 11 (36.4%) with grade 3 AGVHD developed HC and 9 of 11 (81.8%) with grade 4 AGVHD developed HC ($p=0.000$, correlation coefficient 0.644). The onset of HC and AGVHD were temporally related. Both severity of AGVHD and HC predicted for worse survival. An increase of HC incidence was seen in chronic myeloid leukaemia patients but this group had an equivalent survival to other groups when matched for AGVHD. Analysis of other factors possibly causative showed negative correlations for the type of conditioning regimen and for related versus unrelated allogeneic BMT.
We conclude that the urothelium is a target for AGVHD.

RENAL GRAFT REJECTION FOLLOWING SIBLING ALLOGENIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

[1]KL Robinson, [2]G Perry, [2]V D'Intini, [1]A Schwarer, [1]A Spencer

[1]Bone Marrow Transplant Programme and [2]Renal Unit, The Alfred hospital, Prahran, Victoria

We describe the first reported case of solid organ graft rejection following allogeneic peripheral blood stem cell transplantation (AlloPBSCT). A 28 year old female underwent AlloPBSCT from her HLA identical sister for cytogenetic progression of Fanconi's Anaemia. She had successfully undergone a live related donor renal allograft from her father 2 years previously. She was conditioned with ATGAM / Cyclo-TBI and received CsA / methotrexate as GvHD prophylaxis. On day + 9 her renal function deteriorated abruptly and unexpectedly despite being otherwise well. By day +12 haemodialysis was required and severe microangiopathic haemolysis (MHA) had arisen. CsA was ceased and a renal graft biopsy showed severe cellular rejection with superimposed thrombotic microangiopathy. Despite daily plasmapheresis, pulse methylprednisolone, OKT3, FK506 and subsequently micophenylate the patient remained dialysis dependent. The features of MHA persisted but with less severity. At no stage was there evidence of significant acute GvHD. With no functionally significant renal recovery a transplant nephrectomy was performed at day +108. The remaining features of MHA rapidly resolved post-nephrectomy. We speculate that the engrafting third party haemopoiesis produced severe acute rejection of the renal allograft with secondary MHA via a graft versus graft mechanism.

REMISSION OF HLA B27 POSITIVE ANKYLOSING SPONDYLITIS AFTER AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTION FOR RELAPSED HODGKINS DISEASE

R Broady, PI Thompson, R Grigor, PJ Browett

Departments of Haematology, Oncology and Rheumatology, Auckland Hospital, Auckland, New Zealand.

Preliminary data suggests that high dose therapy followed by autologous haematopoietic stem cell transplantation may lead to durable remissions for certain subgroups of severe autoimmune disease. We report a patient with ankylosing spondylitis in whom clinical remission of his underlying autoimmune disorder was achieved following autologous peripheral blood stem cell transplantation for relapsed Hodgkin's disease. The patient, a 34 year old male, was diagnosed with stage IVA nodular sclerosing Hodgkin's disease in August 1995 when he presented with mediastinal and left supraclavicular fossa lymphadenopathy and pulmonary infiltrates. At that time he was known to be HLA B27 positive, with a 4 year history of recurrent neck and low back pain, and 3-4 episodes of iritis per year. Remission was achieved following 4 cycles of MOPP/ABV and 2 cycles of ChlorVPP chemotherapy, but unfortunately he relapsed in August 1997 with right hilar lymphadenopathy. Following 2 cycles of salvage chemotherapy using the ABVD regimen, he underwent successful peripheral blood stem cell mobilization with cyclophosphamide 2 g/m^2 and G-CSF 5 µg/kg/day. In December 1997 he received high dose therapy using the CBV regimen with subsequent reinfusion of autologous peripheral blood stem cells. There was rapid engraftment, and the patient remains in a stable remission 18 months following his transplant. The patients worst affected joints prior to transplantation were his neck and lateral hip areas, requiring voltaren 50-200 mg/day. Visual analogs of pain, morning stiffness and global assessment off voltaren were 3.5, 6.0 and 5.0 respectively (scale 0-10). During the mobilisation period he had an episode of iritis which settled with topical steroid therapy. In the 18 month period since the transplant, he has had no iritis, and no neck, back or hip pain. He has not required any non steroidal anti-inflammatory agents, and his visual analogs of pain, morning stiffness and global assessment are all normal. Prospective studies evaluating the role of high dose therapy and haematopoietic stem cell transplantation in autoimmune diseases are now under way in several centres. This report of a durable remission of ankylosing spondylitis following high dose therapy suggest that this condition may also be amenable to such an approach.

TREATMENT RESPONSE IN MULTIPLE MYELOMA: COMPARISON OF TUMOUR CELL LOAD IN PERIPHERAL BLOOD, BONE MARROW AND PERIPHERAL BLOOD PROGENITOR CELL HARVESTS

LF Lincz[1], RL Crooks[2], SL Way[2], N Granter[1], A Spencer[1,2,3,4]
[1]Hunter Haematology Research Group, Mater Misericordiae Hospital, NSW; [2]Hunter Area Pathology Service, NSW; [3]Hunter Haematology Unit, Mater Misericordiae Hospital, NSW; [4]Alfred Hospital BMT Program, Melbourne, VIC.

Tumour progression was monitored in seven multiple myeloma (MM) patients undergoing a novel oral chemotherapy regimen (cyclophosphamide, idarubicin and dexamethasone; CID) followed by early autologous stem cell transplantation (ASCT). Allele-specific oligonucleotide PCR (ASO-PCR) was used to semi-quantitate the number of tumour cells within the peripheral blood (PB) and PB progenitor cell (PBPC) harvests and compared with results from more conventional disease monitoring techniques of paraprotein levels and morphological bone marrow (BM) assessments. Tumour cells were detected in the PB of all patients at diagnosis but decreased in response to CID therapy. All but two of the 22 PBPC collections contained MM cells, and high levels (above 10%) were statistically correlated with poor overall clinical response to therapy ($p=0.0004$). Despite an apparent equivalence of response based on morphological BM assessment, the levels of MM cell contamination in the PBPC harvests were variable and could not be predicted from individual PB tumour loads calculated prior to mobilisation ($n=6$, $p=0.266$). There was no correlation between the day of leucapheresis collection and the number of contaminating MM cells, $CD34^+$ cells or MM cells per $CD34^+$ cell. Regardless of tumour contamination levels in the PBPC collections, the majority of patients demonstrated post-ASCT clearing of circulating MM cells. This study suggests that levels of circulating MM cells may be the best indication of patient response to treatment and argues against the theory of differential mobilisation of tumour cells and $CD34^+$ cells in response to cytokine treatment.

RETROSPECTIVE REVIEW OF THE FIRST 100 AUTOLOGOUS HAEMATOPOIETIC STEM CELL TRANSPLANTS AT THE ROYAL HOBART HOSPITAL.

RA Harrup, DM Tuck, RM Lowenthal.
Clinical Haematology/Medical Oncology Unit, Royal Hobart Hospital , TAS.

Autologous transplantation using either bone marrow (BM) or peripheral blood stem cells (PBSCs) is commonly utilised in the management of lymphomas and leukaemias and increasingly for palliation in certain solid malignancies. Between March 1981 and June 1999, our centre, which provides the statewide service, performed 100 autologous transplants. The median age was 45.5 years (range 9-65), with M:F 53:47 and 62 from the south, 22 from northwest and 16 from the north of Tasmania. The indications were non-Hodgkin's lymphoma 50%, Hodgkin's disease 10%, breast cancer 12%, leukaemias 9%, multiple myeloma 6%, other solid tumours 11% and rheumatoid arthritis 2%. 46% of all patients are currently alive, median survival 334 days (range 2-3826+). Median time to haematopoietic recovery, defined as time to neutrophils $\geq 0.5 \times 10^9$/L and platelets $\geq 20 \times 10^9$/L unsupported, was 13 days (range 9-35) and 16 days (range 7-58) respectively. Stem cell source for reinfusion was BM 62%, PBSC 33% and combined BM/PBSC 5%. The median duration of cryopreservation was 3 months (range 0-72). Cytokine support was available post transplant to 82% of patients. This study has identified variables predisposing to early haematopoietic recovery, which will enhance the safety and minimise the cost of future procedures.

GRADUATE CERTIFICATE IN APHERESIS NURSING

Dawn Thorp, B Wake, P Casey, R Parkes.
Cancer Centre and Staff Development Department, Royal Adelaide Hospital, North Terrace, Adelaide, South Australia.

The apheresis operator is faced with complex procedural decisions each time an apheresis procedure is performed.
To date in Australia, operators have been prepared by inservice training in the practical aspects of procedures by the technical support personnel from the various machine companies.
The University of Adelaide, in conjunction with Royal Adelaide Hospital, is proposing to offer a Graduate Certificate in Apheresis.
The aim is to give operators an understanding of basic anatomy and physiology, the pathophysiology of diseases treated with apheresis, and the rationale for treatment or for collecting blood products. The course will also explore aspects of vascular access, the choice of replacement fluids, pharmacology, common complications of apheresis and their treatment. Documentation, quality assurance and research issues will be included.
Using a workbook, video-conferences and a two-day workshop means that most of the course can be offered by distance education.
This course is intended to give the participant a theoretical base that will increase the participant's awareness and understanding of this dynamic discipline.
Outline and structure of the course will be presented.

RATIONALISATION OF STEM CELL HARVEST USING CIRCULATING CD34+ CELLS.

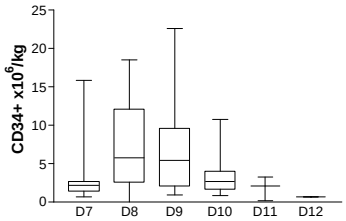
D Thorp, P Dyson, T Hughes.
Haematology Day Centre and Department of Clinical Haematology, Royal Adelaide Hospital and Hanson Centre, Adelaide, South Australia.

In our institution Peripheral Blood Stem Cell Collections [PBSC] are commenced when the patient's/donor's CD34 count has reached a predetermined value. Our aim is to collect sufficient cells for transplantation in the least number of apheresis procedures.
Our standard protocol requires $3x10^6$/kg CD34 cells for transplantation. However, when the stem cells are to be selected a greater starting number is required because there is an approximate 50% loss of cells during the selection process, one research protocol requires $4x10^6$/kg CD34 cells and paediatric hospital requires $5x10^6$/kg CD34 cells.
We examined data from 279 consecutive collections (240 adults and 39 children). Data from five collections was discarded because of missing numbers.
Using the patient's circulating CD34 count and the known collection efficiency of our machines, we can predict the amount of blood to be processed to obtain the desired PBSC for each patient.
By commencing the PBSC when the patients/donor CD34 is >20µl we collected $1x10^6$/kg CD34 cells in 95% of procedures and $3x10^6$/kg CD34 in 56% of procedures in adult patients using the standard two blood volume blood processed procedure.

DOCETAXEL EFFECTIVELY MOBILIZES LARGE NUMBERS OF CD34+ CELLS IN PATIENTS WITH PREVIOUSLY TREATED BREAST CANCER

HM Prince, D Rischin, JF Seymour, M Wolf, H Januszewicz, G Toner, P Chapple, M Brettell, M Quinn, D Wall, L Barber, R Maisano, P Gates. *Blood and Marrow Transplant Service, Div. Haematology and Medical Oncology, Peter MacCallum Cancer Institute.*

The role of high-dose chemotherapy (HDT) with peripheral blood progenitor cell (PBPC) support for patients with breast cancer is under active investigation. We are investigating repetitive HDT in patients with metastatic disease (MBC) and an important prerequisite is the ability to obtain high numbers of CD34+ cells to support this therapy. 25 patients with previously treated metastatic or locally advanced breast cancer were entered in this study. Pts had received a median of 2 prior chemotherapy regimens (range: 1-3). All pts received docetaxel (100mg/m^2) followed by daily G-CSF (10μg/kg). The target total CD34+ count was between 6x10^6/kg (minimum) and 9x10^6/kg (optimum) and used to support 3 cycles of HDT. Peripheral blood (PB) CD34+ cells were monitored daily from day 7 and apheresis commenced when the PB CD34+ cells exceeded 0.5x10^7/L. 27 'sets' of collections were performed in 25 patients with a total of 87 PB CD34+ cell measurements made. The peak day for PB CD34+ cells was D8 with a median PB CD34+ count of 5.7 x10^7/L (range: 0.01-18.5).



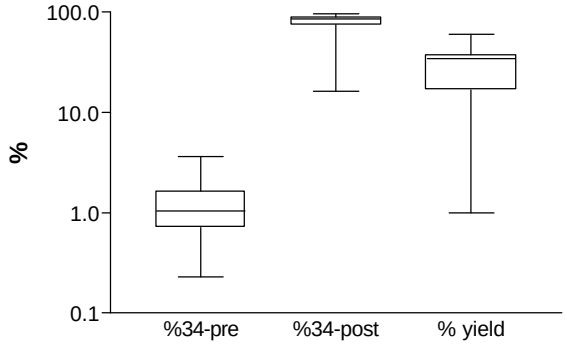
A total of 76 PBPC collections were performed on these 25 pts with the peak autograft CD34+ count on days 8 & 9 (p=0.0012) (left). The median total CD34+ cells collected was 10.1x10^6/kg (range: 1.2-17.9).

The majority of patients required 3 days of collection (n=12) with 10 pts requiring only 2 days collection and 5 pts, 4 days. Two pts had insufficient cells to proceed to HDT. We conclude that docetaxel with G-CSF effectively mobilizes large numbers of CD34+ cells in patients with previously treated breast cancer.

ISOLEX 300i CD34-SELECTED BLOOD CELLS (BC) CAN SUPPORT MULTIPLE CYCLES OF HIGH-DOSE CHEMOTHERAPY: A MULTICENTRE STUDY

HM Prince, J Bashford, P Chapple, M Quinn, DM Wall, M Brettell, L Barber, M Wolf, H Januszewicz, J Seymour, S Juneja, P Gates, R Maisano, MJ Millward, GC Toner.
Autologous Blood and Marrow Transplant Service, Division of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, Melbourne, Victoria and Clinical Haematology and Medical Oncology, The Wesley Clinic, Brisbane, Queensland

We previously reported our results of 8 pts with MBC who underwent BC mobilization and Isolex 300i CD34-selection and demonstrated that such cells could support three cycles of high-dose therapy [HSA,1998]. This protocol was extended across two centres and to date the BCs of 15 pts have undergone CD34-selection. The median pre-selection 34% of the autograft product was 1.0% (0.23%-3.6%) with CD34 content (x 10^6/kg) of 12.2 (3.0-26.9). Following CD34-selection, the median CD34+ cell purity was 85.5% (16.3-96.1) with a median CD34 yield (recovery) of 34.3% (1.0-60.0) (see below). The absolute CD34 recovery was 3.5x 10^6/kg (0.13-9.01).



These CD34-selected cells have been used to support 32 cycles of HDT to date. The median time to ANC>1.0=11d (9-25), plts>20x10^9/L=14d (12-26), >50=19d (14-39) and >100=28d (19-100). Two pts have required the infusion of 'back-up' unselected cells because of delayed neutrophil and platelet recovery. We conclude that Isolex300i CD34-selection results in a relatively CD34-pure product which can support multiple cycles of HDT. However, the loss of two-thirds of the CD34+ cells is the major limitation of this process.

Haemonetics MCS+ For The Collection OF Single Donor Leuka - Depleted On Line Filtered Platelets.

M. Brettell M. Molloy R. McKenna E. Serpell H. Stevens R. Wilke HM. Prince MM. Wolf.

Peter MacCallum Cancer Institute, Ian Cooper Cell Separator Unit, St Andrews Place, East Melbourne Victoria 3002.

Four programmable platelet protocols are available on the Haemometics MCS+. 1) The MCS+ will collect the target number of leukadepleted platelets. 2) Platelets and plasma (LDPLP); leukadepleted platelets and a defined volume of plasma are concurrently collected. 3) Leukadepleted platelets with volume replacement (LDPS), a unit of leukadepleted platelets is collected by MCS+, and donor receives a preprogrammed volume of replacement fluid (saline) during each cycle. 4) Platelets and plasma with volume replacement (LDPLPS), a unit of leukadepleted platelets and a defined volume of plasma are concurrently collected by MCS+. The donor receives a preprogrammed volume of replacement fluid (saline) during each cycle. The leukadepleted platelets and LDPS protocols collect double platelet products that can be split into 2 transfusable platelet collection bags, each maintaining 5 day storage capability. To date 100 Blood Bank donors have undergone platelet collection. The mean platelet count prior to collection was 261 x 10^{9} /L (range 173 – 493 x 10^{9} /L). The mean collection was 5.57 x 10^{11} platelets (range 1.25–10.18 x 10^{11}). Platelet collections were white cell depleted by on line filtration. Machine efficiency based on the available pre collection platelet count was 63% (range 22.5% - 85.6%). 96% of all collections yielded a double dose of platelets.
Furthermore the single needle access was appreciated by the donors and enabled them more freedom of movement to operate the video and TV remote control, or turn the pages of their reading material.

THE USE OF PHERES FLOW™ CATHETER FOR THE COLLECTION OF PERIPHERAL BLOOD PROGENITOR CELLS (PBPC) AND HIGH DOSE THERAPY.

R Wilke, M Brettell, R McKenna, M Molloy, E Serpell, H Stevens, HM Prince, MM Wolf, J Haddad.

Autologous Blood and Marrow Transplant Service and Ian Cooper Cell Separator Unit, Division of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, East Melbourne, Victoria 3002

The efficacy of a new, long term, triple lumen, central venous access device (CVAD) used for stem cell collections and high dose therapy was evaluated. Seven patients had the catheter inserted either into the left or right sub-clavian vein. Seven patients (6 female, 1 male) were enrolled onto our study, with diseases of Ca. Breast (n=6) and chronic myeloid leukaemia (CML), (n=1). The median age of patients was 51 years(range 38-57 years). Median number of days to harvest following insertion was 4 days (range 1-23 days) and median number of days the catheter was in-situ was 31 days (range 3-74 days). The median inlet flow (for apheresis) was 60.4 ml/min (range 0-70 ml/min), with median blood volume processed of 10 liters (range 3822ml-12682ml). A total of 24 apheresis procedures were performed with a mean of 3.4 days per patient (range 3-4 days). Twenty-two procedures were performed on the Cobe® Spectra™ and two were performed on the Baxter CS3000+ cell separator. Over the course of the study, three catheters were removed due to catheter infection, one removed due to pulmonary emboli, and one removed due inadequate mobilization of stem-cells, the patient subsequently coming off high dose therapy (HDT) trial.There were two cases of the catheter failing to draw adequate blood flows to the cell separator. In one, a cathetergram showed no evidence of thrombotic activity or blockage,despite this, Urokinase 5000 units was instilled down all lumens of the catheter and blood flow was re-established. In the second, cathetergram revealed the development of a fibrin sheath, which was subsequently removed under image intensifier control with no further problems encountered. We conclude that the Pheres Flow™ catheter is promising for PBPC collection HDT. Patient accrual will continue.

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PERIPHERAL BLOOD STEM CELL MOBILISATION IN RHEUMATOID ARTHRITIS PATIENTS - A SINGLE CENTRE EXPERIENCE. **Staniforth D, J,Moore, J.Snowden, M. Keir, C. Popoff, E.Coleman, A.Dodds, S.Milliken, P.Brooks and J.Biggs. St.Vincents Hospital, Sydney, NSW.**

Rheumatoid arthritis is a chronic debilitating form of arthritis which has recently been suggested as a potential candidate for PBSCT. We have conducted phase I trials in our centre for this disease since 1996. Initial trials focussed on the safety of GCSF mobilisation in RA patients. Subsequent patients underwent mobilisation with a view to PBSCT with cyclophosphamide conditioning. From July 1996 to May 1999, we performed peripheral blood stem cell collections on 15 Rheumatoid Arthritis patients. All patients received GCSF 10mcg/kg with collection commencing on day 5. The target value of CD34+ cells was 2.0 $x10^6$/kg. All patients were collected on a Cobe Spectra and > 2.5 plasma volumes were processed. Central access was required in two patients. There was no major increase in flares of their disease but some joint discomfort was experienced with > 4 hours on the machine. Mean number of CD34 cells collected on day 5 was 2.98 X 10^6/kg (range 0.74 - 6.69). 12 patients achieved the target value of 2 x 10^6/kg CD34 positive cells in a single apheresis procedure and 3 patients required more than one apheresis collection. 10 of these patients proceeded to PBSCT. In conclusion, PBSC mobilisation can be safely accomplished in rheumatoid arthritis patients.

THERAPEUTIC PLASMA EXCHANGE IN THROMBOTIC THROMBOCYTOPENIA PURPURA - A SINGLE CENTRE RETROSPECTIVE ANALYSIS.

C.Kelly, D.Yokowo, D.Polichronis, K.Taylor, M.Bentley.
Mater Hospital Apheresis/Transplant Unit, Brisbane

Thrombotic thrombocytopenia purpura **(**TTP) is a disease defined primarily by the presence of thrombocytopenia and microangiopathic haemolytic anemia (MAHA) requiring early intensive treatment with therapeutic plasma exchange **(**TPE). Six female patients (pts) have been treated from May 1996 to May 1999. Specifically we analysed diagnostic criteria, time to first TPE, number of TPE, plasma volume exchanged, taper of TPE, other treatment therapy and outcome. Median platelet count was $19x10^9$/L (range 15-63), LDH 1020mmol/L (range 683-1751), creatinine 0.10mmol/L (range 0.08-0.12). Two pts were febrile, 2 pts exhibited confusion. Time to commencement of TPE was 4hrs for 4 pts, 7 and 24hrs for the remaining 2 pts. All pts were treated with corticosteroids and other therapies are listed below. TPE was tapered in most patients. One patient with advanced metastatic breast carcinoma had late recognition of MAHA (>36hrs) and died on the 2nd day of TPE. Patient 4 experienced a grand mal seizure on day 2 of TPE.

Pt.	**Age**	**No of TPE No of Days**	**Replace**	**Other treatment**	**Outcome to date**
1	65	34 / 45	FFP/ Sal.	Vincristine	Relapse
1 (2nd)		34 / 46	FFP / Sal.	-	Relapse
1 (3rd)		19 / 28	FFP / Sal.	Splenectomy	Alive
2	35	16 / 19	CPP / Sal.	Vincristine	Relapse
2 (2nd)		10 / 25	CPP / Sal.	on Steroids	Alive
3	32	8 / 9	CPP/ Sal.	-	Alive
4	38	16 / 23	CPP / Sal.	-	Alive
5	55	2 / 2	CPP / Sal.	Vincristine	Dead
6	48	14 / 22	CPP / Sal.	-	Alive

Our experience supports the efficacy of early intensive TPE in the treatment of TTP and emphasises the safety and tolerability of prolonged TPE in this setting.

Pt No	**Age**	**Pre Plt**	**LDH**	**Bilirubin**	**Creat/Urea**	**Hapt.**	**Fever**	**Abnormal Mental Status**
1	65	33	789	25	0.11/7.9	<0.06	No	Yes - confusion
1(2nd)		110	365	11	0.08/7.7	1.92	No	No
1(3rd)		141	230	10	0.09/5.9	0.74	No	Yes - vague
2	35	19	1751	41	0.12/6.8	0.06	Yes	No - Headache
2(2nd)		132	489	20	0.06/1.2	0.06	No	No
3	32	63	1546	35	0.10/5.7	<0.10	No	No
4	38	15	683	100	0.09/6.0	0.06	No	No (headache)
5	55	17	1250	53	0.08/12.8	0.10	No	Yes - confusion/dec.LOC
6	48	18	764	34	0.09/5.9	0.10	No	No

Patient Number	Time to first TPE	No of TPE / Days	TPVP/P	Replace	Taper	Toxicities	Other treatment	Outcome
1	24hrs	34 / 45	1.0-1.8	FFP/ Saline	Yes	Yes	Vincristine, Steroids, Prosorba Column	Relapse
1 (2nd)		34 / 46	0.4-1.9	FFP / Saline	Yes	Yes	Steroids	Alive

1 (3rd)		19 / 28					Steroids Splenectomy	Alive
2	4hrs	16 / 19	1.0-1.5	CPP / Saline	?No	hypotension	Vincristine, Steroids	? Relapse
2 (2nd)		10 / 25	1.0	CPP / Saline	Yes		on Steroids	Alive
3	7hrs	8 / 9	1.0-1.5	CPP/ Saline	Yes	Plasma reaction	Steroids	Alive
4	4.5hrs	16 / 23	1.0-2.0	CPP /Saline	Yes	GM Seizure	FFP, Steroids	Alive
5	4hrs	2 / 2	0.6-1.0	CPP / Saline	N/A	Resp. arrest	Vincristine Steroids	Dead
6	4hrs	14 / 22	0.7-1.7	CPP / Saline	Yes		Steroids	Alive

(including platelet count (PLTC), miciroangiopathic anaemia, fever, renal impairment, abnormal mental state), despite lack of understanding cause and pathophysiology.

At diagnosis all pts had microangiopathic blood film, negative Coombs and haptoglobin <0.1.
One plasma volume was performed on initial TPE and between 1.0-2.0 plasma volumes for susequent TPE.

Comparison between CD34 cell counts and the Immature Reticulocyte Fraction for Stem Cell Mobilisation.

J. Cohen, P. Roeth, D. Rosenfeld, L. Dunlop.
Haematology Department, South Western Area Pathology Services, Liverpool Hospital, Liverpool, NSW.

Patients having peripheral blood stem cells harvested in planning for autologous peripheral blood stem cell transplantation at a later date, first undergo a regime where G-CSF is administered following chemotherapy. The objective is to mobilise CD34 positive cells into the peripheral blood until the level of CD34+ cells is high enough for these cells to be harvested by leukophoresis. At our institution these patients have pre-harvest CD34 counts performed until the absolute CD34+ cell count reaches 15 x 106/L when leukophoresis is commenced. The tests that are also performed include Full Blood Count, Reticulocyte count and the Immature Reticulocyte Fraction (IRF). The Reticulocyte count is performed on the *Cell Dyn 4000* at the same time as the FBC is processed. The technologies for performing the Reticulocyte count utilise both fluorescent flow cytometry along with intermediate light scanner at 7o. The IRF is determined as the fraction of reticulocytes that exceed a predetermined level of RNA fluorescence. Data from 35 patients in whom stem cell mobilisation was attempted will be presented. The findings show that the Immature Reticulocyte Fraction has a valuable role to play as part of the pre-harvest testing. In some patients, the pre-harvest IRF count was very low or even zero, with all of these patients having a very low CD34+ cell count when performed concurrently. It is proposed that the IRF could be used as an initial screen to determine if a CD34 cell count needs to be performed. This could lead to financial benefits to the laboratory, as fewer CD34 cell counts would be required particularly of a weekend and out of hours.

THE PFA-100™ AS A PREDICTOR OF BLOOD LOSS FOLLOWING CORONARY ARTERY BYPASS GRAFTS

J Low, A Delaney.
Haematology Division, Institute of Laboratory Medicine, St Vincent's Hospital and Intensive Care Unit, St Vincent's Private Hospital, Sydney

BACKGROUND: Platelet dysfunction is a major cause of bleeding after cardiopulmonary bypass surgery. The PFA-100™ provides an in vitro quantitative measurement of platelet function in whole blood under high shear conditions. This study investigates the relationship between pre and postoperative PFA-100 and postop bleeding in patients undergoing coronary artery bypass graft surgery (CABG).
METHODS: Blood was taken from patients before CABG and on arrival in the intensive care unit after surgery, for the following tests: PFA-100 closure time (CT) with collagen/epinephrine cartridges, coagulation tests (PT and APTT) and a FBC. Postoperative blood loss into the mediastinal chest drain was recorded hourly, until the removal of the drain. Medications such as preop aspirin and the postop use of aprotinin and blood products, were also recorded.
RESULTS: A preliminary analysis of 36 patients, showed that there was no correlation between either pre or post CT and blood loss as measured in the chest drain (at 1, 3, 6, and 12 h or on removal of the drain) or the duration of bypass.There was also no difference in the median CT between the patients that did or did not receive blood products. However, post CT correlated significantly with post PT ($r = 0.64$, $p = 0.000$), but not APTT and correlated negatively with Hct ($r = -0.43$, $p = 0.009$) and Plt count ($r = -0.48$, $p = 0.004$).
CONCLUSIONS: The PFA-100 CT was not able to predict postoperative blood loss after CABG, but did reflect changes in haemostatic tests and the blood count.

COLLAGEN BINDING ASSAY (VWF:CBA): DETECTION OF VWD AND DISCRIMINATION OF VWD SUBTYPES DEPENDS ON COLLAGEN SOURCE. AN UPDATE.

Emmanuel J Favaloro.
Haematology, ICPMR, Westmead Hospital NSW 2145 Australia.

The VWF:CBA is a relatively new, ELISA-based functional von Willebrand factor (VWF) assay useful in both VWD diagnosis and subtyping (qualitative [ie Type 2] defects exhibit VWF-discordance and give high VWF:Ag to VWF:CBA ratios; 1). We have previously reported that the type of collagen used in the VWF:CBA will influence the assay's utility. The current study updates previous findings. A large number of commercial collagen preparations [n=22], including different batches, have now been tested for their ability to (i) detect VWF [ie VWD], and (ii) discriminate VWD subtypes. Collagen preparations were tested at a range of concentrations and included: Type I, III and IV, various mixtures of these, aqueous preparations, preparations reconstituted from lyophilised stock; original sources range from human placenta to calf skin to equine tendon. Three collagen preparations did not support VWF binding in an ELISA process (ie poor or no detection of VWF; therefore unable to detect VWD). The ability of the remaining collagen preparations to detect VWF was variable, as was their ability to discriminate VWD subtypes. Detection of VWF and discrimination of VWD subtypes was ***not*** mutually inclusive. Some provided excellent detection for VWF, but poor discrimination of Type 2 VWD, while others provided good to acceptable detection ***and*** discrimination. Effectiveness of discrimination of VWD subtypes was also found to be dependent on the type, concentration, and batch of collagen used. Overall best utility was generally achieved using equine tendon preparations, or mixtures of Type I/III collagens. In conclusion: (i) not all collagen preparations can be used effectively in a VWF:CBA (ie to both detect VWD and discriminate VWD subtypes), (ii) once found, a suitable collagen source must be properly evaluated to maximise VWF (and therefore VWD) detection and VWD subtype discrimination.

1. Favaloro EJ & Koutts J. 1997. Pathology, 29: 385-91.

THE VWF-GPIb INTERACTION INDUCES CYTOSKELETAL REORGANISATION INDEPENDENT OF THE GPIbα AND GPIbβ CYTOPLASMIC TAILS

SP Jackson, P Ulsemer, SL Cranmer, CL Yap, S Kulkarni, N Mistry, SM Dopheide, C de la Salle, F Lanza, Y Yuan.
From the Australian Centre for Blood Diseases, Department of Medicine, Monash Medical School, Victoria, Australia, Etablissement de Transfusion Sanguine, Institut National de la Santé et de la Recherche Médicale Unité 311, Strasbourg, France.

Platelet adhesion onto a von Willebrand factor matrix is a multi-step process involving the GPIb/V/IX complex and integrin $\alpha_{IIb}\beta_3$. Once adherent, platelets undergo dramatic cytoskeletal reorganization leading to filopodial extension and the formation of broad lamellipodia, ultimately converting the platelet from a flat discoid morphology to a fully spread form. In this study we have investigated the possibility that the vWf-GPIb/V/IX interaction induces actin polymerization and cytoskeletal reorganization independent of integrin $\alpha_{IIb}\beta_3$. Perfusion of platelets through vWf-coated microcapillary tubes was associated with platelet tethering, rolling and rapid morphological conversion of the cells from flat discs to spiny spheres. Extension of filopodia was also observed in Glanzmann's thrombasthenic platelets excluding a role for integrin $\alpha_{IIb}\beta_3$ in this process. The extension of filopodial was completely abolished by pretreating platelets with cytochalasin D or activators of protein kinase A (PGE_1, theophylline). The ability of the vWf-GPIb/V/IX interaction to induce actin polymerization was confirmed by performing aggregation studies on human platelets or GPIb/IX-transfected cells. Morphological analysis of GPIb/IX-transfected cells adhered on a vWf matrix demonstrated that the vWf-GPIb/IX interaction was sufficient to induce cytoskeletal reorganization leading to the extension of numerous filopodial projections. Studies of GPIbα and GPIbβ cytoplasmic tail truncation mutants demonstrated that the cytoplasmic tail of neither subunit was required for vWf-induced actin polymerization and filopodial extension. These studies demonstrate that the binding of vWf to GPIb is sufficient to induce actin polymerization and filopodial extension in both rolling platelets and GPIb/IX-transfected cells. These cytoskeletal changes appear to be uncoupled from signaling events linked to the cytoplasmic tails of GPIbα or GPIbβ.

DETECTION OF VON WILLEBRAND'S DISEASE 'NORMANDY' (TYPE 2N VWD) IN AUSTRALIA: PRELIMINARY FINDINGS.

Emmanuel J Favaloro*, **John Rowell#, John Lloyd‡, Jerry Koutts*, Mark Hertzberg*.**

*Haematology Department; *ICPMR, Westmead Hospital, NSW 2145; #Royal Brisbane Hospital, Qld 4029; ‡IMVS, SA 5000; Australia.*

von Willebrand's Disease 'Normandy' (Type 2N VWD) is a rare VWD subtype manifesting as a functional defect in von Willebrand factor (VWF)/factor VIII binding. It phenotypically mimics a haemophilia A type disorder and plasma findings often reflect a moderate factor VIII deficiency (ie functional FVIII level is low, but VWF:Ag, VWF:CBA, VWF:RCof relatively normal). Suspicion for Type 2N VWD may be higher when such a finding presents in a female. The diagnosis of Type 2N VWD requires the use of a specific VWF:FVIII binding assay, and may further require genetic analysis. The incidence of Type 2N VWD in Australia is currently unknown. In order to assess this prevalence, we recently undertook to assess plasma samples obtained from 'potential' 2N VWD patients (ie high clinical suspicion of Type 2N VWD; previous plasma studies showed moderate haemophilia A-like trait) as sourced from various Australian haemophilia centres. Subsequent sample retesting involved assessment of VWF (both VWF:Ag and VWF:CBA), factor VIII antigen (FVIII:CAG) and VWF:FVIII binding by specific assays (this was performed by two methods; the first as 'classically' performed [ie VWF by ELISA vs VWF-bound factor VIII using chromogenic assay], the second using a dual stage ELISA process to detect both VWF:Ag and VWF-bound factor VIII by ELISA). In total, some 40 potential Type 2N VWD candidate plasmas were collected and tested. A laboratory pattern consistent with Type 2N VWD was found in only 6 samples, half of which belonged to three siblings from a single family. In conclusion, Type 2N VWD does exist in Australia; however, its prevalence appears to be very low.

PROFOUND THROMBOCYTOPENIA AFTER ADMINISTRATION OF A GLYCOPROTEIN IIB/IIIA RECEPTOR INHIBITOR (REOPRO/ABCIXIMAB).

M Borosak, D A Westerman, CL Smith.

Austin & Repatriation Medical Centre, Department of Clinical Haematology, Heidelberg, Vic. 3084.

Glycoprotein IIb/IIIa receptor inhibitors have been increasingly used after coronary angioplasty, particularly after stent insertion. We present two cases of profound thrombocytopenia after the use of Abciximab (ReoPro).

The first case was an 80 year old man who had percutaneous transluminal coronary angioplasty (PTCA) with stenting for persistent unstable angina. He was given intra-arterial heparin and Abciximab bolus plus infusion. Profound thrombocytopenia (4×10^9/L) was detected within 6 hours of commencement. This was complicated by a right femoral sheath haematoma and significant gastrointestinal bleeding. The patient received a platelet transfusion with a sustained increment (60×10^9/L) and control of bleeding. The second case was a 56 year old man who had PTCA and stenting for post infarct angina. He developed profound thrombocytopenia (3×10^9/L) 15 hours after commencement of Abciximab. He had platelet recovery after 3 days without complication and required no intervention other than cessation of infusion.

The incidence of mild thrombocytopenia ($<100 \times 10^9$/L) after Abciximab is 3.9%, and profound thrombocytopenia ($< 20 \times 10^9$/L), occurs rarely (< 1%), and usually within the first 24 hours. The mechanism is not clear, however exposure of new glycoprotein epitopes with subsequent platelet phagocytosis by preformed has been postulated immunoglobulin. Management of the thrombocytopenia is also ill-defined with controversy regarding the role of platelet transfusion.

A PATIENT WITH HETEROZYGOUS VON WILLEBRAND'S TYPE 2N GENOTYPE AND SEVERE PHENOTYPE.

SE Rodgers[1], NV Lerda[3], GJ Casey[2], EM Duncan[1], DM Quinn[3], RLP Flower[3] and JV Lloyd[1]. *Haematology[1] and Molecular Pathology[2] Divisions, IMVS, Frome Rd, Adelaide and University of South Australia[3], Frome Rd, Adelaide, SA.*

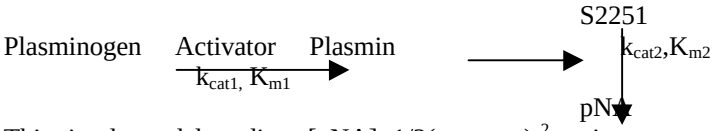
Von Willebrand's disorder type 2N (vWD 2N) is a recessive qualitative disorder, due to reduced binding of factor VIII coagulant activity (FVIII:C) to von Willebrand's antigen (vWf:Ag), which can be detected using a factor VIII binding assay (FVIII binding). There are usually normal levels of vWf:Ag and ristocetin cofactor activity, but reduced FVIII:C. Several mutations have been described in exons 18-24 of the von Willebrand factor (vWf) gene.

We report a family with the first case of vWD 2N detected in Australia. The proband has severely reduced factor VIII binding (3% of normal, reference 62-243), vWF:Ag = 0.29 IU/ml (ref 0.4-1.5), FVIII:C = 0.20 IU/ml by one-stage assay (1-st, ref 0.45-1.75), and 0.04 IU/ml by two-stage assay (2-st, ref 0.40-1.50). Her mother has reduced FVIII binding (37%), vWf:Ag = 0.47, FVIII:C = 0.60 (1-st) and 0.33 (2-st). The father has FVIII binding = 63%, vWf:Ag = 0.39, FVIII:C = 0.71 (1-st) and 0.56 (2-st). DNA analysis of exons 18-24 of the vWf gene revealed no mutations in the father. Mother and daughter were heterozygous for a mutation in exon 18 of the vWf gene (2811G>A, R854Q). No other mutations were found in the proband, whose grossly reduced factor VIII binding is consistent with a homozygote or compound heterozygote. Messenger RNA from her white cells showed the same results as the DNA, excluding a transcription defect. It is likely that the proband has inherited a type 1 mutation from her father, which in combination with the 2811G>A mutation results in a severe phenotype.

Optimisation of methods for the measurement of plasminogen activators

Indra Ramasamy, Em Tran, Albert Farrugia .
Therapeutic Goods Administration Laboratories, PO Box 100, Woden, ACT 2606.

We report our preliminary observations on the optimisation of a colorimetric method for the standardisation and measurement of therapeutic plasminogen activators, used as thrombolytic agents. We first tested the method with tPA (tissue plasminogen activator) on a microtitre plate. The reaction mixture with a total volume of 100 L was composed of 0.05M Tris pH 7.5, and 0.5 mM S2251(H-D-val-Leu-Lys-pNA) and varying concentrations of plasminogen and tPA. In the cascade reaction pNA is formed following:



This simple model predicts: [pNA]=1/2(constant)t^2 ,t=time.
A linear response is observed when [pNA] is plotted against t^2 (fig 1) at any given concentration of plasminogen, and when the slope is replotted against [activator] (fig 2). Our studies show that the kinetic model is valid at plasminogen concentrations of 3.1 M and 6.2 M and tPA range of 1.6-15 IU/mL. We are currently investigating the influence of fibrin monomers on the rate of hydrolysis.

Fig 1

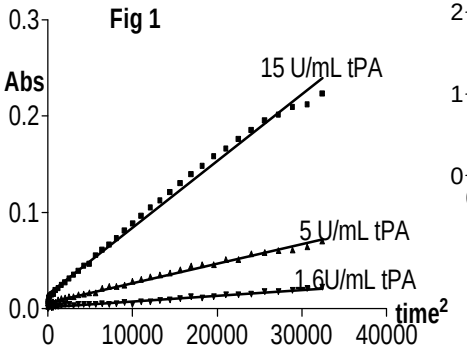
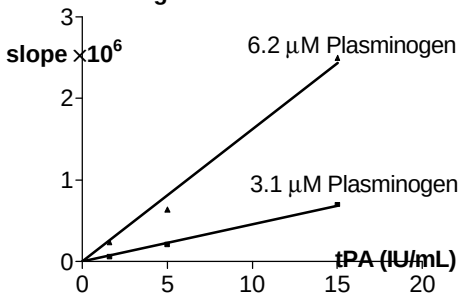


Fig 2



AN UNUSUAL FACTOR IX INHIBITOR

M Seldon, I Goodhew, S Dooley
Haematology, Hunter Area Pathology Service, John Hunter Hospital, Lookout Rd., New Lambton Heights, NSW, 2305

Acquired Factor IX inhibitors are rare compared to acquired Factor VIII inhibitors, and usually occur in association with autoimmune disorders.

We report a case of an acquired inhibitor to Factor IX occurring 2 weeks after an infusion of fresh frozen plasma. A 69 year old man was admitted to hospital because of haematuria, with a diagnosis of a structural renal pelvis lesion. He had had coronary bypass surgery 2 weeks prior at another hospital without any complication or excessive bleeding but had received red cells and plasma peri- operatively. He was well when discharged after 7 days. One week later he noticed haematuria with clots and after an IVP, which showed a pelvi-ureteric lesion, he was admitted to hospital for nephrectomy. He was requiring 4-5 units of blood per week to maintain his Hb. Coagulation studies showed a prolonged aPTT with normal PT, TT, fibrinogen and XDPs. Mixing studies showed full correction of the aPTT. Factor assays showed FIX 20% but with non-parallel lines, with normal FVIII, and FVII. Inhibitor screen was normal. An infusion of FIX concentrate (MonoFIX) was given which corrected the aPTT for a brief period. A further bolus of MonoFIX was given and its half-life studied. The peak level rise was 50% of the expected rise and the level had returned to baseline in under 6 hours. He was commenced on an infusion of MonoFIX, and was immunosuppressed with prednisolone and cyclophosphamide. He received an infusion of IVIg (1.2 gm/Kg). His bleeding settled and his FIX level became supra normal and remained so despite cessation of the infusion. Further laboratory testing detected an inhibitor of FIX. Testing for autoimmune diseases was non-diagnostic, but occurred after IVIg.

This case highlights the laboratory detection of Factor IX inhibitors, their possible aetiology and treatments.

RELATIVE EFFICACY OF LOW DOSE APROTININ & EPSILON AMINOCAPROIC ACID FOR REDUCTION OF BLEEDING AFTER CARDIOPULMONARY BYPASS

N A Marsh, G Bellingham, M M Hales, M J Ray
Department of Pathology, The Prince Charles Hospital, Chermside, Queensland; School of Life Sciences, Queensland University of Technology, Brisbane, Queensland

Cardiopulmonary bypass (CPB) surgery is well known to cause excessive bleeding both intra- and post-operatively. The cause of this bleeding is undoubtedly multifactorial although activation of fibrinolysis plays an important part and thus fibrinolytic inhibitors have been successful in reducing the bleeding. We report here on a double blind, randomised study of aprotinin and epsilon aminocaproic acid (eACA) given to 100 patients undergoing valve replacement surgery. The aprotinin dose was 2 X 10^6 KIU (during surgery) and eACA, 10 g plus 1.25 g/hr for 4 hrs post-bypass. Post-operative haemoglobin loss into chest drainage fluid was used as a measure of bleeding and this was identical in the two groups as were intra- and post-operative transfusion requirements. Measurements were also made of neural damage markers (neuron specific enolase, (NSE) and S100β) and fibrinolytic parameters. Levels of NSE and S100β rose equally in the two groups but importantly, S100β was significantly higher in patients experiencing neurological deficits ($p<0.001$ at 24 hrs post-bypass). This indicates the usefulness of S100β as a marker of neural damage. Of particular interest from fibrinolytic measurements were D-dimer levels, which rose equally in both groups indicating a comparable degree of activation whereas plasminogen fell to a greater extent in the eACA group suggesting greater conversion to plasmin. In fact, α_2–antiplasmin levels rose higher in the eACA group suggesting that the drug has an effect on antiplasmin levels, a previously undescribed phenomenon. Overall, our findings indicate that eACA and aprotinin are equally efficient in reducing post-CPB bleeding although the substantial differences in cost allow considerable savings to be made by using eACA.

ENDOTHELIAL CELL CONTROL OF vWF MULTIMER SIZE

L Xie, CN Chesterman, PJ Hogg.
The Centre for Thrombosis and Vascular Research, School of Pathology, University of New South Wales and Department of Haematology, Prince of Wales Hospital, Sydney

The haemostatic activity of plasma von Willebrand factor (vWF) is a function of multimer size. Only the large vWF multimers are effective in promoting platelet adhesion to a site of vascular injury. We observed that the conditioned medium of cultured human umbilical vein, human microvascular and bovine aortic endothelial cells contained an activity which reduced the average multimer size of plasma or purified vWF and its affinity for collagen. The average multimer size of vWF produced endogenously by human umbilical vein endothelial cells was similarly reduced following secretion. The reducing activity was ablated by pre-treatment with heat or the thiol blocking agents, iodoacetamide, N-ethylmaleimide or E-64, but not by a range of specific serine-, cysteine-, aspartic-, or metallo-proteinase inhibitors. Reduction in vWF multimer size was associated with formation of new thiols in vWF and there was no evidence for additional proteolytic processing of vWF. The reducing activity was associated with a protein with an anionic pI that binds heparin and contains reactive thiol(s). These results suggested that the interchain disulfide bonds that link the vWF homodimers near the N-termini were being reduced by a vWF reductase secreted by endothelial cells. In support of this hypothesis, incubation of vWF with the protein reductants, protein disulfide isomerase and thioredoxin, resulted in formation of new thiols in vWF and reduction in the average multimer size of vWF. In summary, we have evidence that vWF multimer size is regulated by a vWF reductase secreted by endothelial cells. This result may have consequences for normal control of vWF haemostatic activity and perturbation of this control may contribute to the abnormally large vWF multimers associated with thrombotic thrombocytopenic purpura and haemolytic ureamic syndrome.

MYELOMA 101 - THE BASICS

Durie B

Introduction

Multiple myeloma is a relatively rare disease and public awareness is low. Most patients have never heard of myeloma at the time of diagnosis. The first reactions therefore tend to be a combination of fear of the unknown and confusion about all the new information. A trip to the library is frequently not helpful and may even be misleading. Standard medical information is complex and often out of date or not relevant to the situation at hand.

This handbook is intended to improve that situation; to provide a basic understanding of the disease sufficient to allow patients to make informed decisions about treatment choices. The handbook is supplemental to the information given by the doctor. Caregivers, family and friends may also find the information useful.

Although there is no treatment that can cure multiple myeloma, it is an eminently treatable disease. Indeed many patients go on to lead full lives for years even decades after diagnosis. With increasing research the overall outlook for myeloma patients is steadily improving. Knowing more about the disease and understanding what can be done to help, reduces anxieties and makes it easier to come to terms with the diagnosis of myeloma.

Myeloma tends to be a very individual disease. The disease progresses at different speeds and creates a different pattern of problems for each patient. Your doctor will assess your particular situation and recommend the best approach in your case. However, the patient plays a central role in helping make these individual treatment decisions the best they can be. It is important that patients and their families be well informed, ask questions and give serious thought to alternative strategies or options. In this handbook we hope to cover the most important issues to guide management decisions.

What causes myeloma?

There are over 3000 new cases of myeloma in the UK each year representing 15% of blood cancers and 1% of all types of cancer. The incidence varies from country to country from a low 1/100,000 in China to approximately 4/100,000 in most Western industrialized countries. Myeloma is more common in blacks than whites. The male/female ratio is 3:2. The incidence rises with age. Better diagnostic techniques and the higher average age of the general population may in part explain the rising incidence over the last several decades. A trend towards more frequent myeloma in patients under age 55, however, implies important environmental causative factors in the past 3 or 4 decades. Exposures to atomic radiation, petroleum products, pesticides, solvents, heavy metals and airborne particles are major risk factors.

What is myeloma?

Multiple myeloma is a cancer of plasma cells. Plasma cells are normally present in the bone marrow and are responsible for antibody production in response to infection and other immune triggering events. In myeloma, a single defective plasma cell (a myeloma cell) gives rise to the much larger number of myeloma cells which build up in the bone marrow. This process disrupts the normal immune system as well as displacing the normal bone marrow cells. Damage to surrounding bone and soft tissues such as nerves or muscles can also occur. Although the initial growth of myeloma cells may be in response to a triggering infection (e.g. virus infection) the cells literally take on a life of their own and grow in an uncontrolled or incompletely controlled fashion. The exact mechanisms involved remain to be fully explained. However, the myeloma cells certainly divide and grow more frequently than normal plasma cells and develop cancerous properties which enable myeloma cells to invade and damage bone as well as travel through the blood stream to other bone marrow sites. This latter property is what causes myeloma to be "multiple" with what are multiple, bone marrow, ("myelo") tumors ("omas"). Current evidence indicates that the development of myeloma is a multistep, multifactorial process. Thus, not only is there uncontrolled growth of the abnormal plasma cells (myeloma cells) due to DNA damage, but the immune system normally capable of eliminated or blocking cancer development is defective. The dynamic balance of the immune system is therefore shifted in favor of myeloma cell growth.

The normal bone marrow contains <5% plasma cells. In multiple myeloma there are usually >30% plasma cells and this number can increase to over 90% and drastically effect normal marrow and immune functions. Thus a bone marrow sample, typically taken from the back of the pelvic bone, shows the increased numbers of plasma cells visible on microscopic examination. Since the bone marrow involvement can be patchy the exact percentage involvement must be interpreted with some caution. In patients with isolated marrow involvement, called a solitary plasmacytoma (single tumor site), intervening bone marrow sites, including the standard pelvic sampling, are normal. Even with more diffuse disease involving multiple sites, the percentage involvement tends to vary from site to site. The commonest sites of involvement are the pelvis, spine, rib cage, skull plus bones around the shoulders and hips. The amount and activity of the myeloma in these various sites are the major differences between patients. Whereas one patient can have slowly growing disease in a single site another patient can have aggressive bone destruction at multiple sites plus numerous associated medical complications. Careful initial evaluation, or staging, is therefore crucial as a basis for management decisions.

Bone damage is the most serious concern at the time of initial diagnosis. Myeloma cells produce cytokines such as IL-1, IL-6 and TNF, which are substances which can trigger bone cells, called osteoclasts, to destroy surrounding bone. When more than 30% of

the bone has been destroyed, the bone loss is sufficient to show up on x-rays as either thinning (known as osteoporosis) or as dark holes (like swiss cheese) known as lytic lesions. The weakened area of bone can break. This is called a pathological fracture. This is obviously something to be avoided if at all possible. If a fracture does occur it may require urgent treatment including surgical pinning and/or radiation therapy.

The commonest features of multiple myeloma at the time of presentation are discussed below.

1. **Bone pain** is especially common in the middle and/or lower back, rib cage or hips. Pain is mild to severe depending upon the size of the lesion, the speed with which it has developed and whether or not fracture and/or nerve compression have occurred. Typically movement makes the pain much worse.

2. **Fatigue** is also common and is usually proportional to the severity of anemia. Obviously, in addition, the overall impact of the disease can worsen fatigue.

3. **Symptoms of infection** depend upon the site of infection. Pneumonia, bladder or kidney infection, sinusitis and skin infections are particularly common. The reduced immunity in patients with active myeloma predisposes to infection.

4. **Symptoms due to hypercalcemia** (increased blood calcium). Blood calcium levels increase due to a combination of calcium release into the blood stream from destroyed bone and reduced calcium excretion due to kidney damage. Symptoms such as thirst, nausea, constipation and mental confusion occur and can steadily worsen until blood calcium levels are brought under control.

Initial diagnosis of myeloma

Prior to the widespread use of blood and urine testing for screening purposes, some or all of the above symptoms usually led to the diagnosis. Nowadays, abnormalities picked up on routine screening blood and/or urine tests, may lead to diagnosis even before major symptoms have emerged. There is therefore the opportunity to prevent or avoid major bone or kidney damage/and other associated problems.

Investigations to confirm a diagnosis

As for other types of cancer, a biopsy showing the malignant myeloma cells is the cornerstone of the diagnosis. The diagnosis of myeloma is confirmed by:

1. **A bone marrow sample** containing at least 10% plasma cells usually >30% plasma cells. The malignant nature of the myeloma cells is confirmed by the monoclonal pattern (i.e. **all** kappa or **all** lambda light chain subtyping of the myeloma cells), abnormal chromosomes and/or the typical malignant appearance under the microscope when examined by an experienced hematopathologist.

2. **Direct biopsy from a plasmacyloma**. Myeloma can present as single or multiple tumors in bone or soft tissue. Direct biopsy usually shows 90-100% myeloma cells in the tumor confirming the diagnosis of a solitary plasmacytoma or multiple myeloma.

3. **Supportive findings**. If there is no direct biopsy and the bone marrow contains $\leq$ 30% plasma cells, a definitive diagnosis of myeloma requires: **either**

(i) **A high level of myeloma protein** (M-component or monoclonal protein) in the blood and/or urine See **staging** - section below

OR

(ii) **Definite evidence of myeloma on x-ray**. A bone or "full skeletal" survey is required to assess for lytic lesions and/or significant osteoporosis. If there is osteoporosis only, other evidence of bone and/or bone marrow involvement is required. This usually leads to MRI and/or other scanning such as FDG/PET scanning or MIBI scanning.

Borderline cases may require further testing and/or serial monitoring to distinguish between monoclonal gammopathy of undetermined significance (MGUS) or smoldering myeloma both of which require no treatment (see below).

What's NOT myeloma?

Sometimes initial testing can suggest a possible diagnosis of myeloma which is not confirmed by additional testing or monitoring. Ocassionally a monoclonal protein develops in response to some other underlying illness such as an "auto immune" disease (e.g. systemic lupus erythematosis, scleroderma), neurologic disease (e.g. multiple sclerosis, amyotrophic, lateral sclerosis [ALS]) or long standing infectious process (e.g. tuberculosis). **However, what is not myeloma is usually one of the 3 conditions listed below:**

1. **Solitary plasmacytoma**. As already mentioned, the growth of myeloma can start in a single site, most commonly the spine or pelvis. Occasionally the site can occur outside bone in soft tissue. The most common place for a soft tissue plamacytoma is in the head and neck region.

   Patients with a solitary plasmacytoma can have a monoclonal (i.e. myeloma) protein in the blood and/or urine. If a solitary plasmacytoma is removed by surgery and/or eradicated with radiation therapy, then over a few weeks the myeloma protein will completely disappear. This disappearance confirms the diagnosis and argues against multiple myeloma. Unfortunately, this does **not** rule out the possibility of future reoccurrence elsewhere, but typically a remission period measured in years rather than months can be expected following successful treatment of a solitary lesion.

2. **Monoclonal gammopathy of undermined significance (MGUS)**. This is a condition in which the level of plasma cell involvement is low (usually < 10% or certainly <20%) and no growth is occurring (growth or labelling index of plasma cells = 0%). There is no bone damage, blood counts tend to be completely normal and the patient frequently has no symptoms. In this instance no treatment is required. However, careful ongoing monitoring is required. If the disease is stable for 2 years it may remain stable for many years. Still there is a percentage risk (approximately 30%), that myeloma may have developed by the 10 year time point. Obviously it is crucial to diagnose MGUS and avoid unnecessary treatment.

3. **Indolent myeloma**. This is a condition somewhere between MGUS and full blown myeloma. In this instance there is evidence of bone damage and possible blood count and other problems, **but** very slow progression such that minimal or no treatment may be required over long periods. A related condition is "Smoldering" myeloma in which these are no bone lesions, **but** the myeloma protein and bone marrow plasma cell levels are higher than in traditional MGUS. Stable or slowly changing disease is common in this setting also.

Staging and Prognostic Factors

When a diagnosis of myeloma is made it is important to evaluate two aspects:

1. **How much myeloma is there?**

   The traditional method of assessing the amount of myeloma is using the Durie-Salmon staging system. This allows classification of patients into stages I (early disease) to stage III (late disease) and A (normal kidney function) or B (abnormal kidney function).

   For example:

   Stage I A
   - low amount of myeloma
   - normal kidney function

   Stage III B
   - high amount of myeloma
   - abnormal kidney function

This staging system is based upon the number of bone lesions, severity of anemia due to bone marrow replacement with myeloma, level of myeloma protein in the blood and/or urine and serum calcium level.

Typically patients with stage III disease will require more urgent treatment especially stage III B when immediate intervention may be required to protect and/or recover kidney function.

2. **How aggressive is the myeloma?**

   As mentioned earlier, the speed of progression of the myeloma varies from patient to patient. This ranges from progression occurring in a few weeks to several years. The tendency for change is reflected by different tests. For example, growth rate is reflected by the labeling index which gives the number and percentage of myeloma cells which are actively growing and dividing. The serum B_2 microglobulin blood test indicates both the amount and activity of the myeloma. The higher the serum B_2 microglobulin the more active the myeloma. Bone marrow chromosome testing indicates if any myeloma chromosome damage is present. As one might expect, the more damage there is, especially

affected certain chromosomes such as number 11, 13 and 14, then the less likely that standard approaches to treatment will work well. Problems with chromosomes number 5 and 7 suggest damage to the normal bone marrow stem cells or myelodysplastic syndrome (M.D.S.).

There are many tests which can be used if necessary to assess the pattern of disease including tendency for bone damage and/or deposition of myeloma protein in tissues and organs in the form of amyloid deposits.

The standard typing of myeloma by heavy and light chain is also helpful. The commonest types of myeloma are:

- IgG: k or lambda
- IgA: k or lambda
- K or lambda only (Bence Jones myeloma)

Rarer types are:

- IgD: k or lambda
- IgM: k or lambda
- IgE:k or lambda

These types have some prognostic implications. For example, the lambda subtype is more commonly associated with amyloid deposits especially on nerves or in the kidneys. Patients with IgGk myeloma tend to have the overall best survival.

The treatment of myeloma

1. Direct approaches to eradicate the myeloma cells

A. Chemotherapy

Chemotherapy destroys the malignant plasma cells (myeloma cells), with the aim of inducing remission or cure. It involves administering anti-cancer drugs via injection or by mouth (orally). Chemotherapy regimens (treatment programmes) generally stretch over a period of months. Most often, they are administered on an outpatient basis.

The drugs are administered in cycles, giving the patient's immune system and normal bone marrow cells, which are weakened by the chemotherapy, time to recover between cycles. By killing malignant cells, chemotherapy can also relieve many of the symptoms of the disease, including anemia, hypercalcemia, bone destruction and abnormal blood/urine protein levels.

Chemotherapy is said to be effective if it reduces the abnormal blood/urine protein levels and/or the percentage of myeloma cells observed in the bone marrow. It may be considered successful even if it does not induce a full remission (return to normal levels). There are many chemotherapy options involving different combinations and dosages of drugs administered using different protocols (schedules for treatments, diagnostic tests and decisions on future treatment)

Side Effects of Chemotherapy

General side effects of chemotherapy include: hair loss, nausea and vomiting, increased risk of bruising,, bleeding and infection, mouth sores and ulcers. With some chemotherapy agents abdominal cramps, constipation, numbness and tingling in the hands or feet. Fertility may be affected.

Not everyone will experience all the side effects, this will depend on a number of factors, such as the dose given and the person's general health.

B. Radiation Therapy:

This is typically used in a localized area where there is bone destruction and pain. Radiation is also used in an effort to completely eradicate myeloma cells from an area in which a plasmacytoma has been surgically removed. The affected area is exposed to controlled doses of radiation. Radiation can kill malignant cells more quickly than chemotherapy and has fewer side effects. It is therefore used to achieve quicker pain relief, control severe bone destruction and for patients not able to tolerate chemotherapy regimens. It can also be used in conjunction with chemotherapy. Radiation therapy is generally given five times a week over a period of weeks or months. Treatment can normally be on an outpatient basis. Decisions to be made by the radiation therapist with regard to radiation therapy include the dosage, the area to be treated and the period over which the treatment is given.

C. Bone Marrow (BMT) and Peripheral Blood Stem Cell (PBSCT):

Transplants are being evaluated in clinical trials as alternatives to conventional treatment and as potential cures for myeloma. However, no type of transplant is yet definitely curative for myeloma. Typically transplantation involves extremely high dose chemotherapy, sometimes combined with whole body radiation therapy. The therapy is so potent that it destroys all of the patient's bone marrow. Without bone marrow, there is no immune system and no ability to manufacture new blood cells, and the patient cannot live. The marrow transplant procedure replaces the destroyed marrow, rescuing the patient. Thus, the marrow transplant procedure is a means of administering treatment doses that would otherwise prove fatal. It is hoped that by destroying the marrow, all of the malignant myeloma cells will also be destroyed. The transplanted marrow is drawn from a genetically matched donor (allogeneic transplant) or the patient (autologous transplant). When the patient's own marrow is used, it can be purged using chemicals or antibodies designed to remove malignant cells before it is transplanted. Bone marrow and stem cell transplants require preparatory chemotherapy regimens (e.g. VAD chemotherapy) most often administered over a period of months. The transplant itself might involve a hospital stay (weeks to months) followed by a period of reduced activity. Transplants are by far the most aggressive treatment programmes in use today and they also have the highest level of risk. Decisions to be made by the trating physician with regard to bone marrow and/or stem cell transplants include allogeneic (using a sibling's or compatible donor's marrow) vs. autologous (using the patient's marrow), preparatory chemotherapy and radiation therapies and anti-rejection measures. For autologous transplants, decisions must also be made on marrow purging techniques. Transplants remain the subject of much controversy as researchers strive to learn their impact on overall survival, their proper timing and overall role in the treatment of myeloma.

D. Maintenance therapy:

After remission is achieved, maintenance therapy is used to help prolong or maintain remission. Maintenance is used after both conventional chemotherapy and high dose chemotherapy plus transplant.

Many types of treatment have been tested in this setting, however only a few have shown benefit:

(i) **Interferon alpha**: Numerous tudies have shown that alpha interferon can prolong remission especially if an excellent remission is achieved with chemotherapy or transplant. Unfortunately it only delays, but does not prevent relapse. Side effects of fatigue and low grade fever, along with the need for injection three times per week limit the usefulness of interferon.

(ii) **Steroids (prednisone or prednisolone)**: Steroids, such as prednisone tablets, by mouth ca be used to prolong remission. Recent studies have shown that prednisone either 3 times/week or on an every other day basis can both prolong remission and overall survival. Although steroids also have side effects, in general these can be minimized by changes in dose and/or schedule.

(iii) **Supportive care measures**: A variety of supportive care measures can be helpful for maintenance including use of bisphosphonates (e.g. Clodronate, Aredia) which not only help bone disease, but may also prolong remission. Procrit (erythropoietin) by injection once/week improves both hemoglobin and the general quality of remission.

(iv) **New approaches**: The search for better maintenance treatment is a top priority for many myeloma specialists. New protocols testing vaccines or new drugs may be suggested in an effort achieve better results.

2. Treatment directed at relieving symptoms:

Includes administering drugs to control hypercalcemia, bone destruction (resorption), pain and infection.

(i) **Pain**: Most people with myeloma will experience bone pain at some time. Surgery may be used to shrink/eliminate tumors or repair bone damage and/or reduce pain. A whole range of pain medications and procedures (e.g., local anesthetic block) are available to relieve pain. With currently available pain strategies, no patient should suffer needlessly Many centers now have specialized pain management teams.

(ii) **Infection**: Antibiotics and vaccines (e.g., Pneumovax) can play a role in preventing and combating opportunistic infections.

(iii)**Anaemia**: Erythropoietin can be administered to relieve anemia and the symptoms that accompany it (e.g., fatigue).

(iv) **Hypercalcaemia**: Bisphosphonates (e.g., Aredia) can significantly reduce bone damage and provide relief for hypercalcemia in myeloma.

(v) **Other Medications**: Other medications and supplements should be taken with care when under treatment for myeloma. Ideally no additional medications or supplements should be taken without the advice of a physician who is fully familiar with the patient's medical status and treatment program. For example, seemingly innocent over-the-counter anti-inflammatory medications such as ibuprofen can cause kidney damage in some myeloma patients with already reduced kidney function.

What results should one expect from treatment?

1. **Stabilizing**- countering the life-threatening disruptions to body chemistry and the immune system that can occur with Myeloma

2. **Palliative**- relieving discomfort and increasing the patient's ability to

function normally

3. **Remission**-Inducing- lessening the severity of the symptoms, slowing or temporarily arresting the course of the disease

4. **Cure**- achieving a permanent remission (this has rarely, if ever, been achieved and confirmed)

To say it another way, treatment is given to make the patient feel better and function better. It may also control the effects of the disease on normal body function, slow the disease down or halt it temporarily. Remissions can last from months to decades. Experimental treatments aim at a cure, although none has yet been confirmed.

How should one choose among available treatments?

Treatment recommendations are received when the patient is first diagnosed or upon discovery of a relapse. Understandably the patient is upset and often not well-informed about the disease or the treatment options. The situation is generally charged with emotion and does not lend itself to
complex, technical explanations and careful contemplation of alternatives. Most doctors recognize this and focus on getting the patient to accept the more critical next steps, leaving the less critical decisions for a less emotional moment.

If you need to make a treatment decision, the first rule is to stop and think. Other than crisis intervention to deal with acute, life-threatening symptoms, there are few decisions that can't wait long enough for a second opinion or personal reflection. Also, certain treatment decisions can rule out future options. For example, certain types of chemotherapy should not be used if an autologous bone marrow transplant is likely in the future.

This is NOT to suggest that patients refuse critical treatment.

However, it is important to ask your doctor which treatment decisions are critical and which can wait. When the situation permits, take the time to get more than one opinion before beginning a treatment program.

When talking about treatments, start by understanding the objectives. Treatment recommendations typically include multiple components, each with different objectives. Often, certain elements of the treatment program are more urgent than others and require quicker decisions. Others aim more at long-term management of the disease, allowing more time to decide.

Beyond some tried and true stabilizing and palliative treatments. there are very few absolutes in myeloma treatment. For example, bone marrow or stem cell transplantation is not an absolute requirement even when feasible in a young and otherwise healthy patient. Remission inducing treatments, typically chemotherapy programs, can't guarantee results. Doctors have information
on success rates and can use different tests to help choose the programs that have the best odds. The same can also be said for bone marrow transplants, which aim at a cure.

Conclusion:

To make an informed decision, the patient needs to have the facts. Much of what is written about myeloma is written by doctors and researchers to be read by other doctors and researchers. As a result, the literature tends to be very difficult to read for the patient and other concerned lay persons.

As such, the doctor(s) treating the patient bear the burden of patient education. Yet, doctors must take their cues from the patient and family on how far to go on this front. Some patients are fiercely curious and want to discuss all aspects of their situation, treatment and prognosis. Others are overwhelmed and just want to know what to do next.

Most doctors are sensitive to this and will vary their approach based on what they perceive to be the patient's wishes. Patients can shortcut the process by being very explicit about how deeply they want to get into the details of the treatment decision.

Treatment decisions are critically important to the survival and quality of life of the myeloma patient. No matter how comfortable the patient feels with a doctor, it is generally good practice to get more than one opinion before proceeding.

Because the disease is rare, there are a limited number of practitioners and research/treatment centers specializing in myeloma. Doctors understand this and will be helpful in identifying appropriate specialists and making referrals. It is very common to seek a second opinion from a specialist at a research center and to continue to rely on your local referring physician to administer and monitor treatment.

Making good decisions on myeloma treatment requires resourcefulness, careful questioning, serious thought, and courage. But, most of all, it requires that the patient and his/her support group take charge of the process.

What Can I do to help myself?

Ask questions; here are a few examples

1. Treatment program:

Get a complete description:

What are the objectives of the treatment?
What exactly is the treatment?
Over what period will it be given?
What is involved?
How often must the patient visit a medical facility?
Is hospitalization required or a probability?
What is the likely impact on the patient's ability to function (i.e., work and play)?
How do people feel before, during and after treatment?
How long will they be undergoing treatment?
What are typical recovery time frames?
What follow-up or maintenance programs are required?

2. Past Experience:

Find out how well the treatment has worked for others in similar situations. Effectiveness is usually measured in many different ways. The questions to ask here are;

How much experience is there with the treatment?
How many patients have received the treatment?
How long have those patients been followed after the treatment?
What are the chances of achieving a complete or partial remission?
How long have the patients' remissions lasted?
In the event of a relapse, what would the options be (recognizing that these may change in the interim)?
What are reasonable expectations for relieving symptoms such as bone pain, pathological fractures, anemia, fatigue, hypercalcemia?
What factors are seen to influence outcomes?
How long have people who have received the treatment survived?
For newer treatments:
how many of the original group of patients are still alive?

3. Find out about side-effects.

Like most cancer treatments, myeloma treatments generally use strong drugs and other measures aimed at destroying malignant cells and/or re-balancing body chemistry. Typically, there are side effects. Some manifest themselves during treatment. Others may show up well after the treatment is completed. Questions to ask here include:

What side effects have been observed in patients receiving the treatment?
When do they typically occur? How often do theyoccur (i.e., what percentage of patients)?
How serious are the side effects? Are they life-threatening? Are they painful? Are they permanent? How long do they last?
Are there treatments for the side effects? Do they have side effects?

4. Find out about alternatives:

Perhaps, the most important line of questioning is about alternatives. There are always alternatives. You need to ask all of these questions for each of the alternatives:

What are the alternatives to the treatment recommended?

What are the relative pros and cons of the alternatives?

What are the pros and cons of the alternative treatments vs. no treatment?

5. How you can help yourselve/what can I do?

Useful Conacts:

About IMF(UK) and How you can help:

TERMS AND DEFINITIONS

Alkylating Agent: A chemotherapeutic agent such as melphalan (Alkeran) or cyclophosphamide (Cytoxan). Alkylating refers to the way in which these agents cross-link the DNA of Myeloma cells and block cell division.
Amyloidosis: A condition in which Myeloma light chains (Bence Jones proteins) are deposited in tissues and organs throughout the body This occurs more commonly with lambda versus kappa Bence Jones proteins. In patients with amyloidosis the light chain proteins bind to certain tissues such as heart, nerves and kidney rather than being excreted out of the body through the kidneys.
Anemia: A decrease in the normal number of red blood cells, usually below 10 G%, with over 13 - 14 G% being normal. Myeloma in the bone marrow blocks red cell production causing anemia (shortness of breath, weakness and tiredness).
Antibodies: Proteins produced by white blood cells to fight infection and disease.
Appendicular Skeleton: The long bones (i.e. arms and legs) which are attached to spine, chest and pelvis.
Axial Skeleton: The skull, spine and pelvic regions of the skeleton.
Bence Jones: The name used to identify Myeloma protein present in the urine.
The Myeloma or M protein consists of kappa or lambda light chains. The amount of Bence Jones protein is expressed in terms of G per 24 hours. Normally a very small amount of protein (less than 0.1 G per 24 hours.) can be present in the urine, but this is albumin rather than Bence Jones protein. The presence of any Bence Jones protein is abnormal.
Beta 2 Microglobulin: A small protein found in the blood. High levels occur in patients with active Myeloma. Low or normal levels occur in patients with early Myeloma and/or inactive disease. Approximately 10% of patients have Myeloma which does not produce beta 2 microglobulin. For these patients, beta 2 microglobulin testing cannot be used to monitor the disease. At the time of relapse, beta 2 microglobulin can increase before there is any change in the Myeloma protein level. Therefore, 90% of the time, beta 2 testing is very useful for determining disease activity.
Bisphosphonate: A type of drug which binds to the surface of bone where it is being resorbed (eaten into) and protects against osteoclast activity.
Bone Marrow: The soft, spongy tissue found in the center of most bones.
Bone Marrow Aspiration: The removal, by needle, of fluid and cells from the bone marrow.
Bone Marrow Biopsy: The removal, by needle, of a sample of tissue from the bone marrow.
Bone Remodeling: The coordinated, or coupled, activity of osteoclasts (which resorb or destroy bone) and osteoblasts (which create new bone matrix) to form new bone while destroying old bone in a balanced way such that the total amount of bone remains the same.
Calcitonin: A hormone secreted by the thyroid gland which blocks bone resorption temporarily
Calcitrol: An activated form of Vitamin D useful for persons who require extra vitamin D.
Calcium: The mineral which makes up the hard stuff of bone matrix or hydroxyapatite.
CAT (Computerized Axial Tomography) Scan: A computerized Xray study used to detect small areas of bone damage or soft tissue involvement.
Chemotherapy: Treatment with anti-cancer drugs. Clinical Trials: Studies of new treatments.
Consolidation Therapy: A phase of treatment in which an effort is made to improve the response achieved with the initial approach to treatment. Consolidation typically involves either higher dosages or drugs not previously used.
Coupling: The normal coordination between osteoblasts and osteoclasts to maintain a balanced state of bone production and destruction.
Creatinine: A small chemical compound normally excreted by the kidney If the kidneys are damaged the serum level of creatinine builds up resulting in an elevated serum creatinine. The serum creatinine test is used to measure kidney function.
Cytokine: A substance that stimulates growth/activity of a particular type of cell. Cytokines are produced locally (i.e. in the bone marrow) as well as circulating in the blood stream.
DEXA (Dual Photon X-Ray Absorptionmetry): An X-ray study which can measure the amount of bone loss; the best measure of bone density.
Dexamethasone: A steroid given along with other chemotherapy drugs.
Dialysis: When a patient's kidneys are unable to filter blood, the blood is cleaned by passing it through a dialysis machine.
Electropheresis: A laboratory test in which a patient's serum is subjected to a separation technique involving movement in an electric field. The amount of movement is determined by the size and the electric charge of the protein involved. The technique allows both the calculation of the amount of Myeloma protein as well as the identification of the specific M spike characteristic for each patient. Used as a tool for both diagnosis and monitoring.
Erythropoietin: Erythropoietin is a hormone produced by the kidneys. Myeloma patients with damaged kidneys don't produce enough erythropoietin and can become anemic. Injections with synthetic erythropoietin (e.g. Procrit) can be helpful. Blood

transfusion is another alternative, especially in an emergency Synthetic erythropoietin is being used prophylactically before chemotherapy and as supportive therapy after chemotherapy to avoid anemia.
Hypercalcemia: Elevation in the blood calcium level. Common in Myeloma patients and usually resulting from bone destruction with release of calcium into the blood stream. It is often associated with reduced kidney function since calcium can be toxic to the kidneys. For this reason, hypercalcemia is usually treated on an emergency basis using IV fluids combined with drugs (e.g. Aredia), to reduce bone destruction along with direct treatment for the Myeloma.
lgG, IgA: The two most common types of Multiple Myeloma. The G and the A refer to the type of protein produced by the Myeloma cells. The Myeloma protein, which is an immunoglobulin, consists of two heavy chains, for example of a G type combined with two light chains which are either kappa (K) or lambda(~. Therefore the two most common subtypes of Myeloma have identical heavy chains (i.e. lgG kappa and lgG lambda). The kappa and lambda light chains can be produced alone resulting in either kappa or lambda light chain (Bence Jones) Myeloma. The terms heavy and light refer to the size or molecular weight of the protein with the heavy chains being larger than the light chains. Since the light chains are smaller, they are more likely to leak out into the urine resulting in urine Bence Jones protein.
lgD, IgE: Two types of Myeloma, similar to lgG and IgA, which occur less frequently.
Immunofixation: Immunologic method used to identify M-protein type (lgG, IgA, kappa or lambda). The most sensitive routine immunostaining technique, it identifies the exact heavy and light chain type of the M-protein.
Induction Therapy: The initial treatment used in an effort to achieve remission in a newly diagnosed Myeloma patient.
Interferon: A cytokine (or hormone) which is produced normally in response to virus infection. Produced by genetic engineering techniques, synthetic interferon is given as treatment for Myeloma and is used primarily in the maintenance (or plateau phase) to block any re growth of Myeloma and thus delays or prevents relapse.
lnterleukin-6: A cytokine which is a potent stimulus to osteoclast and plasma cell activities.
Lytic Lesion: The damaged area of a bone that shows up as a dark spot on an X-ray when enough of the healthy bone in any one area is eaten away Lytic lesions look like holes in the bone and are evidence that the bone is being weakened.
Monoclonal: Myeloma develops from a single malignant plasma cell (monoclone). The type of Myeloma protein produced is also monoclonal; a single form rather than many forms (polyclonal). The important practical aspect of a monoclonal protein is that it shows up as a sharp spike (M spike) in the serum electropheresis test.
M-protein: An antibody produced by the Myeloma cell and found in the blood or urine: synonymous with Monoclonal protein, Myeloma protein, and M spike.
MRI (Magnetic Resonance Imaging): Magnetic energy rather than X-ray energy, used to obtain an image of the body Gives very fine resolution of soft tissues, especially encroachments on the spinal cord, but is less accurate for bone lesions.
M spike: Another name for M protein. Spike refers to the sharp or spiked pattern which occurs on protein electropheresis when a M-protein is present.
Multi Drug Resistance (MDR): A resistance to standard treatments, it is typically associated with resistance to adriamycin and vincristine, both chemotherapy drugs. The resistance is caused by a build up of the p-glyco protein in the outer cell membrane of the Myeloma cell. This results in drugs, such as adriamycin, being kicked back out of the Myeloma cell instead of building up in the Myeloma cell and eventually killing that cell. Drugs which block this p-glyco protein pump are now in clinical trials (e.g. P5C833, a new cyclosporin analogue).
Nephelometry: A readily available and rapid automated laboratory method to determine the amount of Myeloma protein in the blood. (see "immunofixation" which identifies the type of Myeloma protein). Nephelometry uses a light scattering technique and should be checked against electropheresis to ensure accuracy.
Neutropenia: A reduced level of neutrophils or white blood cells. There are several types of white blood cells, and neutropenia refers to a reduction in the granulocytes, or neutrophils, necessary to adequately combat bacterial infections. Cytotoxic chemotherapy has a tendency to induce neutropenia. In contrast, lymphocytes which are more important in virus infections, tend not to be affected by cytotoxic treatment.
Osteoblast: The cell which produces osteoid which becomes mineralized with calcium to form new hard bone.
Osteoclast: A cell found in the bone marrow at the junction between the bone marrow and the bone. Active Myeloma growth stimulates the osteoclast to destroy bone. This process is called bone resorption. Normally bone resorption is counter balanced by the activity of osteoblasts which create new bone. In Myeloma, osteoblast activity is blocked. The combination of accelerated bone resorption and blocked new bone formation results in lytic lesions.
Osteoid: The protein product which becomes mineralized with calcium to form hard bone.
Osteoporosis: Reduction in bone density typically associated with old age. Diffuse involvement of bones with Myeloma produces what looks like osteoporosis on bone X-ray and bone density measurement.
Pathologic Fractures: Occur in Myeloma-weakened bones which can't bear normal weight or stress.
Plasma Cell: The malignant cell in Myeloma. Normal plasma cells produce antibodies to fight infection. In Myeloma, the malignant plasma cells produce large amounts of abnormal antibodies which lack the capability to fight infection. The abnormal antibodies are the monoclonal protein, or M-protein. Plasma cells also produce other chemicals which can result in organ and tissue damage (i.e. anemia, kidney damage and nerve damage).
Plasmacytoma: A collection of plasma cells found in a single location rather than diffusely throughout the bone marrow, soft tissue or bone.
Plasmapheresis: The removal of certain proteins from the blood.
Platelet: One of the three major blood cells, the others being the red and white cells. Platelets plug up breaks in the blood vessel walls and stimulate blood clot formation. Platelets are the major defense against bleeding.

Progression-Free Survival: The improved survival of a patient that can be directly attributed to the treatment given for the Myeloma. This term identifies Myeloma patients who are in complete remission versus those who have had an episode of relapse (or progression).
Radiation Therapy: Treatment with high-energy rays to kill malignant cells.
Red Cells: The blood cell which contains hemoglobin and carries oxygen from the lungs to all parts of the body. A low level of red cells is called anemia. Red cell production is stimulated by a hormone called erythropoietin. Erythropoietin is produced by the kidneys. Myeloma patients with damaged kidneys don't produce enough erythropoietin and can become anemic. Injections with synthetic erythropoietin (e.g. Procrit) can be helpful. Blood transfusion is another alternative, especially in an emergency. Synthetic erythropoietin is being used prophylactically before chemotherapy and as supportive therapy after chemotherapy to avoid anemia.
Response or Remission: **Complete RemissionlComplete Response (CR)**: Remission and response are used interchangeably. CR is the common abbreviation for both. CR is the absence of Myeloma protein from serum and/or urine by standard testing; absence of Myeloma cells from the bone marrow and/or other areas of Myeloma involvement; clinical remission and improvement of other laboratory parameters to normal. The absence of Myeloma cells and Myeloma protein does not mean that the Myeloma is gone. Sensitive testing methods can detect minute levels of Myeloma. Relapse occurs after complete and partial remission. The time to relapse is influenced by the type of initial treatment as well as the maintenance used.
Partial Remission or Partial Response (PR): PR is a level of response less than CR. In SWOG studies, it has meant >50%<75% response. In other studies it has meant >50% response.
Stable Disease: This describes patients who have some response to treatment, but <50% reduction in Myeloma protein levels. Stable disease is not necessarily bad or sub-optimal (as compared to CR or PR) provided the
Myeloma has stabilized and is not progressing. An acceptable remission (i.e. number of months/years in remission) is not necessarily proportional to the percentage response. With slow moving Myeloma, stabilization can last for many years.
Serum Osteocalcin: A protein produced and secreted by osteoblasts when they are making osteoid. A low level reflects active Myeloma. A higher than normal level reflects more stable Myeloma.
Skeletal Survey/Metastatic Survey: A series of plain X-rays of the skull, spine, ribs, pelvis and long bones to look for lytic lesions and/or osteoporosis.
Sodium Pamidronate (Aredia): This bisphosphonate inhibits osteoblasts. It must be given IV every 3 to 4 weeks, 60 to 90 mg in SOOcc or D5W.
Stem Cell: Normal stem cells give rise to normal blood components, including red cells, white cells and platelets. Stem cells are normally located in the bone marrow and can be harvested for a transplant.
Thrombocytopenia: A reduced level of blood platelets. The normal level is 150 - 250,000. Bleeding problems occur when the platelet level is less than 50,000. Major bleeding is usually associated with a reduction to less than 10,000.
Transplantation: Transplantation: Stem cells are used to rescue the patient's blood-forming potential following very high dose chemotherapy and/or radiation treatment. Transplant is not a treatment, but a method of support to make high dose treatment possible.
Allogeneic: The person donating the bone marrow or stem cells is an HLA identical family member. HLA refers to the Histo-compatibility Locus Antigens used for tissue matching. (Unrelated Allogeneic Transplant: the person donating the marrow is unrelated to the patient.) Autologous: The patient donates his/her own bone marrow or stem cells, prior to treatment, for re-infusion later.
Peripheral Blood Stem Cell (PBSCT): Stem cells are collected from the circulating blood system, not from the bone marrow.
Syngeneic: An identical twin donates the bone marrow or stem cells.
White Cells: One of the three major types of cells in the blood. There are several types of white cells (i.e. neutrophils, lymphocytes and monocytes). Neutrophils are necessary to combat bacterial infection. Neutrophils can drop to very low levels following chemotherapy causing neutropenia. Neutropenia can be prevented or reduced using a synthetic hormone called GCSF (e.g. Neupogen).
Common Tests Used To Evaluate Myeloma:
Amyloidosis: Subcutaneous fat biopsy. If negative, Congo red staining of bone marrow, kidney or rectal biopsy can be carried out.
Blood: Routine blood counts, renal and liver function tests, chemistry panel tests, LDH, Myeloma protein level, serum beta 2 microglobulin, CRP and peripheral blood labeling index.
Bone Marrow: Used to make the diagnosis and to monitor the disease status.
Bones: Routine skeletal survey (X-rays), MRI and/or CT scanning for questionable areas. More expenmental tests are whole bocy MIBI and/or PET scanning.
Urine: 24 hour collection for measurement of Bence Jones protein and creatinine clearance.

MATCHED UNRELATED DONOR (MUD) MARROW TRANSPLANTATION USING CYCLOSPORIN, METHOTREXATE AND OKT3 AS GVHD PROPHYLAXIS

C Chow, DJ Gottlieb, KF Bradstock, MS Hertzberg, M McGurgan, P Stavros, I Kuzmanic.

Blood and Marrow Transplant Unit, Westmead Hospital, Sydney.

Increased rates of acute and chronic GVHD are a major factor in increased morbidity and mortality of patients receiving marrow transplantation from HLA matched unrelated donors. OKT3 is a murine monoclonal antibody to the T3 (CD3) antigen of human T cells. We retrospectively analysed data of 25 consecutive patients who received MUD transplants for haematological malignancy between March 1996 and May 1999 and who were given OKT3 in addition to cyclosporin and methotrexate for routine GVHD prophylaxis. 20 recipients were male, 5 female. Mean age of recipients was 28.9 years (range 17-47). Disease categories were AML (7), ALL (6), CML (7), MDS (3) and other (2). Conditioning consisted of Cy/TBI (21) or Bu/Cy (4). OKT3 5mg/day was given intravenously on days +1 to +5, +14 and +28. The majority of patients (16/25) experienced fevers, rigors, nausea, vomiting and diarrhoea following the first dose of OKT3. Hypoxia (4), headache (4), hypotension (3), hypertension (2) and arthralgia/myalgia (2) were also seen. Graft rejection occurred in 1 patient. Neutrophil engraftment (ANC >500) occurred at median 20 days (range 11-34) and platelet engraftment (>20) at median 30 days (range 15-46) post-transplant. Grades 111/IV acute GVHD occurred in 9 of 24 evaluable patients (skin 3, liver 7 and gut 1). Nine of 13 evaluable patients developed chronic GVHD (4 limited and 5 extensive). Four patients had disease relapse following transplant. No cases of post-transplant lymphoproliferative disease were seen. OKT3 can be administered safely following MUD bone marrow transplant and may reduce the incidence and severity of acute and chronic GVHD.

T-CELL DEPLETION FOR MATCHED UNRELATED DONOR TRANSPLANTATION

M Vowels, C Oswald, G Daley, D Ford, P Rowlings, S Russell.

Sydney Childrens & Prince of Wales Hospitals, Randwick, NSW.

Twenty-eight patients aged 0.5 to 18.8 years (median 9 yrs) received a matched unrelated donor marrow (MUD) transplant for the treatment of acute lymphoblastic leukemia (n = 9), acute myeloblastic leukemia (5), myelodysplastic syndrome (3), severe aplastic anemia (6) and other hematologic/oncologic diseases (5) between September, 1985 and May, 1999. Matching at the DR locus was by serologic methods prior to and molecular methods from 1994. GVHD prophylaxis was Cyclosporin and Methotrexate ± steroid for the first 9 patients (non-TCD) and T-cell depletion (TCD) by sheep RBC E-rosetting and Cyclosporin (Csa) for the next 19 patients. Growth factors were not used prophylactically post-MUD, except for 2 patient in the TCD group.

Median follow-up for surviving patients was 105 mths (101-109 mths) in the non-TCD group and 13 mths (2-52 mths) in the TCD group. Engraftment occurred in 27/28 (96%) patients, 9/9 in the non-TCD and 18/19 in TCD group. Median time for neutrophils to 0.5 x 10^9/l was 14 and 21 days and for platelet count to 20-25 x 10^9/l was 19 and 24 days in the non-TCD versus TCD (excluding patients given G-CSF post-MUD) groups respectively. Non-TCD patients had a higher frequency of acute GVHD grade II/IV and III/IV (89% and 55% vs 31% and 0%), chronic GVHD (50% vs 0%) and treatment-related mortality (78% vs 21%), and longer days in hospital from day of transplant (median 46 vs 29) and lower survival (22% $\pm$ 14% vs 64% $\pm$ 12%) compared to TCD patients respectively. There was a 52% reduction in cost of transplant associated with TCD.

TCD and Csa may be a satisfactory method to prevent GVHD in MUD transplants and is associated with a decreased cost, risk of GVHD and IP, and shorter hospital stay and improved survival.

PHARMACO-ECONOMIC IMPACT OF T-CELL DEPLETION IN MATCHED UNRELATED DONOR TRANSPLANTATION

G Daly, A Senner, S Williams, C Oswald, M Vowels.
Department of Oncology and Bone Marrow Transplantation, Sydney Children's Hospital, Randwick, NSW, Australia.

The financial impact of bone marrow transplantation is becoming a major concern for transplant centres worldwide. For this reason we studied the impact of T-cell depletion (TCD) on cost of matched unrelated donor transplants (MUD). Twenty-eight patients aged 0.5 to 18.8 years (median 9 yrs) received MUD for treatment of leukemia (n = 17), myelodysplasia (3), aplastic anemia (6) and other hematologic/oncologic diseases (5) between 1985 and 1999. GVHD prophylaxis was Cyclosporin and Methotrexate ± steroid for the first 9 patients (non-TCD) or TCD using sheep RBC rosetting and Cyclosporin (Csa) for the next 19 patients.

Cost of radiology, pathology, hospitalisation and pharmaceuticals and alimentation were analysed in 1999 Australian dollars. Benefit of treatment was assessed using transplant-related toxicity (TRM), graft versus-host-disease (GVHD) and days in hospital.

Costs of non-TCD and TCD transplants were $4,467 and $2,280, $6,658 and $3,159, $37,723 and $24,387, $107,031 and $44,492, and $155,878 compared with $74,317 for radiology, pathology, pharmacy, hospital and overall charges respectively. This represented a 52% decrease in cost for TCD (p=0.0017). TCD was associated with decreased frequency and severity of acute GVHD grade II-IV and III-IV from 89% and 55% to 31% and 0%, days in hospital from 47 to 29 and TRM from 77% to 22% (p=0.04) compared to non-TCD MUD.

MUD with TCD was associated with a significant reduction in cost. This lower cost may relate to reduced hospital stay, decreased frequency of GVHD and other transplant related complications.

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STATUS OF CORD BLOOD BANKING AND SEARCHING

M Vowels, K Carlton, S Boatwright, R Lam-Po-Tang, S Gordon. *Australian Cord Blood Bank, Sydney Children's Hospital, Randwick and the Australian Bone Marrow Donor Registry, NSW.*

Cord blood (CB) banking commenced in June, 1995 to evaluate collection, processing and freezing. CB was rejected if the volume was < 60ml and/or nucleated cell count (NCC) < 50 $x10^7$. CB was concentrated to less than 60 ml and was frozen in 250ml bags until June,1999 when a 2 bag system was introduced. Donations were low resolution typed at HLA A, B and DR, and typing, volume and NCC made available through the ABMDR and BMDW.

Efficiency of collection was highest with dedicated collectors, next with supervised obstetric nurses and lowest with obstetric nurses. Collection rate increased from 85 donations in 95/96 to 1,300 in 98/99. At 11.6.99, 2,644 donations were collected and processed and 2,437 were banked. Mean volume, NCC and $CD34^+$ count were 98 ml (range 54 -233), $15.6x10^7$ (6.2-52.9) and $3x10^6$ (0.1-16.9) respectively. Over 33% of donors have non-anglo-saxon background. There were 5 searches from 12/97 to 6/98, 35 from 7/98 to 12/98 and 35 from 1/99 to 5/99 when 670, 797 and 1,326 CB's were available for searching. The mean age, Wt, NCC and NCC dose for CB searches were 14.9 yrs (1-58), 35.5 Kg (1-106), $144x10^7$ (1.8-44.4) and $4.88x10^7$/kg (0.5-14.7). 35 HR typings have been requested. Median time from search activation to HR typing request was 6 (0-105) days and from HR typing request to reservation was 21 (11-80) days. 5 CB's have been sent for transplant. One CB was expanded prior to transplant.

The Bank has provided CB's with a NCC >$3x10^8$/kg to over 70% and over 50% of current searches for patients weighing less than 35kg and less than 60kg respectively. It is estimated that a Bank size of 5,000, 10,000 and 20,000 CB's will provide 5 or 6 of 6 HLA A, B and DR low resolution typed CB's for 51%, 70% and over 80% respectively of patients searching.

ABSENCE OF B-CELL LYMPHOPROLIFERATIVE DISORDER AND GRADE III-IV ACUTE GRAFT-VERSUS-HOST DISEASE AFTER E-ROSETTE T-CELL DEPLETION FOR MATCHED UNRELATED DONOR BONE MARROW TRANSPLANTS

PA Rowlings, V Greenstein, D Waldstein, D Ford, R Lindeman, M Vowels.
Leukaemia and Bone Marrow Transplant Research Unit, Department of Haematology, Prince of Wales & Sydney Children's Hospitals, Randwick, N.S.W.

B-cell lymphoproliferative disorder (BLPD) is a well recognized complication following allogeneic bone marrow transplantation (BMT). They occur particularly in the setting of matched unrelated (MUD) or mismatched family member BMT and following the use of anti-T-lymphocyte globulins (ATG) or T-cell depletion of donor marrow as prophylaxis against graft-versus-host disease (GVHD). Incidence of BLPD varies with method of T-cell depletion employed, being lowest following elutriation or use of campath antibodies. The reported incidence following E-rosetting is up to 29%. BLPD post BMT is believed due to Epstein-Barr virus causing unregulated proliferation of donor derived B-cells in the absence of donor and host T-cell inhibition.

Twenty patients received E-rosette T-cell depleted MUD transplants for haematological diseases after conditioning primarily with cyclophophamide, total body irradiation, thiotepa and ATG. The median CD3 cell dose of marrow innoculum was 3.2 x 10^5/kg (0.3-21) and CD19 cell dose 28 x 10^5/kg (0.1-79). Median follow up is 22 mos (range 2-52). 95% of patients engrafted. No patients experienced grade III-IV acute GVHD. Only one patient has developed chronic GVHD to date. There was a trend toward acute GVHD associated with T-cell dose. Patients with none versus any acute GVHD had median CD3 doses of 2.02 (0.3-6.7) and 5.55 (1.3-21) respectively. There were no cases of BPLD.

These patients benefited from reduced incidence and severity of GVHD without the expected high incidence of BLPD reported by others following the use of T-cell depletion and ATG.

PROGRESS REPORT ON THE CYCLO / IDARUBICIN / DEX (CID) REGIMEN WITH EARLY ASCT FOR DE NOVO MULTIPLE MYELOMA

A.Spencer[1], M.Seldon[2], S.F.Deveridge[2], P.Marlton[3], A.Enno[2], R.Cobcroft[3], D.S.Gill[3].

[1]BMT Programme, The Alfred Hospital, Prahran; [2]Hunter Haematology Unit, Mater Misericordiae Hospital, Newcastle; [3]Department of Haematology, Princess Alexandra Hospital, Brisbane.

We have enrolled 26 consecutive patients onto study and initiated treatment in 24 patients (age 31-64 years, median 51.5 years) with 4 q3weekly cycles of oral cyclophosphamide, idarubicin and dexamethasone (CID) (Stage III = 50%, elevated CRP = 50%, elevated ß$_2$M = 33%). Disease type was IgG = 9, IgA = 4, IgD = 3, light chain = 6, non-secretory = 2.To date 91 cycles of CID have been administered with an incidence of grade III or IV neutropenia or thrombocytopenia of 11% and 2% respectively. Response to CID is 70% (CR 10% + PR 60%). To date 20 patients have undergone PBPC harvesting at a median of 4.2 months (range 2.5 - 5.8 months) following diagnosis using IV cyclophosphamide (2g/m^2) with filgrastim from day +5. The first 12 patients received filgrastim 5µg/kg, the dose was then escalated to 10µg/kg for the next 8 patients. Median CD34+ cell yield was 5.8 x 10^6/kg (range 1.12-12.3). Only 1 patient achieved < 2 x 10^6/kg. Patients receiving 10µg/kg vs 5µg/kg filgrastim mobilised greater numbers of CD34+ cells (medians 6.6 x 10^6/kg vs 4.4 x 10^6/kg, respectively; p = .017). All 20 patients have progressed to ASCT utilising single agent melphalan 200mg/m^2 as conditioning at a median of 5.4 months from diagnosis. Overall response rate following ASCT is 89% (CR = 37%, PR = 52%). There have been no treatment related deaths. Overall survival at 2 years is 92% and PFS 79%. We conclude that CID is safe, effective and easily administered therapy for de novo multiple myeloma.

AUSTRALIAN EXPERIENCE WITH ARSENIC TRIOXIDE IN ACUTE PROMYELOCYTIC LEUKAEMIA

D Carney[1], F Firkin[1], I Byard[2], P Cannel[3], A. Concannon[4], S Milliken[4], A Spencer[5], B To[6]

[1]St Vincent's Hospital, Melbourne, [2]North Western Hospital, Burnie, [3]Royal Perth Hospital, [4]St Vincent's Hospital, Sydney, [5]Mater Hospital, Newcastle, [6]IMVS, Adelaide

Arsenic trioxide (As_2O_3) has recently received attention for remission-induction in acute promyelocytic leukaemia (APL) relapsing after treatment with All-Trans Retinoic Acid (ATRA) and/or cytotoxics. We report experience in Australia with IV As_2O_3 alone or with other agents in 9 cases of relapsed APL.

	Relapse Number	Additional Agents	Resp-onse	Length CR	Serious Complications	Arsenic
M 36	3	Nil		Nil	Nil	Nil
M 62	1	ATRA	CR	11 mo	Nil	
F 33	4	Nil		CR	9 mo	Nil
F 28	2	ATRA	CR	7 mo	Nil	
M 66	2	ATRA	CR	4 mo	Nil	
M 43	1	Nil		Death	Nil	Fatal Stroke
M 34	2	Ida		CR	2 mo	Nil
F 42	1	Nil		PR	on tmt	Hepatotoxicity
M 55	1	Nil		CR	1 mo	Nil

In 2 of the 3 cases where As_2O_3 was used with ATRA, the disease had been uncontrolled by ATRA alone. Response was accompanied in 3 by hyperleukocytosis; one developed a fatal CVA, and another severe hepatotoxicity, suggesting adjunctive myelosuppression may benefit such patients. Six have achieved CR, including 2 who had relapsed after allogeneic BMT. Two received 4 subsequent courses of As_2O_3 without development of neuropathy or other significant arsenic toxicity. Such experience indicates considerable efficacy of As_2O_3 as an induction agent in this poor prognosis category of APL.

AN UPDATE ON THE LACE CONDITIONING REGIMEN: TOXICITY, EFFICACY AND INFLUENCE OF FILGRASTIM

I Kerridge[1], S Deveridge[1], M Seldon[1], A Schwarer[2], A Enno[1], A Spencer[2].
[1]Hunter Haematology Unit, Mater Misericordiae Hospital, Newcastle and [2]BMT Programme, The Alfred Hospital, Prahran.

We have utilised the LACE (lomustine, VP-16, cytarabine and cyclophosphamide) conditioning regimen in 48 patients (age 15 – 68 years, median 49.5 years) undergoing autologous stem cell transplantation (ASCT) for refractory or relapsed haematological malignancies (NHL = 40, HD = 4, Acute leukaemia = 4). The last 10 patients received filgrastim from day +1 following reinfusion and were well matched with the initial 38 patients. Of the 44 patients with lymphoma 41% were in CR post-salvage therapy, 45% were in PR and 15% had RD at the time of ASCT. Median CD34+ cell dose was 4.12 x 10^9/litre and sustained engraftment occurred in all evaluable cases. Patients electively receiving filgrastim compared to those not receiving filgrastim demonstrated a shorter duration of neutropenia (14 days vs 9 days) and inpatient stay (26.5 days vs 19.5 days), required less antibiotic therapy (11.5 days vs 5.5 days) and suffered fewer serious (Grade III or IV) infectious complications (32% vs 10%) (all p .05, Mann-Whitney U-test). Immediate significant (Grade III or IV) non-haematological toxicity was minimal – hepatic, n = 3; mucositis, n = 1; diarrhoea, n = 1 and nausea/vomiting, n = 5. No cases of VOD or interstitial pneumonitis occurred and at 12 months there no post-transplant diminution in cardiac or respiratory function detectable. PFS overall is 57% at 2 years and for patients with lymphoma 65%. There have been 2 transplant related deaths. We conclude that LACE is an effective and well tolerated alternative to BCNU or busulphan containing conditioning regimens for ASCT.

DETECTION OF MINIMAL RESIDUAL DISEASE IN PERIPHERAL BLOOD BEFORE CLINICAL RELAPSE, IN B-LINEAGE ACUTE LYMPHO-BLASTIC LEUKAEMIA

MJ Brisco, E Hughes, SH Neoh, P Sykes, M Rice,[1] AA Morley
Haematology & Genetic Pathology, Flinders University & Medical Centre, Bedford Park SA 5042 and [1]Haematology-Oncology, Women's & Children's Hospital, North Adelaide SA 5006

Molecular biology tests can quantify minimal residual disease in acute lymphoblastic leukaemia. In bone marrow, rising levels of leukaemia can be detected weeks or months before clinical relapse, raising the possibility of monitoring patients for early signs of recurrent disease, but monitoring would require marrow to be aspirated quite often. Blood is easier to obtain, and since the tests detect leukaemic cells in blood during induction treatment, when its level is related to that in marrow, we studied whether leukaemic cells could be detected in blood in the period preceding a relapse.
Blood samples have been collected from 93 children and 19 adults, and stored frozen. Following relapse, the marrow sample at relapse, and any blood samples from the preceding 6 months were retrieved, and the DNA extracted. The rearranged immunoglobulin heavy chain gene of the leukaemic cells (from marrow at relapse) was amplified and sequenced, to provide a very specific genetic marker, and PCR primers were designed to the highly variable CDR3 region. Leukaemia in blood was then quantified, by performing limiting dilution PCR. To date, 1 adult (female, age 42, Ph-ve) and 4 children (2 male, 2 female; age 1-6 years; w.c.c. 2-142x10^9/l; Ph–ve) who relapsed have been studied. Twelve blood samples were available, from 2.5-9 weeks prior to relapse, and leukaemia was detected in 8 of these, from $2x10^{-5}$ to 10^{-1} leukaemic cells per normal cell. Leukaemia was detected in all 4 patients whose relapses involved marrow, but not in the fifth, a child who had an isolated testicular relapse. Thus, sensitive molecular tests can detect leukaemic cells in blood before relapse is diagnosed clinically. Ultimately these tests could monitor patients routinely, for early signs of recurring disease.

EFFECTS OF THE CHEMOKINE STROMAL CELL-DERIVED FACTOR-1 ON THE MIGRATION OF PRECURSOR-B ALL CELLS WITHIN BONE MARROW STROMAL LAYERS

KF Bradstock, V Makrynikola, W Shen, DJ Gottlieb.
Dept. of Haematology, Westmead Hospital, NSW, Australia

Acute lymphoblastic leukemia (ALL) blasts undergo migration into layers of bone marrow fibroblasts (BMF) in vitro. However, it has been unclear as to whether this is a selective process mediated by specific chemoattractant molecules, or simply a reflection of the highly motile nature of early B Cell precursors. We further characterized this process using a transwell culture system, in which the 2 chambers were separated by an 8µm diameter microporous membrane. When a BMF layer was grown on the upper surface of the membrane there was an 84.1% reduction in transmigration of the human pre-B ALL cell line NALM-6 into the lower chamber. The involvement of the chemokine Stromal Cell-Derived Factor-1 (SDF-1) in this process was next investigated. Addition of SDF-1 at 100ng/ml into the lower chamber increased transmigration of NALM-6 across the membrane by 2.2 fold, and also induced a 1.4 to 6.1 fold increase in movement of NALM-6 through a BMF layer into the lower chamber. The receptor for SDF-1, CXCR4, was demonstrated by flow cytometry on all 10 cases of precursor-B ALL analysed, as well as on NALM-6. An inhibitory antibody to CXCR4 was able to block the migration of NALM-6 cells into BMF monolayers grown on plastic by 51%, and in 9 cases of ALL by 8-40%, as well as partially inhibit transmigration of leukemic cells through BMF layers along an SDF-1 concentration gradient. These results confirm that precursor-B ALL cells selectively localize within bone marrow stroma in vitro, and that this is partially due to the stromal chemokine SDF-1 binding to its receptor CXCR4 on leukemic cells. SDF-1 may be important in influencing the localization of precursor-B ALL cells in marrow microenvironmental niches.

OVEREXPRESSION OF WT1 +/+ AND -/- ISOFORMS IN THE K562 ERYTHROLEUKEMIA CELL LINE ENHANCES CELL VIABILITY AND DELAYS MEGAKARYOPOIESIS

DP Carrington, T Kromynkh, EM Algar
Department of Haematology and Oncology, Royal Children's Hospital, Flemington Road, Parkville, Victoria, 3052.

The Wilms' tumor gene (WT1) encodes a zinc-finger transcription factor that is expressed as four distinct isoforms, +/+, +/-, -/+ and -/-. It is frequently expressed in leukemic cells, is able to repress differentiation induction of the myeloid lineage, and is proposed to play a role in the proliferation and viability of leukemic cells. In this study we have generated stable cell lines of the erythroleukemia, K562, overexpressing the +/+ and -/- WT1 isoforms from pCI-neo expression constructs. The generated cell lines have extended viability under normal culture conditions indicating a role for +/+ and -/- WT1 isoforms in cell survival. The WT1 overexpressing cell lines did not, however, exhibit altered responses to the induction of apoptosis by the reagents Cisplatin and Adriamycin, or to serum withdrawal. WT1 is rapidly down-regulated during normal haematopoietic differentiation. Megakaryopoiesis, induced by 12-O-Tetradecanoylphorbol 13-acetate, was partially inhibited by the overexpression of both the +/+ and -/- WT1 isoforms in K562 cells. Our results are consistent with the proposed oncogenic role for WT1 in leukemogenesis and also suggest that WT1 may have a role in regulating the differentiation of multiple haematopoietic cell lineages.

REGULATION OF THROMBOPOIETIN mRNA EXPRESSION - AN *IN VITRO* AND *IN VIVO* STUDY

R Sungaran, OT Chisholm, [*]B Markovic, L Khachigian and [+]BH Chong
Centre for Thrombosis and Vascular Research, School of Pathology, [*]School of Safety Science, UNSW and [+]Haematology Department, Prince of Wales Hospital, Randwick, NSW, Australia

Thrombopoietin (TPO), the primary regulator of platelet production is expressed constitutively in the kidney, liver, spleen and bone marrow (BM). However its expression can only be modulated in the stromal cells of BM. We used primary BM stromal cells as a model to study the regulation of TPO mRNA expression in response to various platelet α-granular proteins. We have shown that platelet derived growth factor-BB (PDGF-BB) and fibroblast growth factor-2 (FGF-2) stimulated TPO mRNA expression in both a dose- and time- dependant manner, while platelet factor 4 (PF4), thrombospondin (TSP) and transforming growth factor-beta (TGF-β) suppressed TPO expression. The addition of whole platelet lysate demonstrated a dose-dependant inhibition of TPO mRNA expression an effect that was partially reversed with the addition of an anti-PF4 antibody.

In order to maintain normal haemostasis, the body compensates for any episodes of blood loss by increasing the demand for platelets. However the mechanism whereby platelet numbers are increased remains unclear. We sought to identify a molecular basis for this regulation, therefore we studied the expression levels of TPO mRNA in mice subjected to repeated bleeding. Semi-quantitative RT-PCR was carried out on selected mouse tissues and we found that TPO mRNA expression was clearly upregulated in BM of bled mice compared to the control animals. No differences in TPO mRNA levels between control and bled mice could be detected in the liver and kidney. Interestingly, TPO specific bands were more intense in the spleen of bled animals than control. Our results suggest that TPO expression in murine marrow and spleen may be up-regulated in response to acute blood loss.

INHIBITORY EFFECT OF PF4 ON MEGAKARYOPOIESIS IS DEPENDENT ON VERY FEW AMINO ACID RESIDUES.

CM Vun, H Tao. *Haematology Department, SEALS, POWH, Sydney, New South Wales, 2031, Australia.*

PF4 is produced by megakaryocytes and stored abundantly in the α-granules of platelets. As PF4 is known to inhibit proliferation and differentiation of megakaryocytes *in vitro*, it is suggested that it may exert negative feedback control on megakaryopoiesis. However, the receptor for PF4 remains unknown. The PF4 binding sites which mediate its inhibitory effect have been investigated mostly by peptides based on its C-terminal portion. In physiological condition PF4 exists as a tetramer whose interaction with other biomolecules will be difficult to mimick by peptides derived from fragments of PF4. Therefore, it is important to investigate the binding sites of PF4 using mutant PF4s which still fold to the native conformation. To select the amino acid residues for mutagenesis, the 3D structure of PF4 is examined to identify those residues which are exposed on its surface. Selected residues are mutated using PCR spliced overlap extension and mutagenic mismatch primers. More than 40 PF4 mutants are generated. Using *ex vivo* suspension culture of cord blood $CD34^+$ cells induced to differentiate into megakaryocytes by TPO, the proliferation of megakaryocytes is assessed by BrdU-based DNA synthesis and flow cytometric phenotyping using CD41 monoclonal antibody. Only a few residues on PF4 are essential for the inhibitory action on megakaryopoiesis. Interestingly, some mutants show stronger inhibitory effect than wild-type metPF4. This is probably due to reduced steric hindrance as the implicated residues are mutated to either alanine or glycine. PF4 binds avidly to heparin and heparan sulfate, the later of which is present on megakaryocytes. Although the heparin-binding sites of PF4 are still not absolutely determined, the finding of residues on PF4 essential for megakaryopoietic inhibition different from known heparin-binding sites suggests that a receptor is involved.

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CHARACTERIZATION OF THROMBOPOIETIN-INDUCED NF-κB INTRACELLULAR SIGNALLING PATHWAYS IN MEGAKARYOCYTE.

C Wu, R-L Gao and B H Chong *Haematology Department, Prince of Wales Hospital, Randwick, N.S.W., 2031, Australia*

Thrombopoietin (TPO) is the primary growth factor that regulates megakaryocyte development. TPO mediates its effects by binding to its receptor, c-mpl on megakaryoctes. TPO-c-mpl interaction leads to activation of intracellular signal transduction/transcription factor pathways which are still largely unknown. We have studied TPO-induced NFκB intracellular signal transduction/transcription factor pathways in megakaryocytes.

We have observed that TPO induced a dose-dependent increase in κB binding activity in human megakaryocytic cells, Mo7e/ TPO. Antibody gel supershift assay demonstrated that the TPO-induced κB binding complexes contained components of NFκB transcription factors, Rel A and p50 homodimers. Transient transfection study with pTK CAT (2x κB) reporter gene containing a NFκB consensus sequence suggests that the TPO-induced κB-dependent transcriptional activity can be attributed predominantly to Rel A homodimer. We further found that the TPO-induced NFκB activity is mediated by two signal transduction pathways. First, a novel pathway that involves phosphorylation of Bcl-3 by protein tyrosine kinase (PTK), binding of phosphorylated Bcl-3 to p50 and hence removing p50/p50's inhibition towards RelA/RelA. Second, a pathway that includes IκBα phosphorylation by protein kinase C (PKC) resulting in the nuclear translocation and activation of Rel A homodimer.

In conclusion, our data indicate that NFκB pathways, which are well described in immune cells such as lymphocytes, play a role in TPO-mediated gene regulation in megakaryocytes.

GINSENOSIDES STIMULATE PROLIFERATION OF HUMAN MEGAKARYOCYTIC PROGENITORS BY UP-REGULATION OF GATA-BINDING ACTIVITY

R Gao, C Wu, J Jin, H Tao, BH Chong.
Haematology Department, Prince of Wales Hospital, Sydney, New South Wales 2031, Australia

Ginseng is a medicinal herb that has been used as a regenerative tonic in China for several thousand years. We have previously reported that ginsenosides (GS) purified from the extract of ginseng roots, stimulate the growth of erythroid and myeloid progenitors in bone marrow culture, and significantly increase colony formation of BFU-E, CFU-E and CFU-GM. In this study, we investigate the effects of GS on megakaryocytic progenitors using semi-solid and liquid culture of CD34+ haematopoietic stem cells derived from normal human bone marrow and cord blood under serum-containing serum-free conditions. In serum-containing cell culture, GS (0-100 μg/ml) increased megakaryocyte progenitor cell numbers and CFU-Meg colony formation, and enhanced the proliferative effects induced by thrombopoietin (TPO) and IL-3 in a dose-dependent manner ($p<0.01$). A maximal effect was observed at 20 μg/ml. In serum-free culture, GS enhanced TPO-induced megakaryocyte growth but it has no effect by itself. We then explored the possibility of GATA transcription factors playing a role in mediating the growth enhancing effects of GS. When megakaryocytic cells (MO7e, CHRF-288 and Meg-01) were stimulated by GS, Northern blot analysis showed that GATA-2 mRNA was increased by 1.9, 1.8 and 2.4 fold respectively (but GATA-1 mRNA levels remained low with or without GS stimulation) and electrophoresis mobility shift assay (EMSA) reviewed increased GATA binding activity. Antibody supershift assays indicated that the GS-induced GATA binding activity was due to GATA-2 protein. UV cross-linking study demonstrated that the GATA-binding complex appeared as a 50 kD band corresponding to the size of GATA-2. In conclusion, our data suggest that GS stimulates megakaryocyte progenitor cell growth by enhancing the effects of TPO and/or IL-3. This effect may be mediated by GATA-2.

A PICTURE OF MEGAKARYOPOIESIS EMERGES THROUGH THE FOG.

Melissa Holmes, Helen Tao, Jeremy Turner*, Archa Fox*, Orin Chisholm, Merlin Crossley*, and Beng Chong.
*Centre for Thrombosis and Vascular Research, School of Pathology, UNSW, Kensington 2052, Australia. *Department of Biochemistry, University of Sydney, NSW 2006, Australia.*

FOG (Friend-Of-GATA) is a transcription factor identified in mice which plays a vital role in haematopoiesis; the embryonic lethality of the FOG knockout is due to severe anaemia with a pronounced block in erythroid cell maturation and the total absence of megakaryocytes. We used a PCR approach with degenerate primers to identify FOG-like genes in humans. This has resulted in the isolation of a novel factor, hFOG-2, from the human erythroleukemic cell line K562[1]. The full-length hFOG-2 cDNA, isolated using RACE strategies, encodes a 128 kDa protein and contains eight zinc fingers. The relationship between hFOG-2 and FOG can be seen not only at the amino acid level but also with their transcription factor partners: interaction domains for GATA-1[2] and the co-repressor mCtBP2[3] are conserved in hFOG-2 and we have shown that this new FOG also interacts with these factors. Thus, we have identified a second member of the mammalian FOG family.

FOG is known to be a GATA-1 cofactor and together they activate the haematopoietic-specific promoter, NF-E2. Using several reporter constructs in NIH3T3 cells, we now show that hFOG-2 can either enhance or repress GATA-mediated activation depending on the promoter. These studies also resulted in the identification of a repression domain in hFOG-2 and the demonstration that repression is dependent on the integrity of the mCtBP2-interaction motif.

Unlike FOG which shows restricted expression, hFOG-2 was detected in cell lines representing different haematopoietic lineages and also in numerous tissues including skeletal muscle and heart. Due to the conservation of key residues involved in FOG interaction within all mammalian GATA family proteins, we predict that hFOG-2 may also play a role outside the haematopoietic system.

1. M. Holmes, J. Turner, A. Fox, O. Chisholm, M. Crossley, and B. Chong (1999) J. Biol. Chem. (in press)
2. A. Fox, C. Liew, M. Holmes, K. Kowalski, J. Mackay, and M. Crossley (1999) EMBO J. 18: 2812-2822
3. J. Turner and M. Crossley (1998) EMBO J. 17: 5129-5140

CLINICAL OUTCOMES OF HUMAN HERPESVIRUS 6 INFECTION AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

N Woodward, S Durrant, J Faogali, T Sloots, J Morton.
Bone Marrow Transplant Unit, Royal Brisbane Hospital, Herston Rd Herston, Queensland 4029.

Human herpesvirus 6 (HHV-6) has been associated with numerous manifestations after allogeneic bone marrow transplant (BMT) including delayed engraftment and cytopenias, graft-versus-host disease (GVHD), pneumonitis, fevers and rash and meningoencephalitis. We prospectively evaluated 27 patients for 120 days (range 35-120) post allogeneic BMT. HHV-6 culture and polymerase chain reactions (PCR) positivity were assessed in peripheral blood mononuclear cells. 24 patients (89%) were anti-HHV-6 positive prior to BMT. Patients were evaluated with a median of 12 specimens each with HHV-6 culture and PCR. 15 patients became culture and PCR positive, 6 patients were PCR-positive alone and 1 patient had an isolated HHV-6 culture. In patients positive by both tests, HHV-6 PCR positivity appeared significantly earlier than culture positivity (p=0.03).
18 patients had asymptomatic reactivations. 3 patients had bacterial culture negative fever and rash preengraftment, and 1 patient had fever, delayed engraftment and haemorrhagic cystitis at the time of HHV-6 positivity in the absence of other cause. No statistically significant association was observed between HHV-6 positivity and interstitial pneumonia, GVHD, steroid usage or cytopenias. 24 of 27 patients were randomized to receive either oral acyclovir or oral valacyclovir for cytomegalovirus prophylaxis with no significant differences in incidence of HHV-6 positivity or clinical events between treatment groups. In summary, there appears to be a high incidence of asymptomatic HHV-6 reactivation post allogeneic BMT, with self-limited fever and rash being the main clinical manifestation in a smaller proportion of patients.

SINGLE INSTITUTION OUTCOMES OF TREATMENT OF SEVERE APLASTIC ANAEMIA

N Woodward, S Durrant, L Lockwood, EA Gillett, J Rowell, J Morton.
Bone Marrow Transplant Unit, Royal Brisbane Hospital, Herston Rd, Herston, Queensland, 4029

In severe aplastic anaemia (SAA), the treatment of choice for young patients (pts) with a matched sibling donor (MSD) is allogeneic bone marrow transplant (BMT) with immunosuppressive therapy (IST) reserved for older patients and those without an MSD. We reviewed case records of 27 consecutive pts with SAA treated between 1989 and 1999. 12 pts received IST alone, and 15 pts underwent BMT. Pts receiving IST were significantly older at presentation (median 42 vs 22 years, p=0.0008). All pts treated with IST received antithymocyte globulin (ATG) resulting in 3 complete and 6 minimal responses. 7 pts died, 6 from complications of pancytopenia. 5 pts remain alive at a median of 1736 days (range 190-3576) with a median Karnofsky score of 90%. 1 pt progressed to myelodysplastic syndrome. Pts treated with BMT had significantly better survival (100% vs 36% for IST, p=0.002) and became transfusion independent significantly earlier (p<0.0001). All were multiply transfused pre-BMT. Median time to neutrophil engraftment was 17 days (range 13-24) and platelet engraftment was 19 days (range 10-68). 2 patients required second transplant with sustained engraftment achieved in both. 8 of 15 developed acute GVHD (Grade III/IV n=1) and 4 chronic GVHD (1 extensive). 12 of 15 have discontinued immunosuppression at a median of 365 days after BMT (range 160-1361). All pts remain alive at a median of 1414 days after BMT (range 82-3618) and 2025 days after diagnosis (range 1194-3612), with a median Karnofsky score of 100%. 3 pts developed solid tumours and 3 pts carried pregnancies to term post BMT. We conclude that young pts treated with allogeneic BMT for SAA have excellent survival and minimal morbidity.

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A RANDOMISED STUDY OF VALACICLOVIR AS PROPHYLAXIS AGAINST CYTOMEGALOVIRUS INFECTION AND DISEASE IN BONE MARROW TRANSPLANT RECIPIENTS

S Durrant, R Herrmann

Valaciclovir International Bone Marrow Transplant Study Group; Bone Marrow Transplant Unit, Royal Brisbane Hospital, Herston, Queensland, Australia, 4029

High dosage of aciclovir has been shown to reduce the risk of CMV infection and improve survival in allo. BMT recipients. Valaciclovir, a prodrug of aciclovir, has better oral bioavailability and the aim of this randomized, double-blind, study was to compare aciclovir with valaciclovir as prophylaxis for CMV infection and disease. All patients were initially treated with IV aciclovir at a dose of 500mg/m^2 x 3/day starting 5 days before BMT and continuing to 28 days after BMT or discharge. After the IV phase the patients received either oral valaciclovir 2g x 4/day or oral aciclovir 800mg x 4/day for a duration of 18 weeks. Pre-emptive therapy based on virus isolation, antigenemia, or PCR was allowed in the study.

746 patients were enrolled in the study, 376 patients were randomised to valaciclovir and 370 to aciclovir. 725 patients (97.2%) were evaluable for efficacy; 366 patients in the valaciclovir arm and 359 in the aciclovir arm. The two patient groups were comparable for important characteristics. Patients receiving valaciclovir had a significantly longer time to development of CMV viremia and detection of CMV in urine. The overall incidences of viremia were 26% and 38% in the valaciclovir and aciclovir but there were no differences in the risks for CMV disease (3.6% and 5.6%) or survival (76%) and 75%) respectively. There were also no differences in the incidence or types of adverse events seen in the two groups. In particular, the risks for thrombotic microangiopathy were the same in the two groups.

Valaciclovir is more effective than aciclovir for prevention of CMV infection and might be useful in prophylactic strategies against CMV disease in allogeneic BMT patients.

DETERMINATION OF APC RESISTANCE IN ANTICOAGULATED AND LUPUS POSITIVE PATIENTS.

L Ivey, J Thom, J Ivey, R Baker.
Coagulation Unit, Haematology Department, Royal Perth Hospital, and University of Western Australia, Perth, Australia.

Clotting based APC assays have limitations when testing patients on oral anticoagulant (OA) therapy or with a lupus anticoagulant (LA). Predilution in FV deficient plasma facilitates testing of patients on warfarin and phospholipid rich RVV based methods have been shown to be the most suitable method when testing patients with a LA. We evaluated a phospholipid rich RVV based clotting test (Gradileiden V test - Gradipore, Australia) in a large patient cohort and determined its sensitivity to the FV Leiden mutation. We also examine whether normal plasma can be used to dilute plasma from warfarinised patients without compromising sensitivity to the FV Leiden mutation.

1956 plasmas were studied including congenital Protein C (5), and Protein S deficiency (5), LA (26), FV Leiden heterozygote (102), and homozygote (5), warfarin (54), standard heparin therapy (31) and normal healthy controls (21). Molecular analysis was performed on all samples.

The effect of FV Leiden concentration on the APC ratio was examined by determining the APC resistance of a homozygous plasma serially diluted in six sources of normal plasma (NP). The relationship is non linear and dependent on the initial APC ratio of the chosen source of NP. APC resistance is demonstrated in the varying sources of NP in dilutions of 1/4 (25% FVL) to 1/32 (3% FVL). A 1/2 dilution in pooled NP is recommended for patients on OA therapy because the test remains sensitive at levels of 25% FVL and is the dilution routinely used for other applications in a coagulation laboratory.

Our results show the Gradileiden FV test clearly predicts the presence of Factor V Leiden in a large cohort of patients. The method offers advantages when testing patients with a LA and patients receiving warfarin providing a 1/2 predilution step in pooled NP is performed. Pooled NP does not affect the sensitivity of the test to the mutation, is routinely used in coagulation laboratories, and is considerably less expensive than FV deficient plasma.

Can We Predict and Prevent Thrombosis?

Ross Baker. Thrombophilia and Haemophilia Unit, Royal Perth Hospital, Department of Medicine, University of Western Australia.

Substantial progress has recently been made in the understanding of the interaction of genetic and environmental factors that precipitate thrombosis. Familial clustering of venous thrombotic events has led to the discovery that over 70% of these patients have a pro - thrombotic blood abnormality. They include Factor V Leiden, Prothrombin 20210 G>A and the methylenetetrahydrofolate reductase (MTHFR) variant. We found the healthy Australian prevalence range around 4% for both Factor V Leiden and the Prothrombin gene mutations to 12% with the homozygous MTHFR variant. They can increase the relative risk of VTE by up to 8 fold. The oral contraceptive pill further increases this risk to over 50 fold. Two or more different but co - existing abnormalities are common which substantially increase the thrombotic risk. In almost half the cases, venous thrombosis is precipitated by circumstantial risk factors such as pregnancy, oestrogen containing medication, immobility, surgery and extended travel.

Occlusive platelet rich thrombus formation following rupture of the atherosclerotic plaque causes premature MI and stroke also has a familial aggregation. We have not found on a population basis an impact of Factor V Leiden, prothrombin gene or MTHFR mutation on MI or stroke. However we have shown other blood abnormalities such as raised plasma homocysteine levels and the Kozak platelet glycoprotein 1bα polymorphism to be common and independent risk factors for stroke by 8 and 2 fold respectively.

Prevention of thrombosis relies upon identifying the index case for these abnormalities and identifying 1st degree relatives who have a similar predisposition. This ensures that appropriate preventative strategies are adopted such as identification and modification of other risk factors, appropriate thromboprophylaxis in high risk situations or simple vitamin supplementation.

RECENT ADVANCES IN THE TREATMENT OF THROMBOPHILIA

P A Ockelford, *Auckland, N.Z.*

The major recent advance in the treatment of venous thromboembolism (VTE) is confirmation that primary outpatient therapy with low molecular weight heparin (LMWH) can be safely and effectively used in the routine hospital setting to treat both venous thrombosis (VT) and pulmonary embolism (PE). This approach is cost effective. Current evidence does not suggest important clinical differences between the available LMWHs when used either once or twice daily. Studies are now underway to evaluate the minimal ATIII binding pentasaccharide in initial treatment of VTE. Fibrinolytic therapy produces early benefits from enhanced clot dissolution but longerterm advantages are yet to be proven. Secondary warfarin prophylaxis is highly effective in presenting recurrent VTE events with a relatively consistent 90% risk reduction achieved when short term and longerterm therapy are compared. The risk of recurrence (2 - 10%) after discontinuing warfarin prophylaxis depends on the duration of treatment since the event and the presence or absence of a definable risk factor. Decision models have been applied to calculate years of continued treatment needed to prevent one fatal event when patient age, precipitating event and time elapsed since the index thrombosis are considered. These suggest that six months of secondary warfarin prophylaxis is acceptable for older patients, those with a transient risk factor and patients with the factor V Leiden. In younger patients, where the burden of therapy is compatible with individual's lifestyle, 12 months therapy is more effective than three months but this observed benefit (7.5% v 1.5% recurrence; r.r. 0.2) is not sustained two years after the event. Alternative strategies other than merely prolonging treatment duration are thus required to prevent late recurrences in idiopathic VTE. Long term and perhaps indefinite therapy is also required for the inherited thrombophilias and antiphospholipid syndrome but the optimal duration and anticoagulant intensity remains to be defined.

THE ROLE OF HAEMOPOIETIC SERPINS IN REGULATING CELL GROWTH AND DIFFERENTIATION

PB Coughlin

Department of Structural Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK.

In recent years it has become apparent that a distinct class of intracellular serpins exists. We have studied the first of a new class of intracellular serpin (murine haemopoietic serpin2A) which is most closely related to human antichymotrypsin. It lacks a cleavable secretion signal, possesses a unique predicted bait sequence of Cys-Cys and has a C-terminal extension containing two further cysteine residues.

Serpin2A is expressed in primitive haemopoietic cells and down regulated upon differentiation. It is also upregulated in lymphoid cells upon T-cell activation. Immunofluorescence and confocal microscopy studies show prominent nuclear localisation as well as cytosolic distribution. This pattern of expression and subcellular localisation suggest that serpin2A plays a role in regulating important events in cell growth and/or apoptosis.

> The amino acid sequence of serpin2A indicated that it would function as a protease inhibitor although the reactive site loop does not allow us to predict its physiological target protease. The Cys-Cys motif at the reactive site together with its cysteine containing C-terminal region further suggest that the biological activity of the protein will be sensitive to redox conditions and, consistent with this, circular dichroism studies show that serpin2A structural stability is redox sensitive.

In order to investigate possible roles of serpin2A in haemopoiesis we have used the yeast-2-hybrid system to identify novel interacting proteins. We have screened libraries derived from mouse testis and human T-cells and a number of potential interacting proteins have been identified. In keeping with our preliminary observations the potential interactors include genes involved in cell cycle regulation and redox sensitive proteins. Surprisingly, the screens have not identified any target proteases. Further studies will clarify the relationship between redox conditions, conformational state and the interaction of serpin2A with inhibitory and non-inhibitory targets.

EFFECTS OF BMT CONDITIONING ON ACUTE GRAFT-VERSUS-HOST DISEASE: THE EMERGING ROLE OF CYTOKINE SHIELDS.

Geoff Hill

Mater Medical Research Institute, South Brisbane, Australia

Donor T lymphocytes are known to play a critical role in acute graft-versus-host disease (GVHD) and the graft-versus-leukemia (GVL) effect after allogeneic BMT. Therefore, the separation of GVHD from GVL by manipulation of donor T lymphocytes is difficult. Recently, the intensity of BMT conditioning has also been shown to play a critical role in subsequent GVHD severity, primarily by amplifying GVHD of the gastrointestinal (GI) tract and the subsequent inflammatory cytokine storm (ie endotoxin induced TNFα and IL-1 production). This concept predicts for a new GVHD prophylaxis strategy involving the use of cytokine shields to protect the GI tract from damage by both conditioning and GVHD. Two cytokines, Interleukin-11 (IL-11) and Keratinocyte Growth Factor (KGF), show particular promise in murine BMT models. Both cytokines prevent early GVHD of the GI tract and reduce serum endotoxin and TNFα levels after allogeneic BMT. This is associated with marked reduction in GVHD mortality and morbidity. IL-11 also has potent anti-inflammatory properties resulting in type 2 T lymphocyte polarisation associated with reduced IL-12 production. KGF does not appear to affect donor T lymphocyte differentiation. Neither cytokine affects anti-host cytotoxic T lymphocyte function after BMT. As would be predicted, IL-11 and KGF do not inhibit the GVL effect despite the marked reduction in GVHD. The ability of these cytokine shields to separate GVHD and GVL suggests they may provide an important adjunct to standard GVHD prophylaxis.

INFUSION OF EXPANDED CD34+ SELECTED CELLS CAN ABROGATE POST MYELOABLATIVE CHEMOTHERAPY NEUTROPENIA IN PATIENTS WITH MULTIPLE MYELOMA.

J. J. Reiffers, C. Cailliot, B. Dazey, I. McNiece, P. Duchez, A. Pigneux, J. Caraux, T. Cousin, B. Bussière and J.M. Boiron**.** *BMT Unit, CHU de Bordeaux, Pessac, France; Amgen S.A., Paris; Dept. of Cell Therapy, Blood Center, E.T.S.A, Bordeaux, France; Univ. of Colorado HSC. Denver, CO.*

The objective of this study is to assess whether infusion of cultured CD34+ peripheral blood cells is safe and can reduce post-myeloablative chemotherapy pancytopenia. Eleven patients (pts), who were candidates for high-dose chemotherapy (HDC) and autologous transplant have been enrolled to date. PBPC's were collected after chemotherapy and filgrastim. From the total leukapheresis product, a median of 4.0×10^6 CD34+ cells/kg were cryopreserved, without further processing. The remaining cells were CD34+ selected, cryopreserved and, before planned HDC, cultured for 10 days (D-10 to D0) in Teflon bags containing serum-free medium supplemented with 100 ng/mL each of r-metHuSCF, r-metHuG-CSF and PEG-rHuMGDF (Amgen). After HDC; [melphalan 140 mg/m^2 + TBI (6 pts) or melphalan 200 mg/m^2 (5 pts)], expanded cells and unprocessed PBPC' s were infused on D0 and D1, respectively and the patients received r-metHuG-CSF (Amgen) S.C. (5 µg/kg/d) beginning at D0.

N = 11	median	range	fold
Unmanipulated CD34+ cells (10^6/kg)	4.0	(1.4-5.3)	
Total nucleated cells (10^6/kg) after culture	178.5	(37-355)	34.3
CD34+ cells (10^6/kg) after culture	10.1	(2.5-16.7)	2.6
Days with ANC < $0.5x10^9$/L	2	(0-7)	
Days with platelets < $20x10^9$/L	1	(0-4)	
Time to recover ANC > $0.5x10^9$/L	D5	(0-D8)	
Time to recover platelets > $20x10^9$/L	D9	(0-D13)	
Days with platelets transfusion	1	(0-4)	
Days with RBC transfusion	0	(0-3)	
Days with febrile neutropenia	0	(0-4)	

These preliminary results suggest that *ex-vivo* expansion of CD34+ cells is reproducible. It is to our knowledge the first report of abrogation of neutropenia in patients treated with highly myeloablative chemotherapy.