

ABSTRACTS

**HAEMATOLOGY SOCIETY OF AUSTRALIA
AND NEW ZEALAND**

PERTH, WESTERN AUSTRALIA

25-28 JULY, 2000

1.

CURRENT CONCEPTS OF COAGULATION. George J. Broze, Jr. Division of Hematology, Washington University School of Medicine, St. Louis, Missouri, USA.

In the current scheme, tissue factor plays a critical role in the initiation of coagulation. Plasma gains access to tissue factor exposed at subendothelial locations at the site of a wound. Factors VII and VIIa in plasma bind this tissue factor, and the factor VIIa/tissue factor catalytic complex activates limited quantities of factor X and factor IX. Additional local activation of factor VII may be produced by factor VIIa (auto-activation) or through feedback activation by other proteinases (e.g. factor Xa, factor IXa, thrombin). Some factor Xa generates thrombin that produces the local activation of platelets and the critical cofactors V and VIII. TFPI dampens the clotting process by producing factor X-dependent feedback inhibition of the factor VIIa/tissue factor complex. Persistent and amplified production of factor Xa and thrombin then proceeds through the actions of factor IXa with its cofactor VIIIa. Factor XIa, perhaps activated by thrombin or through other means, produces additional factor IXa to supplement that initially generated by the factor VIIa/tissue factor complex before its inactivation by TFPI.

Based on the clinical phenotype of individuals with severe factor VII deficiency it is clear factor VIIa/tissue factor-mediated initiation of coagulation is important for normal hemostatic function. Individuals lacking factors of the "contact" system, including factor XII, high molecular weight kininogen, and prekallikrein do not bleed. Thus, contact activation is not required for hemostasis, but may contribute to coagulation in certain pathologic conditions. The severe hemorrhagic diathesis seen in hemophilia demonstrates that the amplified and perhaps sustained generation of thrombin by factor Xa, which is produced by factor IXa/VIIIa, is critical for ultimate hemostasis. This augmentation phase of coagulation is required to: 1) overcome the effects of plasma proteinase inhibitors of coagulation enzymes and 2) prevent premature lysis of the clot through thrombin-mediated activation of thrombin-activatable fibrinolysis inhibitor (TAFI, CPU). The factor VIIa/tissue factor-mediated "initiation phase" of coagulation contributes to this secondary phase by priming the system through the stimulation of platelets and the production of factor Va and VIIIa, and by producing the initial factor IXa. Under certain conditions and particularly in locations with high endogenous fibrinolytic activity, additional factor IXa generated by factor XIa is required for hemostasis.

Mouse models of coagulation-related factor deficiencies support the importance of tissue factor initiated coagulation. Tissue factor, factor VII, factor X, factor V and prothrombin null mice die during gestation and/or the peripartum period. Factor VIII and factor IX deficient mice survive gestation, but display a severe hemorrhagic diathesis postpartum. Factor XI and TAFI null mice, however, appear asymptomatic in the unchallenged state, perhaps reflecting a lower fibrinolytic potential in the mouse. Complete deficiency of TFPI the major regulator of the initiation phase of coagulation, or protein C, which plays an important role in limiting the augmentation phase of coagulation, lead to lethal disseminated intravascular coagulation. Consistent with the multigenic nature of thrombophilia, the combination of modest defects in the regulation of the initiation phase and augmentation phases of coagulation, heterozygous TFPI deficiency and the factor V Leiden genotype, produce near absolute mortality in mice.

2. VON WILLEBRAND'S DISORDER. Dr. Paul Giangrande, Oxford Haemophilia Centre, UK

Diagnosis of VWD:

VWD is the commonest inherited disorder of haemostasis and the diagnosis is undoubtedly overlooked. Screening programmes of target groups, such as women with menorrhagia, may be useful in identifying cases. The diagnosis requires consideration of both the clinical history as well as laboratory data. No single test will suffice to establish the diagnosis. A number of variables influence VWF levels, including age, gender, blood group, pregnancy, physical exertion and oestrogen therapy and people with borderline results deserve repeat testing. In addition to the usual screening tests, the PFA-100 has recently proved to be a sensitive screening instrument. An ELISA test using a monoclonal antibody directed against a platelet-binding Gp-Ib binding site was widely used (at least in Europe) until recently, when it was shown to be unreliable in detection of type 2 VWD. Many laboratories have now returned to using functional assays based on ristocetin-induced platelet aggregation. However, an ELISA assay based on binding of VWF to collagen has been increasingly adopted. An important advantage of this test is that it identifies primarily high molecular weight forms of VWF and thus the VWF:CBA/VWF:Ag ratio may be used to subtype the disorder. Multimer analysis has been generally regarded as providing the definitive method but it is not generally available and analysis is often difficult. Type 2B VWD is associated with aggregation of platelets with a low concentration of ristocetin (0.5 mg/ml or less). Type 2N VWD may be mistaken for mild haemophilia, and factor VIII binding tests are required for diagnosis. Once a case of VWD has been identified, it is important to offer testing to relatives. Secondary VWD associated with hypothyroidism has been described, which responds to thyroxine treatment.

Treatment of VWD:

DDAVP will raise the VWF and factor VIII levels 3-5 fold after a dose of 0.3 µg/kg. However, tachyphylaxis is observed with repeated doses. A baseline level below 10 IU/dl will not be associated with satisfactory response. Hyponatraemia and arterial thrombosis are recognised complications, and the drug is best avoided in both the elderly and children under 2. DDAVP may also be effective in type 2N VWD, although the half-life of factor VIII will be short. The use of DDAVP in type 2B VWD is controversial, but there is now increasing evidence that it may be used safely. No recombinant VWF concentrate is available yet. All blood products used in VWD should be subjected to virucidal treatment and cryoprecipitate should no longer be used. Concentrates used for the treatment of patients unresponsive to DDAVP which are widely used in the USA, Europe and Japan are 8Y (BPL, UK), Haemate P (Aventis Behring), Alphanate (Alpha Corporation) and VHP-VWF (LFB, France). The content of VWF in these products is usually available, and VWF levels of >100 IU/dl peri-operatively are recommended and a level of not less than 50 IU/dl in the immediate post-operative period. Many centres simply monitor the factor VIII level although this is merely a surrogate marker. Tranexamic acid alone is very good in controlling bleeding from mucosal surfaces, eg epistaxis and menorrhagia. An intranasal spray preparation is now available which may be useful for menorrhagia. The levels of VWF and factor VIII rise significantly during pregnancy and haemostatic support is rarely required during pregnancy. However, there is undoubtedly a higher risk of both primary and secondary post-partum haemorrhage in VWD and the factor VIII and VWF levels should be checked a few days after delivery. DDAVP should only be used with caution during pregnancy.

3.

BLOOD VESSELS FROM BONE MARROW. Julie H Campbell, Chih-Lu Han, Johnny L Efendy and Gordon R Campbell *Centre for Research in Vascular Biology, University of Queensland, Brisbane, QLD 4072 Australia*

We have shown that inserting a length (up to 80mm) of polyethylene tubing into the peritoneal cavity of rabbits, rats, mice and dogs induces the formation of a granulation tissue capsule over 2-3 weeks. This is a general reaction to a foreign object in the peritoneal cavity and acts to seal it from the rest of the body, just as pearl nacre forms around an irritant piece of sand in an oyster. The capsule consists of myofibroblasts and the collagen matrix they have produced covered by a single layer of mesothelial cells. The mesothelial cells are derived from the lining of the peritoneal cavity and possesses anti-thrombotic properties similar to endothelial cells. The capsule can be removed from the polyethylene tubing and everted by cutting one end then pulling it back over itself (like taking off a sock) such that the mesothelium now lines the lumen. The resulting tube of living tissue (minus polyethylene tubing) now resembles a blood vessel. As such, 20mm lengths have been successfully grafted by end to end anastomoses into the severed carotid artery (rabbit) or abdominal aorta (rat) of the animal in whose peritoneal cavity it was grown. Successful arteriovenous shunts (femoro-femoral) have also been achieved in the rabbit. To date, patency of up to 12 months has been demonstrated by Doppler ultrasound. Histologically, by 3-4 months after transplantation to a high pressure arterial site, the myofibroblasts have differentiated into smooth muscle-like cells volume fraction of myofilaments not significantly different from cells of the nearby artery. Functionally, by 6 weeks they respond to vasoconstrictor agents in organ bath studies, and relax in response to acetylcholine. Elastic fibres have formed within the 'media' and the outside surface has developed a thick 'adventitia' complete with vasa vasorum. While the source of the 'endothelium' (mesothelial cells) is clear, the origin of the granulation tissue myofibroblasts that subsequently become 'smooth muscle-like' cells is not known.

To address this question we inserted pieces of tubing or boiled blood clot from another species (any foreign body induces the granulation tissue response) into the peritoneal cavity of rats, rabbits and mice then harvested the capsule at different times. In the first 2 or 3 days, rounded cells were seen attached to the surface. These cells stained with labelled antibodies to Ly5 (CD45) showing that they were derived from haemopoietic cells of the bone marrow. Ultrastructurally they resembled either resident peritoneal macrophages or less differentiated cells of bone marrow origin. Mesothelial cells were also evident. By day 6, the capsule was already quite thick and composed of the same rounded cells plus matrix covered by a continuous layer of mesothelium. By this time, some of the cells deep in the capsule had begun to take on the appearance of fibroblasts and only a few cells stained with antibodies to Ly5. Cells resembling macrophages and fibroblasts were both present alongside others with features of both cell types. By 2 weeks nearly all cells in the capsule were spindle-shaped, layered as in the media of a blood vessel and contained peripheral bundles of myofilaments. They stained with labelled antibodies to α -smooth muscle actin but none of these cells stained for Ly5, an antigen that is known to disappear as these cells differentiate.

To check the bone marrow origin of these cells, female C57BL/6 mice expressing the Ly5.2 variant on the surface of their haemopoietic cells were X-irradiated to destroy bone marrow, then immediately transfused with 10^6 nucleated bone marrow cells taken from the femur and tibia of a congenic strain of male mice expressing the Ly5.1 variant. Four weeks later, flow cytometry of female mouse blood with Ly5.1 antibodies confirmed successful engraftment (80-99%) of male marrow. Tubing was then placed into the peritoneal cavity of these female mice and the capsule harvested 14 days later. *In situ* hybridization with a Y-chromosome probe confirmed the male donor, and thus bone marrow origin of the elongated cells that formed the capsule and thence differentiated into smooth-muscle-like cells. In similar chimeric mice, many of the α -smooth muscle actin containing cells that formed the neointima following scratch injury were derived from cells of the bone marrow, questioning the origin of cells in restenotic lesions in human arteries following angioplasty.

4.

Antiphospholipid antibody-associated thrombosis:- Warfarin, Aspirin or Both??? Timothy A Brighton, Staff Haematologist, St George Hospital, Kogarah and University New South Wales, Kensington, Sydney, NSW. Australia.

The management of patients with antiphospholipid antibodies (aPL) and thrombosis is a poorly defined area of clinical research. The purpose of this overview is to summarise the available literature and to identify key issues that require urgent examination by prospective clinical trials.

Several characteristics of aPL-associated thrombosis deserve emphasis. Firstly the laboratory definition of aPL remain difficult, contentious and poorly standardised. Lupus anticoagulants (LA) and anticardiolipin antibodies (aCL) remain the principle aPL of clinical importance, although anti-beta 2-Glycoprotein (β_2 GPI) and anti-Prothrombin (FII) antibodies are also often assayed. Secondly, aPL may be associated with immune (e.g. SLE) and non-immune (e.g. idiopathic, drug-induced, infections, malignancy) clinical disease. The thrombotic risk of aPL may vary depending on the clinical association. Thirdly, aPL have been associated with venous and arterial thrombotic disease. Additionally various foeto-maternal complications, in particular recurrent fetal loss, are at least partly attributable to thrombosis of placental vessels. Fourthly, a detailed pathophysiological understanding of aPL-associated thrombosis has eluded definition. The term "antiphospholipid" is a misnomer given recent studies demonstrating antigenic specificity of these antibodies toward phospholipid-binding proteins such as β_2 GPI and FII. It is not currently possible to distinguish thrombogenic from non-thrombogenic aPL, either on clinical or laboratory characteristics. The uncertainty of the mechanisms of thrombosis contributes to the controversy regarding causality and also undermines the clinical significance of aPL. Fifthly, the current definition of the Primary Antiphospholipid Syndrome (PAPS) is all-inclusive, circular and perhaps has little relevance when considering patients with only thrombosis (no thrombocytopenia or fetal loss) and aPL.

There is no doubt that aPL are associated with thrombosis in patients with and without definable immune disease. Approximately 30-40% of patients with SLE have either aCL or LA and usually both. The relative risk of thrombosis in retrospective and recent prospective studies of patients with SLE is between 3 and 10-fold. Recent prospective studies in patients without definable immune disease have also found similar associations for aCL and LA (far fewer studies) for venous and arterial thrombotic disease. In pregnant women with SLE, it is well accepted that aPL is a marker for adverse fetal and maternal outcomes. In pregnant women without immune disease, while not conclusive it seems likely that aPL are associated with fetal loss, intrauterine growth retardation and even preeclampsia.

Given the poor understanding of aPL-associated thrombosis and the apparent void of rigorous clinical research, therapeutic decisions regarding anticoagulation remain difficult. The acute management of arterial or venous thrombotic complications does not substantially differ from the standard considerations of age, site of occlusion and the presence of coexisting disease. In both arterial and venous aPL-associated thrombosis there appears to be a propensity for recurrence once anticoagulation is ceased. The presence of IgG aCL at any titre was significantly associated with recurrence in large prospective studies of venous thrombosis. Evidence in arterial disease is more anecdotal. Clearly some patients, perhaps predominantly those with immune disease, require high intensity anticoagulation. However given the risks of haemorrhage, this should be reserved for patients failing conventional-intensity anticoagulation. Aspirin alone anecdotally seems insufficient as secondary prophylaxis for venous and arterial thrombotic disease. Randomised studies in fetal loss have demonstrated efficacy of heparins, which are the anticoagulants of choice. The addition of low dose aspirin, while commonly practiced, remains unproven. Well-designed prospective studies are urgently required to make improvements to the treatment of patients with aPL-associated thrombosis.

5.

OBSTETRIC ASPECTS OF Antiphospholipid ANTIBODIES. B.N.J. Walters. *King Edward Memorial Hospital Women and Royal Perth Hospital WA*

The association of antiphospholipid antibodies with arterial and venous thrombosis has been recognised for sometime. In addition to these complications, this thrombogenic disorder has dramatic effects in pregnancy, for both mother and baby. The incidence of fetal loss is increased at all stages of pregnancy, manifest as miscarriage, mid trimester abortion and fetal death in utero. For the mother, in addition to venous thromboembolism and arterial events, there are substantial risks in thrombotic and other complications of the pregnancy syndrome, pre eclampsia. These risks carry over into the postnatal period and have implication for contraceptive practice.

Antiphospholipid antibodies occur in pregnancy in a number of scenarios. They may be present in women with known autoimmune disease. The antibodies may have been discovered in the process of evaluation for thrombophilia in women with previous thrombotic events. Thirdly, antibodies may have been discovered subsequent to investigation of previous adverse pregnancy outcomes. Finally, in some asymptomatic patients abnormalities are discovered by serendipity. The approach to antithrombotic antiplatelet and immunotherapy varies with the clinical history of each patient, both outside and within pregnancy. This presentation will discuss diagnosis of obstetric antiphospholipid syndrome and treatment of pre-existing antiphospholipid problems in patients with and without known autoimmune disease. Treatment options include anti platelet therapy, anticoagulation usually by means of low molecular weight Heparin, immunomodulation by high dose Gamma Globulin infusion and other less utilized modalities. The implications for the developing fetus and new born will be canvassed. Prognostic features of these disorders will be discussed, in addition to the therapeutic aspects.

6.

Treatment options for low-grade lymphoma. Gunnar Juliusson, Dept of Hematology, University Hospital, Linköping, Sweden

The group of indolent lymphomas contains several different entities, the most common being follicular lymphoma and chronic lymphocytic leukaemia. Mantle cell lymphoma is an entity important to recognize, since although response may follow initial therapy, refractory progression occurs early, and survival is poor. On the other hand, marginal zone lymphoma in the stomach, which is associated with *Helicobacter pylori* infection, is easily managed with anti-bacterial treatment alone. The major subgroups have had a similar outcome for decades, with treatment based on alkylating agents. Such treatment may well reduce symptoms and induce remissions, but has not showed to prolong survival. More intensive anthracyclin-containing combination therapy may prolong response duration. Interferon alpha has been studied extensively together with and following chemotherapy. As maintenance following intensive combination chemotherapy IFN leads to a prolongation of remission duration, and in one study also survival was improved. During the 1990's, purine analogues were introduced. Single agent therapy with fludarabine produce tumour responses, but was not clearly better than alkylator-based treatment. With the combination fludarabine-mitoxantrone-dexamethasone improved responses, including molecular responses were achieved, and randomized studies are evaluable soon. Rituximab, a humanized chimeric anti-CD20 antibody has a significant apoptosis-inducing activity in follicular lymphoma. Anti-CD20 antibodies have also been used as carriers for radioactive isotopes. High dose chemotherapy with or without total body irradiation and supported with autologous stem cells have been studied. It was shown that patients given autologous bone marrow treated in vitro with a cocktail of anti-B-cell antibodies and complement following high dose cyclophosphamide and total body irradiation had a significantly better outcome if the purging procedure resulted in a total depletion of tumor cells from the harvest in vitro before reinfusion. A randomized study comparing purged and unpurged stem cells with chemotherapy only was intended, but the inclusion rate was insufficient. Overall, it has not been clearly shown that autologous transplantation improves outcome for patients with follicular lymphoma, with the exception for patients who have had transformation to high-grade lymphoma. In this situation high-dose therapy with autologous transplantation could be considered standard care. Allogeneic transplant may well turn out to be an option, since such transplants may be performed with reduced intensity conditioning suitable for elderly patients, and since graft-versus-lymphoma is active in eradicating lymphoma. However, there are still problems with controlling graft-versus-host disease, as well as the long-lasting post-transplantation immune suppression.

7.

THE CBB GENE AND HEMATOPOIESIS. Trevor Blake, Hong Tran, and P. Paul Liu, National Human Genome Research Institute, NIH, Bethesda, MD

CBFB and *CBFA2* (a.k.a. *AML1*, *PEBP2aB*, and *RUNX1*) form a heterodimeric DNA-binding factor that regulates expression of many genes important in hematopoiesis. *CBF α 2* is the DNA-binding subunit while *CBF β* associates with *CBF α 2* and enhances its DNA-binding affinity. In mouse knock-out models, both *Cbfa2*^{-/-} and *Cbfb*^{-/-} embryos die in midgestation from a combination of definitive hematopoiesis defects and CNS hemorrhage.

To study the role of *CBFB/PEBP2B* in normal development we isolated the zebrafish homolog gene. Zebrafish *cbfb* is highly similar to mammalian *CBFB/PEBP2B* and its protein product can associate with mammalian *CBF α 2* and enhance its DNA binding. Zebrafish *cbfb* is expressed in early hematopoietic tissues in the developing embryo, as is *scl/tal-1*. In addition, *cbfb* is expressed in Rohon-Beard cells, cranial nerve ganglia, hindbrain, retina, branchial arches, jaw, and fin buds. The expression of *cbfb* in hematopoietic tissues is abolished in several "bloodless" mutants, confirming that *cbfb* is expressed in hematopoietic cells.

Results from our zebrafish studies cannot distinguish the roles of *cbfb* and *scl* during hematopoiesis. On the other hand, results from mouse knock-out models indicate that *Scf* is required for primitive as well as definitive hematopoiesis while *Cbfb* and *Cbfa2* are only required for definitive hematopoiesis. To study the relationship between *Cbf* and *Scf* during hematopoiesis, embryos were produced by crossing *Scf-lacZ* mice with *Cbfb-MYH11* mice, which harbors a leukemogenic fusion gene that dominant-negatively suppresses *Cbf* function. Analysis of the embryos suggests that either *Scf* is upstream of *Cbf* or they function in independent pathways.

8.

STI571: A TYROSINE KINASE INHIBITOR FOR THE TREATMENT OF CML VALIDATING THE PROMISE OF MOLECULARLY TARGETED THERAPY. Brian J. Druker, Moshe Talpaz, Debra Resta, Bin Peng, Eliasbeth Buchdunger, John Ford, Sofia Fernandes Reese, Hagop Kantarjian, Renaud Capdeville, Charles L. Sawyers. Oregon Health Sciences University, Portland, OR, USA; MD Anderson Cancer Center, Houston, TX, USA; Novartis Pharmaceuticals, East Hanover, NJ, USA and Basle, Switzerland; University of California, Los Angeles, CA, USA.

The Bcr-Abl fusion protein, resulting from a (9;22) chromosome translocation, causes several types of leukemia. The 210 kDa form of Bcr-Abl is present in virtually all patients with chronic myelogenous leukemia (CML) and a 185 kDa variant is present in approximately 20% of acute lymphoblastic leukemia patients. The transforming function of Bcr-Abl requires tyrosine kinase activity of these Bcr-Abl fusion proteins, which is elevated as compared to c-Abl. Thus, Bcr-Abl is an ideal candidate for a molecularly targeted therapeutic agent and an inhibitor of the Bcr-Abl kinase would be predicted to be an effective and selective therapeutic agent for CML. STI571 (formerly CGP57184B) was synthesized at Novartis Pharmaceuticals by identifying a lead in a high throughput in vitro screen for tyrosine kinase inhibitors and optimizing its activity for specific kinases. STI571 functions through competitive inhibition at the ATP binding site and shows a high degree of specificity for the Abl, PDGFR and Kit tyrosine kinases. It induces either growth arrest or apoptosis specifically in Bcr-Abl expressing hematopoietic cells with no obvious effects on normal cells or in cells transformed by other tyrosine kinase oncogenes.

Based on anti-leukemic activity in several pre-clinical models and a lack of significant toxicity in animals, a phase I clinical trial was conducted in CML patients who had failed other treatment options. All patients in the chronic phase (n=31) have achieved hematologic remissions once therapeutic dose levels were achieved. With prolonged therapy (5 months or greater), a growing fraction of these patients have cytogenetic responses, including several individuals with complete disappearance of the Ph chromosome. STI571 also has remarkable activity as a single agent in CML blast crisis and Ph + ALL patients. Although responses tend not to be durable, 20% of myeloid blast crisis patients have ongoing responses between 6 months and one year. As virtually all patients with CML express Bcr-Abl and the Bcr-Abl protein is unique to tumor cells, this disease has provided an ideal opportunity to test the concept that drugs targeted against a tumor-specific abnormality will have therapeutic utility. Ongoing studies are directed at optimizing the use of this agent.

9.

The Biology and Treatment of Chronic Lymphocytic Leukaemia. Gunnar Juliusson, Dept of Hematology, University Hospital, Linköping, Sweden

Chronic Lymphocytic Leukaemia (CLL) is the commonest leukaemia type, and has a variable clinical spectrum and prognosis. The phenotype includes the B-cell markers CD19 and CD20, but also CD5. CD23 can be used to distinguish CLL from mantle cell lymphoma. The commonest chromosome abnormality is a deletion at 13q14, close to but not including the retinoblastoma gene. Major efforts have been made to identify and characterize a suggested tumour suppressor gene at the minimal deleted region, but so far no candidate gene has proved to be significantly involved. Trisomy 12 is the next common abnormality, which is found mainly in CLL cell clones without del(13)q. p53-deletions and 11q-deletions are less common, but identify patients with poor survival. Recently, mutation analysis of immunoglobulin genes have identified one CLL subtype with naïve B-cells and atypical cytology, unmutated Ig-genes and poor prognosis, and a second subtype with post-germinal mutated Ig-genes and a better outcome. Other parameters indicating better prognosis besides low clinical stage include low CD38 expression, low serum beta-2-microglobulin, and low plasma levels of soluble CD23. CLL is a disease characterized by immune deficiency, including secondary hypogammaglobulinaemia. Bacterial airway infections are most common in patients with early disease. Autoimmune complications directed to blood cells are not uncommon, especially DAT-positive haemolytic anaemia is a significant problem mainly in advanced and heavily pretreated disease. Standard therapy includes alkylating agents, mainly chlorambucil, for which many different schedules are available. Still no newer treatment has consistently shown to give an improved survival as compared to single drug chlorambucil. However, treatment with the purine analogues, mainly fludarabine, leads to an improved response rate and a longer disease duration. Purine analogues cause T-cell deficiency, which increases the risk for opportunistic infections, as well as autoimmune haemolysis. Recently, studies of combination chemotherapy including purine analogues and alkylating agents have been performed. The humanized chimeric anti-CD20 monoclonal antibody has been used alone and together with chemotherapy. Still, durable complete remissions are still an exception. Autologous stem cell harvest used for support of high-dose chemotherapy might lead to prolonged remissions and molecular eradication of minimal residual disease. However, it is still not clear which patients, if any, benefit from this procedure, since most patients eventually relapse. Allogeneic transplantation has recently been successfully performed with reduced intensity conditioning, which opens up new possibilities for elderly patients. It is clearly documented that CLL-cells may be eradicated by donor T-cells through the graft-versus-leukemia effect, and since this antitumor activity may be everlasting, this is a possibility for cure. However, the major problems with allogeneic transplantation, i.e., graft-versus-host disease and posttransplant immune suppression, still remains using 'mini-transplants'. With improved control over specific donor T-cell activity we might in the future see reduced transplantation-related mortality and thus an improved survival of CLL patients.

10.

MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA. Paula Marlton. *Princess Alexandra Hospital Brisbane*

Studies of minimal residual disease (MRD) in Acute Myeloid Leukemia (AML) are important for several reasons. They may provide us with information on which rational individualized therapeutic decisions can be made to improve the outcome for patients with AML. They may also provide important insights into the biology of AML again with potential clinical impact. Little is known for example about the kinetics of the leukemic cell burden beyond remission induction therapy or during the development of relapse. It is clear from data already available that the traditional view of curative therapy totally eradicating the leukemic clone is too simplistic. Emerging MRD data indicate that factors beyond leukemia cell eradication such as the immune response and other host factors are likely to play important roles in maintaining remission status.

Techniques available to analyse MRD range in sensitivity, complexity and practical applicability. Methodologies include conventional cytogenetics, fluorescent in situ hybridization (FISH) or molecular cytogenetics, multiparameter flow cytometry and PCR based techniques. This session will review data available for various approaches and focus on recent developments in PCR techniques for MRD detection in AML.

PCR approaches include qualitative or quantitative assays; RNA- or DNA- based assays; and end point or real-time assays. Some (although not all) qualitative RNA-based end-point PCR (RT-PCR) assays of specific sensitivity can be predictive of relapse in APL. This has not held true for similar assays for rearrangements of genes encoding subunits of CBF (core binding factor) which characterize inv(16) and t(8;21) AML. Emerging data indicate however that quantitative PCR approaches can yield predictive information in AML with AML1-ETO expression. Both end-point and real-time quantitative assays appear useful in this setting. DNA-based assays including PCR and FISH methods have the advantage over RNA-based assays of identifying all cells with the rearrangement regardless of expression status. Genomic PCR has not been widely utilized however because of technical constraints in amplifying the variable and often very large segments of DNA involved in the common rearrangements. Preliminary work demonstrates that this is feasible in CBF-MYH11 rearrangements and may provide useful MRD information.

FISH techniques for detecting specific structural abnormalities are hampered by a relative lack of sensitivity and often high level of background false positivity. Some data support its potential usefulness in MRD monitoring however and its role remains undefined in large prospective series. For patients without a defined molecular rearrangement suitable for PCR or FISH detection, more generic markers of disease are being explored for MRD detection. Expression level of genes such as wt-1 or multi-colour flow cytometry are alternative approaches in such patients. Ultimately, the potential to gain biologic insight into the kinetics of relapse may be greatest using a variety of MRD approaches, which may also yield highly predictive clinical information.

11.

DETECTION OF MINIMAL RESIDUAL DISEASE BY FLOW CYTOMETRY. Dario Campana, *Departments of Hematology-Oncology and Pathology, St. Jude Children's Research Hospital, and University of Tennessee, Memphis, Tennessee, USA*

Flow cytometric detection of MRD is based on the identification of immunophenotypic combinations expressed on leukemic cells but not on normal hematopoietic cells. It affords the detection of one leukemic cell among 10,000 normal bone marrow cells, and can be currently used in approximately 90% of cases of acute lymphoblastic leukemia (ALL) and 75% of cases of acute myeloid leukemia. We have used high-density DNA microarrays to compare the gene profile of leukemic cells to that of their normal counterparts. This approach allowed us to identify new markers of leukemia that can be used for MRD detection in ALL. Methods for rapid exchange of flowcytometric data should facilitate exchange and review of MRD data obtained in different centers.

We recently analyzed data of a prospective study of MRD in 195 children with newly diagnosed ALL in clinical remission. Bone marrow aspirates (n=629) were collected at the end of remission induction therapy and at weeks 14, 32 and 56 of continuation therapy. Detectable MRD at each time point was significantly associated with a higher relapse rate ($P < 0.001$). Patients with high levels of MRD at the end of the induction phase ($\geq 1\%$) or at week 14 of continuation therapy ($\geq 0.1\%$) had a particularly poor outcome. The predictive strength of MRD remained significant even after adjusting for adverse presenting features, excluding patients at very high or very low risk of relapse from the analysis, and considering levels of peripheral blood lymphoblasts at day 7 and day 10 of induction therapy. The incidence of relapse among patients with MRD at the end of the induction phase was $68\% \pm 16\%$ (SE) if they remained MRD⁺ through week 14 of continuation therapy, compared with $7\% \pm 7\%$ if MRD became undetectable ($P = 0.035$). The persistence of MRD until week 32 was highly predictive of relapse (all four MRD⁺ patients relapsed vs two of the eight who converted to undetectable MRD status; $P = 0.021$). Thus, sequential monitoring of MRD by flow cytometry provides highly significant, independent prognostic information in children with ALL.

Since MRD can also be monitored by PCR amplification of antigen receptor genes in patients with ALL, and the two techniques yield highly concordant results we are currently using the two methods in tandem for risk-assignment. This allows MRD monitoring in all patients, and should limit the occurrence of false-negative findings due to immunophenotypic shifts or clonal evolution.

12.

ISOLATION OF HUMAN PERIPHERAL BLOOD DENDRITIC CELLS FOR CLINICAL APPLICATION. Derek Hart, Maria Gillece, David Munster, Slavica Vuckovic, Alejandro J López. Mater Medical Research Institute

Purpose of the Study Isolate blood dendritic cells (DC) for potential use in immunotherapy. **Methods** Current methods of expansion and enrichment of monocyte derived DC (Mo-DC) populations, require prolonged in vitro culture in the presence of exogenous growth factors. Furthermore the resulting Mo-DC have significant phenotypic (and potentially functional) differences to circulating DC. We have investigated the feasibility of purifying blood DC populations by a series of simple enrichment procedures, including positive selection with the monoclonal antibody CMRF-44, that do not require extensive culture in vitro. The resulting DC population relates closely to the physiological precursor population and may be functionally superior to Mo-DC. We undertook the isolation of these cells by sequential steps of enrichment from PBMC obtained from buffy coat or pheresis MNC preparations. **Results** After a short incubation, the cells were centrifuged over a Nycodenz gradient, a method previously used to enrich DC from the blood (J. Immunol. Methods 184:84. 1995). The interface population was labeled with biotinylated CMRF-44 antibody (Blood 89:3708.1997) followed by streptavidin coated magnetic beads, and positive selection (Miltenyii MACS). This procedure enriches CMRF44⁺ CD19⁻CD14⁻ DC fifty fold to a purity $>25\%$. Further enrichment to 80% purity was achieved by negative selection using CD14 plus CD19 specific antibodies prior to the positive selection step. Additionally to CMRF-44, other mAb identifying DC such as CMRF-56, have been produced and used in isolation protocols. **Conclusions** We describe the phenotypic and functional characteristics of these DC in comparison to conventional preparations. We propose further investigation of the potential of this routine blood DC purification platform as a basis for controlled trials of DC vaccination as therapy for cancer.

13.
NEW THERAPEUTIC STRATEGIES IN BONE MARROW TRANSPLANTATION. Rainer Storb, M.D. Fred Hutchinson Cancer Center and the University of Washington Seattle, Washington, USA

Almost simultaneously, work by several groups of investigators has brought about changes in the way hematopoietic stem cell allografts are being carried out to treat patients with hematological diseases. The focus has shifted away from attempts at eradicating malignant cells through high-dose chemoradiation treatment toward using the stem cell donor's T lymphocytes for that purpose via an allogeneic graft-versus-tumor effect. While most of the nonmyeloablative transplant regimens used in these efforts are still fairly intense and toxic, a radical departure from conventional transplantation focuses on the almost exclusive use of immunosuppressive agents with little toxicity to establish the allografts. For patients with hematologic malignancies, the success of the procedure rests entirely on the allogeneic graft-versus-tumor effect induced by donor T cells. As for nonmalignant diseases, the procedure can be used to establish a stable state of mixed donor/host hematopoietic chimerism which, in itself, may be sufficient to cure disease manifestations.

14.
OVERVIEW ON THE THERAPEUTIC USE OF DENDRITIC CELLS. Gavin Cull. Department of Haematology, Princess Alexandra Hospital, Brisbane, Australia.

Dendritic cells (DC) are bone marrow-derived professional antigen presenting cells which are essential for the induction of a primary immune response, utilising their unique capacity to stimulate T cells which have had no prior contact with antigen. Their capacity to take up, process and present soluble antigen in the context of MHC and appropriate co-stimulatory molecules allow them to perform this specialised function. This potent antigen presenting capacity makes them an ideal candidate for immunotherapeutic strategies aimed at initiating or enhancing an immune response against tumour antigens. However, they are present in very low numbers in blood and tissues and this has made their acquisition for therapeutic purposes difficult. In recent years this problem has been overcome by techniques which generate large numbers of functional DC by the in-vitro culture of peripheral blood monocytes or blood and marrow-derived CD34 cells. This has led to a rapid growth in development of basic scientific discovery and technology in the field of immunotherapy. A large number of clinical trials applying these advances in a range of human tumours have been reported or are in progress.

Generation of an effective anti-tumour immune response in-vitro or in-vivo requires several components. These include an antigen which is specific for the tumour, an antigen-presenting cell (APC) capable of presenting this tumour antigen to T cells and activating a tumour-specific cytotoxic T-lymphocyte (CTL), and the ability of this CTL to then kill the native tumour cell. Tumour-specific and tumour-restricted antigens which can act as a target for immunotherapy have been identified. In particular, B-cell malignancies lend themselves naturally to this approach by the production of tumour-specific immunoglobulin, or idiotype, by the malignant cells. It has been demonstrated that anti-idiotypic immune responses can be generated in patients with lymphoma and myeloma using idiotype-primed dendritic cells. These responses have included the development of idiotype-specific CTL and, in the case of lymphoma, a limited number of clinical responses have been observed. Chronic myeloid leukaemia also presents itself for this form of immunotherapy by the production of the tumour-specific fusion protein, bcr-abl, and tumour-specific immune responses have been reported using dendritic cell-based vaccination schedules. In the non-haematological setting, melanoma is the most extensively studied and there is considerable data demonstrating tumour-specific immune responses using a variety of dendritic cell-based strategies. These immune responses have been associated with clinical benefit though the overall response rate has been modest.

Vaccination with tumour-antigen primed DC can generate tumour-specific immune responses but the evidence for significant clinical benefit remains limited. The challenge now is to improve the immunological response rate and convert these to sustained clinical responses. Issues relating to the vaccination protocol such as the optimal type of DC, the best method to prime these DC with tumour antigen and the most effective schedule are being investigated. The discovery of additional tumour antigens and examination of methods to enhance their immunogenicity may afford further improvements. Finally, the detection of in-vivo immunosuppressive factors which prevent the development of a clinically relevant anti-tumour immune response may lead to strategies to overcome these immune deficits.

15.

Anti-angiogenic and vascular targeting agents - molecular mechanisms and potential clinical applications. **Russell Bassar, Departments of Haematology and Medical Oncology, Royal Melbourne Hospital, Parkville, and Western Hospital, Footscray, Victoria, Australia**

Therapies that inhibit the growth of new blood vessels, so-called angiogenesis inhibitors, offer considerable promise as anticancer agents. The link between angiogenesis and tumour progression and spread was first established some 30 years ago by Judah Folkman, who noted that without new blood vessels, many tumours only grow to a few millimetres in size. He also found that while a tumour may stay small, its cells continue to proliferate, a situation brought about by a balance between cell the rates of proliferation and apoptosis. These observations led to the concept of an "angiogenic switch", a complex process by which a tumour mass expands and overtakes the rate of internal apoptosis by developing blood vessels, thereby changing into an angiogenic phenotype. Evidence has emerged this change is the result of a shift in net balance of stimulators and inhibitors of angiogenesis within the tumor microenvironment, in which the inhibitors were downregulated. It is now recognised that the growth of most tumours, and probably haematological malignancies, and the formation of metastases are dependent on this process.

Angiogenesis is influenced by a number of specific humoral factors. Perhaps the most important is vascular endothelial growth factor (VEGF), which is secreted by normal tissues and tumour cells in response to ischaemia and is critical for the proliferation and survival of endothelial cells. VEGF contributes to tumour growth both by stimulating angiogenesis and by increasing vascular permeability. In preclinical studies, inhibition of VEGF-induced angiogenesis has resulted in reduction of tumour growth. One approach used is sequestration of VEGF with antibody. Another is inhibition of VEGF receptor signalling. There are several receptors for VEGF, and these are largely confined to endothelial cells. They have been isolated and cloned, and demonstrated to possess tyrosine-kinase activity within the cytoplasmic domain. This activity is responsible for initial signal transduction following ligand binding. Inhibitors of the tyrosine kinase of VEGF receptors *flt* and *kdr* have been identified and characterised by a number of groups. Early clinical trials of VEGF inhibition by both of the above mechanisms have shown biological activity and hints of anti-tumour effect.

A number of different agents have been shown to inhibit angiogenesis in a non-specific manner. The most promising of these is thalidomide, which was recently shown to be effective in patients with myeloma. Other agents are currently under investigation.

An alternative to inhibiting angiogenesis is to specifically target the endothelium of newly formed blood vessels, taking advantage of phenotypic and structural differences from normal vasculature. The integrity of neovasculature is highly dependent on the maintenance of the three-dimensional shape of the endothelial cells, in the absence of the peri-vascular structures present in normal vessels. A tubulin cytoskeleton is responsible for maintaining the shape of neo-endothelial cells. Agents which disrupt tubulin will significantly affect endothelial cell shape, leading to vascular occlusion. A new class of tubulin-binding drugs produce dramatic ultrastructural change in endothelial cell shape with minimal cytotoxicity *in-vitro*. Studies in xenograft models demonstrate dramatic vascular occlusion and infarction of tumours.

In summary, the vasculature of tumours represents a potentially very important target for therapeutic intervention. Important development challenges remain in the identification of usable markers of activity for these agents, and in the design of initial clinical studies.

16.
INV(16) AND LEUKEMIA. Lucio Castilla, Neeraj Adya, and P. Paul Liu. National Human Genome Research Institute, NIH, Bethesda, MD

Chromosome 16 inversion is one of the most frequent chromosomal abnormalities in human acute myeloid leukemia and generates a fusion gene *CBFB/PEBP2B-MYH11*. We have previously shown that *CBFB-MYH11* dominantly interferes with the normal function of *RUNX1* (a.k.a. *CBFA2*, *AML1* and *PEBP2aB*) and blocks definitive hematopoiesis during embryogenesis.

To study the mechanism of leukemogenesis in the presence of the *CBFB-MYH11* gene, we characterized chimeric mice generated with ES cells containing the *Cbfb-MYH11* knock-in gene. A selective blockage of myeloid and lymphoid differentiation by the *Cbfb-MYH11* gene was identified. Moreover, around 50% of chimeras developed leukemia and lymphoma at an age of 12-26 months. By ENU mutagenesis we showed that *Cbfb-MYH11* is critical for the development of acute myeloid leukemia, but additional genetic events are needed for full transformation. Using retroviral mutagenesis, we are identifying genes that cooperate with *Cbfb-MYH11* for leukemogenesis.

Using the cDNA microarray technology, we have been profiling gene expression in primary leukemia cells containing *inv(16)*. A 6.7k human cDNA microarray was used for this purpose. Gene expression patterns among a panel of M4Eo *inv(16)*+ leukemia cases were compared with those of M4 leukemic cases without *inv(16)* and of normal bone marrow. Those gene expression changes which are unique to the *inv(16)*+ cases will be studied further.

Identification of target genes downstream of *CBFB-MYH11* and cooperating genetic events necessary for leukemogenesis will help us understand better the oncogenic mechanism of *CBFB-MYH11* and may provide new therapeutic targets.

17.
CHEMOTHERAPY AND DONOR LEUKOCYTE INFUSION FOLLOWED BY INTERFERON-ALPHA FOR RELAPSED MALIGNANCY AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION. Jeff Szer, K Kannan, A P Schwarzer, A Spencer, A P Grigg. Bone Marrow Transplant Service, The Royal Melbourne Hospital, VIC 3050 and Bone Marrow Transplant Program, Alfred Hospital VIC 3181

Interferon-alpha (IFN) is known to promote graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (allo BMT). This property may also be used to enhance a graft-versus-leukaemia effect (GVL) after donor leukocyte infusion (DLI), a mode of therapy increasingly offered to patients relapsing after allo BMT. The aims of this study were to examine the efficacy and toxicity of IFN therapy given after DLI in patients with acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), acute lymphoblastic leukaemia (ALL), acute undifferentiated leukaemia (AUL) and multiple myeloma relapsing after allo BMT. Twenty-seven patients between October 1996 and September 1999 (16 AML, 4 ALL, 3 CML, 3 Multiple Myeloma, 1 AUL) who relapsed after allo BMT were treated with chemotherapy followed by DLI; IFN was subsequently given to patients without significant GVHD or rapidly progressive disease. The outcome after DLI with regard to remission rate, disease free survival (DFS) and GVHD were analysed. Eighteen patients received IFN following DLI, 14 of whom developed significant GVHD (grade II 8/14; IV acute or extensive chronic); thereafter GVHD resolved with cessation of IFN alone in 4, but 10 required systemic immunosuppression. Twenty-three patients were given chemotherapy and DLI as initial treatment of relapse; 10 achieved CR, in four only after the onset of GVHD. The other 4 patients received chemotherapy and DLI as a consolidation of a chemotherapy-induced remission. The CR was durable only in patients with CML (3/3) and AML (4/8). IFN induced GVHD in the majority of patients receiving DLI. The induction of GVHD and GVL by this approach produced excellent results in 3 patients with CML and modest results in AML, but appeared to be less effective in myeloma and ALL.

18.

PRIOR CRYOPRESERVATION OF EX VIVO EXPANDED CORD BLOOD CELLS IS NOT DETRIMENTAL TO ENGRAFTMENT AS MEASURED IN THE NOD-SCID MOUSE MODEL. Julie A Wood¹, Chris G Milross², Cathryn M Collins², Jamie Case¹, Robert E Nordon³, Alison M Rice¹. ¹*Children's Cancer Institute Australia for Medical Research*, ²*Radiation Oncology, Prince of Wales Hospital*, ³*Graduate School of Biomedical Engineering, University of New South Wales, Randwick NSW, Australia*

Small collections of cord blood (CB) make transplantation of adult patients difficult, if not impossible, and engraftment, even in small children, can be quite slow. Cytokine mediated expansion has been proposed to facilitate engraftment. As CBs are frequently frozen in one aliquot, patients are transplanted with unmanipulated cells, from which an aliquot has been reserved. This aliquot is expanded for 10 days and then infused into the patient. Data shows that engraftment is better when expanded cells are infused on day 0 rather than day 10 (Shpall et al, McNeice et al). Additionally, the functional quality of the expanded product cannot be assessed pre-transplant, as the patient is treated and given the first dose of cells prior to expansion. Expansion and subsequent cryopreservation of CB upon collection would allow assessment of the quality of the expanded product pre-transplant, giving the clinician a more informed choice. We questioned whether cryopreservation of expanded cells would compromise their ability to engraft the NOD-SCID mouse. CB CD34⁺ cells were incubated for 7 days with SCF+FL+MGDF. Half of the expanded cells were cryopreserved prior to transplantation. Post-thaw nucleated cell recovery was 94%, whilst recovery of day 14 progenitors was only 17%. NOD-SCID mice were transplanted with either freshly expanded or expanded CD34⁺ cells which had been frozen. Blood, bone marrow (BM) and spleen were monitored for human engraftment. Despite the loss of day 14 progenitors among the thawed expanded cells there was no difference in the rapidity of human engraftment. BM of mice transplanted with thawed expanded CD34⁺ cells showed significantly higher levels of human engraftment (13.90±3.82%) than mice transplanted with fresh expanded CD34⁺ cells (1.68±0.93%, p=0.0064). These results suggest that prior cryopreservation of expanded CB cells is not detrimental to the ability of the cells to engraft in NOD-SCID mice.

Shpall EJ, Quinones R, Jones R, Bearman S, Cagnoni P, Giller R, Nieto Y, McNiece I. Transplantation of cancer patients receiving high dose chemotherapy with ex vivo expanded cord blood cells. *Exp Hematol* 1999, 27:308a
McNiece I, Jones R, Bearman S, Cagnoni P, Nieto Y, Shpall EJ. Transplantation of ex vivo expanded PBPC after high dose chemotherapy results in decreased neutropenia. *Exp Hematol* 1999, 27:332a

19.

SITE-DIRECTED MUTAGENESIS OF PLATELET GLYCOPROTEIN IB-ALPHA DEMONSTRATING RESIDUES INVOLVED IN THE SULPHATION OF TYROSINE 276, 278 AND 279. A. Sasha Tait¹, Jing-Fei Dong², José A. López², Ian W. Dawes³ and Beng H. Chong¹. *Haematology Department, Prince of Wales Hospital, NSW, 2031*¹, *Baylor College of Medicine, Houston, TX, USA*², and the *School of Biochemistry and Molecular Genetics, UNSW, NSW, 20523*.

The sulphation of tyrosine is an important post-translational modification in molecules involved in protein-protein interactions. The interaction between platelet glycoprotein (GP) Ib-alpha and plasma von Willebrand factor (vWf) is crucial in maintaining haemostasis in arterial flow. Three tyrosine residues in GPIb-alpha have been previously reported to be sulphated and the sulphation has been shown to be required for vWf-binding. This investigation has identified specific residues in GPIb-alpha that are involved in regulating tyrosine sulphation. It is known that the region where the sulphation occurs is highly acidic. The carboxylic acids between glutamic acid 270 and aspartic acid 297 were changed by site-directed mutagenesis to the neutral amino acids, glutamine and asparagine, respectively. The mutations were expressed in CHO cells previously transfected with GPIb-beta and GPIX. Static binding studies were performed to determine what affect the mutations had on vWf-binding. Metabolic labeling of the cells was performed to determine if the mutant receptor contained sulphated tyrosine residues. Mutations to glutamic acid 270 (E270Q) and aspartic acid 283 (D283N) produced a receptor without tyrosine sulphation. vWf-binding was decreased by 50% in the E270Q, and 70% in the D283N cells. Other mutations affected vWf-binding without affecting sulphation. This is the first demonstration of specific amino acids involved in regulating the sulphation of tyrosine residues in the platelet GPIb-alpha receptor.

20.

THE ETS FAMILY OF TRANSCRIPTION FACTORS AND REGULATION OF THE MEGAKARYOCYTE SPECIFIC GENE GPIX DURING MEGAKARYOCYTE DIFFERENTIATION. M Eisbacher, LM Khachigian, BH Chong. The Centre for Thrombosis and Vascular Research, University of New South Wales and Department of Haematology, Prince of Wales Hospital, Sydney Australia

The regulation of lineage restricted gene expression during megakaryocyte (MK) development is poorly understood. Glycoprotein (GP) IX is a MK specific gene expressed toward the latter stages of MK development and is believed to play an essential role in stabilising the formation of the GPIb-IX complex on the MK/platelet surface. This study used phorbol-12 myristate 13-acetate (PMA) induced differentiation of leukaemic Dami cells as a model system to investigate the regulation of GPIX gene expression during MK differentiation. Northern blot analysis revealed that GPIX mRNA expression increased within 12h of exposure to PMA (100nM) and remained elevated up to 48h. Induction of GPIX mRNA was also dose dependent. Pre-treatment of Dami cells with cycloheximide abolished PMA inducible GPIX expression suggesting the requirement of new protein synthesis. Analysis of cell surface protein expression by flow cytometry confirmed these findings with increased surface expression after 5 days. To determine the transcriptional basis of these observations 600bp of the GPIX promoter was isolated and cloned upstream of a luciferase reporter construct. Dami cells were transiently transfected with the reporter and incubated with PMA. PMA produced a 4-5 fold increase in GPIX dependent reporter activity. To localise the PMA response elements we constructed nested 5' deletions of the parent promoter using exonuclease III and PCR. Transient transfection of these constructs demonstrated a significant decrease in both basal and PMA-inducible gene expression mediated by the region between -65 and -18 upstream of the transcriptional start site. This region contains a consensus nucleotide recognition element 5'CTTCCT3' for the Ets family of transcription factors. Mutation of the Ets binding site (EBS) in both the parent promoter and a shorter construct abolished PMA-inducible expression, further confirming the importance of this site. The Ets family member Fli-1 has previously been shown to bind the EBS of the GPIX gene and is able to transactivate the GPIX gene in non-haematopoietic cells. Work performed in our laboratory has shown that although Fli-1 is able to transactivate the GPIX gene in non-haematopoietic cells, over-expression of the same Fli-1 construct in Dami cells dramatically represses PMA-induced GPIX reporter activity, suggesting that Ets members other than Fli-1 may regulate GPIX in Dami cells. Ets family members are known effectors of the Mitogen activated protein kinase (MAPK) signalling pathway. To investigate the upstream signalling events associated with PMA-induced expression of the GPIX gene, Dami cells were pre-incubated with the MAPK kinase inhibitor PD98059 or the p38 inhibitor SB202190. Pre-incubation with increasing amounts of PD98059 inhibited PMA-inducible GPIX reporter activity in a dose dependent manner. In contrast SB202190 had no significant effect. These results demonstrate the requirement of the upstream activation of MAPKK for PMA-inducible expression of GPIX. This study demonstrates the capacity of PMA to modulate GPIX expression in MK cell lines and the potential importance of specific Ets factors and the MAPK signalling pathway in MK differentiation. A greater understanding of the regulation of MK specific gene expression during differentiation may provide further insights into the controlling elements of lineage commitment.

21.

STRUCTURE-BASED DESIGN: THE ACTIVATION OF ANTITHROMBIN III BY HEPARIN AND ITS INTERACTION WITH FACTOR XA AND THROMBIN J.C. Whisstock¹ A.M. Lesk² & R.N. Pike¹. ¹*Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia.* ²*Cambridge Institute of Medical Research. Wellcome / MRC building, Hills Road, Cambridge UK.*

Antithrombin is a serpin under conformational control. Interaction with the sulphonated polysaccharide heparin induces expulsion of the Reactive Centre Loop (RCL) and enhanced affinity for the cognate proteinases thrombin and factor Xa. The recently determined X-ray crystal structure of antithrombin bound to a specific pentasaccharide has allowed a detailed analysis of the mechanism of antithrombin activation by heparin. These data reveal the role of different saccharide units within the pentasaccharide in inducing a series of structural shifts that lead to RCL expulsion.

Efficient inhibition of thrombin by antithrombin is mediated *via* ternary complex formation between antithrombin, thrombin and full length heparin. Heparin pentasaccharide is not long enough to bridge between antithrombin and thrombin and only induces a 2-fold increase in the inhibitory potential of antithrombin vs. thrombin. In contrast, pentasaccharide alone is sufficient to induce a 300-fold increase in the inhibitory activity of antithrombin vs. factor Xa. To try to explain these data we have built molecular models of the encounter complex between antithrombin-thrombin and antithrombin-factor Xa. We have identified specific differences in the active sites of thrombin and factor Xa that may account for the ability of heparin pentasaccharide alone to mediate efficient inhibition of factor Xa.

22.

NEW DRUG DISCOVERIES: RECOMBINANT RECEPTORS, X-RAY CRYSTALLOGRAPHY AND RATIONAL DRUG DESIGN TARGET Fc RECEPTORS. PM Hogarth*, M Powell*, B Matthews§, T McCarthy§, G Pietersz*, T Bradford*, T Garrett§. *Austin Research Institute, Kronheimer Building, Austin Campus A&RMC, Studley Rd, Heidelberg, 3084, Australia; §Biomolecular Research Institute, 343 Royal Pde, Parkville, 3052, Australia.

We have used recombinant receptors together with x-ray crystallography and structure based drug design to develop a range of compounds that inhibit Fc receptor dependent platelet activation.

Structure based, or rational drug design is emerging as a powerful approach to the generation of new drugs. As the human genome project draws to a close, the 100,000 or so proteins are potential targets for the development of new pharmaceuticals. The structure of few of these proteins is currently known, but as efforts to produce these improve and as the technologies of structure determination advance, there will be a rapid expansion of knowledge of protein structure and function. The determination of the three dimensional structure of proteins allows the precise definition of active sites of proteins, and therefore the design of small molecule inhibitors. We have applied this approach to the design of new anti-inflammatories and platelet function modifying drugs. Immune complexes are potent activators of platelet function through the Fc γ RII. Fc γ RIIa is the only IgG receptor of platelets and immune complex binding for this receptor results in rapid receptor phosphorylation leading to platelet activation and aggregation, both in experimental models and in pathological disease processes. The Fc γ RIIa is a transmembrane glycoprotein, consisting of two extracellular domains, a membrane spanning region and cytoplasmic tail. The cytoplasmic tail contains immunoreceptor tyrosine activating motifs and is one of the few immunoreceptors to contain such a signalling motif in the ligand binding chain, in stark contrast to the IgE receptor, Fc ϵ RI, the T cell receptor and most other immunoreceptors where such signalling motifs are in non-covalently associated signalling subunits.

We adopted two strategies to generate potential new pharmaceuticals. The first using recombinant soluble ectodomain from Fc γ RII, and the second, structure based drug design. Recombinant soluble forms of Fc γ RIIa were engineered and contain the two extracellular ligand binding domains. The experiments *in vitro* indicate that the soluble receptor was able to inhibit immune complex induced activation of platelets and established the principle that interfering with the immune complex Fc:receptor interaction offers a new approach to the generation of drugs designed to protect platelets from this form of destruction. The use of x-ray crystallography and rational or structure based drug design offers an alternative to the use of large molecular weight protein receptor entities as pharmaceuticals. Previously successfully designed drugs include Relenza and those targetting HIV protease, all of which are directed at inhibiting enzyme functions. The greater difficulty inhibiting protein:protein interactions, which usually involve large featureless surfaces, present difficulties in this approach to the development of new drugs. However, the Fc receptor structure provides a geometrically and chemically suitable target area. The extracellular domains of Fc γ RIIa were expressed in six different systems and receptor produced in insect cells crystallised and the structure of these extracellular domains solved at 2Å resolution. The receptor appears as a dimer bringing together the Ig binding surfaces of each mo

These data indicate that the design of highly specific receptor antagonists is possible. This work heralds a new era in the development of drugs for the treatment of inflammatory diseases.

23.

HEPARANASE: A NOVEL NEW TARGET FOR ANTI-CANCER AND ANTI INFLAMMATORY DRUGS. Chris Parish, Craig Freeman and Mark Hulett *Division of Immunology and Cell Biology, John Curtin School of Medical Research, ANU, Canberra, ACT 2601*

Heparan sulfate (HS) is an important component of the extracellular matrix (ECM) and the vascular basement membrane (BM) which function as barriers to the migration and vascular extravasation of metastatic tumour cells and leukocytes. Cleavage of the HS chains by heparanase activity produced by invading cells assists in the disassembly of the ECM and facilitates cell migration. Heparanase therefore represents an excellent target for the development of drugs to inhibit the passage of cells through the BM.

Recently we developed a simple and rapid assay for heparanase activity that has facilitated purification of the enzyme to homogeneity, cloning of the enzyme and screening for enzyme inhibitors (1). Our findings indicate that the same heparanase enzyme is expressed in platelets, T lymphocytes and metastatic tumour cells with normal heparanase expression being restricted to the placenta and lymphoid organs. However, the enzyme is upregulated in metastatic tumour cells. Exhaustive studies have so far revealed only one heparanase sequence, consistent with the view that this enzyme is the dominant endoglucuronidase in mammalian tissues.

In parallel studies sulfated oligosaccharide-based inhibitors of heparanase activity have been developed (2). A lead compound, phosphomannopentaose sulfate (PI-88), is an effective inhibitor of angiogenesis, tumour metastasis and inflammation. The drug has successfully completed a Phase I clinical trial in healthy volunteers and is currently being tested in cancer patients.

1. Hulett, M.D., Freeman, C., Hamdorf B.J., Baker, R.T., Harris, M.J. and Parish, C.R. (1999). Cloning of mammalian heparanase, an important enzyme in tumour invasion and metastasis. *Nature Medicine* 5, 803-809.

2. Parish, C.R., Freeman, C., Brown, K.J., Francis, D. and Cowden, W.B. (1999). Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel *in vitro* assays for angiogenesis and heparanase activity. *Cancer Research*, 59, 3433-3441.

24.

AN EVALUATION OF SPECIFIC GENETIC VARIANTS OF FACTOR V, PROTHROMBIN, METHYLENETETRAHYDROFOLATE REDUCTASE, FIBRINOGEN AND PLASMINOGEN ACTIVATOR INHIBITOR-1 AS PREDICTIVE MARKERS OF DEEP VENOUS THROMBOSIS AFTER ELECTIVE LOWER LIMB ARTHROPLASTY. DDF Ma¹, KF Cheong¹, JE Joseph¹, A Dominguez¹, B Courtenay², M Neil², M McGrath³. *Departments of Haematology¹, Orthopaedics², and Vascular Medicine³, St Vincents Hospital, Victoria St Darlinghurst, NSW 2010.*

A number of studies have demonstrated that variants of factor V (G1691A, FV Leiden), prothrombin (G20210A), methylenetetrahydrofolate reductase (MTHFR C677T), B β -fibrinogen (-455G \rightarrow A) and plasminogen activator inhibitor-1 (PAI-1 -675 4G/4G) are associated with increased risk of thrombosis. Lower limb arthroplasty carries a significant risk of post-operative venous thromboembolism in spite of prophylaxis, and the role that these gene variants may play is not fully defined. In this ongoing prospective study, testing of the above genetic variants in successive patients undergoing elective total hip arthroplasty (THA) and total knee arthroplasty (TKA) was performed. Mutations of factor V, prothrombin, MTHFR, B β -fibrinogen, and PAI-1 genes were identified by PCR-based assays and restriction analysis. All patients also underwent routine lower limb duplex ultrasonography approximately 7-10 days post operatively.

To date, 194 consecutive patients have been enrolled in the study and an interim analysis will now be described. THA was performed in 115 (59%) patients, and TKA was performed in 79 (41%) patients. The incidence of post operative deep venous thrombosis was significantly higher following TKA (57%, n=45) compared to THA (10.4%, n=12; p<0.0001). The incidence of the prothrombin (G20210A) and factor V (G1691A) genotypes was 2.6% and 6.2% respectively. There was no significant difference in prevalence of either genotype between DVT and non-DVT patients, or THA and TKA patients with DVT. No patients were found to be homozygous for either of these genotypes. The homozygous MTHFR (C677T) genotype was found in 11.3% (n=22) of all patients, and its frequency was significantly higher in TKA patients with DVT (20%, n=9) compared to TKA without DVT (2.9%, n=1; p<0.05 Fisher exact test). The B β -fibrinogen (-455G \rightarrow A) homozygous genotype was detected in 4.6% all patients. There was a slightly increased incidence in THA patients with DVT (16.7%, n=2 of 12) compared to THA patients without DVT (3.9%, n=4 of 103), however this difference did not reach statistical significance. The PAI-1-675 4G/4G homozygous genotype was isolated in 25.3% of all patients, and there was no significant difference in prevalence between DVT and non-DVT patients, or THA and TKA patients with DVT.

In summary, the preliminary findings from this study show that the homozygous MTHFR (C677T) genotype may be a significant factor in determining which patients will develop ultrasonographic evidence of DVT following TKA. It is also possible that the B β -fibrinogen (-455G \rightarrow A) homozygous genotype may provide an increased risk factor for DVT, but more patients will need to be studied. Further follow-up and recruitment of patients is continuing, and an examination of correlation with clinical evidence of DVT and other risk factors (eg. age, obesity, type of anaesthesia) will also be performed.

25.**AN EVALUATION OF APC-RESISTANCE AS A RISK FACTOR FOR POSTOPERATIVE DEEP VENOUS THROMBOSIS AFTER ELECTIVE LOWER LIMB ARTHROPLASTY. J Low¹, DDF Ma¹, A Dominguez¹, B Courtney², M Neil² and M McGrath³. Departments of Haematology¹, Orthopedics² and Vascular Medicine³, St Vincent's Hospital, Victoria St, Darlinghurst, Australia 2010.**

Lower limb arthroplasty carries a significant risk of post-operative venous thromboembolism in spite of prophylaxis. Targeting more intense prophylaxis to patients with higher risk may reduce the rate of thrombosis and reduce therapy related complications. APC-resistance and the FV Leiden mutation have recently been identified as such potential risk factors. However, the clinical usefulness of pre-operative screening is unproven. This study is part of an ongoing prospective study of specific genetic variants in successive patients undergoing elective total hip arthroplasty (THA) and total knee arthroplasty (TKA).

Preop-operative APC-resistance was measured by 2 different commercially available kits - Chromagenix Coatest (CXAPC) and Gradipore Gradileiden V (FVL). Anticoagulated patients were excluded from the study and dilution of test plasma in FV deficient plasma was not done in order to detect APC-resistance not related to FV Leiden. CXAPC and FVL ratios were expressed as normalised ratios. APTT, fibrinogen and kaolin clotting time (KCT) were also measured. Post-operative DVT was detected by lower limb duplex ultrasonography performed 1-2 weeks after surgery.

To date, results from 144 patients have been analysed. There was no significant difference in the mean CXAPC, median FVL or the median of any of the other coagulation variables measured, between the 36 patients that developed DVT and the 108 non-DVT patients (see Table). There was also no significant difference in the coagulation variables between the group of 95 THA patients (9 or 9.6% with DVT) and the group of 49 TKR patients (27 or 55% with DVT).

	DVT (n= 36)		No DVT (n=108)		
CXAPC	0.87	(±0.33)	0.90	(±0.30)	NS
FVL	1.04	(0.95 – 1.13)	1.00	(0.91 – 1.09)	NS
APTT	34	(32 – 35)	33	(30 – 34)	NS
KCT	74.2	(66.7 – 83.9)	72.7	(64.9 – 79.6)	NS
Fibrinogen	3.5	(3.27 – 3.84)	3.39	(3.05 – 3.89)	NS

Results are expressed as medians (25th – 75th percentile range) except for CXAPCC (mean ± SD)

13 of the patients in this study were found to be heterozygous for FV Leiden. The FVL method was found to be superior to the CXAPC in distinguishing between heterozygotes and normals. FVL ratios were 0.30 - 0.36 for heterozygotes , and > 0.64 for normals. CXAPC ratios for heterozygotes (0.61 – 0.86) overlapped with normals (>0.71).

Our interim analysis does not support the hypothesis that APC-resistance is a significant risk factor for DVT in this group of patients. Further studies are underway.

26.**THROMBOEMBOLIC DIATHESIS IN PULMONARY EMBOLISM: RELEVANCE OF ACTIVATED PROTEIN C RESISTANCE. ML Kaley-Zylinska, A Bennet, PA Ockelford, Venous Thromboembolism Unit, Haematology Dept, Auckland Hospital Auckland, New Zealand**

Consecutive patients presenting to the Thromboembolism Unit at Auckland Hospital have undergone evaluation after six months of secondary warfarin prophylaxis to detect an underlying thrombophilia. The patients at clinical presentation have been divided into those with DVT +/- PE or PE alone. In this review the patients with isolated pulmonary embolism have been considered as a subgroup, and the results of thrombophilia testing have been compared with the findings in those with deep venous thrombosis with or without associated PE. Isolated PE was defined as a clinical presentation with symptoms of PE, confirmed by objective testing, and with normal limb examination or negative limb ultrasound if there was clinical suspicion of a lower limb thrombosis. The majority of patients had a high probability V/Q scan. Thrombophilia testing was performed in 48 of the 67 patients with PE available for analysis. In this group there were no individuals with Antithrombin III, Protein C or Protein S deficiency. Activated protein C resistance / Factor V Leiden was observed in 8.8% (compared 29.4% for DVT) and the prothrombin mutation was identified in 5.9% (cf 12.5% DVT). The incidence of APCr/ FVL mutation in patients with isolated PE is significantly ($p = 0.01$) less than that observed in patients with co-existing DVT. In contrast, in those patients considered to have clinical thrombophilia on the basis of a family or personal history of VTE, the incidence of activated Protein C resistance due to the Factor V Leiden is no different (30%) than those presenting with DVT. The reduced association of FVL with new isolated PE is consistent with previous reports of a lesser frequency of isolated PE as a presentation for patients with this mutation. The mechanism and significance of this observation remains uncertain.

27.**A RANDOMIZED DOUBLE BLIND PLACEBO CONTROLLED TRIAL OF LOW-MOLECULAR WEIGHT HEPARIN VERS PLACEBO TO PREVENT VENOUS THROMBOLIC EVENTS AFTER CAESAREAN SECTION: A PILOT STUDY. Burrows RF¹, Gan ET¹, Gallus AS², Wallace EM¹. ¹Monash University, Monash Medical Centre, ²Flinders Medical Centre**

Objective: To pilot a protocol for a national multicentre randomized trial in which a low molecular weight heparin (Fragmin) will be compared to placebo in prevention of venous thrombotic events immediately and for up to 6 weeks post caesarean section.

Methods: Consenting patients having had an emergency or elective caesarean section were commenced on study medication 6-24 hours post operatively. The study medication, Fragmin 2500iu or saline, is given sc once daily for 4 or 5 days post operatively depending on their length of stay. Patients are reviewed in hospital for operative outcomes and contacted at 2 and 6 weeks postoperatively. Any outcomes are then followed up and confirmed.

Results: The study ran for 5 months, July 1 - November 31 1999. During this time there were 1185 deliveries and 289 caesarean sections. Of the 141 patients given information 76 (54%) consented, overall 26.2% of caesarean section patients entered the trial. The demographics and major outcomes are presented below. All patients were follow-up to 6 weeks post operatively.

Demographics of patients randomised in this trial

N	Fragmin		Control		p
	39		37		
AGE (years)	31.7	(4.8)	31.3	(5.5)	0.7
Gestation (weeks)	38.38	(2.4)	37.95	(2.84)	0.5
Weight (kg)	81.7	(17.2)	79.9	(14.0)	0.7
Elective caesarean	26/39	66.7%	20/37	54.1%	0.3
Emergency caesarean	13/39	33.3%	17/37	45.9%	0.3
Epidural	6/39	15.4%	8/37	21.6%	0.6
Spinal	33/39	84.6%	24/37	64.9%	0.06

Outcomes of randomized patients

N	Fragmin			Placebo			p
	39			37			
Antibiotics post-op	2/39	5.1%	(0.6-17.3)	1/37	2.7%	(0.1-14.2)	0.5
Transfusion	0/39	0%	(0-9.0)	1/37	2.7%	(0.1-14.2)	0.5
Major bleed	0/39	0%	(0-9.0)	0/37	0%	(0-9.5)	-
Major wound disruption	0/39	0%	(0-9.0)	0/37	0%	(0-9.5)	-
Major reaction	0/39	0%	(0-9.0)	0/37	0%	(0-9.5)	-
Major bruising	0/39	0%	(0-9.0)	0/37	0%	(0-9.5)	-
DVT/PE on leaving hospital	0/39	0%	(0-9.0)	0/37	0%	(0-9.5%)	-
DVT/PE 2 weeks	0/39	0%	(0-0.9)	0/37	0%	(0-9.5)	-
DVT/PE 2-6 weeks	1/39	2.7%	(0.1-13.5)	0/37	0%	(0-9.5)	1.0

Conclusion: The 26% recruitment, a sample size estimate that is reasonable given the thrombosis rate of 1.3% seen in this pilot, the absence of negative operative outcomes and our ability to contact participants 2 and 6 weeks postoperatively, indicates that this study is feasible.

28.

THE RISK OF APROTININ IN ASSOCIATION WITH FACTOR V LEIDEN DURING CARDIOPULMONARY BYPASS: INVESTIGATION USING AN EX VIVO MODEL Linden M.D.†, Schneider M.‡, Erber W.N.† †Haematology, The Western Australian Centre for Pathology and Medical Research, Nedlands and ‡Department of Anaesthesia, Fremantle Hospital, Western Australia.

Previous studies have shown laboratory evidence suggesting aprotinin may cause a significant increased risk of peri-operative thrombotic complications in patients with Factor V LEIDEN. It has been suggested that this is due to its demonstrated ability to competitively inhibit activated protein C (APC) function in vitro. Confirmation of this effect in vivo has been difficult due to the relatively low population frequency of Factor V LEIDEN (4.0% in western Caucasian populations) leading to small patient cohorts. Furthermore limitations of treatment and blood sampling under cardiac surgical conditions impede laboratory investigation of clinical samples. Therefore an ex vivo model was developed to mimic the effects of cardiopulmonary bypass with the exclusion of the patient. This model allows for a more flexible approach to pharmacological management and sampling than would be feasible in a theatre situation. Blood was collected by venesection of 2 normal donors and 2 Factor V LEIDEN heterozygotes into ACD bags at 37 C. One of each of normal and Factor V LEIDEN heterozygote bloods were treated with aprotinin and the others with placebo (saline). Heparin was administered to each bag before it was recalcified and added to a pump prime containing crystalloid saline, further heparin and aprotinin/placebo. The blood was then circulated at 2L/min at 30-32 C through a modified cardiopulmonary bypass circuit with a paediatric oxygenator. Blood samples, drawn at specific intervals were analysed in duplicate for APC Ratio, antithrombin concentration, and heparin concentration. After 60 minutes of circulation the heparin was neutralised by protamine sulphate injections. Results showed a decrease in APC Ratio for both Factor V LEIDEN and normal bloods with the addition of aprotinin. This decrease was not observed in the placebo treated bloods. All bloods exhibited a decrease in APC Ratio at the commencement of cardiopulmonary bypass. After the commencement of cardiopulmonary bypass the APC Ratio of the Factor V LEIDEN blood treated with aprotinin had decreased by 36% to 0.62 (reference range 1.9-4.0). This extremely abnormal value may represent an increased risk of peri-operative coagulation. Analysis of heparin concentration showed an average 51% decrease in heparin concentration at the commencement of cardiopulmonary bypass. This was greater than the predicted decrease due to haemodilution with crystalloid (19%). These data suggest that heparin was removed or consumed by some mechanism of cardiopulmonary bypass. Plasma antithrombin levels decreased at the commencement of bypass. This was consistent with the predicted decrease based on haemodilution (58%). Thus antithrombin was not consumed during the experiment. The data from this model predicts an increased risk of peri-operative thrombosis due to inhibition of APC function in cardiac surgical patients heterozygous for the Factor V LEIDEN mutation who receive aprotinin. Further investigation of this phenomenon is required to determine if the benefits of aprotinin may be offset by potential risk in these patients. The ex vivo model employed was an effective tool for the investigation of the haemostatic effect of aprotinin, heparin and protamine. This model may be exploited for other applications such as the investigation of novel or emerging agents prior to clinical trial.

29.

PRELIMINARY EVALUATION OF POLYMERASE CHAIN REACTION (PCR) METHOD FOR THE DIAGNOSIS OF INVASIVE FUNGAL INFECTION. WN Patton¹, J Scotter², T Anderson², L Jennings², M Schousboe², W Chan¹, J Stevens¹, P Ganly¹. *Departments of Haematology¹ and Microbiology², Canterbury Health Laboratories, PO Box ISI, Christchurch, New Zealand.*

Invasive fungal infections (IFI) remain an important cause of morbidity and mortality amongst immunocompromised patients and invasive aspergillosis (IA) has become the leading infectious cause of death following allogeneic bone marrow transplantation. Current methods of diagnosis of IFI are unreliable, prophylactic measures are only partially effective, treatment strategies are usually empirical and available drugs for IA are potentially toxic or expensive. Improved methods for the diagnosis of IFI are needed and a PCR based approach has the potential to greatly enhance the specificity, sensitivity and speed of diagnosis of IFI and to target anti-fungal therapy more effectively. One such technique has described the detection of fungal pathogens in the blood of selected patients with febrile neutropenia (J Clin Microbiol 1997; 35: 1353-60) and we have developed a similar method with design modifications. Important principles include the amplification of a target DNA sequence within the 18S rRNA gene highly conserved amongst common fungal pathogens; measures to isolate fungal DNA from within the cell wall and to separate it from human DNA; and the use of PCR ELISA for the sensitive, semi-quantitative detection and species identification of the digoxigenin labelled PCR product. Extraction of DNA from *Aspergillus* species and contamination issues have proved problematic but the assay is now operable. Preliminary data has shown positive PCR results in two patients (CGL allograft recipient and case of AML) with clinically proven IA (12 out of 27 samples tested), whereas samples have been PCR negative in 10 normal controls and in one autograft recipient without any evidence of fungal infection. In one of these cases of proven IFI samples (n=3) were available two weeks prior to the onset of therapy with systemic amphotericin B (amph B) and these were all PCR positive. One further patient (ALL induction) gave positive results for IA in 7/8 samples tested prior to amph B therapy for *Candida Krusei* septicaemia. The remaining patient tested, a case of myeloma with renal failure and proven aspergillus infection of CAPD fluid, was also PCR positive for IA in blood and CAPD fluids. These preliminary results are most encouraging and data from further testing of a bank of prospectively collected samples of patients with and without proven IFI will be presented.

30.

HIGH SUCCESS RATE IN USE OF LIPID-BASED AMPHOTERICIN PREPARATIONS FOR RESCUE OF PATIENTS WITH HAEMATOLOGICAL MALIGNANCIES WHO HAVE SUSPECTED OR CONFIRMED FUNGAL INFECTION AND ARE INTOLERANT OF OR REFRACTORY TO CONVENTIONAL AMPHOTERICIN B. R P Herrmann, P K Cannell, R I Baker, A L Barr, and M Trent, Haematology Department, Royal Perth Hospital, Wellington Street, Perth, Western Australia, 6000

Conventional Amphotericin B (c-Amb) has been the mainstay of treatment of systemic fungal infection for 40 years. Renal toxicity varying from electrolyte abnormalities to renal failure is seen in virtually 100% of patients on protracted dosing. The availability of amphotericin B encapsulated into liposomes or bound to other lipid carriers has resulted in greatly reduced toxicity and increased therapeutic index.

Two preparations are available approved for marketing in Australia, Ambisome and Abelcet.

Forty six patients with haematological malignancies were treated in our institution with either preparation over a period of 3 years for suspected (n=30) or confirmed (n=16) systemic fungal infection. At Royal Perth Hospital it is a requirement that patients demonstrate 50% rise in serum creatinine above baseline, 50% for below baseline of creatinine clearance, inability to maintain potassium, sodium or magnesium balance or anaphylaxis before such preparations are used.

The median absolute neutrophil count at onset of liposomal potassium was $0.5 \times 10^9/L$ ($0.0-7.5 \times 10^9/L$) and median white cell count at the end of treatment was $3.4 \times 10^9/L$ ($0.1-34 \times 10^9/L$). Complete resolution of fungal infection was obtained in 24 of 30 (80%) of those with suspected fungal infection and 12 of 16 (75%) are those with confirmed fungal infection.

These results indicate that successful salvage can be obtained with lipid-based amphotericin preparations in patients with highly suspected or confirmed systemic fungal infection and the use of lipid-based preparations should be considered early in those with confirmed infection to diminish toxicity and optimise conditions for the patient's survival, particularly as protracted therapy may be required.

31.

A REVIEW OF THE EFFICACY AND TOXICITY OF LIPOSOMAL AMPHOTERICIN PREPARATIONS (\pm ITRACONAZOLE) FOR THE TREATMENT OF INVASIVE FUNGAL INFECTIONS. M MOLONEY, M SLAVIN, HM PRINCE, JF SEYMOUR, MM WOLF, EH JANUSZEWICZ, N HETHERINGTON, N TREVASKIS, M DOOLEY.

Departments of Pharmacy, Infectious Diseases and Haematology, Peter MacCallum Cancer Institute (PMCI), East Melbourne, Victoria, Australia.

Patients (pts) with invasive fungal infections in the setting of neutropenia or immunosuppression, have a poor response to treatment with conventional amphotericin B deoxycholate (CAB). Additionally, optimal dose and treatment duration of CAB are often compromised by nephrotoxicity. Liposomal amphotericin formulations can be used in this setting due to their more favourable toxicity profile, and possibly greater efficacy, but are significantly more expensive. The features and outcomes of all pts who received treatment with liposomal amphotericin (Ambisome[®], AMB; or Abelcet[®], ABLC) at PMCI from 1 – 12/99 were retrospectively reviewed. 12 pts received 13 courses of treatment. The underlying disease was ALL (3 pts), AML (4), CLL (3), variant hairy-cell leukaemia (1), non-small cell lung cancer (1) and non-Hodgkin's lymphoma (NHL) (1). Organisms responsible were *Aspergillus* species (6 pts), *Aspergillus* plus *Mucor* (1 pt), *Mucor* (1 pt), suspected *Aspergillus* (4pts), 2 of whom were later found to have *Legionella pneumonia* at autopsy. All pts had pulmonary involvement by the infection. 10 pts had received prior antifungal therapy with CAB at a dose of 1mg/kg/d, for a mean duration of 7.2 days (range 2 - 17). Liposomal amphotericin was initiated due to CAB- induced nephrotoxicity in 8 cases; mean change from base line serum creatinine (SeCr) of 0.10 mmol/L (range 0.04 - 0.13), mean peak SeCr 0.16 mmol/L (0.1 - 0.22). 4 pts commenced liposomal amphotericin due to progression of fungal infection despite CAB treatment. The mean dose of AMB was 3.9mg/kg/d (2-5mg/kg/d, 10 pts), and for ABLC was 5mg/kg/d (3 pts). 1 pt received ABLC followed by AMB. The mean duration of treatment was 27 days (range 3 - 86). Of the 10 pts with proven or strongly suspected infection, 5 were cured (mean follow-up 10 months; range 5 - 16) and 1 pt subsequently underwent allogeneic bone marrow transplant with antifungal cover without reactivation of fungal infection. 1 pt improved and remains on itraconazole after 8 months. 4 pts died; 1 from progressive *Mucor*, 1 of relapsed disease (ALL), 1 from progressive *Aspergillus* and 1 of bowel perforation due to neutropenic enterocolitis. The median duration of treatment with liposomal amphotericin in the 5 cured pts was 39 days (22 – 63), and the underlying disease was; ALL (2 pts), and AML, CLL and variant hairy-cell in 1 case each. Two of the cures were achieved after courses of oral itraconazole (2 weeks and 13 months continuing course respectively). Of the 8 pts who developed nephrotoxicity with CAB, 3 manifested further deterioration during treatment with AMB; baseline / peak SeCr were 0.14 / 0.15, 0.09 / 0.16, and 0.20 / 0.22 mmol/L, respectively. 2 pts experienced back pain upon infusion of AMB. 1 pt experienced a rigor and 1 had intractable vomiting after ABLC. The total cost of liposomal amphotericin was approximately \$360,000 (mean of \$51,790 per cured pt). Guidelines for liposomal amphotericin usage at PMCI for the treatment of proven or suspected invasive *Aspergillus* include treatment failure of CAB or nephrotoxicity (50% increase in SeCr or SeCr > 0.17 mmol/L) resulting from a course of CAB. For *Mucor* infections, the initial use of liposomal amphotericin may be warranted. We conclude that the response rate for liposomal amphotericin preparations in patients refractory to, or intolerant of, CAB is high and treatment of stable or responding *Aspergillus* infection with liposomal amphotericin may be shortened by the initiation of itraconazole.

32.

LOW DOSE VALACICLOVIR FOR THE PREVENTION OF CMV DISEASE POST ALLOGENEIC BONE MARROW TRANSPLANTATION. Irving I Williamson J, Sloots T, Siebert D, Mollee P, Morton J, Durrant S. Irving I, Department of Bone Marrow Transplantation Royal Brisbane Hospital Herston Rd HERSTON Queensland 4029

Cytomegalovirus (CMV) disease remains a potentially fatal complication of allogeneic Bone Marrow Transplantation (BMT). The optimal regimen for CMV prophylaxis has not yet been agreed upon. Both ganciclovir prophylaxis and intravenous aciclovir followed by high dose valaciclovir (8 grams per day in 4 divided doses) have been shown to be efficacious in reducing the incidence of CMV disease post BMT. However both these strategies have failed to deliver any survival benefits Low dose valaciclovir (2 grams per day in 2 divided doses) has not been studied in the setting of allogeneic BMT.

There is a similar lack of consensus over which methodology to apply to CMV surveillance. Antigenaemic (eg. the pp65 antigen test) and PCR methods have been shown superior to culture based methods with the role of quantitative PCR unclear.

Eighty-five (85) consecutive allografts performed at the Royal Brisbane Hospital (RBH) between August 1998 and January 2000 were reviewed. Forty (40) were fully matched sibling transplants and 45 were Matched Unrelated Donor (MUD) or mismatched transplants. All 40 sibling allografts received aciclovir 1500mg/m² per day in 3 divided doses from D-1 to D+21 followed by valaciclovir 2 grams per day in 2 divided doses until D+100. Thirty-five (35) of the 45 MUD/Mismatched group received this regimen of aciclovir/valaciclovir while 10 received prophylactic ganciclovir. All patients underwent CMV surveillance testing every 2 weeks from D+28 until D+84 and then monthly until day D+140 with the pp65 antigen test, qualitative plasma PCR and quantitative plasma PCR (Roche Diagnostics).

Overall, 17 of the 85 patients (20%) displayed CMV positivity on PCR at some point. Ten (10) of these 17 suffered CMV disease with 5/10 currently alive. Other matched sibling allografts 4/40 (10%) had positive PCR testing with 2 of these 4 suffering CMV disease. In the MUD/Mismatched group 13/45 (29%) had a positive PCR test for CMV. Ten (10) of these 13 suffered CMV disease.

In all cases of CMV disease the plasma PCR test was positive before the pp65 antigen test. The quantitative PCR test mirrored both the quantitation from the pp65 test (when applicable) and the clinical state of the patient.

In summary, low dose valaciclovir prophylaxis results in rates of CMV disease similar to published reports-which use either ganciclovir or high dose valaciclovir CMV prophylaxis. If validated in future studies this approach utilising low dose valaciclovir could yield significant cost savings and clinical benefits to patients undergoing allogeneic bone marrow transplantation. The plasma PCR test for CMV is more sensitive to the pp65 antigen test for CMV surveillance. Both the pp65 antigen test and the quantitative PCR test predicted the severity and outcome of CMV disease. Quantitative PCR of plasma may therefore be useful for both CMV surveillance and the monitoring of CMV disease.

33.

LOW DOSE ACICLOVIR PROPHYLAXIS IS INEFFECTIVE FOR THE PREVENTION OF HSV INFECTIONS FOLLOWING TRANSPLANT AND CHEMOTHERAPY. C Fernandes, M Slavin, HM Prince, JF Seymour, H Januszewicz, M Wolf, M Moloney. Departments of Pharmacy, Infectious diseases and Haematology, Peter MacCallum Cancer Institute, East Melbourne, Victoria

Introduction: The efficacy of aciclovir prophylaxis for herpes simplex virus (HSV) reactivation in patients (pts) following high-dose therapy and autologous blood cell transplantation (ABCT) or chemotherapy for haematological malignancies has not been extensively evaluated. The emergence of aciclovir-resistant HSV and breakthrough infections have been reported infrequently at commonly used doses such as 400mg PO bd. The Haematology Unit policy at PMCI has been to use low dose aciclovir prophylaxis of 200mg PO bd. Therefore we retrospectively evaluated the incidence of clinically suspected and microbiologically proven breakthrough HSV infection in haematology unit pts for the period Jan 1999 to Jan 2000.

Methods: A population of 90 consecutive pts undergoing ABCT or chemotherapy who received prophylactic aciclovir (200mg PO bd or 1mg/kg IV bd) was evaluated. Only pts with positive baseline HSV serology or those with 'unknown' HSV status at the commencement of therapy received aciclovir prophylaxis. Outcome measures were the incidence of (a) HSV treatment based on clinical diagnosis of 'breakthrough' infections (b) a HSV 'breakthrough' confirmed microbiologically by PCR, IF and/or culture and (c) response of breakthrough infections to treatment-doses of aciclovir.

Results: Of the 90 pts (50% male) evaluated, treatment comprised: ABCT in 51, chemotherapy without BC support in 39. The median age was 51 yrs (range 18 - 80). Breakthrough HSV infection was clinically suspected in 27 cases (30%), all of whom received treatment dose aciclovir for HSV 1 or 2 infection. Of these 27 pts, mucositis was evident in 25 and neutropenia $< 1.0 \times 10^9/L$ in 22. Treatment comprised; high-dose chemotherapy and ABCT in 16, chemotherapy alone in 10. Microbiological specimens were not sent in 9 pts. Among investigated pts ($n = 18$), 11 (61%) were confirmed HSV positive on PCR, IF or culture (HSV1 = 8, HSV2 = 3). Applying this rate of microbiological confirmation to the entire cohort of pts, yields an overall incidence of definite infection of 18%. Resistance was not clinically evident as lesions resolved in all treated patients. The rate of clinically suspected infection did not vary according to treatment group ($P = 0.51$).

Conclusion: Low dose aciclovir prophylaxis (200mg PO bd) appears to be inadequate in this patient population with an unacceptable 18% incidence of confirmed breakthrough infections. Given the limitation of current HSV assays and the need to treat 30% of patients (clinical diagnosis alone) the incidence of 'breakthrough' infections may indeed be higher.

34.

RELENZA® (ZANAMIVIR) FOR PRESUMED INFLUENZA INFECTION IN HAEMATOLOGY PATIENTS: THE PETER MACCALLUM CANCER INSTITUTE EXPERIENCE IN THE WINTER OF 1999. C Fernandes, HM Prince, M Slavin. Departments of Pharmacy, Infectious Diseases and Haematology, Peter MacCallum Cancer Institute, East Melbourne, Victoria

Background: Influenza outbreaks occur worldwide during the winter season leading to significant morbidity and mortality in high-risk patients (pts) such as those with compromised immune systems. Zanamivir (Relenza®) is a selective inhibitor of influenza A and B virus neuraminidases, which results in the prevention of viral spread across the mucous lining of the respiratory tract. Zanamivir treatment should be initiated within 48 hours from onset of symptoms since it can shorten the duration of infection and reduce infectivity. Whilst a nasopharyngeal aspirate (NPA) can be performed prior to treatment, results may be influenced by delays in transport to the laboratory and incorrect analysis may reduce the sensitivity of this test. There were three positive NPA's reported in the immediate time period prior to the use of zanamivir.

Methods: Pts with clinically suspected influenza infection received zanamivir 10mg (2 oral inhalations) twice daily for 5 days. NPA was performed and treatment initiated prior to result of NPA.

Results: Four pts were clinically diagnosed and treated within a two-week period and two pts (D, E) developed symptoms whilst inpatients. All pts were residing on a communal ward. All pts were nonsmokers and had flu-like symptoms accompanied with fever and one pt (C) had concurrent pneumocystis carinii pneumonia (PCP). Empirical systemic antibiotics were administered concurrently in all pts except E. All chest x-rays were clear with the exception of patient C. All subsequent NPA were negative. No adverse effects of the zanamivir were reported.

Discussion: The aim of empiric therapy is to treat suspected pts with 'high risk' factors based on clinical assessment rather than waiting for the NPA results. Although NPA's performed on these pts were all negative; they had a clinical syndrome associated with influenza. The negative NPA's may have been a consequence of the difficulties in obtaining an adequate nasal epithelium sample and a delay in laboratory analysis of the aspirate. The development of accurate and rapid virus detection techniques [i.e. PCR (polymerase chain reaction) assays. 'bedside' immunoassays] will provide better and prompt laboratory diagnosis.

Conclusion: Zanamivir is well tolerated and was associated with a rapid resolution of influenza symptoms in this group of immunocompromised pts. There was no progression to lower respiratory tract infection. The drug is now available on the hospital formulary for use in Influenza A and B viral infection. A prospective analysis of the drug will be conducted in the 2000 winter season in association with the trial of newer influenza detection kits and PCR assays.

Patient	Treatment Date	Age (yrs)	Sex	Condition	Neutropenia during treatment	Days to resolution of symptoms
A	19/08/99	69	F	AML(M ₂)	Yes	1
B	4/08/99	56	F	Myeloma	No	1
C	7/08/99	18	M	Lymphoma	No	4
D	8/08/99	57	F	PBSCT-BREAST	No	2
E	19/12/99	25	M	ALL	Yes	1

35.

A NOVEL XK GENE MUTATION IN A PATIENT WITH MCLEOD SYNDROME AND UNUSUAL MUSCLE PATHOLOGY. HJ Iland, SG Supple, MH Barnett, JD Pollard, AND F Yang. The Kanematsu Laboratories, The Institute of Haematology and The Institute of Clinical Neurosciences, Royal Prince Alfred Hospital, Camperdown, NSW and The Department of Medicine, University of Sydney, NSW.

A 29 year old male with a history of elevated creatine kinase and necrotising myopathy, which had been diagnosed and treated as polymyositis, underwent haematological and neurological review. Prominent red cell acanthocytosis was noted, and a diagnosis of McLeod syndrome was postulated. Kell antigen expression was reduced, but anti-Kx antibodies were unavailable for definitive diagnosis. An open quadriceps muscle biopsy demonstrated grouped necrotic fibres accompanied by striking mononuclear cell infiltrates positive for CD68. Muscle pathology of this type has not been reported previously in patients with McLeod syndrome. The patient's XK gene, which codes for the Kx antigen, was then PCR-amplified and sequenced, and a novel point mutation was identified in exon 3. This mutation, a TGG-to-TAG transition at position 1023, generates a new in-frame stop codon. The predicted product is therefore a truncated XK protein of 313 amino acids, compared with 444 in the normal length protein, and lacks the last two of ten putative transmembrane domains as well as eight of the ten putative cytoplasmic serine kinase phosphorylation sites. Normally the XK and Kell proteins exist as a disulfide-bonded complex on the cell surface. The disulfide bond is formed between Kell cysteine residue 72 (Cys72) and XK Cys347; the latter is located on the extracellular loop between the ninth and tenth XK transmembrane domains. Our data suggest that the McLeod syndrome in the patient reported here may be the result of failure to form a disulfide-linked membrane-associated XK-Kell complex due to loss of XK Cys347. A rapid screening test based on amplified restriction fragment length polymorphism at position 1023 of XK has been established for analysis of family members.

36.

THROMBOPOIETIN DERIVED AUTOLOGOUS CRYOPRESERVED PLATELET SUPPORT FOR PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANTATION. Mark Bentley¹, Kerry Taylor¹, Cathryn Kelly¹, Debra Taylor¹, Ben Leach², Robyn Rodwell¹, Robyn Minchinton², Sue Wright¹, Debra Fontana³, Kim Jardim-Surmin³, Dianne Baldry¹, Janet Coulston¹, April Fitzsimmons¹, Katrina Williams¹, Anne-Marie Kerwick¹, Silvana Lanzalone³.

¹Haematology Department Mater Hospital South Brisbane Australia ²Platelet Reference Laboratory ARCBS

³Pharmacia & Upjohn Sydney Australia and Milan Italy

Thrombopoietin (rhTPO) is a naturally occurring, glycosylated polypeptide that stimulates the differentiation of bone marrow stem cells into megakaryocytes and ultimately platelets. The potent biological effect of rhTPO (Genentech, Inc, CA, USA) could be utilised in cancer patients undergoing high dose chemotherapy (HDCT) and peripheral blood progenitor cell transplantation (PBPC) to allow the production, collection and cryopreservation of autologous platelets for subsequent transfusion to support the period of marrow hypoplasia. rhTPO-derived cryopreserved autologous platelets may have a number of potential advantages in comparison to homologous volunteer-donor platelets. This product would avoid potential infectious complications and transfusion-associated graft-versus-host disease, obviate the need for leucodepletion filters and premedication and prevent or circumvent platelet alloimmunisation. Cryopreservation of platelets may also provide a product with enhanced haemostatic capabilities and thereby improve overall outcomes of HDCT and PBPC. The aim of this randomised, open label, single centre study is to explore the feasibility and the efficacy of autologous platelet support obtained with two dose schedules of rhTPO administered to patients undergoing HDCT and PBPC for haematological and non-haematological malignancies. Patients receive either two doses of rhTPO 1.2mcg/kg intravenously four days apart or a single dose of rhTPO 2.4mcg/kg at the time of platelet recovery (platelets $>100 \times 10^9/L$) following PBPC mobilisation and collection. Patients with previously cryopreserved PBPC are also permitted to receive rhTPO if disease progression or relapse indicates the need to proceed to HDCT and PBPC. The primary end points of the study are the ability of these two dosing schedules to mobilise and cryopreserve adequate platelet numbers and the efficacy of subsequent autologous platelet transfusions during HDCT and PBPC to produce adequate corrected count increments (CCI) and prevent or control bleeding episodes. Additional objectives include a detailed analysis of in vitro measures of platelet function, including turbidoaggregometry, platelet function analysis and platelet surface glycoprotein patterns. Seven patients with a median age of 58 years (range; 50-70) have been enrolled in the study since commencement in December 1999. The indications for HDCT and PBPC included relapsed or primary refractory diffuse large cell non-Hodgkin's lymphoma (four patients), multiply relapsed follicular lymphoma (one patient), myeloma (one patient) and large cell transformation of follicular lymphoma (one patient). These patients had received a median of two prior treatment regimens (range; 1-3) prior to PBPC mobilisation. Six patients underwent successful PBPC mobilisation utilising high-dose cyclophosphamide and G-CSF; one patient failed to mobilise adequately, however had an adequate stem cell dose for PBPC which had been cryopreserved three years earlier in first complete remission. Four patients were randomised to receive two doses of rhTPO 1.2mcg/kg and three received 2.4mcg/kg as a single dose. Platelet apheresis was scheduled to begin when platelet counts rose above $600 \times 10^9/L$ or day 12 if that target level had not been reached. A median of 3 platelet aphereses per patient (range; 2-4) were performed to yield a median platelet collection of 25.97×10^{11} (range; 17.81-42.33) for cryopreservation. Five patients with a median age of 58 years (range; 51-70) have now successfully completed HDCT and PBPC, four of these with entirely autologous platelet support. No haemorrhagic complications were experienced in any of the patients using a prophylactic transfusion trigger of $<20 \times 10^9/L$. Detailed analysis of the results of rhTPO mobilisation, autologous platelet cryopreservation and processing and the efficacy and safety of autologous cryopreserved platelets to support HDCT and PBPC will be presented.

37.

CRITICAL ROLE FOR PAI-1 IN TUMOUR CELL TRANSENDOTHELIAL MIGRATION. Evelyn L Douglas¹, Belinda C Edmonds¹, Nicholas WR Wickham¹. ¹Department of Haematology and Oncology, The Queen Elizabeth Hospital, Woodville South, South Australia, Australia.

Plasminogen activator inhibitor 1 (PAI-1) inhibits the production of the serine protease plasmin. PAI-1 levels are increased in the plasma and tumour tissue of many cancer patients particularly in those with more advanced disease. This suggests that PAI-1 plays a role in the development of metastatic disease. As PAI-1 is known to be produced by endothelial cells, the process of transendothelial migration became a focus for our studies as tumour cells migrate twice through the blood vessel wall during metastasis. The aim of this study was to investigate the role of (PAI-1) in tumour cell transendothelial migration.

Three different approaches were taken to determine the relationship between PAI-1 and the ability of tumour cells to transmigrate. First, PAI-1 antigen levels were measured in the supernatant and extracellular matrix (ECM) of cultures of human umbilical vein endothelial cells (HUVEC), the colon cancer cell lines SW480 and SW620, and in co-cultures of HUVEC and tumour cells using enzyme-linked immunosorbent assay (ELISA). HUVEC produce large amounts of PAI-1 (3405 ng/mL \pm 195 at 72 h). The two colon cancer cell lines differ in their ability to produce PAI-1, with SW480 cells producing small amounts of PAI-1 (315 ng/mL \pm 5.5) and SW620 cells producing almost no PAI-1 (41 ng/mL \pm 0.18). In addition, co-culture with tumour cells reduced PAI-1 secreted by HUVEC by 32% for SW480 cells and 70% for SW620 cells after 72 hrs of co-culture. Co-culture with SW620 tumour cells caused a 97% reduction in HUVEC PAI-1 levels in the ECM. The two cell lines have different abilities to reduce HUVEC PAI-1 levels with SW620 colon cancer cells being more potent than SW480 cells.

Secondly, the ability of tumour cells to transmigrate was determined using a two-chamber transwell system including a Matrigel coating over the porous membrane separating the two compartments with a confluent HUVEC monolayer over this. Tumour cells were added to the upper chamber and the number of tumour cells that had migrated to the underside of the membrane after 72 hours was analysed by counting the cells in 9 central fields of view (100x mag.). A variation of this model included the absence of the HUVEC monolayer to determine the effect of the endothelial cells on the migration of the tumour cells through the Matrigel layer. Larger numbers of SW480 colon cancer cells migrated through the transmigration model than SW620 cells (130 cells \pm 15.5 versus 112 cells \pm 28.5). SW480 cells, which produce their own PAI-1, are more capable of transmigration in the absence of the HUVEC monolayer (549 cells \pm 189 in the absence of HUVEC versus 130 cells \pm 15.5). SW620 cells, which produce almost no PAI-1 themselves, appear to require the HUVEC to be present in order to migrate through the Matrigel layer (85 cells \pm 7 in the absence of HUVEC versus 112 cells \pm 28.5).

Finally, the direct effect of PAI-1 on tumour cell transmigration ability was shown by adding anti-PAI-1 antibody to the Matrigel and medium of the transwells. The anti-PAI-1 antibody reduced the number of tumour cells which transmigrated. The effect of the antibody was the greatest with SW480 cells migrating through the model without the HUVEC monolayer (only 113 cells \pm 5.5 migrated when the antibody was present compared with 549 \pm 189 when it was absent), although the effect was still seen in the presence of HUVEC (28 cells \pm 9 versus 130 cells \pm 15.5). The antibody had a similar effect on SW620 cell transmigration; 66 cells \pm 18.5 with anti-PAI-1 versus 112 \pm 28.5 (in the presence of HUVEC) and 60 cells \pm 9.5 with anti-PAI-1 versus 85 \pm 7 (in the absence of HUVEC).

The two cell lines tested reduce the amount of PAI-1 produced by the HUVEC, whilst at the same time appear to require a certain amount of PAI-1 to migrate through the transmigration model efficiently. The reduction of PAI-1 activity led to a smaller number of tumour cells transmigrating through the model. PAI-1 appears to be necessary for the optimal invasiveness of tumour cells so further exploration into its role in tumour cell transendothelial migration would be beneficial for the development of possible therapies against metastasis.

38.

THE CHEMOKINE RECEPTOR CXCR4 ENHANCES INTEGRIN MEDIATED IN VITRO ADHESION AND FACILITATES ENGRAFTMENT OF LEUKEMIC PRECURSOR-B CELLS IN THE BONE MARROW. W Shen, LJ Bendall, DJ Gottlieb, KF Bradstock. Westmead Millennium Institute, Westmead Institute for Cancer Research.

We have previously demonstrated that acute lymphoblastic leukemia (ALL) blasts migrate into layers of bone marrow fibroblasts (BMF) in vitro using the b1 integrins VLA-4 and VLA-5. The chemokine SDF-1 and its cellular receptor CXCR4 influences ALL migration. One potential mechanism for this is through SDF-1-mediated induction of adhesion through b1 integrins. Treatment of pre-B cell line NALM6 and primary acute lymphoblastic leukemia (ALL) cells with SDF-1 resulted in a doubling of adhesion to fibronectin and laminin, and VCAM-1, but had no effect on binding to collagens I or IV. The integrins VLA-4 and VLA-5 were involved in adhesion to fibronectin and VLA-4 mediated adhesion to VCAM-1. Antibodies to CXCR4 and pertussis toxin inhibited SDF-1-induced adhesion on these substrates. Overnight treatment of NALM6 cells with SDF-1 resulted in a 15 fold reduction in the expression of CXCR4, which returned to control levels over a 72 hour period. SDF-1-treated NALM6 cells with downregulated CXCR4 expression demonstrated a reduced capacity to engraft into the bone marrow of NOD/SCID mice 24 days after injection. The homing of SDF-1-treated cells to the bone marrow 24 hours after injection was also reduced by $72 \pm 16\%$ compared to control cells. The importance of the b1 integrin VLA-4 in the homing of NALM6 to the bone was demonstrated using a subline of NALM6 (4A1) which lack this integrin. 4A1 cells almost completely fail to home to the marrow 24 hours after injection and are almost undetectable in the marrow 24 days after injection. Together these data show that SDF-1 and CXCR4 are involved in regulation of b1 integrin function. The function of these integrins, especially that of VLA-4, are important for the localisation of pre-B cells to the bone marrow in vivo.

39.

BREAST CANCER CELL ASSOCIATED FIBRONECTIN INDUCES THE RELEASE OF MATRIX METALLOPROTEINASE-2 FROM THE SURFACE OF BONE MARROW FIBROBLASTS. Sonia Saad, DJ Gottlieb, KF Bradstock, LJ Bendall. Westmead Millennium Institute, Westmead Institute for Cancer Research

Breast cancer is one of the most common forms of cancer in women but despite being responsive to hormonal manipulation and chemotherapy, relapse following treatment is common, particularly in patients presenting with metastatic disease. One of the most common sites of metastasis is bone and bone marrow, which may provide a favorable microenvironment for the growth of tumor cells and act as a reservoir of disease, allowing further hematogenous spread. There is a strong correlation between tumor invasion and metastasis and the expression of matrix metalloproteases (MMPs), which are mostly associated with adjacent normal fibroblasts. Most members of the MMP family consist of three well-conserved domains: an amino terminal propeptide; a catalytic domain; and a hemopexin-like domain. MMP-2 and MMP-9 are unique among MMPs due to the inclusion of three fibronectin type II domains within their catalytic domains. The three fibronectin type II repeats, also termed the collagen binding domain (CBD), bind native collagen. This interaction is believed to be responsible for binding of MMP-2 to the cell surface. We have previously shown that the co-culture of breast cancer cells and bone marrow fibroblasts in vitro results in an increase in the levels of MMP-2 found in the culture supernatant. Direct contact between the two cell types is required for optimal increases in MMP-2 levels, although a small component is mediated by soluble factors. Here we demonstrate that MMP-2 is derived from the surface of the bone marrow fibroblasts. We believe that MMP-2 is associated with the bone marrow fibroblasts by binding to b1-integrin associated native collagen through its CBD. Breast cancer cell-associated fibronectin elutes MMP-2 from the bone marrow fibroblasts via its collagen binding domain. This demonstrates a mechanism through which breast cancer cells can induce a rapid increase in soluble MMP-2, potentially facilitating their invasive potential.

40.

LONG-TERM ENGRAFTING UMBILICAL CORD BLOOD CELLS ARE PRESERVED AFTER EX-VIVO CULTURE IN A NON-CONTACT SYSTEM. Ian Lewis Division of Haematology, IMVS.

We describe stroma-based and stroma-free cultures that maintain long-term engrafting hematopoietic cells for at least 14 days ex vivo. Umbilical cord blood (UCB) CD34+ cells were cultured in transwells above AFT024 feeders with fetal-liver-tyrosine-kinase (FL) + stem-cell-factor (SCF) + interleukin- (IL)-7, or FL + thrombopoietin (Tpo). CD34+ progeny were transplanted into NOD-SCID mice or preimmune fetal sheep. SCID repopulating cells (SRC) with multilineage differentiation potential were maintained in FL-SCF-IL7 or FL-Tpo containing cultures for up to 28 days. Marrow from mice highly engrafted with uncultured or expanded cells induced multilineage human hematopoiesis in 50% of secondary but not tertiary recipients. Day-7 expanded cells engrafted primary, secondary and tertiary fetal sheep. Day-14 expanded cells, while engrafting primary and to a lesser degree secondary fetal sheep, failed to engraft tertiary recipients. SRC that can be transferred to secondary recipients were maintained for at least 14 days in medium containing glycosaminoglycans and cytokines found in stromal supernatants. This is the first demonstration that ex-vivo culture in stroma-non-contact and stroma-free cultures maintains long-term engrafting cells, defined by their capacity to

engraft secondary or tertiary hosts. Although differences exist between two commonly used xenogeneic transplant models, they may measure human progenitors at a similar stage of differentiation.

41.

USE OF CELL TRACKING TO STUDY THE RELATIONSHIP BETWEEN THE ABILITY TO ENGRAFT THE NOD/SCID MOUSE AND THE LEVEL OF PROGENITOR DEVELOPMENT. Ramirez C.D.¹ Nordon R.E.² Rice A.M.¹

¹*Stem Cell Biology Program, Children's Cancer Institute Australia for Medical Research, NSW Australia;*

²*Graduate School of Biomedical Engineering, UNSW, Australia*

Cytokine-mediated expansion has been proposed as a means of increasing the total cell dose and improving the rate of engraftment. However, little is known about the relationship between the ability to and the rate of engraftment, the stage of primitive progenitor cell development and the early phenotypic changes that occur following growth factor (GF) activation of haematopoietic stem cells.

In this study we used a high resolution cell division tracking method to determine the correlation between the number of cell divisions and 1) the expression level of GF receptors and lineage markers in vitro and 2) engraftment potential in vivo. In initial experiment, MACS enriched CD34⁺ cells were stained with the intracellular tracking dye 5-(-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) and sorted from 10/256 channel wide CFSE gates on the left (L) and right (R) of median CFSE fluorescence. CFSE stained CD34⁺ cells were then cultured with SCF, MGDF, Flt3-L and \pm IL-3 and analyzed by flow cytometry at 1, 2 and 3 days of culture.

We characterized the following cell surface molecules with respect to divisional history were a) proliferation markers (CD38/CD71) b) putative stem cell markers (CD34/Thy-1), c) lineage specific markers (CD33/CD19/CD41/CD71/CD61) and d) growth factor receptors (CD117- c-kit receptor and CD130-IL-6 receptor associated). CD38, CD71, CD61, CD41 and c-kit were up-regulated after incubation with SCF+MGDF+Flt3-L+IL-3 with respect to cell division. CD34 and Thy 1 expression were down-regulated in successive divisions. Expression of CD130 and CD19 were below the sensitivity of detection. By day 3, R cells had undergone 5 cell divisions and L cells, 4 divisions. It would appear L cells were more primitive than R cells since the cells underwent fewer divisions and showed higher levels of primitive stem cell marker expression. C-kit expression was increased in the first division, but was down-regulated thereafter. CD41 and CD61 had their highest level of expression in second generation progeny. Cells cultured with SCF+MGDF+Flt3-L factors had fewer divisions by day 3 than those cultured with SCF+MGDF+Flt3-L+IL-3. However the phenotype did not differ significantly between the 2 populations.

Experiments investigating engraftment potential used MACS enriched CD34⁺ cells stained with CFSE sorted from the centre of the peak. Cells were cultured with SCF+MGDF+Flt3-L+IL-3 and on day 3 only cells that had divided were transplanted into NOD/SCID mice. In later experiments expanded divided cells were separated into division 1, division 2 and division 3 respectively. Controls included mice transplanted with unexpanded cells on day 0 and all expanded cells (undivided and divided cells) on day 3. NOD/SCID mice were sacrificed 6-7 weeks after transplantation. Bone marrow and spleen were analyzed for human engraftment by flow cytometry. In the first series of experiments human cells were detected in BM and spleen of all mice transplanted suggesting that divided cells retain the ability to engraft. All mice engrafted including mice transplanted with divided cells. In the second series of experiments we investigated the engraftment potential of a cell population that had undergone a defined number of divisions. There was no engraftment in mice transplanted with undivided cells or cells that had divided once. Low levels of human cells were seen in mice transplanted with division 2, 3, unexpanded and expanded cells. These results need to be confirmed due to very low number of cells transplanted for mice receiving undivided and division 1 cells.

Cell tracking provides novel methodology for studying the relationship between the ability to engraft and the level of progenitor development. Our findings suggest that despite maturation of the cells induced by cytokine stimulation, divided cells retain the ability to engraft the NOD/SCID mouse.

42.

CliniMACS CD34-SELECTED CELLS TO SUPPORT HIGH-DOSE THERAPY. HM Prince, D Wall, P Chapple, M Quinn, M Brettell, D Haylock, JF Seymour, M Wolf, H Januscewicz, G Richardson, T Joyce, R Maisano, D Rischin
Blood and Marrow Transplant Service, Peter MacCallum Cancer Institute, Victoria, Australia

Background: We have previously demonstrated that CD34-selected blood cells using the Isolex300i device can support 3 repetitive cycles of high-dose therapy (HDT). We performed a similar study utilizing the cliniMACS device. Methods: Ten patients (pts) with advanced breast cancer had blood cells (BC) mobilized with docetaxel (100mg/m²) and G-CSF (neupogen; 10µg/kg/d) sc. BCs were harvested and then processed using the cliniMACS CD34-selection device and equally divided into 3 separate bags for cryopreservation. A bag of unmanipulated cells was also collected on a separate day, divided and cryopreserved. Pts subsequently received 3 cycles of HDT with cyclophosphamide (4g/m²), thiotepa (300mg/m²) and paclitaxel (175mg/m²) followed by BCs. The planned design was for pts to receive CliniMACS CD34-selected cells to support each of the 3 cycles of HDT (i.e 1/3 for each cycle). If however, hemopoietic recovery was delayed after the 1st cycle, 1/3 of the unmanipulated cells could be infused following the 2nd cycle and the remaining cliniMACS-selected cells (2/3) were used to support the 3rd cycle. Results: 8 pts have commenced the HDT phase to date and 21 cycles of HDT completed. Only one pt was able to be supported with CD34-selected cells for all 3 cycles. The remaining pts (n=7) required unmanipulated cells to support the 2nd cycle. Two of these 7 pts also required infusion of back-up unmanipulated cells because of delayed engraftment with CD34-selected cells. The median number of CD34-selected cells (x10⁶/kg) infused per cycle was 1.1 (0.65-2.6) and unselected cells was 2.1 (1.7-2.8). There was no correlation between the dose of CD34-selected cells infused and neutrophil/platelet recovery. When hemopoietic recovery was compared between cycles of HDT supported by CD34-selected (n=14) and unmanipulated cells (n=7) there was a significant slowing with the CD34-selected cells; time to ANC >0.5 (x10⁹/L) = 13d v 9d, >1.0 = 14d v 10d, plt > 20 = 18d v 13d, >50 = 25 vs 17d (all p values <0.0001). Conclusion: The use of CliniMACS selected CD34+ cells in the doses utilized in this study results in significantly prolonged hemopoietic recovery after HDT. These findings may also have implications for the design of allogeneic mini-transplant and ex vivo expansion studies.

43.**GCSF INHIBITS PERIPHERAL BLOOD CD14 MONOCYTE-DERIVED CYTOKINES ON THE DAY OF STEM CELL COLLECTION. J.Moore, V.Nink, J.Zaunders, D.D.F. Ma, Departments of Haematology and the Centre for Immunology, St. Vincent's Hospital, Sydney, NSW.**

The increasing use of peripheral blood stem cells (PBSC) for haematopoietic transplantation has focussed attention on the immunological difference of PBSC compared to bone marrow stem cells. Despite the PBSC product containing 10 times more T cells and 50 times more monocytes there appears to be no increased incidence of acute GVHD. It is hypothesised that there is immunomodulation associated with GCSF mobilisation possibly mediated by monocytes. To address this issue, we have performed CD14 monocyte cytokine analysis on normal controls without GCSF (n=18), normal allogeneic stem cell donors on day 5 of GCSF (n=7) and Rheumatoid arthritis (RA) patients undergoing stem cell mobilisation for subsequent autologous PBSCT (n=6) on day1 (pre GCSF) and day 5 of GCSF. Heparinised whole blood was incubated with 1µg LPS (Sigma, Australia) for 4 hours at 37°C then lysed, permeabilised and stained intracellularly with PE labelled anti-TNF, IL1, IL6 antibodies (Fastimmune Cytokine System, Becton Dickinson, San Jose, CA). Cell surface staining with CD14-FITC was used to identify monocytes and unstimulated and unlabelled controls were run on all specimens. Whole blood flow cytometry was used in this situation because it may more accurately reflect circulating cells in physiological and disease states. Baseline cytokine expression was similar between normals and Rheumatoid arthritis patients. After 5 days of GCSF, there was a highly significant reduction (by Mann Whitney U test) for all 3 cytokines despite a similar percentage of circulating peripheral blood monocytes in both normal donors (Table 1) and RA patients (Table2). Our data demonstrate that GCSF attenuates the intracellular expression of these pro-inflammatory cytokines which may help explain the unexpected similar incidence of GVHD despite high numbers of T cells and monocytes in the allogeneic setting. In Rheumatoid arthritis patients these findings may partly explain the low incidence of flares in these patients using GCSF for PBSC mobilisation.

Cytokine	% Expression in CD14 monocytes on Day 1 GCSF	% Expression in CD14 monocytes on Day 5 GCSF	P value
IL1	93.7±4.3	59.1±12.5	0.0005
TNF	76.3±18.7	22.2±12.1	0.0005
IL6	68.4±18.3	30.2±8.9	0.0005

Table 1 - Normal controls (n=18) and normal donors on day 5 of GCSF (n=7)

Cytokine	% Expression in CD14 monocytes on Day 1 GCSF	% Expression in CD14 monocytes on Day 5 GCSF	P value
IL1	91.2±4.3	59.1±12.5	0.0005
TNF	59.6±18.4	19.1±14.4	0.002
IL6	66.7±16.1	26.9±21.9	0.008

Table 2 - Rheumatoid arthritis patients (n=6) on day 1 and day 5 of GCSF

44.

ALLOGRAFTING IN MYELOPROLIFERATIVE DISORDERS. Rainer Storb, M.D. Fred Hutchinson Cancer Center and the University of Washington Seattle, Washington, USA

Myeloproliferative disorders and myelofibrosis are stem cell disorders which are incurable by conventional therapies but are amenable to therapy by allogeneic hematopoietic stem cell transplantation. Best results are generally achieved if transplants are carried out early in the course of the disease (refractory anemia) while patients with more advanced disease (>RAEB) may experience recurrence of their underlying disease after transplantation. Long-term event-free survivals in patients with refractory anemia given transplants from HLA-matched related or unrelated donors are on the order of 65%, while in patients with more advanced disease, event-free long-term survivals of 20-30% have been observed. Conditioning regimens involving targeted busulfan along with the immunosuppressive agent cyclophosphamide appear to give the best results both for patients with refractory anemia and >RAEB as well as patients with myelofibrosis. For older patients, nonmyeloablative protocols involving fludarabine, 200 cGy total body irradiation, followed by postgrafting immunosuppression with mycophenolate mofetil and cyclosporine are being explored.

45.

INCREASED CIRCULATING PLATELET-LEUKOCYTE COMPLEXES AND PLATELET ACTIVATION IN PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME, SYSTEMIC LUPUS ERYTHEMATOSUS AND RHEUMATOID ARTHRITIS. JE Joseph¹, P Harrison¹, IJ Mackie¹, D Isenberg², SJ Machin¹. Haemostasis Research Unit¹ and Rheumatology Department², University College London, UK WC1E 6HX.

Thrombosis is known to occur in patients with antiphospholipid antibodies (APA), although the exact mechanisms responsible remain unclear. Platelet activation may play a pathogenic role and in order to investigate this possibility, sensitive flow cytometric techniques were used to assess platelet activation in 20 patients with primary antiphospholipid syndrome (PAPS) and 30 patients with systemic lupus erythematosus (SLE) (14 of whom had secondary APS). Platelet surface CD62p and CD63 expression, PAC-1 and annexin V binding, platelet-leukocyte (platelet-granulocyte [PL-G], platelet-monocyte [PL-M] and platelet-lymphocyte [PL-L]) complexes and platelet-derived microparticles were measured using flow cytometry, and plasma levels of soluble P-selectin (sP-sel) were assayed by ELISA. Platelet-leukocyte complexes were also measured in 10 patients with rheumatoid arthritis (RA) who were APA negative. The median values for all tests are summarised in the following table.

	CD62 (%)	CD63 (%)	PAC-1 (%)	Ann V (%)	MP's ($\times 10^6$ /ml)	sP-sel (ng/ml)	PL-G (%)	PL-M (%)	PL-L (%)
Controls	1.2	3.0	3.7	3.0	0.35	18.8	3.5	5.5	3.0
PAPS	1.3	5.2	7.5	3.2	0.45	41.4	3.7	7.6	2.9
SLE	1.6	3.1	3.6	3.1	0.40	32.1	4.6	9.2	3.7
RA	-	-	-	-	-	-	4.3	7.9	3.4

Median platelet CD63 expression was significantly higher in PAPS patients compared to controls ($p=0.007$), as well as SLE patients with and without secondary APS ($p=0.03$ and $p=0.002$ respectively). Median levels of PAC-1 binding were significantly higher in PAPS compared to controls ($p=0.007$) and SLE patients without APS ($p=0.015$). Median values for platelet-granulocyte and platelet-monocyte complexes were significantly higher in SLE patients compared to both controls and PAPS patients, and platelet-monocyte complexes were significantly increased in PAPS patients compared to controls. Platelet-granulocyte and platelet-monocyte complexes were also significantly higher in 10 patients with rheumatoid arthritis (RA) and no evidence of APA, when compared to controls. Plasma soluble P-selectin levels were significantly higher in both PAPS and SLE patients compared to controls. There was no significant difference in median platelet CD62p expression, annexin V binding and platelet microparticle numbers between the three groups, although some individuals had elevated levels. We conclude that there is evidence of increased platelet activation in PAPS, and to a lesser degree, in SLE. Since platelet-leukocyte complexes were significantly higher in SLE than in PAPS, and they were also elevated in patients with RA, it appears that mechanisms apart from platelet activation alone are likely to be responsible for their formation and circulation.

46.

A WHOLE BLOOD FLOW CYTOMETRIC METHOD FOR QUANTITATION OF CIRCULATING PLATELET MICROPARTICLES AND PLATELET ACTIVATION. S. Smith¹, A. Trickett^{2,6}, F. Harlow³, L. Peters⁴, G Davis^{3,6}, M Brown^{5,6}, Y.L. Kwan^{1,2,6}, T.A. Brighton^{1,2,6}. ¹Haematology SEALS St George Hospital Kogarah Australia, ²Clinical Haematology, Division of Cancer Care Services, St George Hospital, ³Dept. Women's Health, St George Hospital, ⁴Becton Dickinson, Australia, ⁵Nephrology Department, St George Hospital, ⁶University of New South Wales, Kensington. Australia

Introduction:-Platelet activation can readily be assessed by flow cytometric methods seeking the surface expression of platelet activation markers. Common markers include CD62 (* granule P-selectin), CD63 (lysosomal membrane protein), and Annexin V (calcium dependent binding to phosphatidylserine). Additionally, upon platelet activation the platelet plasma membrane fragments with the liberation of platelet-derived microparticles (PMP). Increased proportions of circulating PMP have been reported in a variety of disease states such as ITP, TTP and HIT. Methods for absolute quantitation of PMP have been lacking.

Objective:-To develop a whole blood flow cytometric method to measure absolute numbers of PMP whilst simultaneously measuring other markers of platelet activation.

Methods:-PMP in this study were defined as platelet-derived particles (CD61 positive) of 0.2-0.5* μ m in size. Fluorescent polystyrene beads (Duke Scientific, CA) of 0.2, 0.5 and 1.9 * μ m size were used daily to calibrate side scatter (SSC) as a means of measuring particle size. Experiments were performed to determine the best method of blood collection in order to minimise in vitro platelet activation. Anticoagulated whole blood was diluted into a series of six tubes for each patient: one for quantitation of platelets and PMP, two as antibody controls, and the remaining three to measure various markers of activation. Each tube contained CD61-PerCP (Becton Dickinson, Australia) directed against platelet glycoprotein GPIIIa (CD61) and either FITC or PE conjugated anti-Glycophorin A to identify platelet/RBC complexes that may be present. Platelets and PMP were positively identified by SSC and CD61 positivity. To the quantitation tube a known quantity of 0.5* μ m polystyrene beads (excitation 469nm / emission 509nm) was added. The three activation tubes contained CD62-PE (Becton Dickinson Australia), CD63-FITC (Immunotech, France) and Annexin V-FITC (Pharmingen, CA) respectively. The control tubes contained isotype and manufacturer matched control antibodies to CD62 and CD63. Data from each tube was acquired on a Becton Dickinson FACSort with a 488nm argon laser. For the quantitation tube, acquisition was set to stop once at 2000 bead events. Any CD61 or bead event was stored for subsequent analysis. For the remaining tubes 10,000 CD61 events were collected. Data analysis was performed using Attractors software (Becton Dickinson, Australia). Quantitation of platelets and PMP was derived from the number of platelets counted in proportion to the number of beads counted after correction for doublets, triplets and quadruplets. The platelet count was compared to that obtained from an EDTA tube on an Abbott Cell Dyn 3500 or Coulter STKS. The percentage of CD62, CD63 and Annexin V positive events were recorded.

Results:-Experiments determined that in vitro platelet activation was minimised by blood collection under no or light tourniquet, with collection of an EDTA vacutainer followed by the platelet flow tube (Greiner vacutainer, 3.8% sodium citrate with 6.3 *M Prostaglandin E1 (Cayman Chemicals, MI) to inhibit platelet aggregation). Acquisition within 1-2 hours of sample collection was possible without significant in vitro platelet activation. Time from collection to sample acquisition averaged 30 minutes, and acquisition time for samples with platelet counts > 100 x 10⁹/l required 10 minutes. The Attractors software was configured to provide automated analysis. Inter-operator reproducibility studies demonstrated an acceptable CV (<3%). Correlation between flow and automated platelet counts was good (Automated Impedance count = 1.007*Flow count, r² = 0.69).

Conclusion:-We report an easy, reproducible flow cytometric based method to quantitate PMP and platelet activation. This should prove useful in assessing the contribution of platelet activation in various disease states.

47.

EXPERIENCE WITH ENZYME-LINKED IMMUNOSORBENT ASSAYS TO DETECT FACTOR VIII ANTIBODIES IN HAEMOPHILIA A. M. Ling, E. M. Duncan, S. E. Rodgers, and J. V. Lloyd. Haematology Division, IMVS, Frome Rd, Adelaide, SA 5000.

In haemophilia antibodies may arise that bind to and inactivate the coagulant function of factor VIII (FVIII), and they are commonly referred to as "inhibitors". These antibodies can be detected and quantitated by methods based on their interference with coagulation eg the Bethesda method. The assessment of anti-FVIII antibodies by a coagulation-based assay will not detect those directed solely against epitopes not involved in FVIII function whereas ELISA can detect both inhibitory and non-inhibitory antibodies. We compared ELISA to the Bethesda method, in a study of a group of patients with haemophilia. Our aim was to determine whether there was a relationship between the results of the two assays and whether it was possible to detect antibodies by ELISA in any patients negative by the Bethesda method, as evidence for antibodies against non-functional epitopes. Because it has been proposed by others that haemophilia patients with the intron 22 inversion frequently develop non-inhibitory antibodies, we also assessed the results for a sub-group of patients with that mutation.

Plasma from 52 haemophilia patients (34 severe, 5 moderate, 6 mild and 7 acquired) and 20 normal controls were studied. ELISA was performed using two different preparations of recombinant FVIII: unformulated, pure FVIII (U-rFVIII) generously supplied by Baxter and the commercially-available, formulated FVIII (F-rFVIII) Recombinate (containing stabilisers including albumin), also produced by Baxter. Bethesda assays were performed using the standard method with the source of FVIII for incubations being a pool of normal plasma buffered at pH 7.4, as recommended for the Nijmegen modifications.

Of the 52 patients in the study, 26 had measurable inhibitor levels by Bethesda assay, ranging from 0.7 to 700 BU/ml, and 26 had no inhibitor detected. Using U-rFVIII in the ELISA, all patients positive by Bethesda were positive by ELISA. In addition, three samples that were negative by Bethesda were positive by U-rFVIII ELISA whilst the remaining 23 were negative by both methods. The three patients with discrepant results do not have any clinical suspicion of a factor VIII antibody. A different pattern of results was seen using F-rFVIII in the ELISA. Again, all patients positive by Bethesda were positive by the F-rFVIII ELISA method. However only one patient who was negative by Bethesda was positive by F-rFVIII ELISA, whilst the remainder were negative by both Bethesda and F-rFVIII ELISA. Therefore there was a slightly higher number of samples with antibodies detected by ELISA compared to the Bethesda method, suggesting a very small proportion of patients develop non-inhibitory antibodies. The U-rFVIII ELISA detected non-inhibitory antibodies in three patients whereas the F-rFVIII ELISA only detected one of these patients as positive. The reasons for the difference in sensitivity between the two recombinant FVIII preparations used in ELISA are not known. However these results suggest the formulation of FVIII used in ELISA can influence the outcome of results in comparative studies.

When the sub-group of 21 patients with severe haemophilia and the intron 22 inversion is considered separately, 48% (10/21) were positive by U-rFVIII ELISA, 43% (9/21) were positive by F-rFVIII ELISA and 43% (the same 9/21) were positive by Bethesda assay. Therefore in this small group with the inversion, there was only one with a non-inhibitory antibody, detected using ELISA with U-rFVIII. In this study the frequency of inhibitors in intron 22 patients is similar when results for detection by the Bethesda method and ELISA are compared.

48.

CURRENT DIAGNOSIS OF von WILLEBRAND DISORDER IN AUSTRALIA – TIME FOR A CHANGE? James Thom, Emmanuel Favalaro[#] and Ross Baker*. *Coagulation Unit, Royal Perth Hospital, WA* and *#Special Haemostasis Unit, Westmead Hospital, NSW. On behalf of the ASTH Emerging Technologies Group.*

Seven lyophilised samples were distributed to 19 Australian laboratories, mostly expert and experienced in testing for von Willebrand's disorder (vWD). The samples were taken from six patients with well-defined vWD or haemophilia and a normal healthy control with no bleeding history. Each laboratory was asked to perform analysis for von Willebrand factor (vWf) with their usual techniques and invited to suggest a possible diagnosis. Returns were received from all participants. The results were discussed at a dry workshop held in Perth which was attended by most participants.

All laboratories performed a one-stage factor assay for FVIII, and measured antigenic and functional vWf by a variety of techniques. Most laboratories appropriately interpreted their own results, however coefficients of variation for both functional and antigenic vWf assays were commonly greater than 20%. The normal sample in this study was correctly identified by the majority of participants but was classified as vWD or possible vWD by 25%. A sample from a patient with type 1 vWD disorder and a significant bleeding history was considered normal by 70% of participants.

Most centres understood the complexities involved in the diagnosis of vWD and expressed caution before classifying or sub-typing solely on the basis of laboratory results.

The variable clinical manifestations seen in different patients with similar vWf levels, fluctuations in response to various physiological factors and imprecision in laboratory testing combine to produce significant diagnostic uncertainty for vWD. Even with optimised techniques it may be difficult to assign a definite diagnosis of vWD with values in the 30 – 70% range. However these patients may have excessive bleeding with surgery, and in women have a bleeding tendency

resulting in menorrhagia and iron deficiency anaemia. It is proposed that we should consider the vWf level a graded risk factor for a bleeding tendency rather than have absolute cut-off values for a definitive disorder.

49. IMPROVING THE MANAGEMENT OF DEEP VEIN THROMBOSIS IN AUSTRALIA. Julian Cooney, Ross Baker
Thrombosis and Haemophilia Service, Department of Haematology, Royal Perth Hospital, Perth, Western
Australia

The proven safety and efficacy of low molecular weight heparin in the initial treatment of deep venous thrombosis (DVT) together with non-invasive imaging investigations, thrombophilia testing and outpatient management has revolutionised management in Australia. However, there are concerns that with varying opinions regarding management, practitioners may not be adequately treating lower limb venous thrombosis. Between February and April 1999, we performed a return postal survey of 815 medical practitioners who would see patients presenting with DVTs. We gave four clinical cases of lower limb venous thrombosis- A) Post partum calf thrombophlebitis B) Calf vein thrombosis in pregnancy C) Symptomatic popliteal vein thrombosis after total hip replacement D) Spontaneous extensive lower limb thrombosis in a young man. We asked the treating doctor to reply how they would manage each case. This included the use of anticoagulants, type and duration of therapy, outpatient care and thrombophilia screening. Overall response was 453 (55.6%). There were two groups- (i) All medical staff at a large teaching hospital (700 beds)-Royal Perth Hospital (274 replies out of 445- 61.6%) (ii) A randomly selected group of General Practitioners in Western Australia (179 replies out of 370- 48.4%). Subgroup analysis was performed, looking into the age of the medical practitioner, the number of cases of deep venous thrombosis treated per year, and the practice type. In the cases (A) to (D) above, the proportions choosing to anticoagulate as part of their initial management were 25.2%, 45.5%, 78.6% and 93.7% respectively. There were significant differences in responses by Doctors older than 50 years, those consulting on less than five DVT cases per year, and the General Practitioner group. These included the use of outpatient management, thrombophilia screening, duration of anticoagulation and further imaging investigations. We have identified significant variations of practice in the management of symptomatic DVTs. This includes avoiding the use of anticoagulants in some cases, where previous trials have suggested efficacy and safety. We recommend ongoing discussion with medical practitioners in Australia about lower limb venous thrombosis, with the aim of establishing optimal management guidelines. A large community based DVT trial is needed to help clarify areas of controversy.

50. DENTAL EXTRACTIONS IN WARFARIN TREATED PATIENTS. B Gibbs and P Ockelford, Oral Health Unit and
Department of Haematology, Auckland, New Zealand.

In warfarinised patients presenting for dental extractions management options include: discontinuing warfarin; substituting heparin for warfarin temporarily; reducing warfarin to subtherapeutic levels or maintaining anticoagulant treatment at a low therapeutic INR. Normal haemostasis is a balance between fibrin deposition and dissolution within the mouth. Saliva contains large amounts of plasminogen activator which contributes significantly to reduced clot stability and increases the potential for bleeding after oral surgery. Systemic antifibrinolytic therapy does not result in detectable inhibitory levels within the saliva but local administration of tranexamic acid mouthwash reduces salivary clot lysis for two hours following administration. We have used the regimen of Sindet Pedersen, irrigation of the extraction socket with 5% tranexamic acid solution immediately after tooth removal followed by 10 mls 5% aqueous tranexamic acid mouthrinse for two minutes four times a day for seven days, in 100 consecutive warfarin treated patients undergoing dental extraction. The INR at operation was 1.9 - 4.0 and the mean number of extractions 4.5 (range 1 - 27) per patient. Aspirin was discontinued seven days preoperatively. There was no abnormal bleeding in 62% of patients but in 30 of our subjects the bleeding which occurred (usually in the first 24 hours) was controlled at home by local pressure. Only eight individuals required a dental assessment for local therapy on an outpatient basis. No patient required admission. Bleeding risk correlated with the removal of maxillary teeth and more severe periodontal disease. It is increased in smokers and those using ASA even when the antiplatelet drug is discontinued seven days preoperatively. It is independent of INR especially if < 3.0. Dental extractions are now routinely and safely performed using this protocol in patients with an INR < 4.0. Consideration is being given to reducing the severity of periodontal disease prior to surgery and stopping antiplatelet agents 10 days before the extractions are performed.

51.

PROTEIN Z AND THROMBOSIS. George J. Broze Jr., Division of Hematology, Washington University School of Medicine, St. Louis, Missouri, USH.

Protein Z (PZ) is a vitamin K-dependent plasma protein whose structure is very similar to coagulation factors VII, IX, X and protein C. In contrast to these serine protease zymogens, however, PZ lacks proteolytic activity. Instead, PZ binds to activated factor X (factor Xa) in a Ca²⁺-dependent fashion at phospholipid surfaces and serves as cofactor for the inhibition of factor Xa by a previously unidentified plasma protein called PZ-dependent protease inhibitor (ZPI). In the presence of phospholipids and Ca²⁺, the rate of factor Xa inactivation by ZPI is enhanced >1000-fold by PZ (t_{1/2} < 10 sec. vs. 210 min). The combination of PZ and ZPI dramatically delays the initiation and reduces the ultimate rate of thrombin generation in mixtures containing prothrombin, factor V, phospholipids, and Ca²⁺. In similar mixtures containing factor Va, however, PZ and ZPI do not inhibit thrombin generation. Thus, the anticoagulant action of PZ and ZPI presumably must precede the activation of factor V and formation of the prothrombinase complex or, perhaps, follows the consumption of prothrombin at local sites.

Besides inhibiting factor Xa in a PZ-dependent manner, ZPI also rapidly inactivates factor XIa in a process that does not require PZ, phospholipids or Ca⁺. Inhibition of factor XIa by ZPI is not affected by the presence of high molecular weight kininogen and is enhanced by heparin (t_{1/2} 25 sec. vs. 50 sec.). As is typical for members of the serpin superfamily of proteinase inhibitors, ZPI is proteolytically cleaved and inactivated during its inhibition of factor Xa and factor XIa. ZPI activity is consumed during the coagulation of plasma in vitro through the actions of factor Xa (with PZ) and factor XIa. PZ gene-deleted mice have a grossly normal phenotype in the absence of a challenge. When combined with the homozygous factor V Leiden [FV(X/X)] genotype, however, PZ deficiency causes intrauterine and perinatal thrombosis and a consumptive coagulopathy that leads to near absolute mortality. The genetic combinations FV(X/X)IPZ(+/-) and FV(RI+)IPZ(-/-) produce smaller, though significant, reductions in survival. The intensification of the thrombotic phenotype in FV Leiden mice produced by PZ deficiency is consistent with recent human data showing that a combination of prothrombotic traits significantly increases the risk of thrombosis and underscores the multigenic nature of thrombophilia. The risk of thrombosis associated with PZ and ZPI deficiency in humans remains to be determined.

52.

New strategies to achieve haemostasis. Dr. Paul Giangrande. Oxford Haemophilia Centre, UK

When bleeding is the consequence of a specific defect of haemostasis, the aim of treatment is the correction of that single defect. However, in many cases bleeding is due to the presence of multiple disturbances in the clotting mechanism and a broader approach is required.

Desmopressin (DDAVP) is well known to boost levels of factor VIII and von Willebrand factor but also shortens the prolonged skin bleeding time in patients with renal failure, liver cirrhosis and congenital or acquired platelet function defects, including aspirin-induced platelet dysfunction. Administration of DDAVP is associated with enhanced expression of P-selectin (CD 62) on both platelets and endothelial cells. DDAVP has also been shown to reduce blood loss in patients without bleeding disorders in the setting of a variety of surgical procedures, including cardiac surgery. A concentrated nasal spray preparation is now available.. There are several reports of arterial thrombosis associated with the use of desmopressin, including myocardial infarction Another potential risk is that of water intoxication with resultant hyponatraemia and seizures, and children under the age of 2 are particularly vulnerable. It should only be used with caution in pregnancy.

Aprotinin is a polypeptide of 58 amino acids with a molecular weight of 6512 which inhibits the action of several serine proteases, including trypsin and plasmin. Aprotinin is widely used to inhibit fibrinolysis during cardiac surgery and orthotopic liver transplantation but has also been shown to be of value in major orthopaedic surgery The reduction in blood requirement is particularly useful for patients who are Jehovah's witnesses or who have rare blood groups or antibody combinations, such that it is difficult to secure compatible units of blood. It is also effective in operation normally characterised by particularly large blood losses, such as those in patients taking aspirin and patients undergoing cardiac transplantation. Allergic reactions may occur and repeated exposure may even result in anaphylactic reactions: the reported incidence of anaphylactic is from 0.3 to 0.6% after a single exposure, rising to almost 5% with prior exposure. There is evidence that severe reactions are mediated by IgE, and pre-operative screening for the presence of aprotinin-specific IgE antibodies may be of some value in identifying patients at risk Aprotinin is extracted on a commercial basis from bovine lung and concern has been expressed about the potential for transmission of prions. However, the product is derived from cattle in BSE-free countries and *in vitro* experiments involving spiking of material with mouse-associated scrapie agent have demonstrated an 18 log reduction of the added prions during the manufacturing process. The issue of whether the use of aprotinin is associated with an increased risk of vein graft thrombosis in cardiac bypass surgery is still not entirely resolved.

Fibrin sealants have been developed to combat blood loss in a variety of settings. The basic ingredients of the various preparations are human fibrinogen and thrombin, which form a film of fibrin upon mixing. Other agents such as factor XIII, aprotinin or tranexamic acid may be incorporated to enhance clot stability. They have been proven to reduce blood loss and the requirement for blood transfusion in a variety of operations, including liver transplantation, and knee and hip arthroplasty.

Recombinant factor VIIa (NovoSeven) has recently been licensed in many countries for the treatment of both acquired haemophilia and congenital haemophilia A and B associated with inhibitory antibodies. It is particularly valuable in the rare cases of haemophilia B with inhibitors, where infusion of plasma-derived concentrates may be associated with serious allergic reactions. However, the agent is also being hailed as a universal haemostatic agent, of value in a variety of conditions including liver disease, reversal of warfarin, thrombocytopenia, congenital platelet defects and even post-surgical bleeding. The human gene is expressed in baby hamster kidney cells, and is grown in a medium free of human and bovine material. It has a short half-life of approximately 2 hours and needs to be given by frequent bolus injections. It is theoretically possible to monitor the plasma VIIa levels after infusion by monitoring the factor VII level in diluted patient plasma, or by monitoring the VIIa level using a clotting-basic method using a mutant tissue factor which is selectively deficient in promoting factor VII activation. However, most units simply monitor treatment by keeping the prothrombin time shortened by 3-4 seconds. NovoSeven is an extremely expensive material and this undoubtedly limits its clinical use.

Microvascular bleeding associated with **massive blood transfusion** is a consequence of depletion of viable platelets, fibrinogen and other labile coagulation factors. The aims of haemostatic support are maintain the prothrombin time and APTT < 1.5 x control. A platelet count of less than 50 x 10⁹/l should serve as a threshold for platelet transfusion, although a higher threshold of 100 is appropriate in cases of CNS injury or multiple trauma. Fresh frozen plasma should suffice to correct deficiencies of all factors, but cryoprecipitate is a valuable source of fibrinogen. Solvent/detergent treated plasma is now available. Although subjected to virucidal treatment which will inactivate enveloped viruses, it is derived from pooled plasma and is significantly more expensive.

Uncontrolled studies suggest that infusion of **protein C concentrate** may improve outcome in septicaemia, especially meningococcal infections. Protein C is involved in regulation of inflammation, and acquired deficiency may occur in

overwhelming sepsis which is typically associated with widespread purpuric lesions. Randomised studies are now planned.

53.

NON-FIBRINOLYTIC FUNCTIONS OF THE PLASMINOGEN SYSTEM. Herren, T., Burke, T.A., Plow, E.F. Joseph J. Jacobs Center for Thrombosis and Vascular Biology, and Dept. of Molecular Cardiology, The Cleveland Clinic Foundation, Cleveland, OH 44195, U.S.A.

Although human leukocytes bind plasminogen with low affinity, their binding capacity is high and can be readily modulated. One of the most extensively characterized pathways for modulation of plasminogen receptor expression occurs when U937 monocytoid cells are stimulated with PMA; the stimulated cells can be separated into adherent and non-adherent populations, and plasminogen binding is selectively upregulated in the non-adherent cells and downregulated in the adherent cells. To gain insight into the mechanisms involved, these cell populations were harvested, lysed, and cell membranes were prepared by differential centrifugation. After separation by SDS-PAGE, plasminogen binding to individual membrane proteins was detected by ligand blotting with ¹²⁵I-plasminogen and quantitated by densitometry of the autoradiograms. Of the numerous membrane proteins, ~15 exhibited plasminogen binding activity in each of the cell populations. Enolase and annexin II, two known plasminogen binding proteins, were identified by Western blotting, but their levels in the membranes from the adherent and nonadherent cells were not differentially regulated. The most notable difference in plasminogen binding between the membrane preparations was associated with a 17kD protein. Plasminogen binding to this protein was highest in the non-adherent and lowest in the adherent cells. This 17kD protein was purified by preparative SDS-PAGE followed by affinity chromatography on plasminogen-Sepharose. N-terminal sequencing of tryptic peptides showed homology of the amino acid sequence to histone H2B, a protein with a C-terminal lysine. Although H2B is regarded primarily as an intracellular protein, its expression on the cell surface of leukocytes is regulated, has been linked to apoptosis, and may be of importance in the pathogenesis of autoimmune diseases and AIDS. Indeed, FACS analyses not only verified its surface expression but also demonstrated upregulation of its cell surface on non-adherent U937 cells. Furthermore, an antibody to the C-terminus of H2B reduced plasminogen binding to the U937 cells substantially. Surface expression of H2B was also observed for human neutrophils, under conditions where their plasminogen binding capacity was dramatically upregulated. Taken together, these results suggest that remodeling of cell surfaces influences the expression and/or exposure of membrane proteins, including ones which can serve as plasminogen binding sites. H2B represents a plasminogen binding protein that contributes substantially to the modulation of the plasminogen binding capacity of leukocytes.

54.

EFFICACY OF THALIDOMIDE IN THE TREATMENT OF RELAPSED AND REFRACTORY MYELOMA. BW Baker & PJ Browett, on behalf of the New Zealand Leukaemia Study Group. Haematology Dept., Palmerston North Hospital, and Dept. of Molecular Medicine, University of Auckland, New Zealand.

Although thalidomide was initially introduced as a sedative in the late 1950s, the disastrous consequences of its use by pregnant women led to its withdrawal from the market in 1961. Very restricted access to this drug has continued and it has been used successfully in a variety of mucocutaneous disorders including chronic GVHD. Suggested modes of action include inhibition of angiogenesis, inhibition of TNF- α , IL-6 and IL-12 production, alterations in T cell function and modification of surface adhesion molecules on lymphocytes. The demonstration of prominent bone marrow vascularisation in multiple myeloma led Singhal and colleagues to administer thalidomide to 84 heavily pre-treated patients with refractory disease. A total response rate of 32%, including some complete responses, has been reported in these patients (N Engl J Med 1999, 341:1565). In March 1999, a supply of thalidomide (Sauramide 100mg capsules) was obtained from Penn Pharmaceuticals, UK. As at 31 March 2000, a total of 27 patients with myeloma have received this agent in New Zealand under an individual patient approval scheme developed with the support of Medsafe, a branch of the Ministry of Health. Information on all 27 patients has been obtained from the treating physicians by circulating a written questionnaire. The median age of patients in this series was 61 (range 37-81) with 19 of the 27 patients male. The median time interval from diagnosis to treatment with thalidomide was 39 months and patients had been treated with a median of two prior regimens (range one to four). Three patients had previously undergone high dose therapy and stem cell transplantation. Of the 27 patients, 24 had refractory or progressive disease and three were considered unsuitable for, or had refused further chemotherapy. The starting dose of thalidomide was 200mg/day, with dose increases at 1-2 weekly intervals to a maximum dose of 800mg/day, although to date no patient has received > 500mg/day. Two patients received additional steroid therapy, but no other chemotherapy agents were given in conjunction with thalidomide. Data on efficacy was available for 16 of the 27 patients at 31 March 2000. Of the remainder, five patients had just commenced treatment and remain on thalidomide, four died before efficacy could be assessed, while treatment was stopped within one month because of side effects in two. One patient (6.3%) had a >90% reduction in paraprotein level; three (19%) had a 50-89% reduction; five (31%) had a 25-49% reduction; four (25%) had a 0-25% reduction and three (19%) had progressive disease, giving an overall response rate of 56% using the criteria of Singhal et al. All responses were observed on doses between 200mg/day and 500mg/day. The duration of response ranges from one to 12+ months, with eight of the 13 patients showing a paraprotein reduction of >25% remaining on thalidomide at 31 March 2000. Side effects were assessable in 23 of the 27 patients. Drowsiness was experienced by ten patients, necessitating dose reduction in one and cessation of therapy in one; constipation occurred in 11, resulting in cessation in four; skin reactions were noted in five, with dose reduction in two and cessation in two; peripheral neuropathy has been documented in two patients, leading to dose reduction in one. In this relatively high-risk group of patients with myeloma, useful responses were obtained in a significant proportion of patients with generally tolerable side effects. These results are consistent with other studies of this agent in pre-treated patients, and further studies are required to better define the role of this drug in the overall treatment strategy for patients with multiple myeloma.

55.

A PHASE II TRIAL OF THALIDOMIDE IN PATIENTS WITH MULTIPLE MYELOMA (MM) FOLLOWED BY INTRON-A™ (INTERFERON ALFA-2B) THERAPY. James J Biagi, H Miles Prince, Max Wolf, Henry Januszewicz, John Seymour, Andrew Grigg, Kate Lillie, Paul Mitchell. Department of Haematology, Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia

Background: Interest in thalidomide as an anti-angiogenic agent has prompted testing in a number of malignancies, including multiple myeloma (MM). The largest clinical trial to date (Singhal et al; NEJM 1999) reported on 84 patients, 76 of whom had relapsed after high dose therapy. After treatment with thalidomide in doses up to 800 mg/day, overall response rate (ORR) was 35%, and the 12 month event free (EFS) and overall survival (OS) were 22% and 58%, respectively.

Study Objectives: To determine the toxicity profile of thalidomide ± interferon-α (IFN) in patients with relapsed and/or refractory MM, and to determine ORR, EFS and OS. Concurrent laboratory investigations will assess MVD characteristics and other prognostic parameters (β2M, LDH, C-RP).

Study Design: Phase II open label study, with planned enrollment of 60 patients.

Treatment: Thalidomide 200 mg/d x 14 d; dose escalation by 200 mg/d every 2 weeks to maximum 800 mg/d; if SE's develop, dose reduce by 100 mg; at 12 weeks commence IFN 3 MM U sq tiw (dose reduce to 1.5 MM U tiw); continue study medications while tolerated and until progression.

Summary: Of twenty patients enrolled, four discontinued therapy due to dose-limiting side effects. Initial response data is limited due to short follow-up interval, but suggests substantial improvement in disease-related parameters. Updated data will be presented.

Patient Characteristics:	Number of Patients at diagnosis: (unless stated in brackets)
Number of Patients	60 planned / 22 enrolled / 21 evaluable
Age	range (41-83) / median (65 yrs)
Male:Female ratio	14:7
ECOG Performance Status	WHO 0 (5) / WHO 1 (9) / WHO 2 (7)
Years of Disease	Max. (9) / Min. (1) / Median (3)
Number of Previous Treatments	Range 1-6 / median 3
Response: most recent treatment	
Durie-Salmon Stage at Relapse	CR 2 / PR 6 / SD 6 / PD 2 / unknown = 4
M Protein IgG/IgA/IgD	
Serum β2-M > 6mg/l	I = 2 / II = 10 / 3 = 8 / unknown = 1
C-R Protein > 3 mg/l	
Serum Cr. > 0.13 mmol/l	13 / 4 / 1
B-J protein > 0.15 g/24 hr	5
Haemoglobin < 100	12
Chr. Del 13 / complex changes	2
	5
	6
	3 / 1
Results to Date:	Number of Patients:
Maximum Tolerated Dose, mg: 200 / 400 / 600 / 800 mg	
Side Effects Profile:	8 / 7 / 2 / 4
Constipation Gr 1 / 2 / 3	
Neuropathy Gr 1 / 2 / 3	4 / 12 / 3
Fatigue Gr 1 / 2 / 3	3 / 1 / 2
Other Gr 1 / 2 / 3	8 / 5 / 0
Outcomes: PR / SD / PD	2 / 5 / 2
Withdrawal from Study:	5 / 14 / 2
Due to: PD / SE / Death	7 Total
Response for patients with PR: (% decrease for Pts 01, 07, 11, 13, 17)	2 / 4 / 1 (disease related) Pt. 01: CRP 52% Pt. 07: serum Ig 82%; LDH 42% Pt. 13: serum Ig 67% Pt. 11: serum Ig 30%; BJP 82% Pt. 17: serum Ig 39%; CRP 45%
CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease; SE = side effects; Gr = grade; Pt. = patient	

56.

CYTOGENETICS OF MULTIPLE MYELOMA. LJ Campbell, Victorian Cancer Cytogenetics Service, Melbourne.

There is increasing interest in the significance of cytogenetic abnormalities in Multiple Myeloma (MM). Success rates for conventional cytogenetic analysis in MM range from 80-97% and the abnormality rates range from 20 to 60%, approximately 40% in most series. Common abnormalities include hyperdiploidy in 30 to 50% of abnormal cases, with extra copies of 3, 5, 7, 9, 11, 15 and 19 consistently observed, monosomy or partial deletion of 13, structural abnormalities of chromosome 1 and involvement of the 14q32 breakpoint, the site of the IgH gene. Abnormalities of 14q32 include the t(11;14) seen in mantle cell lymphoma and the t(8;14) seen in Burkitt's lymphoma. Since January 1998, the Victorian Cancer Cytogenetics Service has received samples from 85 patients with MM for cytogenetic analysis. Sixty-nine cases were successfully karyotyped (81%) with an abnormality rate of 36%. We added IL-4 to cultures in 58% of cases (commencing in 1999) and the abnormality rate increased from 22.5% to 44% although no significant improvement in the success rate was seen ($p=0.9$). Of the 25 abnormal cases observed, 8 were hypodiploid and 15 hyperdiploid with 75% of these having chromosome counts between 50 and 58. The commonest abnormalities were loss of a sex chromosome (12 cases), structural abnormalities of 1 (12), monosomy of 13 (11), trisomies of 3 (10), 5 (10), 15 (9), 9 (8) and 11 (7) and abnormalities of 14q32 (8) including 2 cases of t(11;14). Less frequently cited abnormalities such as trisomy 21, loss of 20 or del(20q) and deletions of 8p were also seen. Abnormalities of 11q were seen in only 2 cases, excluding the t(11;14) cases. The finding of any cytogenetic abnormality has been associated with stage III disease, > 30% bone marrow plasmacytosis, high levels of b2 microglobulin and the presence of lytic bone lesions. Plasma cell percentages ranged in our cases from 0 to 94% with a median of 28 % and 35 cases (41%) had a plasma cell percentage of > 30%. There was no correlation between the presence of > 30% plasma cells in the marrow and a successful result. The presence of > 30% plasmacytosis did however significantly increase the likelihood of finding an abnormal karyotype ($p=0.001$). Monosomy 13 or del(13q) has been found by some but not all authors to correlate with a short survival and abnormalities of 11q have also been associated with a poor prognosis. Alternately, hyperdiploidy has been linked to longer survival. Thus, there is intriguing data appearing regarding the prognostic potential of cytogenetics in multiple myeloma (MM), but more information is needed to determine its usefulness.

57.

VARIATIONS IN TRAIL-INDUCED APOPTOSIS OF EASILY OBTAINED MYELOMA CELLS ARE NOT RELATED TO TRAIL RECEPTOR EXPRESSION OR PRIOR CHEMOTHERAPY. A Spencer¹, L.Linoz², T-X Yeh¹. ¹BMT Programme, The Alfred Hospital, Melbourne, Victoria & ²Hunter Haematology Research Group, Mater Misericordiae Hospital, Newcastle, NSW.

TNF-related apoptosis-inducing ligand (TRAIL) shares significant homology with CD95 (Fas) ligand and has the ability to induce apoptosis in sensitive cells through a caspase mediated pathway. We have previously demonstrated TRAIL-induced apoptosis of the myeloma (MM) cell lines NCI H929 and RPMI 8226 and the FasL-sensitive Jurkat T-cell ALL line. Resistance to TRAIL-induced apoptosis was seen with the lymphoblastoid cell lines (LCL) U266 and MC/CAR. Two different forms of TRAIL - purified human recombinant soluble TRAIL (S-TRAIL; comprising residues 1-14-281, Biomol) and a leucine zipper construct of TRAIL (LZ-TRAIL, Immunex) were used with the latter demonstrating maximal apoptosis induction of sensitive lines at lower concentrations (500 ng/ml) than the former (1 µg/ml). We have now measured TRAIL-induced apoptosis of freshly obtained bone marrow (BM) MM cells from 16 patients at varying stages of their disease. Furthermore, we have examined the relationship between TRAIL-receptor expression (TRAIL-R1, R2, R3, R4 and osteoprotegerin) and TRAIL-induced apoptosis in sensitive and resistant cell lines and 5 MM patients utilising RT-PCR and surface immunostaining of purified MM cells. Mononuclear (MNC) BM cells were obtained from 16 MM patients and incubated for 24 hours with S-TRAIL (n = 8) (2 µg/ml) or LZ-TRAIL (n = 8) (1 µg/ml) with appropriate non-TRAIL incubated control cultures. The number of viable MM cells following incubation with or without TRAIL was determined by staining with anti-CD138 and subsequent analysis on an EPICS profile II. The relative TRAIL-induced reduction in MM cells was then calculated from the percentage of MM cells observed post-incubation using the formula: untreated minus TRAIL-treated/untreated. BM samples from 6 of 16 (38%) patients demonstrated a significant (>10%) relative reduction in the number of MM cells following TRAIL incubation (range 1% - 59%). This did not correlate with prior therapy, as a reduction was seen in 2 of 5 samples obtained from patients at diagnosis, 2 of 5 samples following standard-dose chemotherapy and 2 of 6 samples following high-dose chemotherapy. Concordance between TRAIL-receptor mRNA detection and cell surface expression was shown in the 2 MM cell lines and 2 LCL. Furthermore, all 4 cell lines and 5 MM patient samples tested (2 sensitive, 3 resistant) demonstrated mRNA expression of the intra-cellular death domain containing TRAIL-R1. Variable expression of the 2 decoy (TRAIL-R3 and R4) and soluble (osteoprotegerin) receptors was seen and this did not correlate with TRAIL sensitivity. We conclude that MM cell expression of death effector receptors for TRAIL is insufficient to confer sensitivity to TRAIL-induced apoptosis but that in a significant minority of patients, irrespective of prior therapy, MM cells are sensitive to TRAIL-induced apoptosis. The investigation of TRAIL as an adjunct to presently available therapies for MM is justified.

58.

CYTOPLASMIC TGF- β AND IL-10 ARE RESPONSIBLE FOR DENDRITIC CELL DYSFUNCTION IN MYELOMA PATIENTS WITH ACTIVE DISEASE. RD Brown, B Pope/ A Murray, W Esdale, J Gibson and DE Joshua. Institute of Haematology, Royal Prince Alfred Hospital, Camperdown, NSW.

It is ironic that immunotherapy protocols are being widely evaluated as a novel form of therapy for patients with multiple myeloma since these patients respond poorly to other forms of immunisation. The long term goal of our studies is to determine more favourable conditions for idiotype immunisation in patients with myeloma. We have studied high potency dendritic cells and the conditions for maximal expression of the costimulatory molecules CD80 and CD86 which determine the nature of the T cell response. High potency dendritic cells (DCs) were defined as CMRF 44+, PI-, CD19-, CD14- cells according to the standard assay (BLOOD 89:3708). The number of DC progenitors in peripheral blood was 0.05-0.8% of the mononuclear fraction. The expression of the costimulatory molecule CD80 (B7-1) was upregulated by huCD40LT (Immunex) + rIL-2 on normal DC. However CD80 upregulation on the DC of patients with myeloma was significantly reduced in patients in stable disease (n=93 and absent in those with active disease (n=7). Neutralisation studies using monoclonal antibodies identified TGF β and IL-10 as the inhibitors involved and the addition of rTGF β to normal DC caused a similar defect to that seen in the myeloma patients. As the serum TGF β and IL-10 levels in these patients were not significantly different from normal (TGF β range 0 - 32pg/1 and IL-10 range 0 - 20 pg/l) further studies are required to quantitate cytoplasmic TGF β and IL-10 levels in DCs, plasma cells and T cells to determine the relationship between these factors and immune paresis. These studies demonstrate that DCs collected from patients with active disease would be inhibited in their antigen presenting capacity and thus be poor cellular vectors for immunotherapy. A biological modifier like huCD40LT may be necessary to upregulate CD80 expression to avoid further T cell tolerance/anergy.

59.

PRIMARY NON-HODGKIN'S LYMPHOMA OF THE TESTIS: A RETROSPECTIVE ANALYSIS SPANNING THREE DECADES. B Solomon, JF Seymour, MM Wolf, EH Januszewicz, A Wirth, HM Prince. Departments of Haematology & Radiation Oncology, Peter MacCallum Cancer Institute, East Melbourne, VIC.

Primary non-Hodgkin's lymphoma (NHL) of the testis is an uncommon clinical presentation, representing 5% of testicular tumours and just 1% of all cases of NHL. There are no prospective or randomised studies to provide prognostic information and guide treatment. However, prior retrospective studies have described an aggressive clinical course and a distinct pattern of extranodal relapse. Therefore we have retrospectively analysed all patients (pts) seen at PMCI from 1972 - 98 with previously untreated NHL (excluding lymphoblastic or Burkitt's) whose presenting complaint was a testicular mass. 26 cases were identified from the medical records database, and one excluded due to a concomitant myeloproliferative disorder, leaving 25 for further analysis. The median age was 69 yrs (range 45 - 82) with involvement of the left testis in 16 (64%), and right in 13 (52%) (bilateral in 4; 16%). Disease stage was; I in 14 (56%), II in 8 (32%), and IV in 3 (12%).

Other features at baseline: Performance Status 2 in 8%, elevated LDH in 15%, IPI score > 2 in 24%, median serum albumin 36 g/L (range 24 - 45). Diagnosis was established by orchidectomy in 24 cases and histology was diffuse in all pts (large-cell or mixed). One pt had concomitant paraneoplastic nephrotic syndrome. 24 pts received additional therapy post-surgically; including chemotherapy in 18 (median 5 cycles; anthracycline-containing in 13), XRT in 11 (encompassing inguinal, iliac and paraaortic nodes in 8, and the contralateral testis in 5), with 5 pts receiving both modalities. 2 pts received CNS prophylaxis with IT methotrexate, neither of whom has relapsed within the CNS to date. 22 of 25 pts (88%) attained CR, with 2 early deaths and 1 case of resistant disease. 13 of 22 responding pts have relapsed from CR, with a continuous pattern evident up to 10 yrs, and a median remission duration of 5.2 yrs (10 yr RFS rate 36 + 12%). At relapse 3 pts had symptomatic hypercalcemia and the dominant sites of failure were extranodal (91%), involving the contralateral testis (3 pts - none had received XRT to the remaining testis), cerebral parenchyma (2), lung (3), skin (1), bone (2) and liver (1). 1 additional pt subsequently developed leptomeningeal disease (cumulative incidence CNS disease 13%). 19 pts have died, 4 from unrelated causes, and with follow-up beyond 20 yrs, the median overall survival is 4.4 yrs, and cause-specific survival (CSS) is 4.6 yrs (10 yr rate 40 + 12%). Within the entire cohort, factors associated with an inferior CSS were; serum albumin < 35 g/L (median CSS 13.3 vs 1.4 y; P = 0.007), advanced stage, and lack of anthracycline-containing chemotherapy (both P = 0.14). IPI score, age, application of XRT and year of diagnosis were not significant. Among pts with stage I / II disease (n = 22), albumin < 35 g/L (median CSS 13.3 vs 1.6 y; P = 0.037) and no anthracycline-containing chemotherapy (P = 0.10) remained predictive. There was a trend for improved outcome with 6 cycles of chemotherapy compared with fewer (median CSS 13.3 vs 4.2 yrs). **In conclusion**, this analysis confirms the reported demographics and aggressive nature of primary testicular NHL, even when clinically localised, and has identified serum albumin as a strong prognostic indicator. Relapse risk persists up to 10 yrs and frequently involves widespread extranodal sites including the CNS and contralateral testis, limiting the value of abdomino-pelvic XRT. We suggest that optimal therapy, even for clinically localised disease, should include (1) 6 cycles of anthracycline-based chemotherapy, (2) prophylactic XRT to the contralateral testis, and (3) CNS prophylaxis, although the parenchymal distribution of recurrences makes the most effective means of achieving this problematic. These data have been submitted to the

International Extranodal Lymphoma Study Group multicentre analysis and will hopefully contribute to the recognition of specific prognostic indices and the development of novel investigational treatment strategies.

60.

POLYMORPHISMS IN THE INTERLEUKIN-10 (IL-10) GENE PROMOTER INFLUENCE SUSCEPTIBILITY TO NON-HODGKIN'S LYMPHOMA. Cunningham LM*, Chapman CML*, Dunstan R^a, Joske DJL** Department of Haematology, The Western Australian Centre for Pathology and Medical Research (PathCentre), Queen Elizabeth II Medical Centre, Western Australia. ^a School of Biomedical science, Curtin University of Technology.

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine which inhibits T cell response and enhances B cell proliferation and differentiation. Expression of IL-10 is transcriptionally regulated and has been shown to be under genetic control. A number of polymorphisms have been identified in the IL-10 gene promoter, including three biallelic substitutions at positions -1082, -819 and -592 numbered relative to the transcription start site. *In vitro* studies have revealed that the GG genotype at position -1082 and the GCC haplotype (G at -1082, C at -819 and C at -592) are associated with high IL-10 protein expression. This study tested the hypothesis that polymorphisms in the IL-10 gene promoter, particularly those which affect protein expression, may be of importance in determining susceptibility to Hodgkin's and non-Hodgkin's lymphoma. DNA was extracted from 112 patients with lymphoma and samples were genotyped for polymorphisms in the IL-10 gene promoter at positions -1082, -819 and -592. Genotype results were correlated with lymphoma subtypes according to the REAL system of classification. Genotype frequencies at position -1082 are summarised in the table below:

Genotype	Controls (n=162)	Low grade NHL (n=22)	Int. grade NHL (n=39)	High grade NHL (n=24)	HD (n=27)
AA	41 (25.3%)	6 (27%)	13 (33.3%)	13 (54.2%)	9 (33.3%)
AG	80 (49.4%)	13 (59%)	20 (51.3%)	8 (33.3%)	12 (44.4%)
GG	41 (25.3%)	3 (14%)	6 (15.4%)	3 (12.5%)	6 (22.2%)

Frequency of the low IL-10 producing AA genotype was increased in all grades of NHL and HD, although this difference was only statistically significant in the high-grade sub-type ($p = 0.017$, Chi-Square). More than 50% of patients with high grade lymphoma were found to have the low IL-10 producing AA genotype and the odds ratio for the association between this genotype and high grade lymphoma was 3.3 (95% CI 1.3 – 8.3). Similarly, the frequency of the lower IL-10 producing ACC and ATA haplotypes were increased in all grades of NHL and HD, although again this was only statistically significant in the high grade subgroup ($p = 0.02$, Chi-Square). Odds ratio for the association between the ATA and ACC haplotypes and high-grade lymphoma was 2.2 (95% CI 1.12- 4.49). We conclude that polymorphisms in the IL-10 gene promoter influence susceptibility to lymphoma, particularly high grade or aggressive forms of NHL. Further investigation is required to fully elucidate the role that this cytokine plays in lymphomagenesis.

61.

CONVENTIONAL PROPHYLACTIC INTRATHECAL CHEMOTHERAPY IS INEFFECTIVE FOR THE PREVENTION OF CENTRAL NERVOUS SYSTEM (CNS) RECURRENCE IN PATIENTS WITH HIGH-RISK INTERMEDIATE-GRADE NON-HODGKIN'S LYMPHOMA (NHL). SL Chua, JF Seymour, J Streater, MM Wolf, EH Januszewicz, HM Prince. *Departments of Haematology, & Pharmacy, Peter MacCallum Cancer Institute, East Melbourne, VIC.*

CNS relapse is a devastating and usually fatal complication of NHL. The efficacy of CNS prophylaxis is established in patients (pts) with Burkitt's or lymphoblastic NHL. In contrast, for pts with "intermediate-grade" NHL the role of CNS prophylaxis is less clear. Factors such as involvement of bone marrow (BM) or multiple extra-nodal (EN) sites, an elevated serum LDH, or a high International Prognostic Factors Index (IPI) score can identify pts at increased risk of CNS recurrence who require CNS-directed prophylaxis. This usually consists of intrathecal (IT) methotrexate (MTX) \pm Ara-C. Although routinely used, the efficacy of such IT prophylaxis is unproven. To examine this question, we analysed the outcome of all pts with newly diagnosed intermediate-grade NHL receiving IT prophylaxis from 1/91-10/99 at Peter MacCallum, comparing their incidence of CNS relapse with that expected without prophylaxis using a published predictive index. 25 pts were identified from pharmacy records and all were evaluable. Histology was diffuse large cell in 23, anaplastic and angiocentric in 1 case each. Immunophenotypic lineage (n=23) was; B in 18, T in 4, and NK in 1. Other pt features; male 68%, median age 54yrs (range 15-74), stage I_E in 7, IV in 18 (median 2 EN sites, BM involved in 10), B-symptoms in 11, high LDH in 67%, high β_2 -M in 64%, PS \geq 2 in 28%, and median IPI score of 3 (0-5). No pts had clinical evidence of CNS involvement at diagnosis and in all cases negative cytology was obtained from the initial CSF examination (median 15d from diagnosis); median WBC count 0 (0-16) $\times 10^6/L$, protein 0.31 (0.2-1.39) g/L. Systemic treatment with anthracycline-based chemotherapy was used in all pts (median 6 cycles; range 1-8) and included high-dose MTX \pm Ara-C in 6 pts. 3 pts also received consolidative local XRT. Response to systemic therapy was CR in 56%, PR 32%, and NR 12%. IT CNS prophylaxis was given via lumbar puncture alone in 23, used a ventricular reservoir in 2 pts, was concurrent with systemic therapy in 21, and followed this in 4. The median number of IT treatments was 5 (1-12) and comprised MTX \pm steroid in 14, together with Ara-C in 11. Median MTX dose per injection was 12mg (10-15), and Ara-C was 50mg (40-100). 2 pts also received prophylactic cranial XRT. Complications of IT prophylaxis led to cessation in 1 pt and included; headache in 3, arachnoiditis 1, and back pain 1. The median interval from diagnosis to first IT injection was 14d (range 1-241). With a median follow-up of 34 Mo the overall survival rate is $36 \pm 11\%$ at 3yrs and isolated CNS disease as the first relapse site developed in 5 pts, with 1 additional pt developing CNS disease after systemic relapse. 5 of the 6 CNS relapses occurred < 1 yr from diagnosis (1 at 26 Mo) and involved; spinal cord 2, brain parenchyma 2, leptomeninges 4. The overall 3yr actuarial CNS-relapse rate was $32 \pm 11\%$. Although the power to detect significant differences is low, factors associated with a trend for a higher CNS-relapse rate were; B lineage, delay of ≥ 14 d from diagnosis to first IT injection, and systemic treatment lacking HD MTX \pm Ara-C (each $P \leq 0.16$; 3yr CNS-relapse rate $\sim 50\%$) Disease stage, pt age, serum LDH, no. EN sites, IPI score, no. IT injections, baseline CSF protein, and BM involvement were not predictive (each $P \geq 0.2$). Based on a published predictive index (Ann Oncol 9:191, 1998), the expected number of cases of CNS relapse in our cohort without prophylaxis is 2.56. We have seen 6 instances despite prophylaxis, which is significantly higher than expected (Observed / Expected ratio 2.3; $P = 0.035$). Thus, there is no evidence that IT CNS prophylaxis, as applied here, was effective at reducing the incidence of CNS relapse in this high-risk cohort. These data suggest that if conventional IT chemotherapy is used, it should be commenced as early as possible in the treatment program (≤ 14 d). However, alternative approaches such as the use of long-acting drug formulations (e.g. Depot Ara-C) or the incorporation of systemic HD MTX \pm Ara-C, either in primary therapy (e.g. Hyper-CVAD) or as adjunctive therapy appear more promising and merit prospective evaluation in a multicentre setting.

62.**PHASE II STUDY OF DICE CHEMOTHERAPY IN PATIENTS WITH LYMPHOMA James J Biagi, H M Prince, C Smith, E Abdi, M Leahy, C Folkson, M Wolf, H Januszewicz, J Seymour, J Stone, B Dale, Dept of Haematology, Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia, on behalf of the ALLG**

Background: The optimal treatment for patients with non-Hodgkin' s lymphoma (NHL) who fail front-line combination chemotherapy remains a therapeutic challenge, prompting ongoing clinical trials to elucidate more effective combination therapies. Current salvage regimens for relapsed aggressive NHL produce a 23-37% CR if a CR to initial therapy was achieved. In refractory disease, however, CR rates do not reach 20%, with long-term DFS < 10%.

Rationale and Objectives: Ifosfamide (Ifos) as a single agent in the setting of refractory disease demonstrates an ORR up to 47%. It has been incorporated into combination regimens. The largest study to date used MIME to treat 208 patients with indolent and aggressive NHL. Results included an ORR of 60% with 24% CR rate. DICE, another combo regimen incorporating Ifos, was reported in 1995 for heavily pre-treated, relapsed or refractory patients. The ORR was 67% with 23% CR rate. The objective of our study was to determine the effectiveness of a modified DICE regimen in patients with relapsed or refractory NHL, chronic lymphoproliferative disorders (LPD) and Hodgkin's disease (HD). The original DICE protocol was modified for two main reasons: 1) dosing and schedule were modified to reduce the inpatient requirement for intravenous (iv) infusion from four days to one; 2) a lower dose regimen was added to include treatment of older patients (>55 yrs). The endpoints of this study were response rates, toxicity, progression-free survival (PFS) and overall survival (OS). In this paper, we present available responses and toxicities.

Treatment Schedule	Patients >55 years:	Patients <55 years:
Dexamethasone	10mg QID orally D1 - D4	10mg QID orally D1 - D4
Ifosfamide	2.5g/m ² IV D1	4gm/m ² IV D1
Cisplatin	50mg/m ² IV D1	100mg/m ² IV D1
Etoposide	50mg IV D1	100mg IV D1
Etoposide	100mg orally D2-D4	200mg orally D2-D4
MENSA	1g/m ² pre/post/ ifos D1/D1x2	1.6g/m ² pre/post ifos D1/D1x2D

The planned treatment is monthly for 6 cycles, or until disease progression or patient withdrawal.

Results: This trial has recently completed accrual of 39 of the planned 40 patients. Herein we present response rates and toxicity data. Treatments were well tolerated, with acceptable toxicities and minimal delays or dose reductions. Results on disease free and overall survival endpoints are pending final data analysis. We conclude that DICE is an effective regimen in relapsed/refractory lymphoma with promising response rates in this poor prognosis group of patients.

63.

RADIOIMMUNOTHERAPY WITH IODINE-131 ANTI-CD 20 CHIMERIC MURINEMUMAN MONOCLONAL ANTIBODY (RITUXIMAB) FOR RELAPSED OR REFRACTORY NONHODGKINS LYMPHOMA: COMPASSIONATE PATIENT USAGE IN AUSTRALIA IN 1999. Turner JH, Leahy MF, Ng P, Claringbold PG, Davidson D, Webb M and Cordingley FT. Fremantle Hospital, Fremantle, Western Australia.

The majority of patients with NHL are not cured by chemotherapy, radiotherapy or high-dose treatment with autologous bone marrow transplantation or peripheral blood stem cell support. B-cell lymphoma is however a particularly good candidate for radioimmunotherapy because the disease is inherently radiosensitive, and the malignant cells in the blood, bone marrow, spleen and lymph nodes are accessible to monoclonal antibodies.

Rituximab, a chimeric IgG1 antibody which targets the CD 20 antigen, binds to B cell surface antigens which are not shed or modulated and thus allows use of Iodine-131 for anti-CD-20 targeted radioimmunotherapy. Iodine-131 has gamma emission which permits prospective individualised dosimetry calculations to be performed on each patient to minimise the risk of toxicity. The maximum tolerated dose (MTD) for 131I-anti CD-20 radioimmunotherapy has been calculated at 0.75 Gy in dose escalation trials of 131I-Tositumomab (Wahl et al. J Nucl Med 1998, 39 Suppl: 21S-27S) and is calculated for ¹³¹I-Rituximab on standard whole body gamma imaging systems at Fremantle Hospital using a PC based programme to measure retention of radioiodine tracer labelled anti-CD 20 antibody.

At Fremantle Hospital we have developed a semi-automated robust method for preparation of ¹³¹I-Rituximab with consistent radiolabelling efficiency of >96% and preservation of immunoreactivity.

Ten consecutive patients treated at Fremantle Hospital in 1999 were evaluable, having been followed up for at least two months. All patients had relapsed indolent B cell lymphoma. Seven had recurrent follicular lymphoma, two of whom had shown clinical and histological evidence of transformation. Two patients had recurrent small cell lymphoma and one had mantle cell lymphoma. All patients demonstrated CD-20 positive lymphoma. The age range was 45 - 81 years. There were seven females and three males. All had relapsed after at least one standard regimen of chemotherapy. Five had received multiple chemotherapy regimens.

Following treatment at a dose of 0.5 to 0.75 Gy, the majority of patients being treated at the MTD, six patients achieved complete clinical response with continuing remission noted more than six months following radioimmunotherapy. Three patients achieved a partial clinical response sustained for more than six months, one of whom subsequently progressed. Another patient progressed without evidence of clinical response.

Prior to radioimmunotherapy all patients had a platelet count greater than 100/nl and neutrophils greater than 1.5/nl. The only toxicity following treatment was haematological, with platelet and neutrophil nadirs occurring between 40 and 45 days. There was no requirement for haematological support other than a single instance of G-CSF in one patient in whom neutropenia persisted over three weeks.

These encouraging results have prompted a formal Phase II multicentre Australian clinical trial of I-131 Rituximab radioimmunotherapy of non-Hodgkins lymphoma.

64.

INSIGHTS INTO FUNCTION OF THE AML FAMILY OF TRANSCRIPTION FACTORS THROUGH STUDIES IN ZEBRAFISH. ML Kalev-Zylinska, MR Vitas, AM Baas, PS Crosier, KE Crosier Department of Molecular Medicine, University of Auckland, Auckland, New Zealand

The family of AML genes is characterised by a highly conserved Runt domain and members play critical roles in both normal developmental processes and disease. AML1 is required for definitive haematopoiesis and its involvement in the aetiology of several leukaemias is well established. The function of AML2 is largely unknown, but recent evidence supports a role downstream of the TGF-beta signaling pathway. AML3 is critical for bone development and implicated in the autosomal dominant disorder of skeleton formation, cleidocranial dysplasia. The zebrafish is an excellent genetic system for developmental studies. We have isolated cDNAs encoding zebrafish AML1, AML2 and AML3. The amino acid sequences demonstrate that these genes are highly conserved between human, mouse, zebrafish and Xenopus. The expression of AML genes during zebrafish embryogenesis has been studied by whole-mount in situ hybridisation and distinct patterns have been obtained for each gene. AML1 expression is seen in the blood forming areas, lateral plate mesoderm and intermediate cell mass, from 12 hours post fertilisation (hpf). Studies of murine haematopoiesis have provided evidence that AML1 is required for the differentiation of all definitive haematopoietic cells, but not for primitive erythropoiesis. The expression of AML1 in the regions marking the earliest appearance of blood and vascular tissue in the zebrafish suggests an additional role for this gene in primitive haematopoiesis. At 48 hpf, AML1 is expressed in cells populating the ventral wall of the dorsal aorta, that are likely to represent definitive haematopoietic stem cells. Expression is also seen in the retina and hindbrain; reminiscent of the established involvement of other haematopoietic transcription factors in neuronal development. AML2 is strongly expressed in the trigeminal ganglia and Rohon-Beard cells (sensory neurons similar to dorsal root ganglia), suggesting its involvement in neurogenesis. Expression is also observed early, on the yolk sac and later in the ventral tail putative haematopoietic region, raising the possibility of a role for AML2 in blood development. AML3 expression is first seen at 24 hpf, in the pectoral fins and subsequently in the jaw. The patterns of expression observed form the basis for the design of further functional studies. Analysis of haematopoietic mutants is being undertaken and results will be presented. The genetic opportunities offered

by the zebrafish system will enable more accurate delineation of the function of AML genes in the complex developmental pathways of haematopoiesis, neuropoiesis and osteogenesis, and in diseases including leukaemia.

65.

A PHASE II STUDY OF INTERFERON ALPHA (IFN) AND INTERMITTENT ORAL CYTARABINE (YNK01) IN THE TREATMENT OF NEWLY DIAGNOSED CHRONIC MYELOID LEUKAEMIA (CML). P. Mollee¹, K. Taylor¹, C. Arthur², H. Januszewicz³, A. Grigg⁴, T. Hughes⁵, J. Seymour³, K. Bradstock⁶, M. Wolf³, J. Gibson⁷, T. Schwarzer⁸, A. Spencer⁸, P. Browett⁹, T. Hawkins⁹, M. Seldon¹⁰, R. Herrmann¹¹, A. Watson¹², N. Martin¹, S. Shina¹³, S. Wright¹, R. Rodwell¹, J. Coulston¹, D. Taylor¹. ¹Mater Adult Hospital ²Royal North Shore Hospital ³Peter MacCallum Cancer Institute ⁴Royal Melbourne Hospital ⁵Royal Adelaide Hospital ⁶Westmead Hospital ⁷Royal Prince Alfred Hospital ⁸The Alfred Hospital ⁹Auckland Hospital ¹⁰Mater Hospital Newcastle ¹¹Royal Perth Hospital ¹²Townsville General Hospital ¹³Schering-Plough.

YNK01 is an oral precursor of cytarabine and is metabolised to active drug by the liver. Alone or in combination with IFN for CML, YNK01 has produced encouraging haematologic and cytogenetic responses in early trials. We aimed to study the ability of IFN and YNK01 to induce complete haematologic (CHR) and major cytogenetic responses (MCR) as previously achieved with combination IFN and subcutaneous cytarabine (Guilhot. *N Engl J Med* 1997; 337:223), and to assess the safety and tolerability of the combination. Between December 1997 and March 2000, 39 patients with newly diagnosed Ph+ CML were eligible and enrolled on study. The median age was 47 years (range, 17 to 72) with 78% male and 22 % female. At diagnosis Sokal index was 49% low risk, 32% intermediate risk and 19% high risk. Patient's counts were initially controlled with hydroxyurea prior to the introduction of IFN to maximal tolerated dose over 4 to 6 weeks. Combination therapy was delivered in 4 weekly cycles. Each cycle consisted of YNK01 (600mg/d in first cycle) on days 1-10 and continuous IFN. At the end of each cycle, YNK01 dose was adjusted according to toxicity observed during the prior 4 weeks. Haematologic response and toxicity were assessed with each cycle with cytogenetic response assessed every 12 weeks. Patients were required to complete two cycles of combination therapy to be evaluable for efficacy. Eight were considered non-evaluable due primarily to IFN intolerance, leaving 31 patients continuing on trial. After 12, 24, 36 and 48 weeks of combination therapy, the CHR and MCR rates were as follows:

	12 weeks	24 weeks	36 weeks	48 weeks
Evaluable patients	22	21	19	14
CHR	64%	67%	68%	57%
MCR	14%	29%	47%	29%

To date, in evaluable patients, the median time to achievement of MCR has been 24 weeks, and 3 of the patients have achieved a complete cytogenetic response. On an intent to treat basis, the MCR at 36 weeks is 35%. CHR and MCR rates have been similar to IFN/subcutaneous cytarabine studies. Therapy has been prematurely discontinued in 11 of the 31 patients to date due to toxicity (n=8), treatment failure (n=2) and non-compliance (n=1). Such toxicity has included asthenia, gastrointestinal intolerance, cytopenias and hepatitis. Two of these patients have continued on IFN to maintain a MCR. Throughout the study the median daily dose of IFN for evaluable patients was 8 MU (range, 3 to 10) and the median daily dose during the 10 days of therapy of YNK01 for evaluable patients was 600 mg (range, 200 to 1000). This early data indicates that YNK01 in combination with IFN can induce comparable haematologic and cytogenetic responses to parenteral cytarabine and IFN.

66.

EVIDENCE FOR GERM LINE ORIGIN OF CHILDHOOD ACUTE LEUKAEMIA. Ian Morison, Lana Ellis, Anthony Reeve, University of Otago, Dunedin New Zealand.

We hypothesized that the age of onset of childhood acute lymphoblastic leukaemia might reflect a constitutional predisposition. Given the high frequency of deletion involving chromosome 9p in childhood ALL (20-60% of cases) we investigated whether 9p deletion might reflect pre-existing germline gene inactivation. To do this we determined the parental origin of the deleted 9p21 allele in a series of 48 cases of childhood ALL. Ten cases of ALL showed loss of heterozygosity at one or more of the three 9p21 polymorphic markers and in 9 of the 10 cases the lost allele was maternally derived (p = 0.021). In one other small study (Heyman M et al. *Int J Cancer* 1993;54,748) 4/5 cases of childhood ALL also showed loss of the maternal allele (combined probability = 0.0074). The preferential loss of the maternally-derived allele suggests that a parent-specific process modifies the chromosome 9p genome during gametogenesis, and that germline events may play a role in the leukemogenic pathway. Models consistent with the known features of ALL and with the function of known genes in the 9p region will be presented. Our data together with previous data from Heyman et al, provide strong evidence for the involvement of a germline factor which predisposes to a proportion of cases of childhood ALL. Targeted epidemiological studies of the paternal environment in the subgroup of children with 9p LOH may provide a useful way forward to understanding the environmental factors involved in childhood ALL.

67.

ANALYSIS OF VIABLE, APOPTOTIC AND NON-VIABLE LEUKAEMIC BLASTS IN PATIENTS TREATED WITH LOW-DOSE OR CONVENTIONAL CHEMOTHERAPY FOR AML. AE Trickett, A Manoharan, YL Kwan. Clinical Haematology, St George Hospital, Kogarah, NSW 2217.

Introduction: In our institution, elderly patients with acute myeloid leukaemia (AML) are treated with low-dose combination chemotherapy (LDCC) to induce remission without treatment-related morbidity and mortality. LDCC, which consists of cytarabine, etoposide, and mitozantrone or 6-thioguanine, yields overall response rates (complete remission + partial remission) of 83%, which appears to be more favourable than with conventional dose chemotherapy (CDC). There are currently no accurate markers for predicting response to therapy and the reason(s) for the favourable results attained with LDCC are not known, although it has been proposed that lower drug concentrations preferentially induce apoptosis of mitotic cells. Aims: (1) To determine whether LDCC increases the proportion of apoptotic and dead leukaemic blasts in the peripheral blood after commencing therapy. (2) To determine whether quantitation of viable, apoptotic and/or dead blasts could be used as a prognostic indicator for clinical response. Methods: Annexin-V-FITC and propidium iodide were used to differentiate viable, early apoptotic and late apoptotic/necrotic (non-viable) cells by flow cytometry. Leukaemic blasts were identified by expression of CD34 or CD117. Percentage and absolute number of viable, early apoptotic and non-viable blasts were quantitated daily in the blood of patients receiving either LDCC or CDC for AML, both prior to and for up to 6 days after initiation of therapy. Patients: Nineteen patients with AML were studied: 10 following LDCC therapy (at diagnosis = 2, 1st relapse = 3, 2nd relapse = 2, to MDS = 3), and 9 following CDC (at diagnosis = 5, 1st relapse = 4). The median age of patients in the LDCC group was 68 compared to 39 in the CDC group. Results: There was a significant increase in the percentage of early apoptotic and non-viable leukaemic blasts in the blood 3-4 days after commencement of either LDCC or CDC, as compared to the pre-therapy levels. The increase in percentage of early apoptotic blasts 3-4 days after starting treatment was greater in patients receiving CDC. The number of viable leukaemic blasts remaining in the blood 3 days after initiation of chemotherapy was significantly different in LDCC patients who were stratified according to their subsequent clinical response: complete remission median viable blasts $\times 10^9/L$ blood = 0.021 (N = 3), partial remission = 0.1 (N = 4), or no response = 8.4 (N = 3). The relationship was less clear in patients receiving CDC: complete remission median viable blasts $\times 10^9/L$ blood = 0.011 (N = 5), partial remission = 2.65 (N = 1), no response = 0.1 (N = 2). Conclusions: (1) Treatment of AML using LDCC increased the proportion of leukaemic blasts undergoing apoptosis, but to a lesser extent than CDC. (2) Quantitation of the absolute number of circulating viable blasts appears to give an early indication of the clinical response to LDCC.

68.

PARAFFIN SECTION IMMUNOTYPING OF LEUKAEMIAS. S Juneja, L Trute, D Westerman, D Venter, JF Seymour, HM Prince, Royal Melbourne Hospital and Peter MacCallum Cancer Institute, Melbourne.

Paraffin section immunotyping (PSI) of leukaemias is becoming an increasingly useful modality in the diagnosis & detection of residual disease. In certain specific situations it may be the only method for immunotyping leukaemias. PSI also has the potential to detect molecular abnormalities. It involves immunohistologic staining of bone marrow trephine biopsies or clot sections as well as suspected extramedullary myeloid tumours. The technique is applicable on tissues fixed in formalin, Bouin's or B5. The method used in our laboratory is the labelled streptavidin-biotin peroxidase method. The panel of antibodies that is useful in the acute lymphoblastic leukaemia includes CD10, CD79a & CD20 for B-lineage & CD3, F1, CD1a, CD4 & CD8. TdT & CD7 are not lymphoid lineage specific. A useful panel in AML includes anti-MPO ab, lysozyme, CD68, CD61 or (CD41), glycophorin A or C. Biphenotypic acute leukaemias & the rarer subtypes of leukaemias can also be characterized with the above panel & some additional abs. At this stage some antibodies which are useful in flow cytometry like CD13, CD33, CD117 are still not applicable on paraffin sections. PSI is very helpful in the diagnosis & detection of residual disease in hairy cell leukaemia. It can be especially useful in establishing Richter's transformation in CLL. PSI has a valuable role in specific situations in the diagnosis, characterization & monitoring of residual disease in leukaemias. It is likely to have a larger role with the increasing number of antibodies that can be used on paraffin sections as well as greater availability of this method with automated staining machines.

69.

RESPONSES TO INTRAVENOUS AND ORAL ARSENIC TRIOXIDE (As₂O₃) IN ACCELERATED PHASE CML AND MYELOYDYSPLASIA. Frank Firkin, Department of Medicine, St. Vincents Hospital

Safety and efficacy of As₂O₃ therapy has been examined in accelerated phase chronic myeloid leukaemia (AP-CML) no longer responding to interferon therapy, and in severely pancytopenic RAEB. Infusion of 10 mg As₂O₃ daily for 10-12 days in two AP-CML patients produced a fall in WCC with virtual elimination of circulating blasts, and reduction in spleen size. Marrow cellularity and proportion of immature cells was reduced. A moderate increase in annexin V binding to circulating cells occurred during treatment, implicating a possible apoptotic element to the decrease in tumour bulk. Duration of disease suppression was limited to 2-4 weeks, indicating a need for ongoing therapy, and likelihood of a different mode of action in AP-CML than in Acute Promyelocytic Leukaemia cells. IV As₂O₃ treatment in a patient with RAEB also produced a progressive fall in circulating blast cell count, and reduction in marrow cellularity over a period of 14 days. During treatment in all three patients there was a moderate degree of myelosuppression, manifested by a decrease in platelet count and an increase in transfusion requirements. Mildly abnormal liver function tests developed in all patients. Evaluation was also performed of (1) response to oral administration of As₂O₃ as a more convenient mode of drug delivery for protracted therapy, and (2) synergism with other agents. Two patients tolerated long term oral As₂O₃ in a dose of 5 mg daily for up to 120 days. Serum levels ranged between 0.6 - 1.2 mM and disease suppression was observed. Co-administration of oral thioguanine and hydroxyurea, or thioguanine alone produced considerably greater suppressive effects on circulating blast count and spleen size than the oral cytotoxic agents or As₂O₃ alone. This synergistic effect produced marked disease suppressive effects with oral As₂O₃ plus thioguanine doses as low as 40 mg third daily. These findings indicate As₂O₃ has therapeutic potential in AP-CML and in myelodysplasia, and justify further assessment of activity in relapsed and refractory haematological neoplasia. The possibility exists that As₂O₃ has an effect on the neoplastic cells which enhances susceptibility to other cytoreductive agents in Ph chromosome positive disease.

70.

TEACHING UNDERGRADUATE HAEMATOLOGY: THE UNIVERSITY OF SYDNEY APPROACH. Paul Vincent Institute of Haematology and the Kanematsu Laboratories, Royal Prince Alfred Hospital, Camperdown NSW 2050.

Flinders University, and the Universities of Sydney and of Queensland embarked on four-year graduate-entry medical programs, commencing in 1996 (Flinders) and in 1997 (Sydney and Queensland). More recently, the University of Melbourne has also instituted graduate entry for a proportion of its medical student intake. Students are selected on their performances in their undergraduate degree, in the Graduate Australian Medical School Admissions Test (GAMSAT), and at interview. Teaching in the Graduate Programs stresses self-directed, problem-based learning and much of it takes place in small problem-based learning groups. The course comprises four themes that run in parallel and complement each other; these are Basic and Clinical Sciences, Community-Doctor, Patient-Doctor and Personal and Professional Development.

Haematology teaching at the University of Sydney is a prominent part of Year 1 of the graduate medical program. It occupies a 5 week block, and more importantly perhaps it is taught entirely at the 3 clinical campuses. Each week is centred around a clinical case; Week 1, pernicious anaemia; Week 2, thalassaemia and malaria; Week 3, haemophilia; Week 4, chronic lymphocytic leukaemia, and Week 5, venous thrombosis and iron deficiency. Each case provides the basis for the lectures, learning topics, and tutorials that draw on those aspects of the case that illustrate each of the four themes of the Course. Haematology provides excellent material in basic and clinical science (eg, molecular biology), community-doctor (eg, ethics of prenatal diagnosis), personal and professional development (eg, death and dying) and patient-doctor (eg, ward sessions). The block has been generally well received by the students, the first cohort of whom is now in the final year of the course.

71.

ACUTE LEUKAEMIA IN CHILDREN: BMT OR CHEMOTHERAPY? Peter J. Shaw, Marie Bleakley, Loretta Lau, Geoffrey McCowage, *Oncology Unit, New Children's Hospital, Sydney NSW Australia*

The efficacies and toxicities of different treatments require continual re-evaluation. This is exemplified by considering treatment intensification supported by stem cell transplantation in acute leukaemia in childhood. Improvements in the results of chemotherapy must be compared with those of transplantation. There is no doubt that allogeneic transplantation (alloBMT) offers the best control of disease, but this must be weighed against toxicity and the possibility of reserving transplantation as salvage therapy.

Acute lymphoblastic leukaemia (ALL) remains the commonest childhood cancer. Between 1986 and 1992, 664 children with ALL were enrolled on Study V of the Australia and New Zealand Children's Cancer Study Group. The long-term event-free survival (EFS) is stable at 69%. As patients with a WBC of $> 100 \times 10^9/l$ had an EFS of 61%, WBC alone is not a good criterion to select patients for BMT in CR1. High WBC combined with the BFM prednisolone poor response, or the Philadelphia chromosome, and those not entering remission with standard induction therapy are the main subgroups who may be offered alloBMT in CR1. Given the intensity of frontline therapy, many ALL patients who relapse will benefit from alloBMT. At NCH, we treated 56 children with relapsed ALL with an intensive multiagent chemotherapy protocol, New York II; chosen as it complemented the chemotherapy used in CR1. Patients with a matched family donor were offered alloBMT, those without continued chemotherapy. The EFS at 5y is 9% for chemotherapy v. 58% for BMT. Although it is reported that patients who relapse off therapy have a good outcome with further chemotherapy, and BMT be reserved for yet another relapse, there are problems with this approach.

For AML, the position has been the opposite for the past 20 years. AlloBMT in CR1 has been preferred for almost all patients achieving CR1 with a suitable donor. Autologous BMT (ABMT) has been used for those lacking a matched sibling donor. At NCH, our EFS for 20 patients undergoing matched sibling BMT is 82%, for those undergoing ABMT 54%. Part of our success with alloBMT may relate to our use of escalated dose of busulphan given once a day and rapid tapering of cyclosporin for those who do not get GvHD. We have analysed the toxicity and efficacy of single daily dose busulphan in 78 children. The single daily dose is associated with high systemic exposure, high rate of engraftment and low toxicity. These patients have been followed up for 10 years, and show normal linear growth. However, late effects, such as partial alopecia and gonadal dysfunction are seen, and strategies that avoid toxicity associated with myeloablative conditioning would be preferred.

One reason for preferring BMT in CR1 has been difficulty in achieving CR2, but fludarabine-containing regimens may have solved this problem.

We have conducted a meta-analysis of the published literature of alloBMT and ABMT in paediatric AML. Allocation to alloBMT is associated with a reduced risk of relapse and improved disease-free and overall survival. The effect of ABMT is more difficult to gauge. A decision analysis based on published data supports chemotherapy alone for good risk AML patients in first remission, despite a 20% and 30% higher risk of relapse post-chemotherapy compared to ABMT or alloBMT. Despite continued collaboration across national boundaries allowing recruitment of ever larger numbers of patients, as results improve and prolonged follow-up is needed, prospective studies are a long way away from answering questions related to best treatment for paediatric, and adult, AML. Extending meta-analysis of published data to individual patient data could allow us to determine current best treatment within the recognised good, intermediate and poor-risk subgroups of AML patients.

72.

LIGAND BINDING TO GPIIb/IIIa - Y2K STATUS REPORT. Plow, E.F., Haas, T.A., Xiao, Z., Byzova, T.V., Cierniewski, C. Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Cleveland Clinic Foundation Cleveland, Ohio, USA

Antagonism of $\alpha_{IIb}\beta_3$ now has become a primary approach in the strategies to treat acute coronary syndromes. The development of oral "GPIIb-IIIa blockers" with new indications has the potential to greatly expand the utilization of these agents. While the existing and projected uses of GPIIb-IIIa blockers imply a broad understanding of their target and its blockade, there are many fundamental and critical questions regarding this integrin which remain unresolved. This presentation focuses on two of many such unresolved issues which impact on our understanding not only of the structure and function of $\alpha_{IIb}\beta_3$ but also on the efficacy of the GPIIb-IIIa blockers.

Among the most basic issues is the nature and specificity of the ligand binding site within $\alpha_{IIb}\beta_3$. While it is clear that both prototypic peptide antagonists, peptides derived from the C-terminus of the fibrinogen gamma chain (γ -chain peptides) and peptides containing the Arg-Gly-Asp (RGD peptides) sequence, inhibit macromolecular adhesive ligand binding to $\alpha_{IIb}\beta_3$, the relationship and location between these peptide binding sites in $\alpha_{IIb}\beta_3$ is unclear. The relatively low affinity of these two peptide sets for the receptor has been a major impediment to the resolution of this question. To circumvent this limitation, two cyclic peptides, cyclo(S,S)KYGCHarGDWPC (cHarGD), a mimetic of the γ -chain peptide, and cyclo(S,S)KYGCRGDWPC (cRGD), a mimetic of the linear RGD peptides, were synthesized, labeled with fluorescent or radioactive reporters, and their interactions with $\alpha_{IIb}\beta_3$ were analyzed. These studies led to the conclusions that: 1) the two peptides could bind to distant but allosterically interactive sites in $\alpha_{IIb}\beta_3$, 2) the availability of these sites to the peptide ligands was influenced by divalent cations; and 3) occupancy of these two sites imparted different functional responses to the receptor and to platelets. Thus, ligands for $\alpha_{IIb}\beta_3$, including the GPIIb-IIIa blockers, may bind to distinct sites in $\alpha_{IIb}\beta_3$ and may exert different influences on the functions of the receptor.

A second major uncertainty regarding $\alpha_{IIb}\beta_3$ revolves around the existence and importance of other proposed functions of the receptor. Clearly, the primary function $\alpha_{IIb}\beta_3$ rests in its ability to bind fibrinogen and von Willebrand factor binding to $\alpha_{IIb}\beta_3$ and, thereby, to mediate platelet aggregation. Nevertheless, still other functions of $\alpha_{IIb}\beta_3$ may contribute to thrombus formation. One such function depends upon the ability of $\alpha_{IIb}\beta_3$ to bind prothrombin and to influence its activation to thrombin. Prothrombin, but not thrombin, is a ligand for $\alpha_{IIb}\beta_3$ on both resting and activated platelets. While fibrinogen can inhibit prothrombin binding to stimulated platelets, it is unable to compete for its interaction with $\alpha_{IIb}\beta_3$ on resting platelets. Recognition of prothrombin may be dependent upon the exposure of the RGD sequence within the protease domain of prothrombin which resides in close proximity to the catalytic triad. Binding of prothrombin to $\alpha_{IIb}\beta_3$ accelerates its conversion to thrombin by Factor Xa/Va. This consequence of binding can be demonstrated with $\alpha_{IIb}\beta_3$ in a purified form or on platelets in either a resting or stimulated state. As thrombin is formed, it is released from $\alpha_{IIb}\beta_3$ and stimulates platelets to express the prothrombinase complex with greatly amplifies further prothrombin activation and, thereby, thrombus formation. Although prothrombin interacts similarly with resting and activated $\alpha_{IIb}\beta_3$, its recognition by the second β_3 integrin, $\alpha_{IIb}\beta_3$, requires activation for optimal recognition. While various GPIIb-IIIa blockers are all effective in inhibiting fibrinogen binding to $\alpha_{IIb}\beta_3$, they exhibit variable activity in inhibiting the contribution of the receptor to prothrombin activation. These differences may contribute to differences in the efficacy of the various GPIIb-IIIa blockers.

73.

SHEAR-INDUCED PLATELET/VWF INTERACTION. Sacha M. Dopheide, Suhasini Kulkarni, Nayna Mistry, Cindy L. Yap, Yupng Yuan and Shaun P. Jackson. Australian Centre for Blood Diseases, Monash Medical School, Box Hill Hospital Box Hill, Victoria, Australia 3128.

Blood platelets play a key role in maintaining the integrity of the vascular system through their ability to arrest bleeding (hemostasis) and promote repair of injured blood vessels. Considerable progress has been made in the last few years in our understanding of the adhesion mechanisms utilised by platelets to adhere to sites of vascular injury. These advances have been achieved in part through improved imaging techniques that enable real-time assessment of platelet thrombus formation *in vitro* and *in vivo*, and from the study of mice that have undergone genetic manipulation of one or more hemostatic components. These studies have highlighted the complexity of platelet thrombus formation under flow and have demonstrated the importance of synergistic adhesive interactions in this process. In particular, they have helped define the precise role of von Willebrand factor (vWf), and its platelet receptor, glycoprotein (GP) Ib/V/IX, in initiating platelet-vessel wall and platelet-platelet adhesion contacts. Recent studies have demonstrated that GP Ib/V/IX not only maintains the cytoskeletal architecture of resting platelets but also induces actin polymerization and cytoskeletal reorganization in rolling platelets. The ability of the vWf-GP Ib/V/IX interaction to induce remodelling of the actin cytoskeleton may be important for regulating platelet adhesion to the injured vessel wall.

74.

THE DYNAMIC CYTOSKELETON – ITS ROLE IN PLATELET FUNCTION AND HAEMOSTASIS. Simone M Schoenwaelder. From the Australian Centre for Blood Diseases, the Department of Medicine, Monash Medical School, Box Hill Hospital, Arnold Street, Box Hill, Victoria AUSTRALIA 3128

Platelets are small, anucleate blood cells, with a specialised role in haemostasis. These cells respond to vessel wall injury by executing a precise series of responses, designed to arrest blood loss. Platelets initially interact with the injured vessel through the binding of the adhesive protein von Willebrand Factor (vWF) to the platelet surface receptor glycoprotein (GP) Ib/V/IX. This initial interaction is vital for platelet tethering and rolling, especially under rapid blood flow conditions. As a result of the GP Ib/V/IX - vWF interaction, activation signals are transduced into the platelet interior, to activate the major platelet integrin $\alpha_{IIb}\beta_3$. In this active conformation, integrin $\alpha_{IIb}\beta_3$ mediates irreversible adhesion of platelets to subendothelial vWF, and subsequent spreading and aggregation, ultimately leading to the formation of a thrombus. The culmination of these platelet responses results in the arrest of blood loss from the site of vessel injury. Although platelets are critical in the normal process of haemostasis, in certain disease states, such as coronary heart disease these cells contribute to abnormal clot formation that can result in heart attack.

Platelets contain an extensive and very dynamic actin cytoskeleton (CSK). During the course of haemostasis, the platelet CSK undergoes varying degrees of remodelling. Exposure of platelets to vWf on the injured vessel wall induces CSK remodelling leading to dramatic alterations in platelet morphology, transforming the inactive discoid shaped platelet to an activated platelet extending multiple projections (filopodia). In addition to shape change, platelet-subendothelial and platelet-platelet adhesion (aggregation) are also regulated by the platelet CSK, through modulation of adhesion receptor function. After securing platelet adhesion, CSK remodelling regulates the process of platelet spreading through the extension of thin sheets of actin "lamellae", which greatly increase the surface area of adherent platelets and help form a "pseudoendothelium" at the site of vascular injury. This "pseudoendothelium" forms the foundation upon which further thrombus growth and clot formation will occur. In the final stages of haemostasis, the platelet CSK plays an important role in maintaining vessel wall patency by contracting the fibrin clot and shrinking its mass.

Given the fundamental role for the CSK in platelet responses during haemostasis, a better understanding of the mechanisms which initiate and regulate CSK remodelling, is required. One family of enzymes with a well-documented role in CSK remodelling is the Rho family of small GTP binding proteins. The Rho family contains a number of "classical" members which have been well described in the literature, including Cdc42 and Rac, which regulate the formation of filopodia and lamellipodia, respectively. One of the most widely characterised members of the Rho family is RhoA. Activation of this small GTPase results in induction of cell contractility and the formation of stress fibres and focal adhesions. The role of RhoA is thought to lie primarily in maintaining a stable contact between the cell and the underlying substratum. RhoA and its relevant signalling counterparts are all expressed in high levels in platelets, yet despite this, surprisingly little is known about the function of RhoA in these blood cells. One potential hypothesis is that the activation of RhoA, and its subsequent effects on the CSK, may contribute to the formation of stable platelet adhesion contacts, and ultimately in the formation of a platelet thrombus. The formation of these stable RhoA-mediated contacts may be particularly important when considering the flow conditions under which platelets must adhere *in vivo*. This hypothesis is currently being tested in our laboratory.

75.

MISMATCHED ALLOGRAFTS UTILISING ATGAM FOR CONDITIONING AND GVHD PROPHYLAXIS: ACCEPTABLE OUTCOME IN YOUNGER PATIENTS WITH HIGH-RISK DISEASE. A Spencer N Kennedy, J Muirhead AP Schwarzer. BMT Programme, The Alfred Hospital, Melbourne, Victoria

Over the past 3 years we have performed allogeneic bone marrow (n = 6) or peripheral blood stem cell (n = 10) transplantation on 16 patients with predominantly high-risk haematological malignancies using partially mismatched related (sibling, n = 2; non-sibling, n = 6) or unrelated donors (n = 8). Median age of recipients was 34 years (range, 17 - 53). Diagnoses were acute leukaemia (AL) (n = 9), chronic myeloid leukaemia (CML) (n = 6) and progressive myelofibrosis (n = 1). Eight patients were considered high-risk for relapse and 5 as intermediate risk. HLA typing was based on serology for HLA A, HLA B and HLA C and PCR-SSO for DR and DQ. Donors were mismatched at Class I loci (n = 8), Class II loci (n = 7) or both (n = 1). There was 1 detectable mismatch in 1 patients and 2 mismatches in 5 patients. Donor or recipient or both were CMV positive serologically pre-transplant in 14 of 16 pairs. Conditioning was with Cyclo-TBI (n = 14), BuCy (n = 1) or LACE (n = 1). All patients also received ATGAM (Pharmacia & Upjohn) 15mg/kg daily days -6 to -1. (3vHD prophylaxis consisted of cyclosporin A commencing on day -1 and short course methotrexate. Additionally recipients of unrelated transplants received a further 5 days of ATGAM 15mg/kg from day 0 to day +4. All patients engrafted (neutrophils > 0.5 x 10⁹/litre, median day +21, range 13 - 38) and 5 of 16 (31%) experienced 2 grade 3 acute GvHD. Eight patients (50%) were alive at day 100 post-transplant (0 of 4 age >45 years; 8 of 12 age ≤ 45 years, p=.03). One patient relapsed 15 months post-transplant (Ph + ALL). Two of the 7 surviving patients (unrelated donor = 4; related donor = 3) experienced extensive chronic GvHD but presently 0 of 7 have active GvHD. Predicted disease free survival for patients age < 45 years at 3 years is 47% with a median follow-up of 9 months (range 3 - 37 months). We

conclude that patients ≤ 45 years of age lacking a fully matched related or unrelated donor and no other curative therapeutic option should be considered for partially mismatched transplantation.

76.

IMPROVED SURVIVAL OF PATIENTS WITH STEROID REFRACTORY GVHD USING COMBINATION THERAPY WITH ANTITHYMOCYTE GLOBULIN (ATG) AND TACROLIMUS Mollee P, Morton J, Irving I, Durrant S. Bone Marrow Transplant Unit. Royal Brisbane Hospital. Brisbane. Qld.

GVHD is a major contributor to mortality and morbidity after allogeneic stem cell transplantation. Treatment of acute GVHD relies on high dose steroids with refractoriness to this therapy associated with a very poor outcome. We report our experience with the combination of ATG and tacrolimus in the treatment of 21 patients with steroid refractory GVHD transplanted between August 1996 and October 1 1999. Acute GVHD was graded according to the Seattle criteria. Median age at the time of transplantation was 42 years (range, 19 to 52). Diagnoses included chronic myeloid leukaemia (n=7), acute leukaemia (n=6), myeloma (n=3), lymphoma (n=3) and myelodysplastic syndrome (n=2). Stem cell sources included matched related donors (n=13), mismatched related donors (n=2) and unrelated donors (n=6), with 9 utilising marrow and 12 peripheral blood stem cells. Conditioning regimens included busulphan/cyclophosphamide (n=6) and cyclophosphamide/TBI (n=13). All patients except three T cell depleted transplants received cyclosporine/methotrexate GVHD prophylaxis.

Eleven patients developed a maximum of Grade III and 8 Grade IV acute GVHD, all refractory to methylprednisone 2-5mg/kg. The predominant organ system involved was skin (n=7), gut (n=7), liver (n=6) and lung in one case of chronic GVHD. Patients were treated with ATG (15mg/kg for 5 days) and tacrolimus (0.05 - 0.1 mg/kg) in addition to continuation of their high dose steroids and cessation of their cyclosporine. Within one month of treatment, we observed 11 complete responses, 5 partial responses, 1 with no response and 4 with progressive deterioration in one or more organ system. Infectious complications occurred in 16 patients (bacterial n=12, fungal n=4, viral n=6, undefined n=3) in the month following ATG therapy.

Of the 21 patients treated with ATG and tacrolimus, 8 (38%) remain alive. The median follow up of survivors post treatment was 692 days (range, 264-1020). Twelve patients died due to GVHD and/or infection with one death due to TTP. Median survival time from ATG and tacrolimus treatment for non-survivors was 42 days (range, 21-310). Survival following combination therapy was significantly more likely in males (p<0.01). Donor source, age, and organ system involved did not significantly influence outcome. In conclusion, concurrent introduction of ATG and tacrolimus is a promising therapeutic combination for GVHD refractory to steroids and cyclosporine.

77.

PHASE I DOSE ESCALATION TRIAL OF Sm-153 LEXIDRONAM (QUADRAMET) AS A CONDITIONING REGIMEN FOR STEM CELL TRANSPLANTATION. James Morton¹, David MacFarlane², Simon Durrant¹. Divisions of Bone Marrow Transplantation¹ and Nuclear Medicine², Royal Brisbane Hospital.

INTRODUCTION: Disease recurrence remains a major complication of high dose therapy with stem cell transplantation. Attempts to increase the intensity of the conditioning regimen have resulted in reduced rates of disease recurrence but no improvement in survival due to increased regime related mortality. Quadramet is a conjugate of Samarium 153 and EDTMP used in the palliative treatment of metastatic prostate cancer. We hypothesised that this agent would target radiation to bone allowing high dose marrow radiotherapy in addition to standard conditioning regimens without added extra-medullary toxicity. We report the results of an initial pilot study.

METHODS: 10 patients undergoing stem cell transplantation for myeloma or other diseases who could not receive TBI were enrolled. Starting dose was calculated with the aim of delivering a marrow absorbed dose of 35Gy and escalated in groups of 3 patients by increments of 50%. A tracer dose of 800MBq Quadramet was used to determine retained activity for calculation of the subsequent administered dose. The therapeutic dose was administered 14 days later followed by the standard conditioning regimes once the calculated dose rate to the infused elements was ≤ 1 cGy/h.

RESULTS: Administered doses of Quadramet ranged from 18 to 40 GBq. No infusional effects were observed. Post-transplant toxicity and engraftment were as expected. The overall absorbed skeletal dose was calculated at 1.5mGy/MBq administered. There was marked variation in regional skeletal deposition of tracer with highest absorbed doses received by the ribs, pelvis and head. Absorption by the appendicular skeleton was substantially lower, rarely exceeding 10Gy. Responses for patients with multiple myeloma included CR 4/7, VGPR 2/7 and stable disease 1/7.

CONCLUSION: Quadramet can be added to standard conditioning regimes without incremental toxicity and permitting the delivery of high doses of radiation to the axial skeleton. We are continuing dose escalation to 60GBq in addition to 1 O Gy single fraction limb irradiation and Melphalan 200mg/m² (autologous) or Cyclophosphamide 200mg/kg (allogeneic) for patients undergoing stem cell transplantation for multiple myeloma.

78.

FLUDARABINE-MELPHALAN CONDITIONED ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION FOR PATIENTS WITH REFRACTORY OR MULTIPLE RELAPSED HAEMATOLOGICAL MALIGNANCIES. A Spencer G Evans M Kapuscinski, M Prince, AP Schwarzer. *BMT Programme, The Alfred Hospital, Melbourne, Victoria.*

Reduced-intensity conditioned alloPBSCT is being evaluated in multiple BMT centres as a potential strategy to increase the safety and appropriate utilisation of allogeneic stem cell transplantation. Since July 1999 we have undertaken 9 fludarabine-melphalan (Flu-Mel) conditioned alloPBSCT with attenuated graft versus host disease prophylaxis for advanced and/or refractory haematological malignancies. All patients had either chemorefractory or multiply relapsed disease, only 1 was in CR at the time of alloPBSCT. Two had undergone prior autologous PBSCT (3 months and 45 months prior to alloPBSCT). Median age was 50 years (range 32 - 56 years). Diagnoses were AML/MDS, n = 3 (induction failure x 2, CR2 x 1); chemorefractory Sezary Syndrome, n = 2; NHL, n = 4 (chemosensitive relapse x 2, chemorefractory x 2). Patients were conditioned with fludarabine 25mg/m²/day, days -6 to -2 and melphalan 140mg/m² day -2. GvHD prophylaxis consisted of cyclosporin A (CsA) 3mg/kg/day commencing on day -1. Median CD34 and CD3 cell doses were 6.41 x 10⁶/kg and 1.58 x 10⁸/kg, respectively. All patients engrafted with neutrophils > 0.5 x 10⁹/litre and platelets > 20 x 10⁹/litre by a median of 12 days and 11 days respectively. Non-haematological toxicity was minimal, however, 2 patients experienced steroid responsive engraftment reactions both characterised by hyper-pyrexia with associated rash in 1 case and hypoxia / pulmonary infiltrates in the other case. Acute severe GvHD (> grade 3) occurred in 3 patients. The GvHD was steroid resistant in 2 cases and both patients died of opportunistic infections (disseminated aspergillosis and acyclovir resistant HSV, respectively). Autopsy showed that both were in pathological CR at the time of death. One patient with NHL (Primary mediastinal B-cell) progressed and died on day 75 post- alloPBSCT. One patient with Sezary Syndrome after an initial response progressed at day 40. CsA was ceased and within 72 hours he developed hyperpyrexia, cutaneous tumour necrosis and steroid responsive grade III GvHD of the skin. After achieving PR he experienced further disease progression and received DLI on day 128, again with significant disease response. Overall TRM was 22% with 70 % day 100 survival and 6 patients remain alive 14 - 256 days post alloPBSCT. We conclude that Flu-Mel conditioned alloPBSCT with reduced GvHD prophylaxis can generate significant anti-tumour activity against advanced and chemorefractory haematological malignancies with acceptable TRM in poor risk patients.

79.

OUTCOME OF DONOR MARROW TRANSPLANTATION IN ADULTS FROM UNRELATED OR MIS-MATCHED FAMILY DONORS IN THE LATE 1990s. Roberts AW, Grigg AP, Kilvington R, Lee D, Bardy P, Szer J. *The Royal Melbourne Hospital. Parkville. Victoria 3050.*

Marrow transplantation from unrelated or mismatched related donors is potentially curative therapy for patients with otherwise incurable haematological malignancies. Our review of outcomes for patients transplanted in the early-mid nineties at two centres reported 3yr overall survival (OS) and event-free survival (EFS) of 35±6% and 22±6% respectively (ANZ J Med 1997;27). Pretransplant patient characteristics predictive of unfavourable outcome were identified (poor performance status, heavily pretreated patients with advanced disease) and patient selection was modified in light of this. Severe acute graft-versus-host disease (GVHD) was also associated with a poorer outcome. Subsequently molecular typing at class II loci has been routine (matches required at DR and DQ) and molecular typing has been gradually introduced for class I loci. To assess whether overall results consequently improved, we reviewed the outcome of 53 consecutive patients transplanted at our single institution between 1995 - December 1999. All patients received T cell-replete bone marrow as the stem cell source and cyclosporin / short course methotrexate as GVHD prophylaxis. Minimum follow up at analysis was four months and the median follow up for survivors was 25 months. The median age of patients was 38 years (18-55), with 24 being over 40 years (43%). Nineteen patients had CML (13 chronic phase, 6 advanced disease), 14 had AML (all post CR1), 12 had ALL (3 in CR1, and 9 post CR1), and 8 patients had other diseases. Four patients (7.5%) died prior to engraftment and 1 patient (subsequently found to be molecularly mismatched at both HLA-B loci) failed to engraft. Day 100 mortality was 23% (12 of 53) with 1 death due to relapse, 4 due to acute GVHD, and 7 due to other regimen-related toxicities (3 interstitial pneumonitis, 2 haemorrhage, and 2 multi-organ failure). Subsequent to day 100, 16 of 41 at risk have died; 11 of relapse and 5 of transplant-related complications (4 due to GVHD). Acute GVHD occurred in 43/50 evaluable patients with a median onset of 31 days. Fifteen patients experienced grade I acute GVHD and 12 patients grade II. Fifteen patients developed grade III-IV acute GVHD which was fatal in 6. Chronic GVHD was observed in 30 of 41 evaluable patients (73%): 10 limited-stage and 20 extensive, with 5 patients having severe chronic GVHD. Overall survival at 3 years was 35±8%, with an EFS of 32±7%. When compared with the previous series, patients were older, but fewer were heavily-pretreated patients with advanced leukaemia. Overall survival is similar; day 100 mortality and EFS appear to have improved. The major hurdles to long-term disease-free survival continue to be acute GVHD, other regimen-related toxicities and disease relapse. New strategies addressing each of these issues are required.

80.

NON-ABLATIVE ALLOGENEIC STEM CELL TRANSPLANTATION FOR THE TREATMENT OF LOW GRADE HAEMATOLOGICAL MALIGNANCIES. James Morton¹, John Bashford², Cheryl Hutchins¹, Noor Parker³, Brett Teale³, Simon Durrant¹. ¹Royal Brisbane Hospital, ²Haematology Oncology Clinics Australasia, ³ Sullivan and Nicolaides Pathology.

INTRODUCTION: Standard myeloablative conditioning regimes are employed prior to allogeneic stem cell transplantation to kill tumour cells and immunosuppress the host to minimise the risk of graft rejection. A significant component of the curative benefit is derived from the graft-versus-tumour effect. This has led to the investigation of non-ablative immunosuppressive regimes with the aim of establishing donor engraftment and utilising the graft-versus-tumour effect to eradicate residual malignancy. We present our experience of the first 10 patients undergoing CD34 selected non-ablative transplantation.

DONOR: DLI was collected from donors on day -7 and stored in aliquots of 1×10^6 , 3×10^6 , 1×10^7 , 3×10^7 , and 1×10^8 CD3+ cell/kg. Donors subsequently received G-CSF 10µg/kg bd from day -6 with collections performed on day-2 until day0. The pooled collections from day-2 and day-1 were CD34 selected on day0.

PATIENTS; The conditioning regime utilised fludarabine 25mg/m² day-8 to day-4 and cyclophosphamide 60mg/kg day-3,2. Rejection prophylaxis consisted of IV Cyclosporin 5mg/kg from day-1 converted to oral doses from day+14. All patients received post-transplant G-CSF. Patients were followed with disease restaging and chimerism determination on whole blood, CD15 and CD3 fractions monthly from day 28. In the absence of GVHD, patients were administered 1×10^6 CD34/kg on day 30, with withdrawal of cyclosporin from day 60 and incremental DLI or tumour specific T cell therapy from day 90 (based on presence of persisting disease).

RESULTS: Patient details and outcomes are shown in Table 1.

Name	Age	DIS	CD34PRE	CD34POST	CD3PRE	CD3POST	ANC	PLT	STATUS	Chimerism
MM	54	NHL	13.7	8.1	445	0.06	9	11	D+180,CR	Mixed
NC	63	CML	9.6	3.1	326	0.03	10	12	D+180,CR	Delayed Rejection
JW	60	NHL	11	6.9	461	0.01	10	9	D+70, PD	Mixed
LH	68	MCL	6.4	2.9	218	0	10	12	D+63,PD	Delayed Rejection
MH	54	CML	2.5	1.6	220	0	11	12	D+42	Mixed
TH	43	NHL	2.8	2	274	0.01	10	10	D+35, PR	Mixed
LC	49	NHL	9.5	5.1	505	0.02	10	12	D+28	Mixed
JN	62	CML	5.6	4.1	N/A	N/A	8	10	D+21	N/A
VC	60	CML	16.2	9.4	466	0.01	9	10	D+14	N/A
LP	36	CML	N/A	N/A	N/A	N/A	N/A	N/A	D+0	N/A

CONCLUSIONS: The current regimen is well tolerated with negligible nonhaematological toxicity. Prompt donor engraftment has been observed for all patients. Further follow-up will be required to demonstrate sustained engraftment and efficacy of this approach