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Anti-tumour effect in solid tumours, tolerance and immune reconstitution after allogeneic haematopoietic stem cell transplantation

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1 CONTENTS

2 SUMMARY

Both in haematological malignancies and in disseminated solid tumours, re-occurrence of the underlying disease is the main complication. Allogeneic haematopoietic stem cell transplantation (HSCT) increases the chance of cure compared to only chemotherapy in haematological malignancies, but adds the risk of immunological complications such as graft-versus-host disease (GVHD) and severe infections. Immunosuppressive treatment is needed to prevent rejection of the graft and GVHD. The reason for lower relapse incidence after allogeneic HSCT has been attributed to a graft-versus-leukaemia (GVL) effect, which has also been suggested to be present against solid tumours after HSCT.

The possible occurrence of a graft-versus-tumour (GVT) effect in different solid tumours after allogeneic HSCT and the feasibility of a reduced intensity conditioning (RIC) were investigated. Patients with renal cell carcinoma (RCC, n=10), colon carcinoma (CC, n=6), breast cancer (n=1) or a Klatskin tumour of the liver (n=1) were treated with HSCT with a RIC consisting of fludarabine and 2 Gray of total body irradiation (TBI). During the study, four patients died of transplantation-related complications between 45 and 160 days after HSCT and three patients died of tumour progression 92-323 days after HSCT. Almost total tumour regression was seen in the first patient with CC, but he died of pneumonia 4 months after HSCT. Partial responses in the lungs of one additional patient with CC and two patients with RCC, were seen.

Among 624 patients receiving transplants between 1977-1997 at Huddinge Hospital, 254 patients surviving more than 12 months, were retrospectively analysed regarding clinical tolerance, that was defined as the absence of GVHD or rejection after withdrawal of immunosuppression. Patients who did not develop GVHD had discontinued immunosuppression according to the protocols. Children discontinued immunosuppression faster than adults and male recipients with immunised female donors discontinued immunosuppression later. Acute GVHD was associated with longer time to withdrawal of immunosuppression. In multivariate analysis, a high donor age, donation from an immunised female donor to a male recipient, and acute GVHD grades II-IV were associated with longer time to clinical tolerance.

Immune recovery analysed by diversity of the T cell receptor (TcR) and the B cell immunoglobulin heavy chain (IgH) using spectratyping of the third complementarity determining region (CDR3) was analysed in 24 patients after RIC (n=13) and myeloablative (n=11) HSCT. Reconstitution of diversity of the CDR3 region was significantly delayed in the IgH while significantly faster in the TcR after RIC HSCT compared to myeloablative HSCT even though differences were small. Patients in the RIC group were significantly older (54 vs. 42, $p<0.05$) and had slightly more viral infections including asymptomatic CMV infection ($p<0.05$). RIC patients also had a tendency for more chronic GVHD (ns, $p=0.12$). Immune function in vitro was tested by lymphocyte stimulation at 3, 6, and 12 months after HSCT. Decreased responses to CMV and VZV antigens were seen in patients suffering from acute GVHD grade II,

compared to the healthy donors. Patients in the RIC group had significantly lower responses to concanavalin (Con-A), phytohemagglutinin (PHA), and Staph. aureus protein A (SpA) during the first six months after RIC HSCT while patients in the myeloablative group only showed lower responses to Con-A at three months, compared to the healthy donors.

In conclusion, RIC HSCT is feasible in haematological diseases and solid tumours. A GVT effect seems to exist in RCC and CC but has to be enhanced. Most patients receiving HLA-identical stem cells are tolerant within two years after HSCT. The B cell and T cell repertoires are skewed the first year after allogeneic HSCT and immune reconstitution after HSCT with myeloablative and RIC conditioning seem to be comparable. Individual factors such as GVHD, age and infections are probably more important for immune reconstitution than type of conditioning.

3 LIST OF ORIGINAL PUBLICATIONS

I. A graft-versus-colonic cancer effect of allogeneic stem cell transplantation. H Zetterquist, P Hentschke, A Thörne, A Wernerson, J Mattsson, M Uzunel, J Martola, N Albiin, J Aschan, N Papadogiannakis and O Ringdén. Bone Marrow Transplantation 2001; 28(12), 1161-1166

II. Low-intensity conditioning and hematopoietic stem cell transplantation in patients with renal and colon carcinoma. P Hentschke, L Barkholt, M Uzunel, J Mattsson, P Wersäll, P Pisa, J Martola, N Albiin, A Wernerson, M Söderberg, M Remberger, A Thörne and O Ringdén. Bone Marrow Transplantation 2003; 31(4), 253-61

III. Clinical tolerance after allogeneic hematopoietic stem cell transplantation: a study of influencing factors. P Hentschke, M Remberger, J Mattsson, L Barkholt, J Aschan, P Ljungman and O Ringdén. Transplantation 2002; 73(6), 930-936, 2002.

IV. T cell receptor CDR3 repertoire after myeloablative and reduced intensity conditioning allogeneic haematopoietic stem cell transplantation. P Hentschke, B Omazic, J Mattsson, I Näsman-Björk, I Lundkvist, D Gigliotti, L Barkholt, O Ringdén, M Remberger. Manuscript.

V. Reconstitution of the Ig heavy chain CDR3 repertoire after hematopoietic stem cell transplantation with myeloablative or reduced intensity conditioning regimens. B Omazic, P Hentschke, I Näsman-Björk, J Mattsson, V Oxelius, O Ringdén, L Barkholt, J Permert and I Lundkvist. Manuscript.

4 LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
ATG	Anti-thymocyte globulin
BM	Bone marrow
BMT	Bone marrow transplantation
Bu	Busulfan
CC	Colon carcinoma
CD	Cluster of differentiation
CDR	Complementarity determining region
CDR3	Third complementarity determining region
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CNS	Central nervous system
Con-A	Concanavalin A
CsA	Cyclosporine A
CT	Computed tomography
Cy	Cyclophosphamide
DC	Donor chimaerism
DLI	Donor lymphocyte infusion
EBV	Epstein Barr virus
EBMT	European Group for Blood and Marrow Transplantation
FACS	Fluorescence-activated cell sorting
Flu	Fludarabine
G-CSF	Granulocyte-colony stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukaemia
Gy	Gray
HLA	Human leukocyte antigen
HSCT	Haematopoietic stem cell transplantation
HSV	Herpes simplex virus
IBMTR	International Bone Marrow Transplantation Registry
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IFN	Interferon
IL	Interleukin
i.v.	Intravenous(ly)
LFS	Leukaemia-free survival
MC	Mixed chimaerism
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex

MMF	Mycophenolate mofetil
MTX	Methotrexate
MUD	Matched unrelated donor
NK cell	Natural killer cell
OKT-3	Orthoclone, monoclonal antibody against CD3
PB	Peripheral blood
PBSC	Peripheral blood stem cell
PBSCT	Peripheral blood stem cell transplantation
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
RCC	Renal cell carcinoma
RIC	Reduced intensity conditioning
RT-PCR	Reverse transcript-polymerase chain reaction
SAA	Severe aplastic anaemia
SCID	Severe combined immunodeficiency
SpA	Staphylococcus aureus protein A
TBI	Total body irradiation
TcR	T cell receptor
TcD	T cell depletion
TNF	Tumour necrosis factor
TRM	Transplantation-related mortality
VNTR	Variable number of tandem repeats
VOD	Veno-occlusive disease
VZV	Varicella zoster virus

5 GENERAL INTRODUCTION

5.1 HISTORY OF CANCER

Cancer is one of the leading diseases in the world and was estimated to account for about 7 million deaths (12% of all deaths) worldwide in 2000, exceeded by cardiovascular, and infectious and parasitic diseases (WHO 2001). The most common cancer world-wide today is lung-cancer followed by stomach cancer, and breast cancer, by far being the most common cancer among women (Parkin, Pisani et al. 1999). Man has been aware of cancer for thousands of years. The oldest description of human cancer was found in an Egyptian papyrus written between 3000-1500 BC. It referred to tumours of the breast. Hippocrates (460-370 BC) may have been the first to use the term cancer.

During the last hundred years, significant progress has been made in understanding and winning against cancer. Discoveries such as anaesthesia (Warren 1848), X-ray (Roentgen 1895), and the microscope, that was invented already in the 16th century, have been important tools for achieving today's knowledge and therapeutic possibilities. One very important discovery for understanding the origin of cancer and developing diagnostic and curative methods, was the discovery of DNA and its structure in 1953 (Watson and Crick 1953). DNA was found to be the basis of the genetic code that gives orders to all cells. After learning how to translate this code, scientists were able to understand how genes worked and how they could be damaged by mutations.

In the beginning of the 20th century, the only curable cancers were small and localised enough to be completely removed by surgery. Later, radiation was used after surgery to control small tumour growths that were not completely surgically removed. Finally, chemotherapy was added to destroy small tumour growths that had spread beyond the reach of the surgeon and radiotherapist.

5.2 HAEMATOPOIETIC STEM CELL TRANSPLANTATION

Cytotoxic chemotherapy is the primary therapy for all forms of haematological malignancies. Arsenic, which was accidentally found to be able to cure chronic leukaemia, nitrogen mustard and aminopterin, the predecessor of methotrexate, were three of the first used chemotheurapeutic agents (Lissauer 1865; Goldman 1946; S Farber 1948). Irradiation was used to delay the progression of chronic leukaemias already hundred years ago (Pusey 1902). Nowadays there are several chemotherapeutic drugs used to eradicate malignant cells. The major problem of this strategy is the toxicity especially to the bone marrow. The infusion of unaffected stem cells after chemotherapy treatment and/or total body irradiation allows higher doses, which increases the chances of eradicating the last cancer cell. Reinfusion of haematopoietic stem cells harvested from the patient prior to treatment, autologous transplantation, has

been used since the late 1950's. One disadvantage of this procedure is the risk of remaining leukaemic cells in the graft, even when the patient is in remission, causing disease relapse when reinfused to the host (Gale and Butturini 1989).

One of the earliest attempts to use bone marrow (BM) therapeutically appears to have been made by Brown-Seqard and d'Arsonaval, who in 1891 gave BM orally to patients with anaemia (Quine 1896). In 1949 Leon Jacobson showed that mice could be protected from the lethal effects of ionising radiation by shielding their spleens (Jacobson 1949). Lorenz et al showed that intravenous infusion of BM protected against toxic effects of irradiation (Lorenz 1951). The first allogeneic HSCT in humans was reported by Donnall Thomas in 1957 (Thomas, Lochte et al. 1957). The following years about 200 allogeneic HSCTs were performed. Most of these patients failed to engraft and most of the patients died at an early stage after transplantation (Bortin 1970).

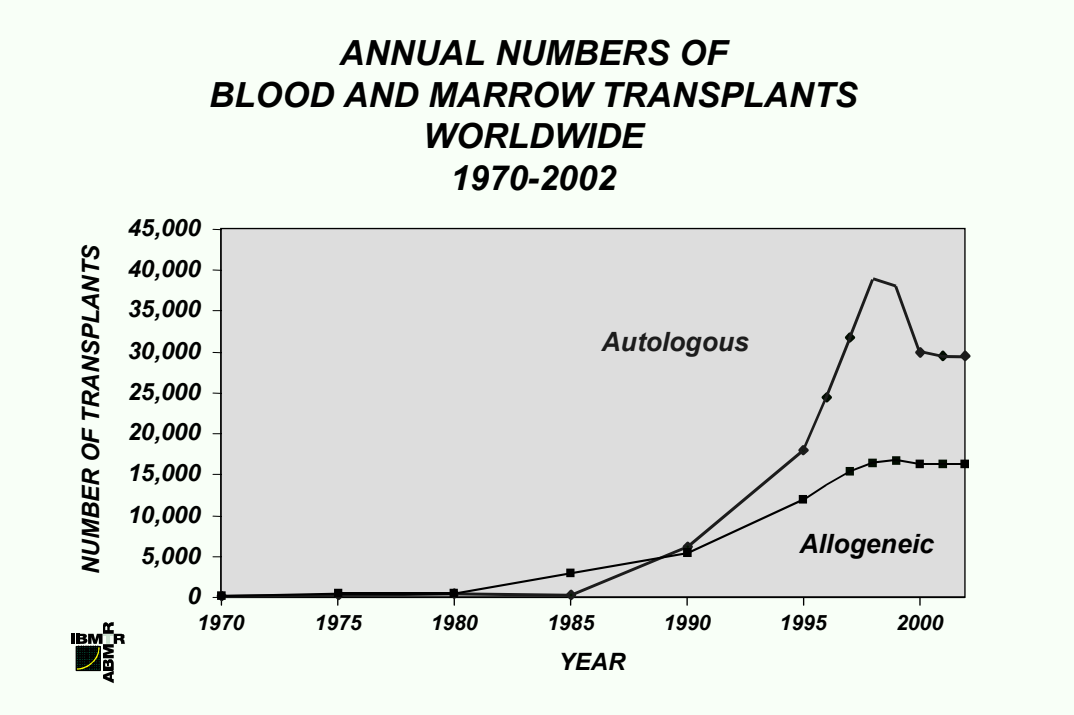


Figure 1. *Annual numbers of blood and marrow transplants worldwide 1970-2002. Reproduced with the kind permission from IBMTR.*

The human leukocyte antigen (HLA) was discovered in the 1950's and was considered to be of importance in the transplantation procedure (Dausset and Nenna 1952; Dausset 1958; Van Rood, Eernisse et al. 1958). Further experiments in dogs showed that the leukocyte antigens were crucial in determining the outcome of an allogeneic graft (Thomas, Collins et al. 1962; Storb, Epstein et al. 1968). The experience that

histocompatibility between the patient and donor is crucial for engraftment and reduction of graft-versus-host disease (GVHD) was a major step towards succeeding with allografts (Bach, Albertini et al. 1968; Dausset, Rapaport et al. 1969; Zinkernagel and Doherty 1974). Improved results were reported in 1975 by the group led by Donnall Thomas who was a pioneer in developing the field of allogeneic HSCT (Thomas, Storb et al. 1975a and b). The first successful BMT using an unrelated donor was performed in 1980 (Hansen, Clift et al. 1980). Since then it has been a rapid increase in the numbers of performed HSCTs worldwide (Figure 1). Today the majority of patients lacking an HLA-identical sibling donor will find a suitable donor among more than seven million HLA-typed donors worldwide (Anasetti, Petersdorf et al. 2001).

There are several indications for HSCT and new indications are constantly investigated. Accepted indications are mainly haematological malignancies, severe aplastic anaemia (SAA), severe combined immunodeficiency (SCID) and inherited metabolic disorders. Autologous HSCT has been used in patients with autoimmune diseases and some case reports with successful outcome have also been reported after allogeneic HSCT (Burt, Slavin et al. 2002). The effect of allogeneic HSCT in solid tumours has been investigated with increasing interest the last years.

5.2.1 Haematopoietic stem cell sources

The function of the haematopoietic stem cell is to restore the complete haematopoietic system and is identified by the CD34 molecule in the cell membrane (Civin, Strauss et al. 1984; Smeland, Funderud et al. 1992). However, the CD34+ cell population is heterogeneous and the pluripotent haematopoietic stem cells constitute only a small percent of the CD34+ cell population (Rusten, Jacobsen et al. 1994). Most haematopoietic stem cells are located in the BM with approximately ten times the amount found in peripheral blood (PB) (Bender, Unverzagt et al. 1991). Until the beginning of the 1990's, BM aspirated mainly from the iliac crest was the only source of stem cells for haematopoietic reconstitution following myeloablative therapy in allogeneic HSCT. This procedure needs sterile conditions and general or spinal anaesthesia. The total fluid volume is normally 500 to 1000 ml depending on the desired amounts of cells. Normally 2-4 x 10^8 nucleated marrow cells per kilogram of body weight of the recipient is desired.

In the 1960's it was shown that low amounts of stem cells were circulating in PB (Goodman and Hodgson 1962; Epstein, Graham et al. 1966). However, the low numbers of stem cells in PB compared to BM has been one of the reasons why the use of peripheral blood stem cells (PBSCs) did not become the method of choice, until the development of better mobilisation strategies the last decade. One important advance in HSCT has been the observation that stem cells can be mobilised from BM into the PB by haematopoietic growth factors (Molineux, Pojda et al. 1990). Since PBSC grafts contain about ten times more T cells than BM, it has been discussion that PBSCT may increase the risk for GVHD, especially when using unrelated donors. This may be one

reason for why the use of PBSC in allogeneic HSCT has lagged behind the use in the autologous setting, in which PBSCs have been used since the beginning of the 1980's (Goldman, Catovsky et al. 1981; Gorin 1986). However, during the last few years there has been a rapid increase in the use of PBSC (Figure 2). Experience has shown similar incidence of acute GVHD, transplantation-related mortality (TRM), relapse, survival, and leukaemia-free survival (LFS), but higher incidence of chronic GVHD, in patients receiving PBSC compared to BM (Ringdén, Labopin et al. 2002). Today, PBSC is the most commonly used stem cell source both in autologous and allogeneic HSCT in Europe and PBSCT is also used with unrelated donors (Ringdén, Potter et al. 1996). Also in HSCT with HLA-matched unrelated donors (MUDs), the use of PBSC has been shown to be as safe as for BM (Remberger, Ringdén et al. 2001). Another stem cell source used recent years is stem cells from umbilical cord blood that contain a lot of immature subsets of haematopoietic progenitor cells (Broxmeyer, Kurtzberg et al. 1991). The lower number of haematopoietic cells may increase the risk of rejection especially in adults, but the risk of GVHD is decreased (Wagner, Rosenthal et al. 1996).

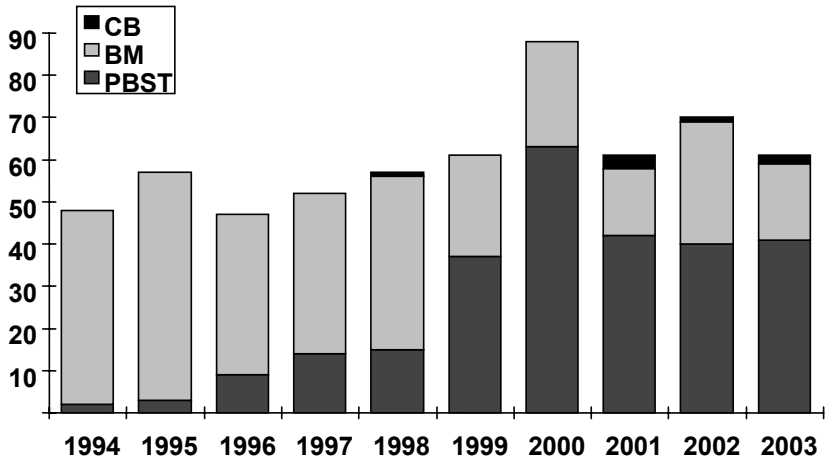


Figure 2. *The numbers of cord blood, bone marrow and peripheral stem cell grafts grafts used at Huddinge University Hospital 1994- 2003.*

5.2.2 Conditioning

The conditioning is the therapy given the days before HSCT and one of its purposes is to eradicate the malignant cells in malignant diseases or the defect cells in metabolic disorders. The "rescue" by allogeneic haematopoietic stem cells allows stronger conditioning regimens compared to if only chemotherapy is given. Higher doses of conditioning therapy increase the chance of eradicating all malignant or defective cells. Toxicity in the lungs and the bowel are the organs limiting even higher doses. The conditioning is also needed to avoid rejection of the transplanted cells. Only in severe immunodeficient patients is conditioning not always needed due to the inability to

reject the graft in these patients (Toren, Nagler et al. 1999). Different combinations of irradiation and/or chemotherapy are used depending on the diagnosis. In malignant diseases, for instance, an anti-cancer effect of the conditioning is wanted while in non-malignant disorders the conditioning needs to be more immunosuppressive.

The most commonly used conditioning in malignant haematological malignancies is cyclophosphamide (Cy) in combination with total body irradiation (TBI), an approach that has been used for more than 30 years (Thomas, Storb et al. 1975a). Today, TBI is commonly given in fractionated doses (fTBI) which decreases the risk of irradiation complications and also allows higher total doses of TBI (Thomas, Clift et al. 1982; Resbeut, Altschuler et al. 1990). In leukaemias that may involve the central nervous system (CNS), i.e. ALL and AML M4/M5, repeated injections of methotrexate are given intrathecally to prevent CNS-relapse. Instead of TBI, busulfan (Bu) is commonly used in combination with Cy. This combination is preferred in AML and in metabolic diseases. Irradiation is avoided in children, especially below three years of age, since they are more sensitive than adults to irradiation complications, especially in the CNS (Ringdén, Bolme et al. 1989; Smedler, Ringdén et al. 1990). Commonly 4 mg/kg/day of Bu during four consecutive days followed by 60 mg/kg/day of Cy during two days is given (Tutschka, Copelan et al. 1987). In metabolic disorders Bu may be followed by higher doses of Cy (Shaw, Hugh-Jones et al. 1986). The two most common protocols using Cy/TBI or Bu/Cy have been compared in several studies and the combination Bu/Cy has been attributed to more transplantation related toxicity and chronic GVHD (Ringdén, Remberger et al. 1999; Gupta, Lazarus et al. 2003). This regimen associated toxicity has been reduced with individualised doses, based on monitoring of Bu concentrations in the blood, to compensate for the variability in pharmacokinetics of Bu between patients (Hassan, Oberg et al. 1991; Hassan, Ljungman et al. 1994).

In non-malignant disorders the main purpose of the conditioning is to prevent rejection. The most common disorder in this group is SAA. Patients with SAA are often immunised by high frequency of blood transfusions, which increases the risk for rejection. Therefore high dose conditioning is needed (McCann, Bacigalupo et al. 1994). Most commonly Cy 200 mg/kg alone or in combination with anti-thymocyte globulin (ATG) is given as conditioning for SAA (Storb, Weiden et al. 1987). In transplantation for SAA with a MUD, TBI is needed to prevent rejection (Deeg, Anasetti et al. 1994). In metabolic diseases different conditioning protocols are used. The majority of these regimens contain Bu (Peters, Shapiro et al. 1998). A common combination is Bu 16mg/kg in combination with Cy 8g/m^2 (Shaw, Hugh-Jones et al. 1986).

Opposite to the strategy to give as much cytoreductive drugs as possible to decrease the risk of relapse, other strategies have been developed during the last decade (Slavin 2000). The experience that patients suffering from GVHD have less relapse of their malignancy has contributed to the insight that the infusion of allogeneic stem cell grafts not only is a “rescue” from permanent aplasia, but also contributes to an anti-leukaemic effect (Sullivan, Weiden et al. 1989). This has lead to the development of less toxic protocols where the anti-leukaemic-effect is the main goal, and the main purpose of the

conditioning is to prevent rejection. These protocols are defined as reduced intensity conditioning (RIC) or non-myeloablative regimens. The intensity of the different RIC protocols varies. One of the pioneers in this field, Dr. Shimon Slavin, has in a variety of malignant and non-malignant blood diseases used a protocol consisting of fludarabine (Flu) 30 mg/m^2 for six consecutive days, oral Bu 4 mg/kg/day for two days and ATG 10 mg/kg/day for four consecutive days (Slavin, Nagler et al. 1998). The Seattle group has used another less intensive protocol with a sublethal dose of 2 Gy TBI followed by immunosuppression with mycophenolate mofetil (MMF) and cyclosporine A (CsA) (Sandmaier, McSweeney et al. 2000). Other groups have used other combinations to prepare for engraftment without total myeloablation (Giralt, Estey et al. 1997; Khouri, Keating et al. 1998; Childs, Chernoff et al. 2000; Kogel and McSweeney 2002).

5.2.3 Immunosuppression

The aim of the conditioning is mainly to eradicate malignant or defective cells and to prevent rejection. The immunosuppressive effect of the conditioning is not sufficiently strong and long-lasting enough to prevent severe GVHD. Different immunosuppressive regimens have been used and the strategy is different depending on the diagnosis, donor type and grade of histocompatibility between the donor and the recipient. The first drug used was methotrexate (MTX) (Storb, Epstein et al. 1970). Monotherapy with CsA has also been used but today the combination of these two drugs is the immunosuppression of choice (Ringdén 1986; Storb, Deeg et al. 1989). Combination of CsA and prednisolone instead of MTX can be used when less toxicity is desired (Forman, Blume et al. 1987). Other immunosuppressive agents such as tacrolimus, MMF and rapamycin have also been investigated.

In HSCT with unrelated and/or HLA mis-matched stem cell donors more intensive immunosuppression is needed to prevent GVHD. Immunosuppression is also needed for a longer time than in HLA-identical sibling transplants. In malignant diseases immunosuppression is withdrawn as soon as possible because prolonged immunosuppression increases the risk of relapse (Carlens, Aschan et al. 1999). On the contrary, protocols for non-malignant diseases contain immunosuppression up to 2 years even in the absence of GVHD.

Different anti-T cell antibodies (i.e. anti-thymocyte globulin, ATG) are immunosuppressive by inactivating T-cells (Filipovich, Krawczak et al. 1985; Willemze, Richel et al. 1992). Thus, ATG given as part of the conditioning acts to prevent rejection. Furthermore, ATG prevents GVHD, since anti-T cell antibodies can be detected up to five weeks after HSCT (Remberger, Svahn et al. 1999). T cell depletion (TcD) of the graft by removing donor T cells in the graft before transplantation is even more effective and provides longer-lasting prevention of GVHD but has the disadvantage of increasing the risk of rejection (Maraninchi, Gluckman et al. 1987). Other disadvantages of TcD are increased risk of relapse, especially in CML patients, and increased incidence of infections (Apperley, Jones et al. 1986; Pirsch and Maki 1986; Marmont, Horowitz et al. 1991).

5.2.4 Supportive care

In addition to prevention of rejection and GVHD, other complications of HSCT have to be prevented and treated. The development of supportive care the last 30 years has been crucial for the much better results in HSCT today.

Isolation of the patient during the aplastic phase is commonly used to prevent exposure to hostile infectious agents (Buckner, Clift et al. 1978). However, the patient's own microorganisms become the greatest threat when the patient's immune system is depressed. This fact has lead to less strict isolation routines and pancytopenic patients may even be allowed treatment at home (Svahn, Remberger et al. 2002). The risk for infections is minimised through antibacterial, antiviral and antifungal drugs. Better diagnostic tools in combination with more effective drugs to prevent and treat infections have increased survival after HSCT. In particular, better strategies to prevent and treat cytomegalovirus (CMV) have been of significant importance (Einsele, Ehninger et al. 1995; Ljungman, Aschan et al. 1998). Also in the field of anti-fungal treatment progress has been made and better diagnostic tools such as PCR-techniques are developed (Tollemar, Ringdén et al. 1993; Einsele, Hebart et al. 1997; Chryssanthou, Klingspor et al. 1999; Ruhnke, Bohme et al. 2003).

Transfusion of blood components is needed in most patients especially during the first two weeks after myeloablative conditioning. Mainly erythrocytes and platelets are given, but granulocytes may be given to patients with severe mucositis or invasive infection (Kerr, Liakopolou et al. 2003). Erythrocytes and thrombocytes are filtered to remove leukocytes, that may contain CMV, and all blood products are irradiated to prevent proliferation of lymphoid cells that may otherwise cause GVHD (Bowden, Slichter et al. 1995). Infusion of parenteral nutrition is of value as nausea, mucositis and GVHD in the gastro-intestinal tract hinders sufficient oral intake in many patients (Weisdorf, Lysne et al. 1987).

Different haematopoietic growth factors to accelerate engraftment have been evaluated. The administration of granulocyte-colony stimulation factor (G-CSF) has been proven to enhance neutrophil recovery but there is no evidence of improved outcome after HSCT. Different studies on G-CSF show various effects on the incidence of GVHD (Ho, Mirza et al. 2003). Since 2002 G-CSF is not used routinely at CAST at Karolinska University Hospital, Huddinge, since our data showed an increased incidence of severe acute GVHD in patients given G-CSF (Remberger, Naseh et al. 2003). A recent multi-centre study in 2223 patients with AML or ALL, showed an increased incidence of acute and chronic GVHD after BMT, leading to reduced survival, but no difference in outcome after PBSCT, when using G-CSF (Ringdén, Labopin et al. 2004). Erythropoietin and thrombopoietin have also been evaluated but have not been implemented in standard treatment after HSCT (Klaesson, Ringdén et al. 1994; Verfaillie 2002).

5.2.5 Chimaerism analysis

The co-existence of residual recipient haematopoietic cells with donor haematopoietic cells following BMT has been known for many years (Santos, Sensenbrenner et al. 1972). This phenomenon is known as mixed chimaerism (MC) (McCann and Lawler 1993). The term donor chimaerism (DC) is used when only haematopoietic cells from the donor are detectable after HSCT. Different methods to analyse the chimaeric status in patients have been used during the last twenty years (Blazar, Orr et al. 1985; Petz, Yam et al. 1987; Lapointe, Forest et al. 1996; Bader, Klingebiel et al. 1999; Mattsson, Uzunel et al. 2000). The basic use of the chimaerism technique is to monitor the engraftment process. In most cases the aim with HSCT is to achieve DC.

Chimaerism is commonly analysed in different cell lineages such as T cells (CD3+), B cells (CD19+), myeloid cells (CD33+), NK cells (CD3-/56+) and haematopoietic progenitor cells (CD34+) (Dubovsky, Daxberger et al. 1999; Bader, Stoll et al. 2000; Matthes-Martin, Lion et al. 2003). The clinical significance of the patient's chimaeric status in different conditions such as GVHD, rejection and relapse has been studied and debated. Some studies have shown a correlation between increased numbers of recipient cells and relapse of acute leukaemia, while other studies have suggested no association of relapse to low levels of remaining recipient cells (Lawler, Humphries et al. 1991; van Leeuwen, van Tol et al. 1994; Bader, Beck et al. 1998). The significance of the chimaeric status in different cell lineages has been studied. For instance, T cell MC seem to be a risk factor for rejection (Dubovsky, Daxberger et al. 1999; Peters, Matthes-Martin et al. 1999). Not surprisingly, in RIC, T cell MC is more common than after myeloablative conditioning (Sandmaier, McSweeney et al. 2000). Consequently, rejection also is more common after RIC. Studies have also shown that patients with T cell MC have significantly lower risk of GVHD (Frassoni, Strada et al. 1990; Mattsson, Uzunel et al. 2001a). That means that MC, if it is GVHD protective, can be beneficial in non-malignant diseases where GVHD is not wanted, while MC in a patient treated for leukaemia may initiate donor leukocyte infusions to enhance the transformation into DC (McSweeney and Storb 1999). The role of MC after RIC HSCT is less explored. A study by Mattsson et al showed that MC is common at the time of acute GVHD in patients treated with RIC HSCT (Mattsson, Uzunel et al. 2001b).

Today, PCR of variable number of tandem repeats (VNTR) is the method of choice for chimaerism analysis. At Karolinska University Hospital, Huddinge, this method has been introduced into clinical practice by Mattsson, Uzunel and Zetterquist (Mattsson, Uzunel et al. 2000). VNTRs or minisatellites are repetitive DNA sequences ranging from 10 to 70 base pairs that are usually not transcribed and the function of these DNA sequences is unclear (Jeffreys, Wilson et al. 1988). Minisatellites are widely spread in the genome, and their high degree of polymorphism give rise to DNA sequences of various lengths, which make them suitable to separate DNA from different individuals (Weber and May 1989). After PCR, the different DNA sequences can be separated and visualised by electrophoresis.

5.3 COMPLICATIONS AFTER HSCT

Following HSCT many different complications may occur. Some grade of regimen-related toxicity is probably inevitable and most patients experience some kind of infectious complication. Relapse and complications associated with GVHD are the greatest causes of death after HSCT.

5.3.1 Graft failure

Crucial for success after allogeneic stem cell transplantation is persistent engraftment of the transplanted stem cells. The frequency of graft failure or rejection varies depending on diagnosis and conditioning. Graft failure most commonly occurs during the first weeks, but late failure may occur, especially after RIC HSCT. In the myeloablative setting, the incidence of rejection is around 2% with an HLA-matched sibling donor and less than 5% if a MUD is used (Hale, Zhang et al. 1998; Remberger, Storer et al. 2002). In protocols using reduced intensity, the rejection frequency is elevated and varies depending on the intensity of the conditioning. Rejection incidence is elevated in patients with SAA that are immunised by multiple transfusions before HSCT (Champlin, Horowitz et al. 1989). T cell depletion increases the incidence of rejection, which is normally prevented by higher doses of immunosuppressive treatment of the recipient (Patterson, Prentice et al. 1986). High stem cell dose has been shown to decrease the risk of rejection (Storb, Prentice et al. 1977; Niederwieser, Pepe et al. 1988)

5.3.2 Relapse

Relapse of the original disease remains the most frequent cause of treatment failure in acute leukaemia patients and the incidence of relapse has been relatively constant for many years (Giralt and Champlin 1994). Relapse incidence mainly depends on disease stage, with the lowest relapse rates for patients in first complete remission. The risk is also higher in patients with ALL compared to other haematological malignancies. In some high-risk leukaemias the prognosis is poor with relapse rates up to 80% (Cortes and Kantarjian 1995). On the other hand, patients with SAA have low incidence of recurrent disease and long term survival is up to 90% with an HLA-identical sibling donor (May, Sensenbrenner et al. 1993; Storb, Etzioni et al. 1994). Diagnosis of relapse is traditionally made by morphological analyses of the BM, laboratory findings in PB, and clinical symptoms and findings. Different criteria are used depending on diagnosis. The appearance of leukaemic cells below the threshold for standard morphological methods is commonly referred to as minimal residual disease (MRD) (Uzunel, Jaksch et al. 2003). The development in recent years of methods for detecting MRD prior to morphological or clinical relapse has increased the possibility of rescuing patients from their leukaemia (Campana and Pui 1995). These methods are using cytogenetic markers to detect submicroscopic numbers of residual cells. Detection of the Philadelphia chromosome by RT-PCR is commonly used in CML and also in some ALL to define molecular relapse (Uzunel, Mattsson et al. 2003). Immunoglobulin and T-cell receptor

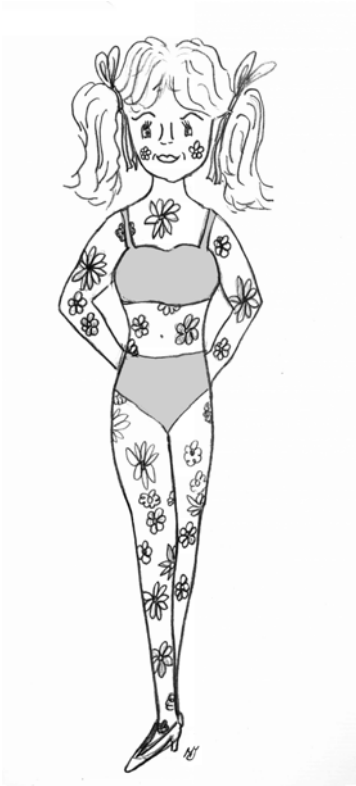
rearrangements as clonal markers are also used (Uzunel, Jaksch et al. 2003). The presence of MC in leukaemia affected cell lineages has also been suggested as an indicator for MRD in ALL, AML and MDS (Zetterquist, Mattsson et al. 2000; Mattsson, Uzunel et al. 2001c).

5.3.3 Graft-versus-host disease

Immunocompetent cells originating from the stem cell graft attack the recipient's tissues by recognising incompatible HLAs and/or minor histocompatibility antigens (mHAs). The process starts by donor T-cells being activated by antigens presented by antigen-presenting cells as macrophages or dendritic cells. Minor histocompatibility antigens are small endogenous peptides that can bind in the groove of HLA molecules (den Haan, Sherman et al. 1995). Several mHAs have been described recently and the expression of some of them has been shown to correlate to GVHD (Goulmy, Schipper et al. 1996; Vogt, van den Muijsenberg et al. 2002). Cytokines play an important role in the development of GVHD (Ferrara, Cooke et al. 1996). A cytokine cascade is initiated by the conditioning. This production and release of inflammatory cytokines increases the cell surface expression of leukocyte adhesion molecules and HLA molecules. This expression in turn stimulates mature donor T cells and recruitment and activation of additional mononuclear effector cells from donor marrow progenitors, which produce additional inflammatory cytokines, thus sustaining the response. Tumour necrosis factor alpha (TNF-alfa) has been shown to be of importance in this initial cytokine cascade and has been shown to correlate to increased incidence of both acute and chronic GVHD (Remberger, Ringdén et al. 1995). The occurrence of GVHD after HSCT has been known since the early days of transplantation and is still the most important complication after HSCT (Grebe and Streilein 1976). Most commonly the skin is affected and other frequent targets are the liver and the gastro-intestinal tract. GVHD has both direct and indirect influence on the outcome after HSCT. Effects on the liver, bowel and lung may lead to liver failure, malnutrition and respiratory insufficiency leading with increasing morbidity. Damage in the skin and mucosa of the mouth and bowel make the patient more vulnerable to infections. Especially chronic GVHD and its treatment are leading to infections.

NK cells are involved in GVHD even though their role is unclear. Increased numbers of NK cells have been found in tissues affected of GVHD (Guillen, Ferrara et al. 1986; Rhoades, Cibull et al. 1993). This indicates that NK cells may contribute to the pathogenesis of the GVHD, but an alternative explanation is that NK cells may inhibit the progression of the GVHD (Borland, Mowat et al. 1983). Recent data suggest that NK cells may protect against GVHD, especially in the mis-matched setting, by killing of recipient antigen presenting cells (APCs), that are important for the development of GVHD (Ruggeri, Capanni et al. 2002; Giebel, Locatelli et al. 2003).

Acute GVHD usually occurs during the first three months after HSCT and is graded I-IV depending on severity (Thomas, Storb et al. 1975a). The mildest form includes skin rash of less than 50% of the body surface while life-threatening grade IV may involve skin, liver and bowel. The incidence of acute GVHD varies depending on several factors. The most important risk factor for GVHD is histoincompatibility between the donor and the recipient (Beatty, Clift et al. 1985). Without immunosuppressive treatment most of the patients, even with an HLA identical sibling donor, would suffer from GVHD, in many cases life-threatening (Sullivan, Deeg et al. 1986). Even with immunosuppressive treatment, the incidence of GVHD can be high, particularly in HLA-mismatched transplants where most patients will suffer from GVHD. Acute GVHD is more common is older patients and older patients are also more sensitive to acute GVHD and its complications (Ringdén and Nilsson 1985). Pretransplant antibodies to CMV, EBV and HSV have been shown to be associated with increased incidence of acute GVHD (Gratama, Weiland et al. 1987; Boström, Ringdén et al. 1990). Another risk factor for acute GVHD is HSCT with stem cells from an immunised female donor given to a male recipient (Gale, Bortin et al. 1987).



A cute GVHD

Chronic GVHD normally develops between three and twelve months after HSCT and differs from acute GVHD not only in the timing after HSCT, but also in its clinical manifestations (Deeg and Storb 1986). Usually, chronic GVHD is preceded by acute GVHD but a few patients without acute GVHD develop de novo chronic GVHD (Storb, Prentice et al. 1983). The same organs as in acute GVHD may be involved and in addition exocrine glands and mucous and serous membranes are often involved causing dry eyes and mouth. The symptoms of chronic GVHD resemble autoimmune diseases' symptoms and are characterised by keratoconjunctivitis, dermatitis, liver dysfunction and prolonged immunodeficiency. In severe cases, subcutaneous fibrosis and contractures may develop (Sullivan, Shulman et al. 1981). Involvement of the airways and lungs can lead to obliterative bronchiolitis, which can be a very disabling condition and with high mortality (Ralph, Springmeyer et al. 1984; Crawford and Clark 1993). Chronic GVHD is associated with marrow depression reflected by thrombocytopenia and anaemia. A delay in immune recovery is also seen which contributes to elevated incidence of bacterial, fungal and viral infections causing significant mortality (Noel, Witherspoon et al. 1978; Atkinson, Storb et al. 1979). The correlation to CMV has been discussed and CMV may probably both precede and follow chronic GVHD (Lönnqvist, Ringdén et al. 1984; Grundy, Shanley et al. 1985). Chronic GVHD has traditionally been graded as limited or extensive but more

clinically relevant may be an overall estimate of the severity expressed as mild, moderate or severe chronic GVHD (Shulman, Sullivan et al. 1980; Lee, Klein et al. 2002).

Treatment of acute and chronic GVHD consists primarily of corticosteroids. Other used agents are CsA, azathioprine, thalidomide and different antibodies such as OKT-3, ATG and IL-2 receptor antibodies (Gratama, Jansen et al. 1984; Herve, Wijdenes et al. 1988; Lim, McWhannell et al. 1988). Psoralen and ultraviolet light (PUVA) as therapy for cutaneous GVHD has shown best responses in chronic GVHD (Vogelsang, Wolff et al. 1996; Furlong, Leisenring et al. 2002). Both in moderate to severe acute and chronic GVHD the outcome is poor. Treatment failure even with powerful immunosuppressive drugs and severe infectious complications are common.

5.3.4 Infections

The combination of an immature immune system and immunosuppressive treatment gives rise to numerous infectious complications that still are a major cause of morbidity and mortality after HSCT (Wingard 1993). Immune recovery is often divided into three phases representing different steps in the development of the immune system and these phases are characterised by different infection patterns.

During the first weeks after HSCT until engraftment gram-positive cocci from the skin and mouth are the most common cause of bacteraemia (Sparrelid, Hägglund et al. 1998). Gram-negative bacteria may cause more severe infections but prophylaxis with ciprofloxacin has decreased the incidence of bacteraemia with gram-negative bacteria (Carlens, Ringdén et al. 1998a). Among viruses, HSV is frequently reactivated during the first weeks after HSCT and disseminated HSV infection is prevented by aciclovir (Watson 1983; Lundgren, Wilczek et al. 1985). Fungal infections with Candida species often occur during the aplastic and neutropenic phase. Most common is oro-esophageal candidiasis but invasive fungal infection with candida or aspergillus may occur. Prophylaxis by fluconazole orally or Amphoteracin B orally or i.v. has been used to prevent candida infection (Quabeck, Muller et al. 1990; Carlens, Ringdén et al. 1998a; Wolff, Fay et al. 2000).

After the neutropenic phase follows a period of about three months characterised by depressed, but recovering, cellular immunity. The most common infection is CMV that is often associated with GVHD (Miller, Flynn et al. 1986). Other viral infections as EBV and adenovirus may occur. The risk of fungal infection is decreased after the neutropenic phase, but late aspergillus infection may occur and is associated with high mortality (Meyers 1990). There is a correlation between invasive fungal infection and GVHD (Tollemar, Ringdén et al. 1989; Jantunen, Ruutu et al. 1997). Treatment of invasive fungal infection, by i.v. administered Amphotericin B or the less toxic liposomal preparation (AmBisome®), has reduced mortality of aspergillus and candida (Ringdén, Meunier et al. 1991). Molecular techniques for early detection of fungal infections, before an invasive infection has been established, have been developed

(Einsele, Hebart et al. 1997). The benefit from empirical treatment, based on these PCR techniques or other non-culture based methods to prevent mortality from invasive fungal infections, is being evaluated (Ruhnke, Bohme et al. 2003). However, the incidence of post engraftment invasive fungal infections, especially invasive aspergillosis, is still high and with considerable mortality (Hebart, Loffler et al. 2000). Pneumocystis carinii and Toxoplasmosis are life-threatening infections mainly occurring 2-6 months after HSCT (Derouin, Gluckman et al. 1986; Bashey, McMullin et al. 1990). With prophylaxis by co-trimazole these infections are very rare nowadays.

During the period beyond 3 months after HSCT both the humoral and the cellular immunity are still impaired. CMV infection has become more common during this late period probably due to better CMV-strategies during the early post-HSCT period (Einsele, Hebart et al. 2000). Invasive fungal infection, mainly aspergillosis, still may occur. Reactivation of VZV is common during this period, especially during chronic GVHD (Steer, Szer et al. 2000).

5.3.5 Toxic side-effects

The dosing of the conditioning is limited by toxicity on the heart, liver, kidneys, lungs and central nervous system. The heart is vulnerable both to irradiation and chemotherapy (von Herbay, Dorken et al. 1988). Cardiac complications of Cy are well documented and cardiac damage may develop if higher pre-transplant doses than 120 mg/kg Cy are given (Kupari, Volin et al. 1990). If other cardiac toxic drugs have been given prior to HSCT, or if Cy is given in combination with TBI, the risk of cardiac damage increases. Lung damage can be caused by Cy, Bu, MTX and other commonly used drugs (Ginsberg and Comis 1982). Even though lungs are commonly shielded to decrease the irradiation dose, damage may develop within a few months after TBI (Gross 1977).

Several drugs used in HSCT patients, like MTX, CsA, ATG and antibiotics can cause liver injury (Wolford and McDonald 1988). Veno-occlusive disease (VOD) of the liver commonly develops within a month after HSCT and is characterised by hepatomegaly, abdominal pain, weight gain (ascites) and jaundice (McDonald, Sharma et al. 1984). The reported incidence varies from 0 to 70% but in a larger material the incidence has been found to be around 5% (Carreras, Bertz et al. 1998). Busulfan has been associated with VOD but monitoring of Bu and pharmacokinetic dose adjustments appear to be useful in reducing the incidence of VOD (Ringdén, Ruutu et al. 1994; Bearman 1995).

Renal insufficiency, defined as doubling of baseline creatinine, is a common complication following HSCT occurring in about half of the transplanted patients (Kone, Whelton et al. 1988). In most cases the insufficiency is mild and intermittent due to prerenal causes like hypovolemia or impaired circulation (Zager, O'Quigley et al. 1989). The most common intrarenal failure is acute tubular necrosis (ATN) caused by nephrotoxic drugs such as CsA, aminoglycosides, aciclovir and amphotericin B

(Kennedy, Yee et al. 1985). TBI is also shown to cause chronic renal failure in some patients (Chappell, Keeling et al. 1988; Cohen 2000).

Neurologic complications during the early post-transplant period are common and in most cases reversible, but may be fatal (Antonini, Ceschin et al. 1998). Chemotherapy, TBI, infections, immunosuppression and other commonly used drugs and chronic GVHD affects the nervous system. Neurologic side-effects from CsA are well known and dose dependent and characterised by tremor and paresthesias while in more severe cases grand mal seizures and cerebellar ataxia may occur (Deierhoi, Kalayoglu et al. 1988; McGuire, Tallman et al. 1988). Aseptic meningitis with headache, nausea and fever has been reported to occur in up to 10% of patients given IT MTX but symptoms usually resolves within a few days (Bleyer 1981; Walker and Brochstein 1988). Leukoencephalopathy that may develop during the first six months post HSCT is a degenerative lesion of the white matter of the CNS caused mainly by irradiation and intrathecal chemotherapy. Characteristic symptoms are slurred speech, lethargy, ataxia, confusion and seizures (Thompson, Sanders et al. 1986; Balis and Poplack 1989).

Diarrhoea following the conditioning is common and is caused by mucosal damage from cytostatics and irradiation (McDonald, Shulman et al. 1986). Alopecia usually follows high-dose chemotherapy and TBI and may be permanent (Ljungman, Hassan et al. 1995; Tran, Sinclair et al. 2000).

5.3.6 Late complications

Cataract is common after HSCT and is mainly diagnosed a few years after TBI but is also seen after conditioning with Bu (Holmström, Borgström et al. 2002). Long-term treatment with corticosteroids and GVHD are also associated with cataract (Deeg, Flournoy et al. 1984).

Secondary malignancies may develop after HSCT and in long-term survivors the incidence is at least four times higher than that of primary cancer in the general population (Lowsky, Lipton et al. 1994). The risk of secondary solid cancer increases with time and young children are at highest risk (Curtis, Rowlings et al. 1997). Lymphoproliferative disorders are most frequent. Among solid tumours malignant melanoma, squamous cell carcinoma, glioblastoma and adenocarcinoma are seen (Bhatia, Ramsay et al. 1996). Most commonly secondary cancers occur within five years after HSCT, but may appear much later (Witherspoon, Fisher et al. 1989). Secondary malignancy of the CNS is a severe but rare complication (Appelbaum and Thomas 1985).

Several studies have reported that survivors of childhood acute leukaemia treated with cytostatics and CNS radiation experience long-term deficits on measures of neuropsychological functioning, IQ and school achievement (Mulhern, Wasserman et al. 1988; Smedler, Ringdén et al. 1990). As irradiation often causes not only cataract

but also mental dysfunction and growth retardation TBI is often replaced by cytostatics in children (Probert, Parker et al. 1973; Pochin 1988; Liesner, Leiper et al. 1994).

Infertility may be caused both by TBI and high dose chemotherapy. Some cases of intensively treated patients that have been able to produce children have been reported (Jacob, Goodman et al. 1995). Most reported pregnancies have been in women going through HSCT due to SAA (Card, Holmes et al. 1980; Sanders, Buckner et al. 1988; Salooja, Szydlo et al. 2001).

5.4 GRAFT-VERSUS-LEUKAEMIA EFFECT

The ability of allogeneic HSCT to eradicate leukaemia is found to be mediated not only by the effects of the high-dose chemotherapy. Early in the era of experimental and clinical HSCT the possibility that allogeneic HSCT eliminates leukaemia through immune-mediated effects was suggested (Barnes, Corp et al. 1956; Mathe, Amiel et al. 1965; Boranic and Tonkovic 1971; Weiden, Flournoy et al. 1979). Several clinical observations are supporting the existence of a graft-versus-leukaemia (GVL) reaction. Today, GVL is not only accepted but also some of the mechanisms are also understood. The insight of how powerful and crucial GVL is for cure in many haematological malignancies has encouraged clinicians and researchers to rely more on this effect and to find methods to enhance the GVL effect.

An early observation in allogeneic HSCT was that patients suffering from GVHD had lower incidence of relapse or even went into remission after relapse (Odom, August et al. 1978; Weiden, Flournoy et al. 1979). Today, it is established that the relapse incidence in leukaemias decreases with increasing grade of acute GVHD, and chronic GVHD is associated with a stronger GVL effect than acute GVHD (Weiden, Sullivan et al. 1981; Ringdén, Labopin et al. 1996). The best LFS is seen in patients with both mild acute and chronic GVHD (Horowitz, Gale et al. 1990). Patients receiving T cell depleted grafts or stem cells from a syngeneic donor have a higher incidence of relapse, compared to HLA identical sibling transplants (Apperley, Jones et al. 1986; Fefer, Sullivan et al. 1987). This supports the idea of the GVL effect being mediated by donor-derived lymphoid cells. Another finding supporting an immune-mediated GVL effect is that immediate withdrawal of immunosuppression in patients with leukemia relapse can induce remissions (Higano, Brixey et al. 1990; Ohashi, Mikoshiba et al. 2003). This has been shown in different leukaemias such as ALL, AML and CML and is most often accompanied by GVHD. Data showing that leukaemia patients with a twin donor has higher relapse incidence than patients with a sibling donor without GVHD after allogeneic HSCT support that the GVL effect can be independent of GVHD (Horowitz, Gale et al. 1990; Ringdén, Labopin et al. 2000). The magnitude of the GVL effect varies between different leukaemias. In a world-wide study Gale et al compared 103 identical-twin transplants with 1030 concurrent HLA-identical sibling transplants matched for prognostic factors (Gale, Horowitz et al. 1994). It was found that patients with AML and CML had the strongest GVL effect from an allo-transplant reflected by a relapse incidence of 16% and 7%, respectively, compared to 53% and

40%, respectively, in AML and CML patients after identical-twin transplants. A similar trend was observed in ALL but was not statistically significant.

Donor lymphocyte infusions (DLI) to enhance the immunological effect of the immunocompetent cells in the graft have been used in different settings. Already in 1982 Storb et al reported lower rejection incidence after the administration of viable donor buffy coat cells following the marrow inoculums in 65 multiply transfused patients with severe aplastic anaemia (Storb, Doney et al. 1982). In another study, DLI given shortly after HSCT to enhance the GVL effect did not significantly decrease relapse incidence but resulted in high TRM because of GVHD and following infections (Sullivan, Storb et al. 1989). Subsequent studies have shown that the GVL effect of DLI exists and is most effective in CML (Kolb, Mittermuller et al. 1990; van Rhee and Kolb 1995). Today DLI is mainly used to re-induce remissions after relapses following HSCT (Drobyski, Keever et al. 1993). The effect of DLI may not be immediate since remissions have occurred 4-12 months after cell infusion (Kolb, Schmid et al. 2003). The best GVL responses have been seen in CML patients having only cytogenetic or molecular evidence of disease (Kolb, Schattenberg et al. 1995; Collins, Shpilberg et al. 1997; Carlens, Remberger et al. 2001). Porter et al reported 73% probability of survival at three years after DLI for relapsed CML (Porter, Collins et al. 1999). In contrast to the better GVL effect after DLI in CML, the relapse rate is higher in CML patients after TcD compared to other haematological malignancies (Goldman, Gale et al. 1988; Aschan, Ringdén et al. 1993). This finding supports that the GVL effect in CML is more important than the possible curative effect from the conditioning. The second best responses of DLI have been seen in patients with recurrent multiple myeloma but the remissions have not been as durable as in CML patients (Lokhorst, Schattenberg et al. 1997; Badros, Barlogie et al. 2001). Also in AML and MDS, a GVL effect of DLI has been seen though with lower rates of lasting responses than in CML (Kolb, Schattenberg et al. 1995; Collins, Shpilberg et al. 1997). Short time from transplantation until relapse has been a poor prognostic factor for response to DLI and survival (Levine, Braun et al. 2002). In ALL, DLI has showed limited benefit (Kolb, Schattenberg et al. 1995; Collins, Goldstein et al. 2000; Carlens, Remberger et al. 2001). The GVL effect induced by DLI seems to be enhanced by IL-2 that is a potent stimulator of effector cells such as T cells and NK cells (Smith 1988; Verma, Bagg et al. 1994; Dunne, Lynch et al. 2001). Recombinant IL-2 has also been used to enhance the effect of DLI (Slavin, Naparstek et al. 1996). Several reports on DLI after HSCT in leukaemias report a correlation between response and chronic GVHD (van Rhee and Kolb 1995). CD8+ depleted DLI has been showed to enhance the graft-versus myeloma effects without increasing GVHD (Bellucci, Wu et al. 2004).

The molecules of the major histococompatibility complex (MHC) are categorised in HLA class I and II. HLA class I is expressed on all nucleated cells and thrombocytes, while HLA class II is expressed in APCs such as dendritic cells, macrophages, Langerhans cells, Kupffer cells, B cells, activated T cells and endothelial cells. GVHD may occur due to disparity between major or minor histocompatibility antigens (Goulmy, Schipper et al. 1996). HLA disparity may trigger a powerful GVL effect, but at the cost of increased TRM due to severe GVHD. On the contrary, NK cells have

been shown to eliminate leukaemia relapse without increasing the incidence of GVHD after mis-matched HSCT (Ruggeri, Capanni et al. 2002). In the HLA class I and II matched setting, targets for GVL effects are probably minor histocompatibility and/or tumour-associated antigens (Goulmy 1997; Mielcarek and Storb 2003). Minor histocompatibility antigens are expressed on some leukaemia cells and can serve as targets for a GVL effect without concurrent GVHD, if their expression is restricted to the haematopoietic tissue (Dolstra, Fredrix et al. 1997).

Cytotoxic T cells and NK cells have been found to be central in the GVL effect. GVL reactions mediated by T cells are MHC restricted, which means that T cells can not recognise antigens unless MHC molecules present them. CD4+ cells are involved in activation of CD8+ T cells and NK cells but can also, when they are activated, directly kill target cells by the Fas/Fas-ligand pathway (Stalder, Hahn et al. 1994). The relative contribution of CD4+ and CD8+ cells has been investigated by different depleting strategies (Champlin, Ho et al. 1990; Jiang, Kanfer et al. 1991; Nagler, Condiotti et al. 1998). On the contrary to T cells, NK cells can act directly against target cells and destroy the stimulating cell by release of perforin and granzyme (Trinchieri 1989). The NK cell is, by its killing inhibitory receptor (KIR), inhibited to attack cells expressing MHC class I. Cells expressing low or no levels of MHC class I or a non-self allele of MHC will activate the NK cell due to the absence of the inhibitory signal (Kärre, Ljunggren et al. 1986). Some cancer cells downregulate MHC molecules, which allows for increased NK cell reactivity against such cancers (Hicklin, Marincola et al. 1999). In animal models, blockade of NK inhibitory receptors have enhanced anti-tumour activity both in vitro and in vivo, suggesting that NK inhibitory receptors can be responsible for diminishing anti-cancer responses (Koh, Blazar et al. 2001). The risk of the inhibition of NK-cell inactivation to self-MHC determinants is breakdown of tolerance leading to autoreactivity. Further, NK cells secrete potent immunomodulatory cytokines such as IFN-gamma, TNF-alfa, IL-2 and IL-12 with mainly increasing inflammatory responses.

5.5 HSCT IN SOLID TUMOURS

Following insights of the potential of the GVL effect in HSCT the idea of a graft-versus-tumour (GVT) effect in various cancers including solid tumours has arisen. Evidence of a GVT effect has been shown in early murine models (Moscovitch and Slavin 1984; Morecki, Moshel et al. 1997). In 1996 two reports of a probable GVT effect in breast cancer were published (Ben-Yosef, Or et al. 1996; Eibl, Schwaighofer et al. 1996). These reports were followed by a report of myeloablative HSCT in ten patients with advanced breast cancer (Ueno, Rondon et al. 1998). Five patients had partial responses and one patient experienced complete regression of metastases suggesting clinical evidence that a GVT effect may occur against breast cancer.

Autologous HSCT has been used to allow high dose chemotherapy in tumours such as breast cancer, lung cancer, neuroblastoma, ovarian cancer, colon cancer, melanoma, sarcoma and glioma (Cheson, Lacerna et al. 1989). In the allogeneic setting, the

advantage would be the GVT effect while the disadvantage is higher TRM. To reduce the risk of TRM in solid tumours RIC HSCT has been chosen. Another argument for RIC protocols in solid tumours is that chemotherapy already has failed in these tumours and the conditioning therefore only is needed to prevent rejection of the stem cell graft. Renal cell carcinoma and melanoma have been chosen to be treated by RIC HSCT as these two cancer types are considered immuno-responsive (Bernhard, Maeurer et al. 1996). Most malignant renal cells express MHC antigens and upregulation of both MHC class I and II is common which make them a possible target for immunological GVT responses (Ohmori, Okada et al. 1995). Also lymphokine activated killer cells have been shown to be part in killing of malignant renal cells (Hattori, Satoh et al. 1995). Results in HSCT for malignant melanoma has been discouraging even though some partial responses have been shown (Kasow, Handgretinger et al. 2003; Blaise, Bay et al. 2004). Childs et al reported complete regression of metastases in a patient with advanced refractory RCC (Childs, Clave et al. 1999a). This was followed by a series of 19 patients with RCC (Childs, Chernoff et al. 2000). In ten of the 19 patients metastatic disease regressed, three had a complete response, and seven had a partial response. The patients who had a complete response remained in remission 27, 25, and 16 months after transplantation. Conditioning consisted of Cy 60 mg/kg on two consecutive days followed by Flu 25 mg/ m^2 on five consecutive days.

Immunological conditions for a possible GVT effect in different tumour types has been recognised in clinical and preclinical studies (Samonigg, Wilders-Truschnig et al. 1992; Linehan, Goedegebuure et al. 1995; Van Pel, van der Bruggen et al. 1995; Rosenberg, Yang et al. 1998). Conditions needed for experimental HSCT in solid tumours are low TRM, measurable effect by computed tomography (CT), or by tumour markers. Results should be compared to a control group not treated with HSCT. Within the European Blood and Marrow Transplant Group (EBMT) protocols for allogeneic HSCT in breast cancer, ovarian cancer, RCC, soft tissue sarcomas, colorectal cancer, melanoma, lung carcinoma and biliary tree adenocarcinoma have been established recent years.

5.6 THE IMMUNE SYSTEM

Early in history it was suggested that infections were caused by small particles and that wounds should be kept clean. Leeuwenhoek described small microorganisms that he saw in the microscope already in the end of the 17th century and the importance of hygiene during deliveries and other medical procedures became obvious during the18th and 19th century. During the 18th and 19th century a few pioneers made the first discoveries that started the science of immunology. Edward Jenner introduced the term vaccination in 1796 when he discovered that protection against human smallpox could be induced by cowpox. Robert Koch and Louis Pasteur made important discoveries in the field of vaccination against bacteria. Emil von Behring and Shibasaburo Kitasato discovered antibodies in 1890. Today, the basics about the organisation of the immune system are discovered. The more we learn about the immune system the more complex it appears to be and even though all different parts of it may finally be discovered the

interaction between the ingredients of this complex system may never be fully understood.

The immune system can be divided into the innate immune system and the adaptive immune system. Initial responses to an infection are non-specific and are mediated by cells belonging to the innate immunity. This system can recognise a diverse array of pathogens and without destroying the host's tissues kill these pathogens once they are recognised. Innate immunity also includes barrier protection of the skin and mucosa, cilia of the respiratory tract, the normal bacterial flora and bactericidal peptides from endogenous bacteria (Boman 1996). The cellular component of the innate immunity is largely dependent upon myeloid cells including mononuclear and polymorphonuclear phagocytes that engulf and destroy pathogens. The mononuclear phagocytes are the macrophages that mature from blood monocytes, and migrate into tissues of the body. Mast cells also play a role in protecting mucosal surfaces against pathogens by direct effects on the surrounding tissues and by recruiting other effector cells (Galli 2000). Among the polymorphonuclear phagocytes the most important and numerous are the neutrophils that are specialised killers by engulfing microbes, while eosinophils and basophils mainly act through release of toxic substances or inflammatory mediators that can activate other cells (Elsbach 1973; Gounni, Lamkhioued et al. 1994; Galli 2000). Cells of the innate immunity have receptors to recognise common surface structures of bacteria and bacterial molecules. Binding to these receptors triggers different responses as phagocytosis or release of inflammatory components. NK cells are large lymphoid cells that are important in the innate immune system (Lanier, Phillips et al. 1986). They can destroy antibody coated infected or malignant cells by interaction with the Fc receptor on the NK cell surface or act directly through characteristic NK mediated cytolytic activity (Santoli and Koprowski 1979). NK cells also secrete cytokines and chemokines that modulate subsequent steps in the adaptive immune response (Biron, Nguyen et al. 1999). In addition, the non-specific defence includes the complement system, acute-phase proteins, and cytokines (Thiel, Holmskov et al. 1992; Brown, Atkinson et al. 1994). Some of these substances can mediate direct effects on infectious agents, promote repair of damaged tissue, or act as mediators between different cells of the immune system.

The adaptive immune system can recognise and respond to particular pathogens that escape the innate immune system. Characteristic features of adaptive immunity are specificity, memory and diversity. The cells having these three characteristics are B and T lymphocytes that have a highly diverse repertoire of antigen-specific receptors that enable the immune system to recognise any foreign antigen. Activated antigen-specific lymphocytes proliferate and differentiate into effector cells that eliminate the pathogen and also leave a number of antigen-specific memory cells that can respond to the same infectious agent even more rapidly by the next encounter. Crucial in adaptive immunity is the exposure of antigens to effector cells by APCs. The dendritic cell may be the most important APC and is highly efficient at presenting antigens to T cells of the adaptive immune system (Ardavin, Amigorena et al. 2004). As in innate immunity, soluble mediators such as cytokines and chemokines play an essential role (Arai, Nishida et al. 1990; Hasegawa and Fujita 2001).

Moreover, the immune system is divided into cellular and humoral immunity. Humoral responses can destroy extracellular microorganisms and can prevent the spread of intracellular infections. Cellular protection mechanisms are needed to destroy cells being infected by intracellular pathogens. Antibodies produced by B lymphocytes are central in recognising pathogens and initiate processes in humoral responses while T lymphocytes are the cells responsible for cell mediated immune responses. Both the T cell receptor (TcR) and the immunoglobulin (Ig) of the B cell achieve their specificity and diversity by similar principles of rearrangement of gene segments (Tonegawa 1983; Borst, Jacobs et al. 1996).

5.6.1 B lymphocytes

B lymphocytes or B cells start their development in the BM where the stroma provide an essential microenvironment (Nagasawa, Kikutani et al. 1994). By rearrangement of Ig gene segments the immature B cell first produce cell surface bound IgM. Then the immature B cell migrate into secondary lymphoid organs as the spleen and lymph nodes where a small proportion of B cells complete their maturation (Liu 1997). The Ig molecule consists of two identical heavy chains (IgH) and two identical light chains and are divided into four isotypes with different structures and functions: IgM, IgG, IgA and IgE (Burton and Woof 1992). The coding unit for the IgH is assembled of one segment from each of the variable (V_H), diversity (D), Joining (J_H) and constant (C_H) genes on chromosome 14 (Tonegawa 1983). Variability of the Ig repertoire is achieved not only by rearrangements and different combinations of IgH and light chains but also by imprecise joining of the Ig gene segments (junctional diversity), random addition or elimination of non-templated nucleotides and by somatic hypermutations (Fanning, Connor et al. 1996). The light chain genes on chromosome 2 and 22 are organised similarly to the IgH genes and are rearranged using the same processes as for the IgH (McBride, Hieter et al. 1982; Grawunder, West et al. 1998). The Ig consists of one constant region and two variable antigen-binding sites. The most variable regions of the IgHs and light chains of the Ig are the complementarity determinig regions (CDR) that form the antigen binding loops (Wilson and Stanfield 1994). Variability of the B cell repertoire have been evaluated in several studies by analysing size heterogeneity of the third complementarity determining region (CDR3) of the IgH, which is formed by the junctions between the V_H-D-J_H gene segments (Desravines and Hsu 1994; Gokmen, Raaphorst et al. 1998). Mature naive B cells recirculate through the lymphoid organs until they encounter their specific antigen that together with co-stimulation from T cells activate the B cells to proliferate (Banchereau, Bazan et al. 1994). The B cells then differentiate into antibody secreting plasma cells or long-lived memory cells (Jacquot, Kobata et al. 1997). Secreted antibodies can participate in host defence by different mechanisms: by antibodies binding and neutralising bacterial toxins, by opsonisation that promotes phagocytosis or by complement activation (Möller and Eklund 1965; Levinsky, Harvey et al. 1978; Cooper 1985; Robbins and Robbins 1986).

5.6.2 T lymphocytes

T cells derive from the BM as the B cells but migrate at an early stage to the thymus where they maturate and go through gene rearrangements to achieve their specificity and diversity (van Ewijk 1991). In the thymus, the T cells differentiate into CD4+ or CD8+ that either will act as helper cells or as cytotoxic killer cells (Davis, Killeen et al. 1993). Unlike B cells, T cells can only recognise antigens if they are presented in the cleft of a MHC molecule. CD4+ T cells are restricted to bind to MHC class II while CD8+ T cells recognise peptides bound to MHC class I molecules (Swain 1983). During the development of immature T cells in the thymus, T cells bearing TcR that recognise self peptide:MHC complexes on thymic epithelial cells are rescued from apoptosis by positive selection while T cells binding to strongly to self-peptides complexed with MHC are removed by negative selection mediated by APCs (Onoe, Gotohda et al. 2003). The TcR has structural similarities to the Ig but is only expressed as a membrane bound molecule that can not be secreted. As in the B cell, the most variable part of the antigen recognising site is the CDR3 (Garcia, Degano et al. 1998). Activated cytotoxic CD8+ T cells recognise pathogen-derived antigens, presented by HLA class I, on the surface of host cells infected with viruses or intracellular pathogens and kill the cell by inducing apoptosis (Squier and Cohen 1994). Apoptosis is mainly induced by secretion of perforin and granzymes or by activation of Fas ligand. CD4+ T cells can be divided into T_H1 and T_H2 cells based on their cytokine expression pattern. T_H1 cells are mainly specialised for activation of macrophages while T_H2 cells activate B cells (Lohoff, Dirks et al. 1989; Stout and Bottomly 1989). Cytotoxic T cells not only induce apoptosis in infected cells but also release cytokines that inhibit viral replication, increase expression of MHC class I in infected cells and activate macrophages (Ramshaw, Ruby et al. 1992).

5.7 IMMUNE RECONSTITUTION AFTER HSCT

After HSCT the immune system is impaired for several months and may never be fully recovered, particularly in elder patients or in patients suffering from chronic GVHD (Atkinson 1990). Among many factors influencing immune recovery time is certainly of most importance since lymphocytes need time to mature even in the absence of complications (Witherspoon, Matthews et al. 1984). Immunosuppressive drugs such as CsA, prednisone and ATG are inhibitory and destructive to lymphocytes. Further, GVHD is not only attacking skin, liver and gastro-intestinal tract but also targets the immune system causing immune depression.

During the first 2-3 weeks after myeloablative HSCT the patient is aplastic until leukocyte recovery is detectable in the PB. Neutrophil granulocytes are the first cells to appear in PB 2-3 weeks after HSCT (Atkinson 1990; Hägglund, Ringdén et al. 1998). These neutrophils have normal phagocytic function but depressed granulocyte chemotaxis until a few months after HSCT (Sosa, Weiden et al. 1980). NK cells appear a few weeks after HSCT and can reach normal levels within two months (Storek, Ferrara et al. 1992). They play an important role as part of the innate immune system until lymphocytes of the adaptive immunity restore, especially against viral infections

(Santoli and Koprowski 1979). Monocytes restore numbers within two months after HSCT and early show many normal functions such as secretion of IL-1 and APC function but chemotaxis and phagocytic functions of macrophages is impaired during 6-12 months (Winston, Territo et al. 1982; Shiobara, Witherspoon et al. 1984; Tsoi, Dobbs et al. 1984; Brkic, Tsoi et al. 1985; Storek, Ferrara et al. 1992). T cell regeneration occurs at much slower rate than that of granulocytes and monocytes (Gratama and Bolhuis 1985). CD8+ T cells may be numerically normal after 1-2 months while the total number of $CD4^+$ T cells is low until at least 6 to 12 months after transplantation, resulting in an inverse CD4+/CD8+ ratio (Zander, Reuben et al. 1985). Even though numerical lymphocyte reconstitution in many cases is achieved during the first months after HSCT, their function within the immune system may still be impaired. The regeneration of the T lymphocyte compartment after stem cell transplantation follows two distinct pathways: peripheral expansion of post-thymic memory cells from the transplant and a later, thymus-dependent regeneration of naive cells derived from prethymic donor stem cells. Reconstitution of an effective T cell function is dependent on education of T cell precursors in the thymus. Thymic regenerative capacity in humans decreases with age, suggesting that thymic-independent pathways of T cell regeneration may predominate during adulthood (Mackall, Bare et al. 1996). Consequently, maturation of T cells is shown to be impaired with increasing age (Mackall, Fleisher et al. 1995). Thus, adults treated with TcD protocols, regenerate T cells primarily via relatively inefficient thymic-independent pathways, resulting in prolonged CD4+ depletion, CD4+ and CD8+ subset alterations and limited TcR repertoire diversity (Mackall and Gress 1997; Fallen, McGreavey et al. 2003).

B cell recovery lags behind T cell recovery with normalised B cell counts 4-8 months after HSCT. Full humoral reconstitution is often delayed until at least one year after HSCT (Ringdén, Witherspoon et al. 1979; Lum 1987). Factors contributing to humoral immunodeficiency after HSCT are absence of hypermutations, delayed occurrence of class-switching and clonal dominance (Näsman and Lundkvist 1996; Suzuki, Milner et al. 1996; Gokmen, Raaphorst et al. 1998). In patients with chronic GVHD, severe humoral immune deficiencies may persist for years, possibly leading to late infections.

After HSCT the patient's immune system is enhanced by a reimmunisation programme similar to that of children (Ljungman, Fridell et al. 1989; Ljungman 1999). This procedure can be carried out 1-2 years after HSCT, when the immune system is mature enough to respond efficiently to vaccination.

5.8 TOLERANCE

The definition of immunologic tolerance is controversial. Commonly, tolerance has been defined as specific absence of a destructive immune response to an antigen. This specific unresponsiveness should also be accompanied by normal immunocompetence. Mechanisms involved in tolerance induction are clonal deletion, clonal anergy, ignorance, immune deviation and suppressor mechanisms (Hilgert 1979; Platt and Bach

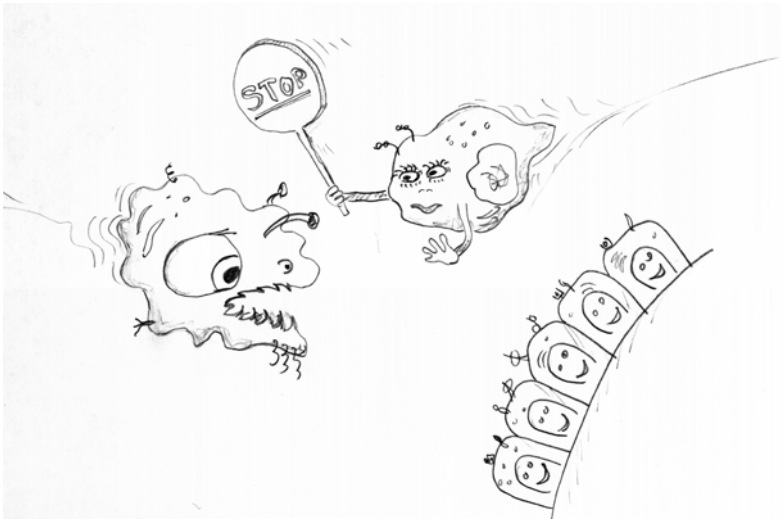
1991; Pleyer, Ritter et al. 2000). Clonal deletion is the basis for central tolerance to self-antigens and is also likely to be most permanent (Sachs 1998). As definitions of immunological tolerance are debated, the term operational tolerance is commonly used to define survival of a graft in the absence of chronic immunosuppressive drugs regardless of the mechanism (Bushell, Morris et al. 1994; Thomas, Neville et al. 1997).

Tolerance to self antigens by clonal deletion of T lymphocytes is achieved in the thymus by thymic epithelial cells and dendritic cells (Cosgrove, Chan et al. 1992; Kruisbeek and Amsen 1996). Transplantation of a new immune system or a solid organ will break the tolerance. In the setting of allogeneic HSCT, tolerance is commonly reastablished within a year after HLA-identical sibling transplants, while tolerance after HLA-incompatible HSCT is more difficult to achieve (Beatty, Clift et al. 1985; Ringdén, Remberger et al. 1995). In contrast to HLA-identical stem cell grafts, HLA-identical organ grafts are seldom available. Theoretically, withdrawal of immunosuppression after transplantation of an HLA-identical sibling graft might be possible. However, rejection of HLA-identical sibling kidney grafts occur even with maintained immunosuppression even though in a low frequency (Burke, Allouch et al. 1994; Moon, Kim et al. 2001). This is probably due to incompatibility in other tissue antigens than those that are analysed. Few reports of total discontinuation of immunosuppression after organ transplantation are found, but trials with low doses of immunosuppression after HLA-identical kidney transplants are reported (Christensen, Grunnet et al. 1998; Bartucci, Flemming-Brooks et al. 1999). Successful organ transplantations without immunosuppression in patients with a syngeneic twin donor have also been performed (Gumprich, Woeste et al. 2002; Liu, Schiano et al. 2002; Sugawara, Ohtsuka et al. 2002). Actually, the first successful kidney transplantation was performed between identical twins (Merrill, Murray et al. 1956).
Several animal models and clinical studies have shown that successful allogeneic HSCT induces tolerance to other tissues or organs derived from the same allogeneic BM donor even in the HLA incompatible setting (Rapaport, Bachvaroff et al. 1978; Sayegh, Fine et al. 1991; Jacobsen, Taaning et al. 1994). Discontinuation of all immunosuppressive treatment without rejection following, was reported in a patient who received a kidney transplant and later an allogeneic BM transplant from the same sibling donor (Gajewski, Ippoliti et al. 2002). Occurrence of donor derived cells at sites outside the organ allograft, microchimaerism, has been suggested to protect from graft rejection (Starzl, Demetris et al. 1992; Rao, Thomson et al. 1994). Scepticism about the significance of this phenomenon has been postulated suggesting that microchimaerism could be an epiphenomenon (Tomita, Khan et al. 1994; Bushell, Pearson et al. 1995). In studies, attempts to create tolerance to transplanted organs by infusion of unmodified BM cells from the same donor have been made (Ascher 1998). Rejection has been decreased but the risk of GVHD remains a major concern in this approach.

One important difference between organ transplantation and HSCT is that in the former condition the patient's immune system is normal and has to accept foreign tissue antigens. The challenge is to prevent rejection without impairing the patient's normal immune function too much. On the contrary, after HSCT the new immune system is impaired and will have to go through maturation, during which tolerance to the host-

tissues can be achieved. T cells are the main target cells for tolerance induction as they are mainly responsible for graft rejection and GVHD. Mechanisms such as clonal deletion and anergy are targeted in studies trying to induce graft acceptance (Tomita, Khan et al. 1994). Focus has also been directed towards regulatory mechanisms from co-stimulatory signals and dendritic cells (Niimi, Shirasugi et al. 2001; Corbascio, Ekstrand et al. 2002). Central tolerance by clonal deletion is the most reliable mechanism of tolerance (Nikolic and Sykes 1996). Tolerance that relies on induction of anergy or suppression of a pre-existing non-tolerant T cell repertoire may be broken or may only be effective in some patients.

Tolerance to xenografts is much more difficult to achieve than tolerance to allografts (Knosalla and Cooper 2002). Attempts to overcome the immunological challenges of xenotransplantation have been made for several years (Terblanche, Dent et al. 1970). A major challenge in this setting is hyperacute graft rejection mediated by alloantibodies (Bennet, Sundberg et al. 2001). If xenogeneic grafts could be used the problem of organs shortage could be solved. So far, progress has been made in animal models. Trials in humans have also been reported (Starzl, Fung et al. 1993; Groth, Tibell et al. 2000). Even though hyperacute rejection, the previously insurmountable obstacle to pig-to-human xenografts, has been overcome in some studies there are still remaining obstacles to overcome (van den Bogaerde and White 1997). Besides the immunological difficulties in xenotransplantation there are also concerns about transfer of potentially dangerous viruses (Groth 1998).



Tolerance is an active process

6 AIMS OF THE PRESENT STUDY

- To study the tolerability of allogeneic HSCT with RIC conditioning in patients with solid tumours
- To evaluate if RIC HSCT can induce an anti-tumour effect in different solid tumours
- To evaluate clinical tolerance after HSCT and prognostic factors.
- To study factors influencing reconstitution of B lymphocytes and T lymphocytes after HSCT, with certain aspects on differences after myeloablative and RIC regimens

7 MATERIAL AND METHODS

7.1 Patients

The patients in this study were transplanted at Huddinge University Hospital 1977-2001. In papers I and II, patients with metastatic solid tumours not curable with conventional therapy were included. Paper III mainly included patients with haematological malignancies, SAA and metabolic disorders while the selection of patients in papers IV and IV was based on type of conditioning protocol. All studies in this thesis were granted ethical permission from the local ethics committee at Karolinska University Hospital, Huddinge. Characteristics of patients and donors in the five studies are presented in Table 1a and 1b.

7.2 Conditioning

Conditioning protocols differed during the studies depending on diagnoses and time period. Cyclophosphamide 60 mg/kg for two consecutive days followed by 10 Gy of TBI with the lungs shielded to receive no more than 9 Gy or Cy in combination with Bu were the most common conditioning regimens in patients with haematological malignancies (Ringdén, Ruutu et al. 1994). Patients with SAA were given high dose Cy and since 1988 ATG prior to HSCT. Patients with metabolic disorders were given Bu 80 mg/m^2/day for four days followed by Cy 2 g/m^2/day for another four days (Ringdén, Groth et al. 1990). Before November 1988 all patients with haematological malignancies were given 8-12 mg of MTX or Ara-C intrathecally twice prior to HSCT and 6 times from day 32 until 102 but after 1988 this treatment was only given to patients with ALL, AML M4 or M5 or with previous CNS leukaemia (Carlens, Ringdén et al. 1998b). Reduced intensity protocols consisted of Flu 30 mg/m^2/day for three or five days, depending on if a matched sibling donor or a MUD was used, followed by either 2 Gy of TBI, Bu 4 mg/kg/day for two days or Cy 30 mg/kg/day for two days. All patients with an unrelated donor were given ATG (ATG-Fresenius® or Thymoglobulin®) or Orthoclone (OKT-3) in addition to the conditioning protocol (Ringdén, Remberger et al. 1998).

Table 1a. Patient and donor characteristics in papers I-V

	I	**II**	**III**	**IV**	**V**
Time period of study	1999	1999-2001	1977-1997	1999-2000	1999-2000
Follow-up time post HSCT	4 months	median 9 months	13 months-18 years	12 months	12 months
Number of patients	1	18	354	23	24
Males/Females	1 male	8/10	221/133	13/10	14/10
Median age (range) at HSCT	77	58 (38-77)	24 (0.6-55)	51 (23-60)	51.5 (23-60)
Diagnoses:					
Haematological diseases					
ALL			81		
AML			80	7	7
CML			95	5	6
CLL			5	1	1
Fanconi anaemia			4		
Lymphoma			6		
MDS			7	4	4
Myelofibrosis			4		
Myeloma			11		
SAA			32		
Essential thrombocytopenia				1	1
Metabolic disorders			29		
Solid tumours					
Breast cancer		1			
Colon cancer	1	6		1	1
Liver cancer		1			
Renal carcinoma		10		4	4
Donors:					
HLA-identical siblings	1	12	259	13	13
HLA-identical related			7		
Mismatched family donor			11		
Matched unrelated donor		6	74	10	11
Mismatched unrelated donor			3		
Males/Females	1 male		198/155	14/9	14/10
Donor age, median (range)	66	48.5 (28-71)	31 (0.5-67)	39 (20-63)	38.5 (20-63)

Table 1b. Conditioning and GVHD-prophylaxis protocols in patients in papers I-V.

	I	II	III	IV	V
Conditioning					
Bu+Cy			62	11	11
Cy			25		
Cy+TBI			263		
Cy+TLI			4		
Flu+Bu				5	6
Flu+Cy				2	2
Flu+TBI	1	18		5	5
ATG or OKT-3	1	6	90	11	12
GVHD-prophylaxis					
MTX			42		
CsA			35		
CsA+MTX			131	9	10
Low dose CsA+MTX			59	8	8
Individual CsA+MTX			64		
CsA+Prednisolone			3		
CsA+MMF	1	18		6	6
T-cell depletion			19		

7.3 Immunosuppressive protocols

Since the first used immunosuppressive drug MTX various immunosuppressive protocols have been developed. The following main protocols used during different time periods are more detailed described in the articles.

I. MTX according to the Seattle-protocol was used from November 1975 to September 1985 (Thomas, Storb et al. 1975a).

II. CsA during the first year post HSCT was used from March 1982 to September 1985 (Powles, Clink et al. 1980; Ringdén, Backman et al. 1986; Aschan, Ringdén et al. 1991).

III. MTX, 15 mg/m^2, given i.v. on day +1, then10 mg/m^2, on days +3, 6 and 11 in combination with CsA, according to protocol II was used from October 1985 to October 1989 and is still given to patients with unrelated donors and/or non-malignant diseases (Storb, Deeg et al. 1986).

IV. T-cell depleted grafts were given to adult leukaemia patients who were randomised either to protocol III or IV from October 1985 to October 1989 (Ringdén, Remberger et al. 1994).

V. Between June 1988 and January 1995, patients with haematological malignancies were given prophylaxis with MTX alone or combined with CsA according to their individual risk of developing GVHD (Aschan, Ringdén et al. 1994).

VI. MTX in combination with low dose CsA has been given to patients with haematological malignancies and HLA-identical sibling donors since 1993. (Carlens, Aschan et al. 1999).

7.4 Chimaerism

We used chimaerism analysis performed by PCR of variable numbers of tandem repeats (VNTRs) to determine the origin of the B and T lymphocytes after HSCT (Mattsson, Uzunel et al. 2001a). Briefly, lymphocytes from PB were separated into CD3+ and CD19+ subsets by using immunomagnetic beads (Dynal, Norway). Thereafter, pre-transplant recipient and donor DNA samples were amplified with different mini-satellite primer pairs to obtain at least one informative locus. PCR analysis was performed with the chosen primer pair on sequential patient samples. PCR samples were separated on 12.5 % nondenaturating polyacrylamid gels in a ready-to-use system from Pharmacia Biotech (Pharmacia Biotech, Uppsala, Sweden). The gels were analysed after 90 minutes of automated silver staining (Pharmacia Biotech).

7.5 Clinical tolerance

After HSCT, most patients experience mild or moderate GVHD, which resolve. When GVHD has disappeared, immunosuppression can be tapered and discontinued. Thereafter, patients can be regarded as clinically tolerant. Immunologic tolerance can be defined as the lack of an immune response against a given antigen in the presence of normal immune responsiveness to other antigens (Deeg, Tsoi et al. 1984). We defined clinical tolerance as the absence of GVHD and rejection with at least one month follow-up after withdrawal of immunosuppression.

7.6 T cell proliferation

Mitogens have the ability to stimulate the synthesis of DNA, RNA and proteins in B cells and T cells (Möller 1970). The in vitro model of lymphocyte stimulation by different mitogens and antigens is a useful method to measure the responsive ability of lymphocytes (Paulin, Ringdén et al. 1987). Different mitogens can selectively activate B or T cell subpopulations or both. Concanavalin A (Con-A) and phytohemagglutinin (PHA) selectively stimulate T cells while SpA can activate both B cells and T cells. We used the mitogens Con-A, PHA and SpA and also viral antigens from CMV, HSV and VZV. Lymphocytes were separated using a centrifugation of heparinised blood on Lymphoprep™ (Nycomed-Pharma AS, Oslo, Norway). The cells were cultured in microtiter plates in 0.2 ml medium consisting of RPMI 1640, AB-serum, penicillin, and streptomycin. DNA synthesis was measured in triplicate samples containing $1.5x10^5$ lymphocytes/well 1 μCi of 3H-thymidine was added to each culture 24 hours before harvesting. We harvested the lymphocytes on the day of optimal response to the various mitogens/antigens. Radioactivity was measured using a scintillation counter and background activity for unstimulated cells was subtracted.

7.7 CDR3 spectratyping

The variability of the CDR3 region in both B cells and T cells results in varying lengths of Ig and TcR transcripts, respectively, with a Gaussian distribution of different CDR3 sizes in healthy individuals (Gorski, Yassai et al. 1994; Gokmen, Bachier et al. 2001). Lymphocytes from PB were separated into CD4+, CD8+ and CD19+ subsets by using immunomagnetic beads (Dynal, Norway). RNA from the lymphocyte subsets was extracted and cDNA was synthesised. An initial PCR, to determine the amount of B or T lymphocyte genomes in each sample, was performed. Then, a second PCR was performed to amplify the CDR3 regions. In this step a 6-fluorescein phosphor amidite (6-FAM) labelled primer was used. Electrophoresis and analysis of the fluorescent PCR products and size standards were performed by CyberGene AB, Sweden, using an ABI Prism 377 automated DNA sequencer, the GeneScan 3.1.2 software and also the Genotyper 2.0 software (Applied Biosystems, Foster City, CA, USA). Electrophoresis was performed on a 36 cm WTR 4,5% polyacrylamid gel, 2400 scans per hour, filter set C. Tamra500 (Applied Biosystems) was used as internal size standard in each sample. The fluorescence intensity of each band on the gel was quantified with the

GeneScan™ software and translated into a histogram displaying the length and intensity of each CDR3 fragment.

The Vß gene in T lymphocytes is divided into 24 subfamilies that are variously expressed in different individuals. First we used 28 Vß subfamily specific primers to analyse the whole Vß region. Then we chose the ten primers corresponding to the most normally expressed Vß subfamilies in six healthy donors and in the studied patients twelve months post-HSCT. At least 8 peaks of different CDR3 sizes and a Gaussian distribution of these peaks should be present in a normal polyclonal expression of a Vß subfamily (Wu, Chillemi et al. 2000). We defined five "scoring groups" ranging from no expression to normal expression: score 0 means no expression of the subfamily; score 1 includes 1-2 peaks; score 2 includes 3-7 peaks; score 3 includes 8 or more peaks; and score 4 includes 8 or more peaks *and* a Gaussian distribution of all peaks reflecting a fully polyclonal expression of the TcR subfamily (Figure 3). The scores for all 10 subfamilies were summarised to a final score of zero to 40 and compared to the scores of six healthy donors.

***Figure 3**. Examples of spectratype scores in four patients: a Gaussian distribution of at least 8 CDR3 size peaks in a subfamily reflects a fully re-established diversity and was given the score 4 (A); at least 8 peaks but with a skewed distribution was given the score 3 (B); 3-7 peaks was given the score 2 (C); and 1-2 peaks reflecting a very poor CDR3 diversity was given the score 1 (D).*

The variability of the CDR3 in the IgH was analysed in the same way as for the TcR Vß chain but one consensus primer spanning the whole V_H region was used. This was possible due to the lower size spread of the IgH CDR3 transcripts. In healthy donors this method resulted in a Gaussian distribution of 25-30 peaks of different CDR3 sizes.

7.8 Lymphocyte subsets, Ig levels in serum and serum Gm allotypes

The numerical recovery of CD4+ and CD8+ T cells, B cells, CD5+ and CD27+ B cells was determined using standardised FACS analysis. IgA, IgG, IgM, IgG1, IgG2, IgG3 and IgG4 levels in serum were determined with nephelometry. FACS analyses and determination of Ig levels in serum was performed at the routine laboratory for clinical immunology, Huddinge University Hospital. Gm allotypes are genetic variants of the immunoglobulin heavy chains of IgG molecules and are inherited in a Mendelian way (Oxelius, Aurivillius et al. 1999). In the Caucasian population there are 10 different genotypes, some of which are common and others very rare. Gm allotypes were used as markers to determine the origin, donor or recipient, of the immunoglobulins circulating

in serum after HSCT. Serum samples were analysed for Gm allotypes in a competitive ELISA(Oxelius 2000).

7.9 Statistical analyses

Data handling and statistical calculations were performed using the software Microsoft® Excel, JMP™ (SAS Institute Inc., Cary, NC, USA) and Statistica (StatSoft®). The method of Kaplan-Meier was used to estimate the probabilities of survival and acute GVHD in paper II and time to discontinuation of immunosuppression in paper III. Differences between groups were compared using the log-rank test. The Mann-Whitney U method was used to compare numbers of days to DC in paper III. The Cox proportional hazards method was used in the multivariate analysis in paper III. In papers IV and V differences in patient and donor age and diversity scores between the groups were compared using the Mann-Whitney U test or Wilcoxon rank-sum test. Fischer's exact test was used to compare differences in incidence of GVHD and infections.

8 RESULTS AND DISCUSSION

8.1 PAPERS I AND II

The application of RIC HSCT in haematological HSCT had been reported to be feasible. This in combination with solitary reports of GVT effects in solid tumours encouraged us to perform this study to investigate the tolerability of the treatment and a possible GVT effect. The strategy of using a reduced toxicity protocol for achieving an immunological platform for a potential GVT effect with acceptable TRM was a logical choice based on experience in other groups. At the time for the start of our study, different RIC protocols were already in use in HSCT for patients with haematological disorders but also in limited extent in solid tumours. The research group at NIH in Washington had used a rather myelosuppressive protocol consisting of Cy and Bu, while the Seattle group reported stable chimaerism in four of five dogs using a minimally myelosuppressive regimen consisting of only 2 Gy of TBI followed by immunosuppression with CsA and MMF (Storb, Yu et al. 1997; Childs, Clave et al. 1999b). Slavin and colleagues introduced a RIC protocol consisting of Flu and Bu (Slavin, Nagler et al. 1998). We were attracted by the very low intensity of the Seattle protocol, but added Flu to decrease the risk of rejection since graft rejection was reported in 20% of 45 patients in a multi-centre study using the minimally myelosuppressive Seattle-protocol (McSweeney, Niederwieser et al. 2001).

In the 18 patients receiving Flu and 2 Gy of TBI all had initial engraftment. Patients with a MUD had a trend for faster T cell engraftment than patients with a sibling donor. This may be due to the more intensive and immunosuppressive conditioning regimen given to patients with a MUD. A graft from a MUD may also induce DC faster than an HLA-identical sibling graft because of the probable higher alloreactivity from a MUD graft. Two patients with a sibling donor had rejected their grafts five and two months after HSCT, respectively. That has lead to a changed conditioning protocol in solid tumour patients following these initial 18 patients, at our centre. All patients below 65 years are now treated with Flu 30 mg/m^2/day for five days followed by Cy 120 mg/kg. Patients above 65 years are still getting conditioning with Flu and 2 Gy of TBI, but now also patients with an HLA-identical sibling donor get Flu for five days instead of three days.

Most patients tolerated the conditioning well and were treated on an outpatient basis. One patient died of ganciclovir toxicity. Two patients suffered from septicaemia. CMV infection was common and four patients developed CMV disease with mild symptoms. Acute GVHD grades II-IV was diagnosed in 9/18 evaluable patients. Four patients suffered from mild chronic GVHD. DLI given to seven patients did neither induce tumour responses nor initiate nor aggravate GVHD.

The first patient, who was the oldest among the 18 patients became DC within three months after HSCT. Rapid decrease in tumour burden was seen clinically and on CT. Unfortunately he died of pneumonia four months after HSCT. Histopathology of

several lymph node metastases from various locations showed central necroses with few surrounding viable tumour cells left. These tumour metastases were too small to have gone into spontaneous necrosis, which is commonly occurring in bigger tumours (Folkman 1974). The presence of HLA class I on the cancer cells supports that a T cell mediated anti-cancer effect may have caused the tumour regression in this patient.

In the following 17 patients going through HSCT for a solid tumour, most patients experienced tumour progression. Five patients died of tumour progression within ten months after HSCT. Responses of small metastases in the lungs were seen in three patients 6-12 months after HSCT. Patient R4 died from GVHD after five months. Metastases, that had progressed during the first months post-HSCT, before the occurrence of severe GVHD, were found to be necrotisised at the autopsy. This finding indicates a GVT effect associated with an ongoing GVHD. Childs et al reported the occurrence of tumour regression following moderate to severe acute GVHD (Childs, Chernoff et al. 2000). They also suggested that the GVT effect in solid tumours is closely associated with GVHD. However, Childs et al also reported responses in some patients without GVHD. In a European multicentre program, 57 patients with RCC (n=25), breast cancer (n=12), melanoma (n=5), ovarian cancer (n=5), soft tissue carcinoma (n=4) and six other solid tumours were recently reported (Blaise, Bay et al. 2004). After RIC HSCT using the Slavin protocol, six partial responses and two complete responses were seen. Responders were three patients with ovarian cancer, two patients with breast cancer, two patients with RCC and one with melanoma. All patients with regression were donor chimaeric and all patients but one experienced chronic GVHD before responses were seen.

8.2 PAPER III

Many studies have evaluated factors influencing GVHD and different protocols to prevent GVHD have been used. The drugs, doses and the time they are used vary among protocols. Reports on compliance of protocols are rare. Therefore, we chose to study the duration of immunosuppression in different patient groups. In most patients clinical tolerance, defined as absence of immunological events after withdrawal of immunosuppression, was established when immunosuppression was discontinued. Some patients still had slight symptoms of chronic GVHD when immunosuppression was discontinued and could not be considered tolerant. In these patients, immunosuppression was mainly discontinued while their remaining symptoms were judged to be probable sequele after a previous GVHD. Therefore the symptoms were not expected to be affected by discontinuation of immunosuppression. Even though these patients may have been tolerant, we could not define them as clinically tolerant. However, these few patients did not affect outcome in the overall analyses of clinical tolerance. Our study showed that most patients had discontinued their GVHD-prophylaxis according to the protocols in the absence of GVHD.

In the univariate analysis, time to tolerance was longer in patients receiving unrelated marrow as compared to those with a sibling donor. This was expected since patients

with a MUD have a higher incidence of acute GVHD grades II-IV and acute GVHD predisposes for chronic GVHD (Storb, Prentice et al. 1983; Ringdén, Paulin et al. 1985; Beatty, Hansen et al. 1991). This suggests that a MUD graft correlates to a higher susceptibility for chronic GVHD which also has been shown in some studies (Kernan, Bartsch et al. 1993; Marks, Cullis et al. 1993; Sanders 2002). However, a study at Huddinge Hospital showed similar incidence of acute and chronic GVHD using MUD BM as with HLA-identical siblings (Ringdén, Remberger et al. 1995). This fact is in concordance with the result in the multivariate analysis that the type of donor did not affect clinical tolerance. After two years, the cumulative proportion of patients with ongoing immunosuppression was similar in patients with related or unrelated stem cell donors. This reflects a similar proportion of patients with ongoing chronic GVHD in the two groups. Not only the time during which immunosuppression is given is of importance. Patients with an unrelated donor get more intensive GVHD-prophylaxis because prevention of GVHD is easier than treating GVHD once it has been established. In this study, we evaluated only the time to discontinuation, without respect to the dose of immunosuppression.

TRM caused by GVHD was high among mismatched patients. Among the 37 transplanted mismatched patients, 14 patients were alive at 13 months. Six of these 13 patients had discontinued immunosuppression and could be considered tolerant. One could assume that the mismatched patients who died from severe GVHD during the first 13 months would not have been tolerant after 13 months, if ever, if they would have survived, which indicates that tolerance is much more difficult to achieve in mismatched HSCT.

In multivariate analysis, high donor age, immunised female donor to a male recipient and GVHD grades II-IV were associated with a longer time to clinical tolerance. This correlates well to other studies evaluating risk factors for acute and chronic GVHD (Gale, Bortin et al. 1987; Carlens, Ringdén et al. 1998b). Accordingly, patients with risk factors for GVHD need more time to discontinuation of immunosuppression.

The importance of single drug prophylaxis with CsA or MTX was analysed even though this may be most of historical interest. Today a combination therapy of these two drugs has become routine in most centres (Storb, Deeg et al. 1986; Ringdén, Horowitz et al. 1993). MTX was correlated to earlier discontinuation. This may be due to the much shorter prophylaxis protocol for MTX. Other studies have shown comparable GVHD protective effect of these two protocols (Ringdén, Backman et al. 1986; Storb, Deeg et al. 1988). Previous studies showed that high donor age or recipient age were risk factors for acute and chronic GVHD (Storb, Prentice et al. 1983; Ringdén and Nilsson 1985; Ringdén, Paulin et al. 1985; Atkinson 1990). In the HLA-identical sibling donor situation, these two factors are closely associated since a young recipient usually has a young donor. In this study donor age was found to be more important than recipient age for time to clinical tolerance.

Chimaerism analysis was only performed in the 13% of patients transplanted during the last two years of the study. After 12 months, 42/46 patients in whom chimaeric analysis

had been performed were alive. In this small group of patients, subgrouping was not possible. No correlation of MC to clinical tolerance could be seen in this group of mixed patients with both malignant and non-malignant diseases. However, a recent study from our centre, with 102 patients with a similar mixture of malignant and non-malignant haematological diseases included, showed that T cell MC was correlated to a reduced risk of acute GVHD grades II-IV after myeloablative HSCT (Mattsson, Uzunel et al. 2001a). Studies in other centres have shown the same results that MC is associated with tolerance. If this is the effect or cause can not be elucidated from these studies.

We have defined clinical tolerance as differing from immunological tolerance. There is a close relation between clinical and immunological tolerance. The latter, defined as failure to respond aggressively to an antigen, involves both target and effector cells. Thus, patients receiving T cell depleted grafts may not get GVHD even if immunosuppressive drugs are not given post transplant. If tolerance is regarded as an active process and not just an immunological unresponsiveness, these patients cannot be considered immunologically tolerant, since their T cell depleted grafts are immunologically defective.

8.3 PAPERS IV AND V

Several studies have investigated recovery of B cells and T cells measured by numerical, morphological and functional parameters. Most studies comparing immune reconstitution after RIC and myeloablative HSCT conclude that both morphological and functional immune recovery is faster after RIC compared to myeloablative HSCT (Morecki, Gelfand et al. 2001; Mohty, Gaugler et al. 2002). We did not find any striking difference between the two groups. In both groups, diversity of the CDR3 region and proliferative responses were impaired after HSCT compared to healthy donors.

At 12 months after HSCT, only patients in the RIC group had recovered TcR CDR3 diversity to a level comparable to healthy donors. In B cells the finding was opposite, indicating faster recovery of the CDR3 diversity of the IgH in the myeloablative group, compared to healthy donors. This indicates that the maturation of the T cell repertoire may be faster after RIC HSCT, while B cell recovery may be slower, compared to the myeloablative group. However, when evaluating clinical factors in each patient a possible explanation for lower diversity in the CDR3 of the IgH could be found. Patients in these studies on B and T cells were the same besides one more RIC patient in the B cell study. The different outcome in the comparison of the TcR repertoire and IgH repertoire indicates that individual factors such as GVHD and age may affect these two systems differently. In the RIC group 11/13 patients had chronic GVHD compared to 6/11 in the myeloablative group ($p=0.12$). It is known that GVHD strongly inhibits humoral immunity, while T cell function is less affected (Atkinson 1990). This may partly explain the discrepancy in results. However, GVHD certainly also affects the T cell compartment negatively as GVHD contributes to thymic damage resulting in

impaired thymic-dependent regeneration (Muller-Hermelink, Sale et al. 1987). Studies have supported the theory of faster reconstitution of the T cell compartment using conditioning regimens that are less toxic to the thymus. A study by Bahceci et al showed that early reconstitution, measured by numbers of T cell subsets and complexity of the T cell repertoire after RIC HSCT using PBSC, is from post-thymic T cell expansion and is unaffected by GVHD (Bahceci, Epperson et al. 2003). Chao et al also suggested that the ability of patients receiving a RIC regimen to recover within a few months is due to preserved peripheral niches in which T cells can proliferate in these patients compared to those receiving ablative regimens (Chao, Liu et al. 2002). They also suggested that thymic recovery is likewise accelerated in RIC compared to myeloablative regimens. Thymys-dependent pathways are certainly of importance even after RIC HSCT as thymus-independent pathways result in a limited repertoire of T cells (Mackall, Bare et al. 1996). A study by Petersen et al showed delayed numerical B cell recovery but similar T cell reconstitution after RIC HSCT with Flu+2Gy of TBI, compared to myeloablative HSCT (Petersen, Ryder et al. 2003). Interestingly, RIC patients in their study were both significantly older and had more chronic GVHD than patients receiving myeloablative conditioning. These results are in parity with our results.

Survival and infection frequency was similar in patients treated with RIC or myeloablative HSCT. In the RIC group more viral infections were seen ($p<0.05$). In a study by Martino et al, the incidence of CMV was higher in patients receiving myeloablative conditioning while the only significant variable associated with a higher risk of infection-related mortality was the development of moderate-to-severe acute GVHD (Martino, Caballero et al. 2001). In our study, both recipient age and donor age were significantly higher in the RIC group (median recipient age 54 versus 42, and median donor age 46 versus 38, $p<0.05$). This is expected because RIC is primarily used in older patients and there is a correlation between patient and donor age when a sibling donor is used. Higher recipient age has been shown to correlate to a higher incidence of viral infections, and also to a higher incidence of GVHD (Storb, Prentice et al. 1983). Most studies show similar or even higher incidence of GVHD in RIC patients. Nachbaur et al suggested that dendritic cell function may be impaired after RIC HSCT (Nachbaur, Kircher et al. 2003). They also suggested an interaction of immunosuppression with dendritic cell function. In a murine model MMF was shown to impair the maturation and function of dendritic cells (Mehling, Grabbe et al. 2000). This may contribute to impaired immune recovery due to delayed antigen presentation by dendritic cells. In our study, MMF was used in five patients in the RIC group and not at all in the myeloablative group. ATG, that both interferes with T cells and dendritic cells, was used in the same extent in both groups (Monti, Allavena et al. 2003). The theory of dendritic cells being part of the impaired immune recovery after RIC HSCT may partly explain our results that showed low IgH diversity and low proliferative responses after RIC HSCT.

HSCT with PBSC instead of BM has been shown to correlate to faster recovery of neutrophils (Champlin, Schmitz et al. 2000). Ottinger et al showed higher levels of naive (CD4+CD45RA+) and memory (CD4+CD45RO+) helper T cells and of B cells

and improved in vitro immune competence after PBSCT compared to BMT (Ottinger, Beelen et al. 1996). This is logical as these grafts normally contain a log more lymphocytes (Weaver, Longin et al. 1994). The infusion of higher lymphocyte numbers is especially correlated to faster functional recovery while absolute numerical recovery is achieved surprisingly fast also after TcD where CD3+ levels are more than 3 log lower than in PBSCT (Bahceci, Epperson et al. 2003). In a study by Storek et al, the rate of severe infections after engraftment was 2.4-fold higher in marrow recipients as compared with PBSC recipients (Storek, Dawson et al. 2001). The difference in the rates of definite infections was greatest for fungal infections, intermediate for bacterial infections, and lowest for viral infections. On the contrary, in a multicentre study comparing 107 patients receiving PBSC from HLA-A, -B, and -DR-compatible unrelated donors to 107 matched controls receiving unrelated BM, no difference between the two groups in bacteraemia, CMV reactivation or disease, and fungal infection was seen (Remberger, Ringdén et al. 2001). We could not do a meaningful comparison of the importance of stem cell source in this material since only four patients were given BM.

9 CONCLUSIONS

- HSCT with RIC conditioning seems feasible in patients with solid tumours. The incidence and degree of toxicity, GVHD and infections were acceptable in our 18 patients with advanced solid cancer tumours. (Papers I and II)

- A GVT effect is present after allogeneic HSCT in patients with solid tumours. Modification of the treatment is needed to enhance the GVT effect. Until the GVT effect can be potentiated, primarily patients with small and slowly growing tumours should be chosen for allogeneic HSCT to give the immunological GVT effect a chance to stop tumour progression or even induce regression. (Papers I and II)

- Clinical tolerance is achieved in most patients receiving HLA class I and II matched haematopoietic stem cell transplants making discontinuation of immunosuppression possible within two years after HSCT in around 70% of patients with a matched related or unrelated donor. (Paper III)

- Spectratyping of the CDR3 region of the B cell IgH and TcR is a useful method in evaluating the diversity of the developing adaptive immune system. (Papers IV and V)

- The TcR and B cell Ig repertoires are skewed under the first year after HSCT. After RIC HSCT with Flu+Bu or Flu+TBI, faster reconstitution of the TcR CDR3 repertoire was seen, while the reconstitution of the Ig H CDR3 repertoire was delayed, as compared to myeloablative HSCT. Individual factors such as GVHD, age and viral infections appeared to be of more significance for immune reconstitution than the type of conditioning. (Papers IV and V)

10 FUTURE PERSPECTIVES

SOLID TUMOURS

Since the first few reports of allogeneic HSCT in solid tumours a few years ago, increasing numbers of allogeneic HSCTs have been performed at different centres. International co-operation and working parties contribute to develop better strategies.

At Karolinska University Hospital, Huddinge the following numbers of patients with solid tumours have received RIC HSCT until February 2004: 16 patients with RCC, nine patients with colon or rectal cancer, two patients with prostate cancer, one patient with Klatskin tumour in the liver hilus, one patient with breast cancer and one patient with soft tissue sarcoma in the kidney. Furthermore, ten patients livertransplanted due to cholangiocarcinoma (n=4), hepatocellular cancer (n=5) or a combination of both these diagnoses (n=1) were treated with HSCT. On February 29 2004, 15/40 patients were alive 0.5-41 months (median 9 months) after HSCT.

Within the co-operation of EBMT, 124 patients with RCC, 106 with breast cancer, 31 with CC, 18 with ovarian cancer, seven with prostate cancer and one with bladder cancer have been reported so far. A GVT effect is considered to have been proven in these diagnoses while RIC HSCT in malignant melanoma has been stopped. Evaluation of the 124 European patients with RCC showed an overall survival of 40% after one year and 30% after two years. When patients were divided into “good risk” and “poor risk” groups according to the number of metastases (0-2/>2), DLI (y/no), Karnofsky score (>70/<70) and chronic GVHD (y/no), a significantly better survival was seen in the “good risk” group whereas all patients in the “poor risk” group had died before 2,5 years after HSCT. This result emphasis the importance of choosing patients with the best chance to benefit of HSCT.

DLI has been widely used in leukaemic relapses and also in HSCT for solid tumours. Desirable is to enhance the GVT effect without enhancing GVHD. A similar strategy to DLI is NK cell infusions. The experience from that approach is so far limited. However, it has been supported that infused activated NK cells may eliminate residual disease in breast cancer (deMagalhaes-Silverman, Donnenberg et al. 2000). Interleukin-2 is commonly used to enhance the GVT effect of NK cells (Soiffer, Murray et al. 1996). Even though NK cell infusions may not enhance GVHD, the simultaneous use of IL-2 may enhance GVHD by affecting other cells, especially T lymphocytes, involved in GVHD (Jiang, Barrett et al. 1997). Wheather pure NK cell or mixed NK/T cell infusions should be used is still to be studied. The occurrence of tumour-specific T cells has been shown in both leukaemias and solid tumours (Caignard, Dietrich et al. 1994; Farace, Orlanducci et al. 1994). Infusion of *in vitro* expanded tumour-specific T cells have been performed but so far results are poor (Sun, Moller et al. 1999). Better methods in obtaining tumour-specific cytotoxic lymphocytes with strong GVT effect are needed and the benefit of NK cells is still to be explored.

Spectratyping has been used to identify that GVT and GVH reactive T cells have different TcR Vß specificities (Dietrich, Caignard et al. 1994; Epperson, Margolis et al. 2001). This fact is encouraging for generating more effective tumour-specific alloreactive T cell clones without enhancing GVHD. In a study by Epperson et al, donor lymphocytes were cultured with leukaemia cells or normal recipient lymphocytes and compared to the skewed repertoires in the patients (Epperson, Margolis et al. 2001). They found similar patterns *in vitro* and *in vivo* and suggested that this method could be useful in predicting functional behaviour of T cell expansions and that GVH reactive Vß families might be eliminated while conserving those being GVL reactive. Different immunosuppressive agents affect the immune system by different mechanisms. After HSCT for solid tumours, some patients seem to progress especially during the most immunosuppressive early post-transplant period. From that perspective, a drug such as rapamycin, that has been demonstrated to possess both immunosuppressing and tumour suppressing abilities simultaneously may be useful in HSCT for solid tumours (Guba, von Breitenbuch et al. 2002; Luan, Hojo et al. 2002).

The role of dendritic cells in GVHD and in the tumour defence after HSCT has been studied with a present focus on using dendritic cells for clinical applications to treat cancer patients (Lundqvist and Pisa 2002; Nachbaur, Kircher et al. 2003). Different vaccination strategies have been used in solid tumours and the possibility of active therapeutic vaccination after HSCT for solid tumours may be a way of enhancing the GVT effect of HSCT (McGee, Price et al. 1999; Zoller and Matzku 2002). Strategies using gene-therapy have been under development in recent years and may contribute to further progression in achieving stronger GVT effects with or without HSCT (Carlens, Gilljam et al. 2000; Fiedler and Wirth 2001). Mesenchymal stem cells (MSC) have been found to be involved in regulatory functions concerning GVHD and GVL effects (Rasmusson, Ringdén et al. 2003). They can reduce the incidence of graft-versus-host disease because of their ability to inhibit T lymphocyte proliferation. The use for MSCs in enhancing GVL/GVT effects without enhancing GVHD is still to be explored.

In summary, desirable for succeeding in curing solid tumours by immunotherapy is the generation of a more specific and stronger targeting of tumour cells. The strategy of performing allogeneic HSCT as a basis for adoptive cell therapy may be necessary, but several anti-tumour strategies are probably needed to prolong survival and cure these patients.

TOLERANCE

Different immunosuppressive regimens are used to compensate for the absence of tolerance after transplantation. With better understanding of the mechanisms being responsible for tolerance development, individualised immunosuppressive strategies being stronger but more specific could be developed both in allogeneic HSCT and in organ transplantation. There are several methods trying to induce clinical or operational tolerance depending on which immunological barriers are present. In the allogeneic HSCT setting the immunological limit so far seems to be haplo-identical transplants

while in the solid organ transplant setting xenotransplantation is the major challenge. The immune system is complex and so is tolerance induction. Approaches to escape mechanisms involved in non-tolerance are certainly different depending on if incompatible MHC-molecules or only mHAs are present. Experiments with co-stimulatory blockades are promising but one could speculate if this approach is specific enough to avoid general immunological impairment (Larsen, Elwood et al. 1996). Strategies involving dendritic cells may reduce GVHD (Zheng, Narita et al. 2004). Combination of strategies may be necessary to obtain lasting tolerance to organ grafts (Sun, Wang et al. 2003).

IMMUNE RECONSTITUTION

A faster recovery of the immune system after HSCT is desirable as infections count for a lot of the transplant related complications following HSCT. Factors influencing immune recovery are thoroughly investigated. Negative as well as favourable factors for immune reconstitution are well defined even though some discrepancies between studies are present. Mechanisms involved in the influence of these factors are only partly understood. Factors such as high age, impaired thymic function, TcD and GVHD are accepted factors associated with delayed immune recovery. The roles of PBSC, conditioning type and chimaeric status are still somewhat controversial. The influence of different factors may be difficult to separate as patient groups are often heterogeneous and various factors are interacting.

The longer time the patient is immune deficient, the longer he will be at risk for different infections. The immune system is depressed both by immunosuppressive drugs and by its naivety after HSCT. It may be difficult to speed up immune reconstitution since time is needed to establish tolerance. The potential risks of rejection and especially GVHD are always there to be handled. Vaccination against measles, mumps, and rubella has been used for many years after HSCT. However, enhanced immunity against more common infections would also be important. CMV still is a major threat for a long time after HSCT, but diagnostic and therapeutic strategies are being developed. Enhancing the immune system to recover faster after HSCT would both prevent infections and might contribute to GVL/GVT responses. General stimulation of the immune system by cytokines is possible. Interleukin-7 is the most potent thymopoietic cytokine identified so far, but may also increase GVHD (Sinha, Fry et al. 2002). Keratinocyte growth factor may also be of clinical interest as it can protect the thymus from damage during the conditioning (Min, Taylor et al. 2002).

DLI enhances the immune system. Finding strategies to enhance immune functions by DLI without increasing GVHD is a challenge. Removal of potentially self-reactive lymphocytes or infusion of specific T cell clones are strategies that have been used (Andre-Schmutz, Le Deist et al. 2002). For instance, virus-specific cytotoxic T lymphocytes against EBV and CMV have been developed (Riddell, Watanabe et al. 1992; Heslop, Ng et al. 1996).

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13 SAMMANFATTNING PÅ SVENSKA FÖR LEKMÄN

(Summary for laymen in Swedish)

Begreppet benmärgstransplantation har de sista åren allt mer ersatts av termen hematopoetisk stamcellstransplantation (HSCT), vilket innebär överförande av blodbildande stamceller. Att termen ändrats beror på att man numer ff.a. extraherar stamcellerna direkt från blodet på liknade sätt som vid en vanlig blodgivning och således inte behöver suga ut benmärg ur bäckenbenet med nålar som tidigare. Blodbildande stamceller kan extraheras från patienten (autolog HSCT) innan den sjuka benmärgen slås ut eller tas från en annan (allogen) givare. Etablerade indikationer för allogen HSCT är högrisk-leukemier, svår aplastisk anemi, immunbristsjukdomar och vissa ovanliga enzymdefekter (ämnesomsättningsrubbningar), som inte kan botas med annan behandling.

Syftet med denna avhandling har varit att studera utmognaden av immunsystemet efter allogen HSCT. Dessutom har vi studerat huruvida en anti-tumöreffekt går att åstadkomma genom allogen HSCT hos patienter med solida tumörer, i första hand njurcancer och koloncancer. Känt är att utmognaden av immunsystemet efter allogen HSCT tar tid och att komplikationer såsom transplantat-kontra-värd reaktion (graft-versus-host disease, GVHD) och infektioner påverkar hur denna utmognad sker. GVHD innebär att vita blodkroppar (lymfocyter) från givaren angriper patientens vävnader, ff.a. hud, lever och tarm. Tonvikt har lagts på jämförelse mellan HSCT med konventionell (myeloablativ) och reducerad förbehandling (reduced intensity conditioning, RIC).

Den första artikeln är en fallbeskrivning av en man med spridd (metastaserad) koloncancer, som behandlades med allogen HSCT. Bakgrunden var vetskapen om att allogen HSCT inte bara innebär räddning av en benmärg, som är utslagen av kraftig cytostatikabehandling, utan också en immunologisk anti-cancereffekt som är tydligt visad ff.a. vid kronisk myeloisk leukemi men även vid akut myeloisk leukemi, akut lymfatisk leukemi och myelom. Patienten genomgick HSCT med reducerad förbehandling 1999 och efter två månader hade kraftig regress skett av lymfkörtelmetastaser ovan ena nyckelbenet och lymfkörtlar vid stora kroppspulsådern (aorta). Patienten dog i lunginflammation fyra månader efter HSCT. Vid obduktionen, som genomfördes inom 24 timmar efter dödsfallet fann man nekroser (döda celler, cellsönderfall) i större delen av tumörerna .

Ytterligare 17 patienter med njurcancer (n=10), koloncancer (n=5), levercancer och bröstcancer genomgick samma förbehandling som den första patienten och samtliga resultaten från de 18 transplantationerna presenteras i den andra artikeln. Förbehandlingen tolererades väl hos dessa förhållandevis gamla patienterna (medianålder=58). Anti-tumör-effekt sågs hos tre njurcancerpatienter och två patienter med koloncancer, dock partiell hos alla utom den första koloncancerpatienten där tumör-regressen var i stort sett total. Förutom funna tumörnekroser i lever och

lymfkörtlar hos en koloncancerpatient sågs ingen tumörregress i lever och skelett medan metastaser i lunga föreföll ha en tendens att bromsas upp och i vissa fall minska hos några patienter. En patient, som avled i allvarlig GVHD, visade sig ha nekroser av metastaser i lungorna medan metastaser i buken hade vuxit till. Slutsatsen av studierna i de två första artiklarna är att behandlingen tolererats förhållandevis väl av de flesta patienterna och att de transplantationsrelaterade komplikationerna (infektioner, GVHD, toxicitet) är acceptabla. Vidare visar dessa experimentella behandlingar att en viss anti-tumöreffekt föreligger, men modifiering av behandlingen för att uppnå kraftigare anti-tumöreffekt är nödvändig och att patienter med långsamväxande tumör är de man i första hand bör inrikta sig på för att en immunologisk anti-tumöreffekten ska ha en chans att hinna bromsa tumörspridning. Önskvärt är att uppnå en kraftigare anti-tumöreffekt utan att få en för kraftig GVHD, vilket är ett välkänt samband även vid elakartade blodsjukdomar.

Den tredje artikeln är en analys av när man kunnat sätta ut förebyggande immunhämmande behandling hos patienter som genomgått HSCT på Huddinge sjukhus mellan 1977 och 1997. Väl känt är vilka faktorer som påverkar risken för immunologiska komplikationer såsom avstötning av transplantatet eller GVHD. Viktigaste faktorn är graden av vävnadskompatibilitet. Flera olika immunhämmande protokoll för att förhindra GVHD har utarbetats under årens gång. Läkemedel, doser och behandlingstid har varierat beroende på sjukdomsdiagnos och grad av vävnadsförenlighet. Vi har studerat hur man i praktiken kunnat följa de immunhämmande protokollen i olika patientgrupper. Vi fann att man vid blodcancersjukdomar hade satt ut immunhämningen inom ett år hos patienter som ej utvecklat GVHD medan man avvaktat upp till två år hos de med icke elakartade blodsjukdomar. Detta då man vet att om GVHD uppstår minskar risken för återfall i blodcancer medan GVHD endast har negativa effekter hos patienter med icke elakartade blodsjukdomar. Dessutom har vi analyserat vad vi definierat som ”klinisk tolerans”, som vi definierat som avsaknad av kronisk GVHD eller avstötning minst en månad efter att immunsuppression satts ut. Utvärdering gjordes först ett år efter HSCT eftersom risken för GVHD är störst under första året. Patienterna utvärderades i grupperna elakartade (maligna) respektive icke-maligna blodsjukdomar. Faktorer som var korrelerade till längre tid till klinisk tolerans var hög donatorsålder, immuniserad kvinnlig givare till manlig donator och akut GVHD grad II-IV.

Den senare delen av avhandlingen handlar om immunologisk utmognad efter allogen HSCT. Utmognaden av B-lymfocyter och T-lymfocyter, vilka är två viktiga typer av vita blodkroppar när det gäller att känna igen och oskadliggöra infektioner, har studerats separat. För att studera T-cells funktionen har använts en metod kallad mitogenstimulering, vilket innebär att man ur blodet extraherar lymfocyter, som sedan konfronteras med s k antigen eller mitogen, vilket är delar av proteiner från t ex virus eller bakterier. Närvaron av antigen/mitogen leder till att normalt fungerande celler stimuleras till att föröka sig (proliferera). Vid förökningen bygger cellerna in den tillsatta aminosyran tritium-Thymidin som är en radioaktivt inmärkt variant av aminosyran Thymidin, som används vid cellens proteintillverkning. Radioaktiviteten som sedan mäts i en beta-räknare (scintillator) ger ett indirekt mått på

stimuleringsgraden. Denna metod har använts länge och är en bra funktionell *in vitro* metod (laboratoriv metod). Som komplement har vi använt en nyare metod som kallas CDR3 (third complementarity determining region) spectratyping. Med denna metod kan man analysera längden på T-cellreceptorns (TcR) respektive B-cellsantikroppens mest variabla del, CDR3. Längden av dessa CDR3 i olika B- och T-celler varierar och uppvisar i ett normalt fungerande immunsystem en längdvariation enligt en Gauss-kurveliknande normalfördelning. Med CDR3 spectratyping har vi genom att extrahera mRNA, kunnat analysera mångfalden bland de T-cellskloner som uttrycks efter HSCT. Efter HSCT är det nya immunsystemet omoget och har inte den mångfald av receptorer som ett utvecklat immunsystem, vilket avspeglar sig i både minskat antal av olika CDR3-längder samt en skev fördelning av dessa. Faktorer såsom förbehandling, infektioner, GVHD och diagnos analyserades samt korrelation mellan resultaten från mitogenstimuleringen och spectratypingen gjordes. Vi fann en något snabbare återhämtning av mångfalden av CDR3-längder hos TcR efter RIC HSCT jämfört med konventionell, fulldos HSCT. Däremot tillväxte T celler sämre efter RIC HSCT än efter konventionell HSCT då de stimulerades, jämfört med friska givare. Motsvarande analys med CDR3-spectratyping gjordes för B-cellsantikroppar som också har en hypervariabel CDR3. Här fann vi en signifikant fördröjning av återhämtningen av variabiliteten hos patienter som genomgått reducerad förbehandling (RIC). Detta bedömdes kunna bero på att patienterna var äldre samt hade fler virusinfektioner i RIC-gruppen samt mer GVHD. Vad gällde absolutnivåer av immunglobuliner av de olika klasserna fann vi ingen skillnad hos patienter med olika förbehandling. Slutsatserna av studierna i de två sista artiklarna är att både T-cellsfunktionen och B-cellsfunktionen är nedtryckta både med avseende på cellantal och funktonalitet. Tid efter transplantation spelar troligen mer roll för återhämtningen än vilken typ av förbehandling som används och det råder ett komplext samspel mellan flera faktorer som påverkar återhämtningen av immunsystemet efter HSCT.

14 PAPERS I-V

Man ska alltid avsluta det man påb