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T cell immunodeficiency and severe herpes virus infections after allogeneic stem cell transplantation

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T cell immunodeficiency and severe herpes virus infections after allogeneic stem cell transplantation.

T cel immuundeficiëntie en ernstige herpes virus infecties na allogene stam cel transplantaties

(met een samenvatting in het Nederlands)

Proefschrift

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Voor mijn ouders

Contents

Chapter 1

Introduction

Introduction

Immune reconstitution in recipients of allogeneic stem cell transplants

After stem cell transplantation (SCT) preceded by a myeloablative-conditioning regimen most haematological lineages regain their normal counts and function rapidly. This is not the case for B and T cell lineages, resulting in a high incidence of opportunistic infections in SCT recipients. Especially CD4+ T cell counts remain severely depressed during the first 6 months post-transplant, thereafter numbers gradually rise[1].

By 3 months post-transplant, recovery of B cell counts is rapidly established, except for patients with chronic graft-versus-host disease (GVHD)[1-5]. The in vitro response of B cells to T cell independent B cell mitogens such as rabbit anti IgM and Staphylococcus aureus Cowen strain, is significantly correlated to total B cell counts and normalizes within the same time period[2,3]. However, the in vivo antibody response to capsular polysaccharide vaccines, which are also T cell independent antigens, is severely impaired in SCT recipients, even when immunization is delayed until 12 months post-transplant. This decreased antibody response to polysaccharide antigens does exist in healthy children of 0-5 years as well[6]. Small et al[2] showed that immunoglobulin (Ig) M production normalized in conjunction with the return of circulating B cells. However, IgG production did not occur until the second year post-transplant, which is a pattern similar to that seen in healthy infants of 0-2 years. Furthermore, during the first year post-transplant B cells showed a similar phenotype and function as cord blood B cells and B cells from healthy neonates. These data support the hypothesis that B cell differentiation post-transplant is recapitulating normal B cell ontogeny[2]. In addition to recapitulation of normal B cell ontogeny, the recovery of B cell function is dependent on T helper cell recovery.

Reconstitution of T cells may result from two different pathways: 1) a thymus dependent production of naive T cells and 2) peripheral expansion of mature T cells[7,8]. Due to thymic involution in adults, the precise role of thymic dependent production of naive T cells in immune reconstitution after SCT is still a matter of debate. T cell immune reconstitution, especially the production of naive T cells, has been studied by different methods. When CD4+ or CD8+ T cells emigrate the thymus they co-express a specific isotype of the CD45 family (CD45RA+). CD45RA+ CD4+ T cells are considered naive CD4+ T cells, however, this marker is not reliable to evaluate naive CD8+ T cell production[9]. After challenge with

an antigen in the periphery these CD4+ T cells convert to a CD45RA- phenotype and become memory T cells[10,11].

Among recipients of fully T cell depleted (TCD) grafts no CD45RA+ CD4+ T cells were detected during the first 200 days post-transplant[12,13]. In these studies, T cell receptor (TCR) diversity was studied by TCR spectratyping as well[14]. TCR diversity was only observed when CD45RA+ CD4+ T cells appeared, which suggests that these cells are thymic emigrants. Furthermore, reconstitution of the TCR repertoire was due solely to the appearance of donor T cells with a random TCR diversity[12,13]. One thymectomized SCT recipient has been studied and results were compared with those of thymus-bearing allogeneic SCT recipients. The regeneration of CD4+ CD45RA+ T cells was strongly impaired in the thymectomized patient compared to the other patients, while CD8+ CD45RA+ T cells regenerated similarly[15].

Whether the abovementioned cell surface marker is an accurate marker for thymic function, has been debated, since research in nude rats showed that T cells may bi-directionally switch between the two isotypes (CD45RA+ and CD45RA-)[16,17]. Furthermore, in HIV-infected thymectomized patients, CD4+ CD45RA+ T cells rose after initiation of highly active antiretroviral therapy, indicating that T cells with this phenotype are not exclusively of recent thymic origin[18]. Recently, Douek et al[19] developed a new method to measure the production of naive T cells. During thymocyte development rearrangement of the TCR gene leads to the excision of circular DNA fragments from genomic DNA[20]. An assay was developed to measure the number of TCR-rearrangement excision circles (TRECs) in peripheral blood lymphocytes. These products are stable, unique to T cells and not duplicated during mitosis[19,20]. Therefore, their concentration in peripheral blood can be used to estimate thymic output[19].

TRECs have been measured in recipients of TCD and unmodified grafts and lower levels were found to be associated with older age, the presence of extensive chronic GVHD and the occurrence of opportunistic infections[21-23]. Earlier, also CD45RA+ CD4+ T cell counts were shown to be related to age in a similar way[13].

Apart from patient age and the occurrence of GVHD, other variables are considered adverse risk factors for T cell recovery post-transplant, such as use of 1) TCD grafts, 2) immunosuppressive therapy, especially serotherapy with Antithymocyteglobulin, to prevent graft rejection or to prevent or treat GVHD, 3) grafts from matched unrelated (MUD) or partially

matched related donors (PMRD) and 4) bone marrow grafts instead of peripheral blood stem cell grafts.

Influence of TCD of grafts on immune recovery Only a few studies have compared immune reconstitution in recipients of TCD grafts with immune reconstitution in recipients of unmanipulated grafts[2-4,24-26]. These studies were largely performed among recipients of related donor grafts and varying ex vivo TCD techniques were used. Recovery of B cell counts and proliferative responses of B cells to B cell mitogens (rabbit anti IgM, Staphylococcus aureus Cowen strain) were not influenced by TCD of grafts[2-4]. When T helper and B cell function was measured in a mitogen-stimulated Ig production assay, Ig production by B cells from recipients of non-manipulated grafts exceeded that of TCD SCT recipients in one study[3], however, no effect of TCD was found by others[2]. TCD did not influence recovery of EBV-specific cytotoxic T cell precursors nor their virus-specific cytotoxic activity[24]. Recovery of total T cell and subset counts was not influenced by TCD either[3,26], although in some studies CD4+ and CD45+ CD4+ T cell subsets showed a slower reconstitution in TCD SCT recipients[4,25]. Furthermore, the proliferative response of T cells to T cell mitogens (phytohemagglutinin, pokeweed mitogen) was found to be significantly lower during the first three months post-transplant in TCD SCT recipients[3]. Apart from one paper describing a higher incidence of CMV reactivations after TCD SCT[4], there is no increase in infectious complications or fatal infections after TCD SCT[3,25,27]. Overall, from these studies it can be concluded that ex vivo TCD of grafts from related donors does not have a major impact on immune reconstitution.

Influence of Antithymocyteglobulin on immune recovery Antithymocyteglobulin (ATG) is often used before SCT with grafts from MUDs or PMRDs to prevent graft rejection. Since ATG could be detected in sera at least during 2 months post-transplant with 25% of the initial peak concentration at day 28-48 [28,29], it is hypothesized that ATG may severely impair T cell recovery. Recently it was shown that total lymphocyte, CD3+ and CD4+ cell reconstitution was significantly lower in transplant recipients of unrelated grafts treated with high-dose ATG (15 mg/kg) as compared to low-dose ATG (7,5 mg/kg)[30]. Furthermore, the rate of viral infectious complications was significantly higher in the high-dose group. In adult recipients of MRD grafts, the use of ATG post-transplant to prevent graft failure resulted in an impaired recovery of CD45RA+ CD4+ T cells, a prolonged inversion of the CD4+/CD8+ T cell ratio, a delay in recovery of normal T cell mitogen responses and an increased incidence of opportunistic infections as compared to patients not treated

with ATG[31]. Patients given ATG showed relatively increased numbers of CD8+ CD28- CD57+ T cells. T cells with this phenotype respond poorly to T cell mitogens and can inhibit the generation and function of virus-specific cytotoxic lymphocytes[31], although this is controversial since others showed these cells to be cytotoxic effector T cells[9].

The results of both studies support the hypothesis that ATG interferes with T lymphocyte recovery[30,31], while B lymphocyte counts and immunoglobulin levels were not influenced by ATG[31].

Influence of donor type on immune recovery It is speculated that T and B cell recovery is related to the degree of HLA matching between host and donor. HLA disparities may alter the capacity of lymphoid progenitors of donor origin to mature within the host thymus or bone marrow. On the other hand, HLA disparities stimulate the occurrence of GVHD. Therefore, patients receiving grafts from unrelated or mismatched related donors will always be treated with more intensive immunosuppression compared to recipients of MRD grafts. A well known characteristic of GVHD itself is a defect in the development of donor derived T cells, which leads to a long lasting T cell deficiency state[32,33]. Dulude et al[33] have demonstrated that GVHD impairs the production of new T cells by the thymus and disturbs the expansion of mature peripheral T cell pools in secondary lymphoid organs. This defective expansion was due to a restriction in the number of functional T cell niches. It is hypothesized that during the acute phase of GVHD, the thymus and secondary lymphoid organs are damaged to such a degree that a prolonged impairment in the development of donor derived T cells will occur. Furthermore, it has been demonstrated that patients with acute GVHD show an increase in CD3+ cell apoptosis, which further impairs T cell dependent immune reconstitution[34].

Altogether, this entanglement of factors makes it impossible to solely analyse the impact of donor type on immune recovery. Despite this, efforts to analyse the influence of donor type were made in several studies[21,23,35-38]. A more prolonged and profound CD3+, CD4+ and CD8+ lymphopenia was seen in MUD recipients compared to MRD recipients. However, the proliferative T cell response to PHA was comparable between the two groups. Furthermore, interference of GVHD with the observed impaired T cell subset recovery in MUD recipients could not be excluded[35]. Niehues et al[38] showed, in the setting of paediatric cord blood transplantation (CBT), that T cell recovery was favourable affected by use of a related donor. In the other studies, no effect of donor type was observed[21,23,36,37]. Recently, immune recovery in unrelated CBT recipients was analysed and compared to data from recipients of

MRD grafts. TREC levels were comparable between the two groups, while TCR diversity was normalized earlier in CBT recipients[39]. Two other studies described earlier[12,13] did study the patterns of CD4+ and CD4+ CD45RA+ T cell recovery and restoration of TCR diversity in recipients of TCD grafts from MUDs and MRDs, respectively. The results were highly comparable in these two reports. One might conclude from these results, that HLA disparities not necessarily result in an altered maturation of lymphoid progenitors of donor origin in the host thymus.

Influence of stem cell source on immune recovery Allogeneic unselected peripheral blood stem cell (PBSC) grafts contain about 10 times more CD4+ naive and memory T cells, CD8+ T cells and B cells[40,41]. Lymphocyte recovery has been shown faster in PBSC recipients[40-43], the difference being most striking for CD4+ naive and memory T cells[40,41,43]. Furthermore, the rate of severe infections after engraftment was significantly higher in bone marrow recipients[43]. However, when ex vivo TCD of grafts was performed with Campath-1 antibodies, the recovery of B and T cells and subsets was comparable among recipients of PBSC and BM grafts[44].

In conclusion, patient age, the occurrence of GVHD and the use of immunosuppressive therapy (especially ATG) are all adverse risk factors for T cell recovery post-transplant. However, the contribution of ex vivo TCD, the use of unrelated or partially matched related donors and the use of bone marrow instead of PBSC is less clear.

The aim of this thesis was:

1 to analyse the impact of high- and low-dose ATG on immune recovery and GVHD among MUD recipients (chapter 2, 3 and 9)

2 to study aspects of EBV and CMV reactivation/infection in recipients of allogeneic SCT:

- ***a*** the predictive value of the EBV DNA viral load for the development of EBV-LPD (chapter 4)
- ***b*** the origin of the EBV strain giving active EBV infection post-transplant (chapter 5)
- ***c*** the benefit of additional B cell depletion of T cell depleted grafts from matched unrelated donors regarding the incidence of EBV-LPD (chapter 6)
- ***d*** the role of patient and/or donor CMV-seropositivity as an adverse risk factor for survival post-transplant (chapter 8 and 9)

3 to review:

- ***a*** the prevention and treatment of EBV-LPD in recipients of bone marrow and solid organ transplants (chapter 7)
- ***b*** the prevention of Cytomegalovirus disease in recipients of allogeneic stem cell transplants (chapter 10)

References

1 Meijer E, Bloem AC, Dekker AW, Verdonck LF. Effect of antithymocyteglobulin on quantitative immune recovery and graft-versus-host disease after T cell depleted bone marrow transplantation: a comparison between recipients of matched related and matched unrelated donor grafts. Transplantation 2003; 75: 1910-1913.

2 Small TN, Keever CA, Weiner-Fedus S, Heller G, O'Reilly RJ, Flomenberg N. B-cell differentiation following autologous, conventional, or T-cell depleted bone marrow transplantation: a recapitulation of normal B-cell ontogeny. Blood 1990; 76: 1647-1656.

3 Keever CA, Small TN, Flomenberg N, Heller G, Pekle K, Black P, Pecora A, Gillio A, Kernan NA, O'Reilly RJ. Immune reconstitution following bone marrow transplantation: comparison of recipients of T-cell depleted marrow with recipients of conventional marrow grafts. Blood 1989; 73: 1340-1350.

4 Martinez C, Urbano-Ispizua A, Rozman C, Marin P, Rovira M, Sierra J, Montfort N, Carreras E, Montserrat E. Immune reconstitution following allogeneic peripheral blood progenitor cell transplantation: comparison of recipients of positive CD34+ selected grafts with recipients of unmanipulated grafts. Exp Hematol 1999; 27: 561-568.

5 Storek J, Wells D, Dawson MA, Storer B, Maloney DG. Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation. Blood 2001; 98: 489-491.

6 Molrine DC, Antin JH, Guinan EC, Soiffer RJ, MacDonald K, Malley R, Malinoski F, Trocciola S, Wilson M, Ambrosino DM. Donor immunization with pneumococcal conjugate vaccine and early protective antibody responses following allogeneic hematopoietic cell transplantation. Blood 2003; 101: 831-836.

7 Mackall CL, Granger L, Sheard MA, Cepeda R, Gress RE. T-cell regeneration after bone marrow transplantation: differential CD45 isoform expression on thymic-derived versus thymic-independent progeny. Blood 1993; 82: 2585-2594.

8 Mackall CL, Fleisher TA, Brown MR, Andrich MP, Chen CC, Feuerstein IM, Horowitz ME, Magrath IT, Shad AT, Steinberg SM et al. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. N Engl J Med 1995; 332: 143-149.

9 Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, van Lier RA. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med 1997; 186: 1407-1418.

10 Clement LT. Isoforms of the CD45 common leukocyte antigen family: markers for human T-cell differentiation. J Clin Immunol 1992; 12: 1-10.

11 Young JL, Ramage JM, Gaston JS, Beverley PC. In vitro responses of human CD45R0brightRA- and CD45R0-RAbright T cell subsets and their relationship to memory and naive T cells. Eur J Immunol 1997; 27: 2383-2390.

12 Dumont-Girard F, Roux E, van Lier RA, Hale G, Helg C, Chapuis B, Starobinski M, Roosnek E. Reconstitution of the T-cell compartment after bone marrow transplantation: restoration of the repertoire by thymic emigrants. Blood 1998; 92: 4464-4471.

13 Roux E, Dumont-Girard F, Starobinski M, Siegrist CA, Helg C, Chapuis B, Roosnek E. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. Blood 2000; 96: 2299-2303.

14 Gorski J, Yassai M, Zhu X, Kissela B, Keever C, Flomenberg N. Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status. J Immunol 1994; 152: 5109-5119.

15 Heitger A, Neu N, Kern H, Panzer-Grumayer ER, Greinix H, Nachbaur D, Niederwieser D, Fink FM. Essential role of the thymus to reconstitute naive (CD45RA+) T-helper cells after human allogeneic bone marrow transplantation. Blood 1997; 90: 850-857.

16 Bell EB, Sparshott SM. Interconversion of CD45R subsets of CD4 T cells in vivo. Nature 1990; 348: 163-166.

17 Sparshott SM, Bell EB, Sarawar SR. CD45R CD4 T cell subset-reconstituted nude rats: subset-dependent survival of recipients and bi-directional isoform switching. Eur J Immunol 1991; 21: 993-1000.

18 Haynes BF, Hale LP, Weinhold KJ, Patel DD, Liao HX, Bressler PB, Jones DM, Demarest JF, Gebhard-Mitchell K, Haase AT, Bartlett JA. Analysis of the adult thymus in reconstitution of T lymphocytes in HIV-1 infection. J Clin Invest 1999; 103: 453-460.

19 Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, Polis MA, Haase AT, Feinberg MB, Sullivan JL, Jamieson BD, Zack JA, Picker LJ, Koup RA. Changes in thymic function with age and during the treatment of HIV infection. Nature 1998; 396: 690-695.

20 Livak F, Schatz DG. T-cell receptor alpha locus V(D)J recombination by-products are abundant in thymocytes and mature T cells. Mol Cell Biol 1996; 16: 609-618.

21 Weinberg K, Blazar BR, Wagner JE, Agura E, Hill BJ, Smogorzewska M, Koup RA, Betts MR, Collins RH, Douek DC. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. Blood 2001; 97: 1458-1466.

22 Hazenberg MD, Otto SA, de Pauw ES, Roelofs H, Fibbe WE, Hamann D, Miedema F. T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events. Blood 2002; 99: 3449-3453.

23 Lewin SR, Heller G, Zhang L, Rodrigues E, Skulsky E, van den Brink MR, Small TN, Kernan NA, O'Reilly RJ, Ho DD, Young JW. Direct evidence for new T-cell generation by patients after either T-cell-depleted or unmodified allogeneic hematopoietic stem cell transplantations. Blood 2002; 100: 2235-2242.

24 Lucas KG, Small TN, Heller G, Dupont B, O'Reilly RJ. The development of cellular immunity to Epstein-Barr virus after allogeneic bone marrow transplantation. Blood 1996; 87: 2594-2603.

25 Lowdell MW, Craston R, Ray N, Koh M, Galatowicz G, Prentice HG. the effect of T cell depletion with Campath-1M on immune reconstitution after chemotherapy and allogeneic bone marrow transplant as treatment for leukaemia. Bone Marrow Transplant 1998; 21: 679-686.

26 Behringer D, Bertz H, Schmoor C, Berger C, Dwenger A, Finke J. Quantitative lymphocyte subset reconstitution after allogeneic hematopoietic transplantation from matched related donors with CD34+ selected PBPC grafts unselected PBPC grafts or BM grafts. Bone Marrow Transplant 1999; 24: 295-302.

27 Schmeiser T, Wiesneth M, Bunjes D, Arnold R, Hertenstein B, Heit W, Kurrle E. Infectious complications after allogeneic bone marrow transplantation with and without T-cell depletion of donor marrow. Infection 1989; 17: 124-130.

28 Baurmann H, Revillard JP, Bonnefoy-Berard N, Schwerdtferger R. Potent effects of ATG used as part of the conditioning in matched unrelated donor transplantation. Blood 1998; 92: S290a. Abstract.

29 Finke J, Bertz H, Schmoor C, Veelken H, Behringer D, Wasch R, Kunzmann R, Heidecker L, Lang H, Meyer-Konig U, Mertelsmann R. Allogeneic bone marrow transplantation from unrelated donors using in vivo anti-T-cell globulin. Br J Haematol 2000; 111: 303-313.

30 Duval M, Pedron B, Rohrlich P, Legrand F, Faye A, Lescoeur B, Bensaid P, Larchee R, Sterkers G, Vilmer E. Immune reconstitution after haematopoietic transplantation with two different doses of pre-graft antithymocyte globulin. Bone Marrow Transplant 2002; 30: 421-426.

31 Small TN, Avigan D, Dupont B, Smith K, Black P, Heller G, Polyak T, O'Reilly RJ. Immune reconstitution following T-cell depleted bone marrow transplantation: effect of age and posttransplant graft rejection prophylaxis. Biol Blood Marrow Transplant 1997; 3: 65-75.

32 Atkinson K. Reconstruction of the haemopoietic and immune systems after marrow transplantation. Bone Marrow Transplantation 1990; 5: 209-226.

33 Dulude G, Roy DC, Perreault C. The effect of graft versus host disease on T cell poduction and homeostasis. J Exp Med 1999: 189: 1329-1341.

34 Lin MT, Tseng LH, Frangoul H, Gooley T, Pei J, Barsoukov A, Akatsuka Y, Hansen JA. Increased apoptosis of peripheral blood T cells following allogeneic hematopoietic cell transplantation. Blood 2000; 95: 3832-3839.

35 Small TN, Papadopoulos EB, Boulad F et al. Comparison of immune reconstitution after unrelated and related T cell depleted bone marrow transplantation: effect of patient age and donor leukocyte infusion. Blood 1999; 93: 467-480.

36 Fujimaki K, Maruta A, Yoshida M, Kodama F, Matsuzaki M, Fujisawa S, Kanamori H, Ishigatsubo Y. Immune reconstitution assessed during five years after allogeneic bone marrow transplantation. Bone Marrow Transplant 2001; 27: 1275-1281.

37 Moretta A, Maccario R, Fagioli F, Giraldi E, Busca A, Montagna D, Miniero R, Comoli P, Giorgiani G, Zecca M, Pagani S, Locatelli F. Analysis of immune reconstitution in children undergoing cord blood transplantation. Exp Hematol 2001; 29: 371-379.

38 Niehues T, Rocha V, Filipovich AH, Chan KW, Porcher R, Michel G, Ortega JJ, Wernet P, Gobel U, Gluckman E, Locatelli F. Factors affecting lymphocyte subset reconstitution after either related or unrelated cord blood transplantation in children - a Eurocord analysis. Br J Haematol 2001; 114: 42-48.

39 Talvensaari K, Clave E, Douay C, Rabian C, Garderet L, Busson M, Garnier F, Douek D, Gluckman E, Charron D, Toubert A. A broad T-cell repertoire diversity and an efficient thymic function indicate a favorable long-term immune reconstitution after cord blood stem cell transplantation. Blood 2002; 99: 1458-1464.

40 Ottinger HD, Beelen DW, Scheulen B, Schaefer UW, Grosse-Wilde H.Improved immune reconstitution after allotransplantation of peripheral blood stem cells instead of bone marrow. Blood 1996; 88: 2775-2779.

41 Storek J, Witherspoon RP, Maloney DG, Chauncey TR, Storb R. Improved reconstitution of CD4 T cells and B cells but worsened reconstitution of serum IgG levels after allogeneic transplantation of blood stem cells instead of marrow. Blood 1997; 89: 3891-3893.

42 Charbonnier A, Sainty D, Faucher C, Arnoulet C, Chabannon C, Blaise D. Immune reconstitution after blood cell transplantation. Hematol Cell Ther 1997; 39: 261-264.

43 Storek J, Dawson MA, Storer B, Stevens-Ayers T, Maloney DG, Marr KA, Witherspoon RP, Bensinger W, Flowers ME, Martin P, Storb R, Appelbaum FR, Boeckh M. Immune reconstitution after allogeneic marrow transplantation compared with blood stem cell transplantation. Blood 2001; 97: 3380-3389.

44 Novitzky N, Davison GM, Hale G, Waldmann H. Immune reconstitution at 6 months following T-cell depleted hematopoietic stem cell transplantation is predictive for treatment outcome. Transplantation 2002; 74: 1551-1559.

Chapter 2

Effect of Antithymocyteglobulin on quantitative immune recovery and graft-versus-host disease after partially T cell depleted bone marrow transplantation: a comparison between recipients of matched related and matched unrelated donor grafts

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Abstract

The effect of Antithymocyteglobulin (ATG) on quantitative immune recovery and graft-versus-host-disease (GVHD) after partially T cell depleted (TCD) bone marrow transplantation (BMT) was analysed in 59 and 32 recipients of grafts from matched related donors (MRD) and matched unrelated donors (MUD), respectively. The conditioning regimen was similar in all patients, except for ATG which was given only to MUD recipients. Thirteen MUD patients were treated with high-dose (20 mg/kg) ATG and 19 with low-dose (8 mg/kg) ATG. During the post-transplant period both CD3+, CD4+ and CD8+ T cell numbers and the incidence of acute and chronic GVHD were significantly lower in MUD recipients compared to MRD patients. MUD recipients treated with high-dose ATG showed the worst T cell and subsets recovery. These data suggest that ATG, often used as part of conditioning regimens in recipients of TCD grafts from MUDs, contributes to the very severe and prolonged T cell deficiency that is typical of these patients. On the other hand, it effectively reduces incidence and severity of GVHD.

Introduction

Allogeneic bone marrow transplantation (BMT) is an established treatment modality for adult patients with haematologic malignancies. However, 70% of patients who might benefit from allogeneic BMT lack an HLA-identical sibling donor (matched related donor, MRD). Therefore, there is an increasing use of HLA-matched unrelated donors (MUD). Unfortunately, transplant-related mortality (TRM) after MUD-BMT is much higher than after MRD-BMT, which is largely due to an increased incidence of opportunistic infections and graft-versus-host disease (GVHD)[1]. The increased incidence of opportunistic infections suggests that after MUD-BMT immune reconstitution is much more impaired, compared to BMT from a matched sibling donor. Several factors may account for the impaired immune reconstitution, such as HLA disparities, the increased incidence of GVHD and the use of more intensive immunosuppression to prevent or treat GVHD.

Here, we set out to unravel the contribution of some of these immunodeficiency inducing factors by studying the recovery of T cell subsets, NK cells and B cells after BMT in patients treated with partially T cell depleted (TCD) grafts from either a MRD or MUD. Both patient groups received a fixed low number of T cells in the graft and a similar conditioning regimen except for Antithymocyteglobulin (ATG) that was only given to recipients of MUD transplants.

Methods

Patients Between August 1997 and March 2001 allogeneic bone marrow transplantation was performed in 104 patients. Thirteen patients could not be analysed; 10 because of early death (< 2 months post-transplant), 1 received donor lymphocytes < 2 months post-transplant, 1 repopulated with blasts, 1 had a graft rejection. In total 91 patients, receiving either bone marrow from a MRD (n=59) or from a MUD (n=32), were studied. Patients with acute leukaemia in first complete remission, chronic myeloid leukaemia in first chronic phase, untreated severe aplastic anaemia were considered low-risk. All patients with other diseases were considered high-risk. The procedures were performed at the Department of Haematology of the University Medical Centre Utrecht. Patients were treated according to

clinical protocols approved by the local investigation review board after informed consent was obtained.

Transplantation procedure Transplantation procedure, monitoring and pre-emptive treatment of CMV reactivations, diagnosis and treatment of CMV disease, HLA typing and partial TCD of grafts were performed as described[2]. In brief, the conditioning regimen consisted of cyclophosphamide (60 mg/kg/day for 2 days), followed by total body irradiation (600 cGy/day for 2 days) with partial shielding of the lungs. ATG (Thymoglobulin™, Sangstat, Amstelveen, the Netherlands) was infused to MUD patients before cyclophosphamide was given. Thirteen patients received a dose of 4 mg/kg/day intravenously for 5 days. Due to a change in national treatment protocols ATG dose was lowered to 2 mg/kg/day for 4 days from April 1999. Nineteen patients received ATG low-dose.

Immunophenotyping Three colour FACS analysis (FACScan, Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA) was performed on heparin-anticoagulated venous blood. To 100 μL of undiluted blood a mixture of three antibodies was added consisting of either CD3-Cy5 (Beckman Coulter, Mijdrecht, The Netherlands), CD4-FITC (BDIS) and CD8-PE (BDIS); CD45-Cy5 (Beckman Coulter), CD3-FITC (BDIS) and CD19-PE (BDIS) or CD45-Cy5, CD3-FITC and a mixture of CD16-PE and CD56-PE (BDIS). After a 20 minute incubation at room temperature the erythrocytes were lysed using FACS lysing solution (BDIS), washed once with wash solution consisting of PBS supplemented with 1% bovine serum albumin and sodiumazide (0.01%), resuspended in 0.5 ml wash solution and analysed by flow cytometry. In every sample a minimum number of 15,000 events were acquired. List mode data were analysed using attractor software (BDIS). Percentages of T (CD3+) , B (CD19+) and NK cells (CD3-, CD16+ and CD56+) in the CD45+ leukocyte population were calculated. Based on the CD3/CD4/CD8 staining the distribution of CD4+ and CD8+ cells within the CD3+ T cell compartment was determined. Normal values were derived from McNerlan et al[3].

Statistics Data are expressed as mean ± SEM. Mean differences between patient characteristics were assessed by Students t-test or Chi-square analysis. Mean differences concerning T, B and NK cell recovery were tested using repeated-measures (RM) analysis and analysis of variance (ANOVA) with group (MRD vs MUD), risk status, CMV serostatus, acute GVHD (aGVHD) and chronic GVHD (cGVHD) as 'between-subjects' or 'fixed' factors, respectively, and age as covariate. All cell counts were log-transformed in order to meet the basic conditions on which the theory of RM-analysis and ANOVA is leaning. ANOVA was used

next to RM analysis, since RM analysis excluded too many cases (due to missing values). Both RM analysis and ANOVA gave comparable results. Levene's test was performed to find out whether the assumption of equality of variance could be maintained. Residuals were checked for normality by means of the Kolmogorov-Smirnov (KS) statistic. The KS-statistic in all cases showed values < 0.2 for the log-transformed cell count residuals. Calculations were performed using SPSS/PC + 10.0 (SPSS Inc, Chicago Il, USA).

Results

Patient characteristics Patient characteristics are described in Table 1. MRD recipients were significantly older (42 ± 1.3 vs 32 ± 1.8; $p<0.001$), received less T cells (1.4 ± 0.1 vs 2.7 ± 0.3 x 10^5/kg; $p<0.001$) and suffered more acute (grade II-IV) and chronic (extensive) GVHD (54% vs 19% and 30% vs 6%; $p<0.01$) compared to MUD recipients.

Immune recovery Analyses with group (MRD vs MUD), age, risk status, patient CMV serostatus, aGVHD and cGVHD as factors revealed that only 'group' and 'CMV serostatus' significantly influenced T cell and subsets recovery during the 6 months post-transplant period. In the MRD group markedly higher values were measured during this period, which was significant by repeated-measures analysis (CD3+ and CD4+ T cells: $p<0.01$; CD8+ T cells: $p<0.05$) and by analysis of variance (Figure 1). During the whole 6 months period no normal values of CD4+ cells were reached in both patient groups and of CD3+ cells in MUD recipients. Patient CMV serostatus also influenced T cell reconstitution. Patients with a positive CMV serostatus showed significantly higher T cell and subsets counts post-transplant compared to CMV-seronegative recipients ($p<0.01$). No interaction between 'group' (MRD and MUD) and 'patient CMV serostatus' was observed. B cell and NK cell recovery was not influenced by any of the factors tested. The incidence of fatal infectious complications was 19% in MUD recipients compared to 12% in MRD recipients (ns).

A subgroup analysis among MUD recipients revealed that none of the aforementioned factors significantly influenced immune reconstitution. MUD recipients treated with high-dose ATG showed a worse CD3+ and CD8+ T cell recovery compared to MUD recipients treated with low-dose ATG, although not significant (Figure 2).

Table 1 Patient characteristics

	MRD (n=59)	MUD (n=32)	P
Age, yr (range)	42 (18-56)	32 (18-52)	p<0.001
Diagnosis			
ALL	3	7	
AML	6	7	
CML	9	11	
SAA	0	4	
MM	16	0	
NHL	11	1	
MDS	0	2	
CLL	1	0	
CMV serostatus R/D			
+/+	20	6	ns
+/-	6	6	
-/+	11	4	
-/-	22	16	
Risk status (%)			
Low	29 (49)	16 (50)	ns
High	30 (51)	16 (50)	
T cell count in graft			
(x 10^5/kg)	1.4 ± 0.1	2.7 ± 0.3	p<0.001
GVHD (%)			
Acute			p=0.001
I/no	27 (46)	26 (81)	
II-IV	32 (54)	6 (19)	
III-IV	3 (5)	0	
Chronic			p=0.008
Lim/no/ne	41 (70)	30 (94)	
Ext	18 (30)	2 (6)	

SAA = severe aplastic anaemia; MM = multiple myeloma; R/D = recipient/donor; Lim = limited; Ext = extensive; ne = non evaluable.

Figure 1

B, T and NK cell numbers during the post-transplant period in recipients of MRD transplants compared to recipients of MUD transplants.
Shaded area represents normal values.
**=p<0.01

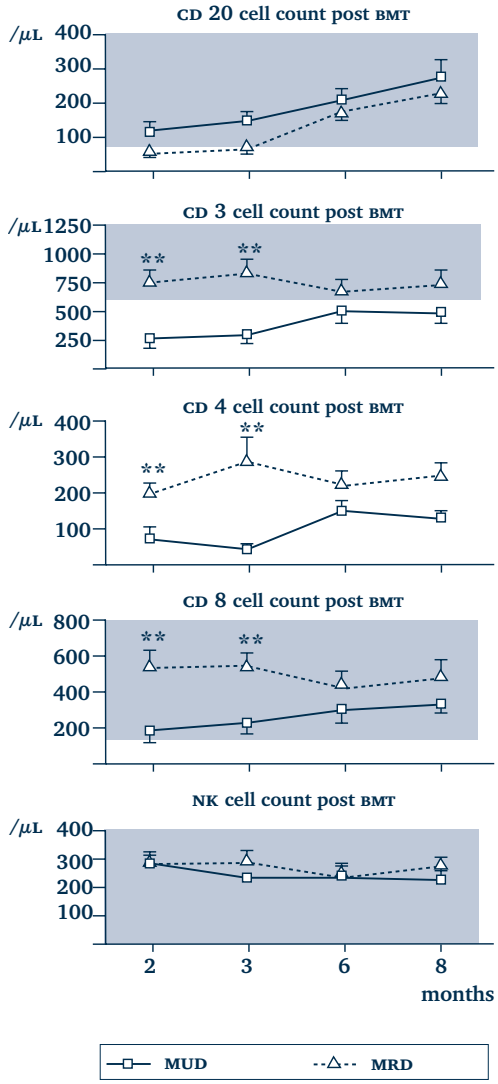
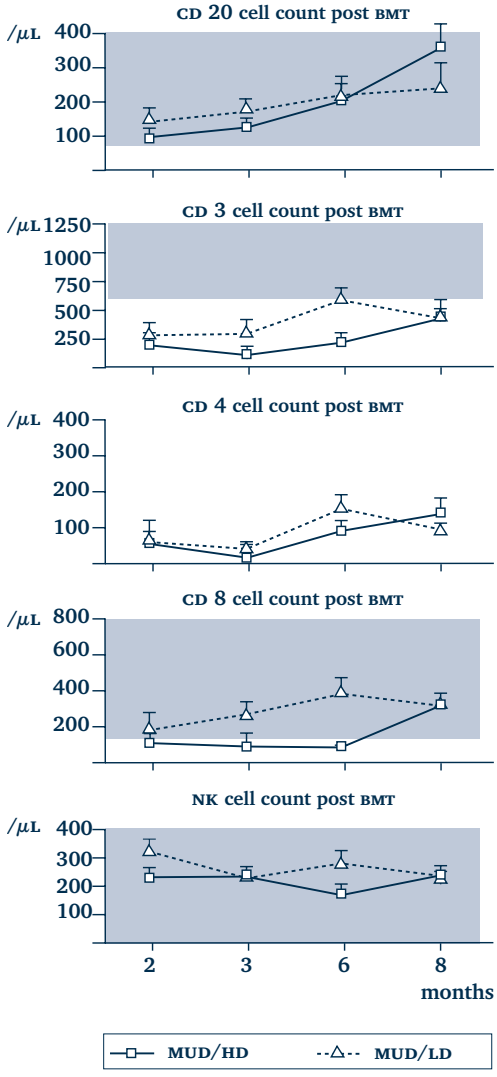


Figure 2

B, T and NK cell numbers during the post-transplant period in recipients of MUD transplants. Patients pretreated with low-dose ATG (MUD/LD) compared to patients pretreated with high-dose ATG (MUD/HD).
Shaded area represents normal values.



Discussion

The results of our study showed a markedly impaired quantitative T cell reconstitution during the first 6 post-transplant months in the group receiving BMT from a MUD compared to patients receiving transplants from an HLA-identical sibling. MUD recipients treated with high-dose ATG had the worst T cell and subsets recovery. These differences between MRD and MUD recipients might explain the well known increased incidence of infectious complications in MUD patients[1].

Two factors may account for the disturbed immune reconstitution among our MUD recipients compared to MRD patients: use of ATG and HLA disparities.

Since ATG could be detected in sera at least during 2 months post-transplant with 25% of the initial peak concentration at day 48 [4], it is hypothesized that ATG may severely impair T cell recovery, which was recently shown to be dose dependent[5].

It is also speculated that T and B cell recovery is related to the degree of HLA matching between host and donor. HLA disparities may alter the capacity of lymphoid progenitors of donor origin to mature within the host bone marrow or thymus. On the other hand, HLA disparities stimulate the occurrence of GVHD. Therefore, patients receiving grafts from unrelated or mismatched related donors will always be treated with more intensive immunosuppression compared to recipients of MRD grafts. A well known characteristic of GVHD itself is a defect in the development of donor derived T cells, which leads to a long lasting T cell deficiency state[6,7]. This entanglement of factors makes it impossible to solely analyse the impact of donor type on immune recovery. Despite this, efforts to analyse the influence of donor type were made in several studies[8-10]. In the first report a more prolonged and profound CD3+, CD4+ and CD8+ lymphopenia was seen in MUD recipients compared to MRD recipients. However, the proliferative T cell response to PHA was comparable between the two groups. Furthermore, interference of GVHD with the observed impaired T cell subset recovery in MUD recipients could not be excluded[8]. In the other studies, no effect of donor type was observed[9,10]. Recently, immune recovery in unrelated cord blood transplant (CBT) recipients was analysed and compared to data from recipients of MRD grafts. T cell receptor (TCR)-rearrangement excision circles (TREC) levels were comparable between the two groups, while TCR diversity was normalized earlier in CBT recipients[11]. Two other reports[12-13] did study the patterns of CD4+ and CD4+ CD45RA+ T cell recovery and restoration of TCR diversity in recipients of TCD grafts from MUDs and MRDs, respectively.

The results in both groups were highly comparable in these two reports. One might conclude from these results, that HLA disparities not necessarily result in an altered maturation of lymphoid progenitors of donor origin in the host thymus.
Based on these data we consider ATG to be the most important negative factor in the reconstitution of T cells and there subsets in our MUD recipients. On the other hand, ATG resulted in a lower incidence and severity of GVHD.

Acknowledgements

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References

1 Marks DI, Cullis JO, Ward KN, Lacey S, Syzdlo R, Hughes TP, Schwarer AP, Lutz E, Barrett AJ, Hows JM et al. Allogeneic bone marrow transplantation for chronic myeloid leukemia using sibling and volunteer unrelated donors. Ann Intern Med 1993; 119: 207-214.

2 Meijer E, Dekker AW, Rozenberg-Arska M, Weersink AJL, Verdonck LF. Influence of Cytomegalovirus-seropositivity on outcome after T cell depleted bone marrow transplantation: contrasting results between recipients of grafts from related and unrelated donors. Clin Infect Dis 2002; 35: 703-712.

3 McNerlan SE, Alexander HD, Rea IM. Age-related reference intervals for lymfocyte subsets in whole blood of healthy individuals. Scand J Clin Lab Invest 1999; 59: 89-92.

4 Finke J, Bertz H, Schmoor C, Veelken H, Behringer D, Wasch R, Kunzmann R, Heidecker L, Lang H, Meyer-Konig U, Mertelsmann R. Allogeneic bone marrow transplantation from unrelated donors using in vivo anti-T-cell globulin. Br J Haematol 2000; 111: 303-313.

5 Duval M, Pedron B, Rohrlich P, Legrand F, Faye A, Lescoeur B, Bensaid P, Larchee R, Sterkers G, Vilmer E. Immune reconstitution after haematopoietic transplantation with two different doses of pre-graft antithymocyte globulin. Bone Marrow Transplant 2002; 30: 421-426.

6 Atkinson K. Reconstruction of the haemopoietic and immune systems after marrow transplantation. Bone Marrow Transplant 1990; 5: 209-226.

7 Dulude G, Roy DC, Perreault C. The effect of graft versus host disease on T cell poduction and homeostasis. J Exp Med 1999: 189: 1329-1341.

8 Small TN, Papadopoulos EB, Boulad F et al. Comparison of immune reconstitution after unrelated and related T cell depleted bone marrow transplantation: effect of patient age and donor leukocyte infusion. Blood 1999; 93: 467-480.

9 Lewin SR, Heller G, Zhang L, Rodrigues E, Skulsky E, van den Brink MR, Small TN, Kernan NA, O'Reilly RJ, Ho DD, Young JW. Direct evidence for new T-cell generation by patients after either T-cell-depleted or unmodified allogeneic hematopoietic stem cell transplantations. Blood 2002; 100: 2235-2242.

10 Weinberg K, Blazar BR, Wagner JE, Agura E, Hill BJ, Smogorzewska M, Koup RA, Betts MR, Collins RH, Douek DC. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. Blood 2001; 97: 1458-1466.

11 Talvensaari K, Clave E, Douay C, Rabian C, Garderet L, Busson M, Garnier F, Douek D, Gluckman E, Charron D, Toubert A. A broad T-cell repertoire diversity and an efficient thymic function indicate a favorable long-term immune reconstitution after cord blood stem cell transplantation. Blood 2002; 99: 1458-1464.

12 Dumont-Girard F, Roux E, van Lier RA, Hale G, Helg C, Chapuis B, Starobinski M, Roosnek E. Reconstitution of the T-cell compartment after bone marrow transplantation: restoration of the repertoire by thymic emigrants. Blood 1998; 92: 4464-4471.

13 Roux E, Dumont-Girard F, Starobinski M, Siegrist CA, Helg C, Chapuis B, Roosnek E. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. Blood 2000; 96: 2299-2303.

Chapter 3

Antithymocyteglobulin as prophylaxis of graft failure and graft-versus-host disease in recipients of partially T cell depleted grafts from matched unrelated donors: a dose finding study

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Abstract

In this study, we set out to evaluate the effect of 3 different Antithymocyteglobulin (ATG) doses on graft failure and incidence of graft-versus-host disease (GVHD) among recipients of partially T cell depleted (TCD) grafts from matched unrelated donors (MUDs). Data of 74 consecutively treated MUD recipients were analysed. Fifty-two, 13 and 9 MUD patients were treated with ATG in a total dose of 8 mg/kg, 6 mg/kg and 4 mg/kg (given from days -8 until -4), respectively. Granulocyte and platelet engraftment was not different between the groups, while graft failure was observed in two patients only (receiving 8 mg/kg and 4 mg/kg ATG, respectively). The cumulative incidence of severe (grade III-IV) acute GVHD and extensive chronic GVHD was 4%, 0%, 44% and 11%, 8%, 44% in groups receiving ATG in a dose of 8 mg/kg, 6 mg/kg and 4 mg/kg, respectively (severe acute GVHD: $p<0.001$; extensive chronic GVHD: $p=0.05$). Based on these findings, we recommend when ATG is used in the setting of stem cell transplantation with (partially) TCD grafts from MUDs, to give a total dose of 6-8 mg/kg. A further decrease in dosage resulted in a highly significant increased incidence of severe acute and extensive chronic GVHD.

Introduction

Despite improvements in HLA typing, graft-versus-host disease (GVHD) is still a major cause of morbidity and mortality after stem cell transplantation (SCT) with grafts from matched unrelated donors (MUDs). T cell depletion (TCD) of grafts is an effective method for prevention of GVHD[1-4]. However, one of the limitations associated with this method is the occurrence of graft failure[3,4]. A large International Bone Marrow Transplant Registry (IBMTR) analysis learned that the risk for graft failure was related to the type of TCD, being lowest when T cell specific techniques were used[3]. Varying strategies are used to prevent graft failure, which is considered to be mediated by residual host T cells having survived the conditioning regimen[5,6]. A method commonly used in the setting of SCT with TCD grafts from MUDs, is the in vivo pre-transplant depletion of host immune cells using Antithymocyteglobulin (ATG) or Campath-1[4]. Several studies also showed effectiveness of in vivo TCD, using ATG or Campath-1, as prophylaxis of GVHD after MUD transplantation[7-18]. The optimal dose of ATG with respect to prevention of graft failure and severe GVHD is not known. The immunosuppressive and direct toxic effects of ATG, however, necessitate the use of the lowest possible dose.

Here, we set out to evaluate the effect of 3 different ATG (Thymoglobulin™, Sangstat) doses on graft failure and incidence of acute and chronic GVHD among MUD recipients of partially TCD grafts.

Methods

Patients For this study data of 74 patients receiving stem cells from MUDs were analysed. Transplantations were performed between January 1999 and May 2002 at the Department of Haematology of the University Medical Centre Utrecht and at the Department of Haematology of the Erasmus Medical Centre Rotterdam, the Netherlands. ATG (Thymoglobulin™, Sangstat, Amstelveen, the Netherlands) was given pre-transplant to prevent graft failure. Because of the immunosuppressive and direct toxic effects of ATG it was decided in November 2001 to decrease the total dose of ATG from 8 to 6 mg/kg in the Utrecht centre and from 8 to 4 mg/kg in the Rotterdam centre. Thirty-three and 19 MUD

recipients were treated with ATG in a total dose of 8 mg/kg, given in 4 days, and were defined as MUD group 1 (treated in Utrecht) and 2 (treated in Rotterdam), respectively. Thirteen and 9 patients received ATG in a total dose of 6 mg/kg and 4 mg/kg (also given in 4 days), respectively, and were defined as MUD group 3 (treated in Utrecht) and 4 (treated in Rotterdam), respectively. Patients with acute leukaemia in first complete remission, chronic myeloid leukaemia in first chronic phase and untreated severe aplastic anaemia were considered low-risk. All patients with other diseases were considered high-risk. Patients were treated according to clinical protocols approved by the local investigation review boards after informed consent was obtained.

Transplantation procedure The transplantation procedure in both centres was identical. The conditioning regimen consisted of cyclophosphamide (60 mg/kg/day for 2 days), followed by total body irradiation (600 cGy/day for 2 days) with partial shielding of the lungs (total lung dose 850 cGy). The graft was infused after the second TBI fraction (day 0). ATG was given in 4 days, from day -8 until day -4, before cyclophosphamide was infused. Post-transplant immunosuppression consisted of cyclosporin which was discontinued within 3 months after transplantation, when no active GVHD was present. Infection prevention for all patients consisted of ciprofloxacin, fluconazole and amphotericin B given orally until granulocyte counts exceeded 500 cells/μl. Cephalothin was given intravenously for 10 days from day +3. Furthermore co-trimoxazole and (val)acyclovir were given orally from day +1 until 12 months post-BMT or longer in case of active GVHD, in a dose of 480 mg b.i.d. and 500 mg b.i.d., respectively. GVHD was classified according to the Seattle criteria[19] and graft failure according to criteria described by Kernan et al and McGlave et al[1,2].

CMV monitoring Until April 2001 CMV monitoring was performed three times a week using a pp65 assay as described[20]. Since then monitoring was performed once a week using a real-time Taqman™ CMV DNA PCR. However, in patients with active GVHD and in patients with a CMV reactivation, monitoring was performed twice a week. CMV reactivation was defined as CMV pp65 antigenemia of $\geq$ 1 positive staining granulocyte/150.000 cells or CMV DNA viral load (VL) of > 1000 copies/ml.

CMV disease Patients with symptoms of pneumonia, gastritis or enteritis underwent bronchoscopy, gastroscopy or sigmoidoscopy, respectively. CMV pneumonia/gastritis/enteritis was defined histologically by typical cytopathic effects and immunohistochemically by immunofluorescence with use of monoclonal antibodies to immediate early CMV antigens in biopsy specimens. When cultures of BAL fluid, saliva, urine and buffy coat were performed

in case of infectious complications, these included always CMV cultures, irrespective of CMV serostatus.

Ganciclovir therapy CMV-seropositive patients who demonstrated CMV reactivation or who were treated with high-dose corticosteroids for acute GVHD grade II-IV received pre-emptive or prophylactic therapy, respectively, with ganciclovir in a dose of 2.5 mg/kg intravenously twice a day for 14 days. When patients were symptomatic (unexplained fever or symptoms compatible with CMV disease), CMV antigenemia/VL was rising or remained positive after 14 days of treatment, ganciclovir dose was doubled or foscarnet treatment was started instead of ganciclovir in a dose of 60 mg/kg twice a day for 14 days. CMV disease was treated with ganciclovir 5 mg/kg twice a day for at least 14 days and continued until symptoms resolved and antigenemia/VL became negative. In case of disease progression or rising antigenemia/VL foscarnet treatment was started instead of ganciclovir in a dose of 60 mg/kg twice a day. Furthermore, treatment with CMV specific immunoglobulins was added to antiviral therapy in patients with CMV pneumonia.

HLA-matching Class I antigens were analysed by serological and low resolution molecular typing with sequence specific primers (SSP). Since July 2000, high resolution molecular typing (with SSP and/or sequence based typing (SBT)) of HLA-A and B antigens was performed as well. Analyses of class II antigens were performed by high resolution molecular typing with SSP and/or SBT throughout the whole study period. The aim was to obtain a fully matched (for HLA-A, B, Cw, DRB1 and DQB1) or, if not available, an one HLA antigen mismatched unrelated patient/donor pair.

BMT In vitro TCD of bone marrow (BM) was performed using the Sheep Red Blood Cell technique (Rotterdam) or the immunorosette depletion technique (Utrecht) as described[20]. Only 3 patients received a peripheral blood stem cell (PBSC) graft (one from group 1, one from group 2 and one from group 3). TCD of these G-CSF stimulated PBSC grafts was performed by positive selection of CD34+ cells (CliniMacs™, Miltenyi Biotec, Bergisch Gladbach, Germany). After these maximal TCD procedures the residual number of T cells was counted and nonmanipulated T cells (from a small BM/PBSC fraction that was set apart) were added to obtain the desired fixed low number of T cells (1-2 x 10^5 T cells/kg recipient weight).

Statistical analysis Differences between groups were compared using Pearson chi-square analyses in case of discrete variables. In case of continuous variables univariate analysis of variance (ANOVA) or Kruskal-Wallis test, whichever was appropriate, was per-

formed. Overall survival (OS) was estimated by the Kaplan-Meier method. Probabilities of transplant related mortality (TRM), relapse related mortality (RRM), acute GVHD and chronic GVHD were calculated by the cumulative incidence procedure; death without TRM, RRM, acute GVHD and chronic GVHD, respectively, being the competing risk. Univariate analyses were performed using the log rank test. Calculations were performed using SPSS/PC+ 10.0 (SPSS Inc, Chicago Il, USA).

Results

Pre-transplant patient characteristics (Table 1). The percentage of HLA antigen mismatched grafts and mean T cell and CD34+ cell counts of grafts were higher in recipients from MUD group 3 (treated with ATG 6 mg/kg) compared with the other groups,

Table 1 Patient characteristics

	MUD group 1 (n=33)	**MUD group 2 (n=19)**	**MUD group 3 (n=13)**	**MUD group 4 (n=9)**	**P**
ATG dose	8 mg/kg	8 mg/kg	6 mg/kg	4 mg/kg	
Age, yr	34	34	35	37	ns
(range)	(17-55)	(18-48)	(18-53)	(19-51)	
Diagnosis, %					
AML	18	26	46	13	
ALL	30	11	15	13	
CML	30	21	23	37	
SAA	0	0	0	13	
Other	21	42	15	25	
CMV serostatus R/D, %					ns
+/+	24	16	15	0	
+/-	15	26	54	38	
-/+	15	11	8	25	
-/-	46	47	23	38	
R/D sex, %					ns
M/F	21	16	8	13	
Others	79	84	92	87	
Risk status, %					ns
Low	18	26	15	0	
High	82	74	85	100	
Mean CD3+ cells[&]	2.2	1.6	2.4	1.9	0.008
(range)	(2-4)	(1-4)	(2-5)	(1-3)	
Mean CD34+ cells[&&]	1.62	1.50	2.76	1.66	0.052
(range)	(0.5-5.9)	(0.55-3.24)	(0.95-10.4)	(0.81-2.96)	
Mismatched graft, %	18	11	46	25	ns

R/D = recipient/donor; [&] = in graft, $x10^5$/kg; [&&] = in graft, $x10^6$/kg.

although this was only significant for mean T and CD34+ cell counts of grafts (p=0.008 and p=0.052, respectively).

Effect of ATG dose on engraftment and GVHD (Tables 2 and 3, Figure 1). Granulocyte and platelet engraftment was not different between the four MUD groups (Table 2). Graft failure was observed in two patients, who were excluded from analyses, one

Table 2 Platelet and granulocyte engraftment

	MUD group 1 (n=33)	MUD group 2 (n=19)	MUD group 3 (n=13)	MUD group 4 (n=9)	P
ATG dose	8 mg/kg	8 mg/kg	6 mg/kg	4 mg/kg	
Platelets > 50 × 10^9/L					
Recovery, %	94	84	100	78	ns
Median days,	26	32	20	41	ns
(range)	(12-208)	(15-112)	(11-52)	(20-158)	
Granulocytes > 500 × 10^6/L					
Recovery, %	100	100	100	100	ns
Median days,	19	23	19	20	ns
(range)	(12-92)	(11-35)	(11-32)	(14-38)	

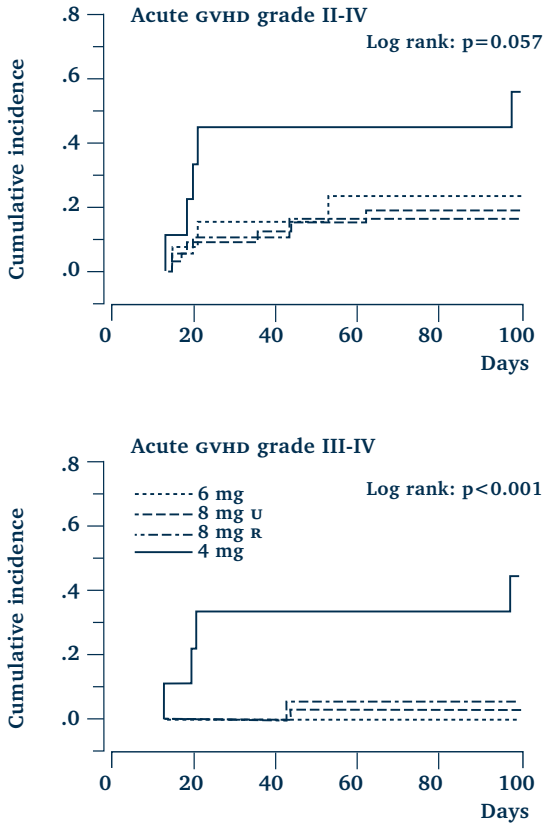
Table 3 Cumulative incidence of acute and chronic GVHD

	MUD group 1 (n=33)	MUD group 2 (n=19)	MUD group 3 (n=13)	MUD group 4 (n=9)	P
ATG dose	8 mg/kg	8 mg/kg	6 mg/kg	4 mg/kg	
aGVHD, %					
II-IV (SE)	18 (7)	16 (8)	23 (12)	56 (17)	0.057
III-IV (SE)	3 (3)	5 (5)	0	44 (17)	<0.001
cGVHD, %					
Any (SE)	24 (7)	26 (10)	23 (12)	44 (17)	ns
Ext (SE)	12 (6)	10 (7)	8 (7)	44 (17)	0.052

Any = limited and extensive.

Figure 1

Cumulative incidence of acute GVHD according to ATG dose.



from MUD group 1 and one from MUD group 4. In recipients of 8 mg/kg ATG, treated in Utrecht (MUD group 1) and Rotterdam (MUD group 2), the incidence of acute and chronic GVHD was highly comparable. A further decrease in ATG dose to 6 mg/kg (MUD group 3) did not significantly increase the rate of acute and chronic GVHD. In the group receiving 4 mg/kg ATG (MUD group 4) 44% of patients developed severe acute GVHD (grade III-IV) and extensive chronic GVHD.

After univariate analyses, no impact of other known risk factors (older age, high-risk disease status, CD3+ and CD34+ cell counts in grafts, positive recipient CMV serostatus, male recipient with female donor, use of an one HLA antigen mismatched graft) on occurrence of GVHD was found. Therefore, multivariate analyses were not performed. Furthermore, data were analysed for centre effect, which could be excluded.

Overall survival and transplant and relapse related mortality (Table 4). Median follow up was 27 (range:17-46), 28 (range:19-38), 11 (range:11-14) and 16 months (range:16-17) for group 1, 2, 3 and 4, respectively. OS and cumulative incidence of TRM and RRM at one year post-transplant are depicted in Table 4.

A higher dose of ATG did not result in an increased TRM nor RRM. One has to remind, when interpreting these data, that patient numbers in group 3 and 4 are very small.

Table 4 Overall survival and cumulative incidence of transplant and relapse related mortality at 1 year post-transplant

	MUD group 1 (n=33)	MUD group 2 (n=19)	MUD group 3 (n=13)	MUD group 4 (n=9)	P
OS, % (SE)	64 (8)	79 (18)	38 (13)	56 (34)	ns
TRM, % (SE)	12 (6)	21 (18)	23 (12)	33 (32)	ns
RRM, % (SE)	24 (14)	0	38 (26)	11 (20)	ns

OS = overall survival; TRM = transplant related mortality; RRM = relapse related mortality; SE = standard error.

Discussion

In this study, we set out to evaluate the effect of 3 different ATG doses (8 mg/kg, 6 mg/kg and 4 mg/kg) on graft failure and incidence of acute and chronic GVHD among MUD recipients of partially TCD grafts. ATG was infused pre-transplant, resulting in in-vivo TCD. Lowering ATG dose from 8 mg/kg to 6 mg/kg did not influence the occurrence of graft failure nor the incidence of GVHD. However, when ATG dose was decreased to 4 mg/kg the incidence of severe acute GVHD grade III-IV and extensive chronic GVHD rose significantly. Low-dose ATG (4 mg/kg) did not affect the incidence of graft failure, suggesting that adequate host immune suppression was achieved.

The effectiveness of ATG (Thymoglobulin™, Sangstat) as prophylaxis of GVHD in recipients of MUD grafts has been described by others[8-12]. In these studies, no in-vitro TCD of grafts was performed and prophylaxis of GVHD consisted of ATG, cyclosporine A and a short-course of methotrexate. The incidence of acute (grade III-IV) and chronic GVHD was highly variable (see Table 5). A dose-response effect was only seen in individual studies, but not between the varying studies. The results of our study are very favourable compared to those described in Table 5, as long as 6-8 mg/kg ATG was used. In other studies[14-18] ATG was derived from a different company (Fresenius). ATG-Fresenius is not equipotent to ATG

Table 5 Effect of ATG on incidence of acute and chronic GVHD

ATG dose (Thymoglobulin™, Sangstat)	Acute GVHD, grade III-IV	Chronic GVHD
15 mg/kg	11%[8]	41%[8]
	7%[9]	0%[9]
7,5 mg/kg	41%[8]	55%[8]
	38%[9]	27%[9]
4,5 mg/kg	0%[10]	27%[10]
	3%[11]*	38%[11]*
	10%[12]	44%[12]
no ATG	54%[10]	57%[10]

* In this report data are presented from recipients of matched related donor grafts (n=42) and alternative donor grafts (n=28), subgroup analysis showed no difference; superscript numbers are references.

(Thymoglobulin™, Sangstat) as used in our study. This makes a direct dose-response comparison between these and our study impossible.

Recently we reported high-dose ATG (20 mg/kg), used before 1999, to be associated with a highly increased incidence of fatal infectious complications compared to intermediate-dose ATG (8 mg/kg) used after 1999[20,21]. Furthermore, total lymphocyte, CD3+ and CD4+ T cell reconstitution was significantly lower in transplant recipients of unrelated grafts treated with high-dose ATG (15 mg/kg) compared with intermediate-dose ATG (7,5 mg/kg)[9]. Another drawback of ATG treatment is a possible increased relapse incidence[8-10,] although this is not supported by data from recent studies of Kroger et al[17] and Duggan et al[12] in which ATG was very effective in reducing the incidence of GVHD without affecting the relapse rate. Duggan et al[12] compared incidence of GVHD, relapse, TRM, OS and disease free survival in recipients of un-manipulated MUD transplants with outcome in recipients of un-manipulated matched related donor grafts. All patients received GVHD prophylaxis consisting of cyclosporine A and short-course methotrexate, while MUD recipients were also treated with low-dose (4,5 mg/kg) ATG given pre-transplant over 3 days. Outcome was highly comparable in both groups. Relapse incidence at 3 years was 45% (SE 7%) in the MUD group and 42% (SE 7%) in the matched unrelated donor group.

In the current study, the highest dose of ATG (8 mg/kg) did not result in an increased TRM nor RRM. However, patient numbers in group 3 and 4 are very small, therefore, these data should be interpreted with caution.

Considering the immunosuppressive and direct toxic effects of high-dose (15-20 mg/kg) ATG, a dose reduction is mandatory. In the setting of SCT with (partially) TCD grafts from MUDs, using limited post-transplant immunosuppression with cyclosporine A, we recommend the use of ATG in a total dose of 6-8 mg/kg.

References

1 Kernan NA, Bartsch G, Ash RC, Beatty PG, Champlin R, Filipovich A, Gajewski J, Hansen JA, Henslee-Downey J, McCullough J et al. Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Donor Program. N Engl J Med 1993; 328: 593-602.

2 McGlave PB, Shu XO, Wen W, Anasetti C, Nademanee A, Champlin R, Antin JH, Kernan NA, King R, Weisdorf DJ. Unrelated donor marrow transplantation for chronic myelogenous leukemia: 9 years' experience of the national marrow donor program. Blood 2000; 95: 2219-2225.

3 Champlin RE, Passweg JR, Zhang MJ, Rowlings PA, Pelz CJ, Atkinson KA, Barrett AJ, Cahn JY, Drobyski WR, Gale RP, Goldman JM, Gratwohl A, Gordon-Smith EC, Henslee-Downey PJ, Herzig RH, Klein JP, Marmont AM, O'Reilly RJ, Ringden O, Slavin S, Sobocinski KA, Speck B, Weiner RS, Horowitz MM. T-cell depletion of bone marrow transplants for leukemia from donors other than HLA-identical siblings: advantage of T-cell antibodies with narrow specificities. Blood 2000; 95: 3996-4003.

4 Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. Blood 2001; 98: 3192-3204.

5 Voogt PJ, Fibbe WE, Marijt WA, Goulmy E, Veenhof WF, Hamilton M, Brand A, Zwann FE, Willemze R, van Rood JJ et al. Rejection of bone-marrow graft by recipient-derived cytotoxic T lymphocytes against minor histocompatibility antigens. Lancet 1990; 335: 131-134.

6 Fleischhauer K, Kernan NA, O'Reilly RJ, Dupont B, Yang SY. Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. N Engl J Med 1990; 323: 1818-1822.

7 Bacigalupo A, Oneto R, Lamparelli T, Gualandi F, Bregante S, Raiola AM, Di Grazia C, Dominietto A, Romagnani C, Bruno B, Van Lint MT, Frassoni F. Pre-emptive therapy of acute graft-versus-host disease: a pilot study with antithymocyte globulin (ATG). Bone Marrow Transplant 2001; 28: 1093-1096.

8 Bacigalupo A, Lamparelli T, Bruzzi P, Guidi S, Alessandrino PE, di Bartolomeo P, Oneto R, Bruno B, Barbanti M, Sacchi N, Van Lint MT, Bosi A. Antithymocyte globulin for graft-versus-host disease prophylaxis in transplants from unrelated donors: 2 randomized studies from Gruppo Italiano Trapianti Midollo Osseo (GITMO). Blood 2001; 98: 2942-2947.

9 Duval M, Pedron B, Rohrlich P, Legrand F, Faye A, Lescoeur B, Bensaid P, Larchee R, Sterkers G, Vilmer E. Immune reconstitution after haematopoietic transplantation with two different doses of pre-graft antithymocyte globulin. Bone Marrow Transplant 2002; 30: 421-426.

10. Anderson RA, Booth KR, Saunders C, Coppes MJ, Russell JA. Low dose antithymocyteglobulin incorporated into GVHD prophylaxis improves GVHD but not other outcomes after pediatric unrelated donor bone marrow transplantation. Blood 2001; 98: S335a. Abstract 5306.

11 Russell JA, Tran HT, Quinlan D, Chaudhry A, Duggan P, Brown C, Stewart D, Ruether, JD, Morris D, Gluck S, Gyonyor E, Andersson BS. Once-daily intravenous busulfan given with fludarabine as conditioning for allogeneic stem cell transplantation: study of pharmacokinetics and early clinical outcomes. Biol Blood Marrow Transplant 2002; 8: 468-476.

12 Duggan P, Booth K, Chaudhry A, Stewart D, Ruether JD, Gluck S, Morris D, Brown CB, Herbut B, Coppes M, Anderson R, Wolff J, Egeler M, Desai S, Turner AR, Larratt L, Gyonyor E, Russell JA. Unrelated donor BMT recipients given pretransplant low-dose antithymocyte globulin have outcomes equivalent to matched sibling BMT: a matched pair analysis. Bone Marrow Transplant 2002; 30: 681-686.

13 Byrne JL, Stainer C, Cull G, Haynes AP, Bessell EM, Hale G, Waldmann H, Russell NH. The effect of the serotherapy regimen used and the marrow cell dose received on rejection, graft-versus-host disease and outcome following unrelated donor bone marrow transplantation for leukaemia. Bone Marrow Transplant 2000; 25: 411-417.

14 Holler E, Ledderose G, Knabe H, Gunther C, Wilmanns W, Kolb HJ. ATG serotherapy during pretransplant conditioning in unrelated donor BMT: dose dependent modulation of GVHD. Bone Marrow Transplant 1998: 21: S30. Abstract.

15 Zander AR, Zabelina T, Kroger N, Renges H, Kruger W, Loliger C, Durken M, Stockschlader M, de Wit M, Wacker-Backhaus G, Bielack S, Jaburg N, Russmann B, Erttmann R, Kabisch H. Use of a five-agent GVHD prevention regimen in recipients of unrelated donor marrow. Bone Marrow Transplant 1999; 23: 889-893.

16 Finke J, Bertz H, Schmoor C, Veelken H, Behringer D, Wasch R, Kunzmann R, Heidecker L, Lang H, Meyer-Konig U, Mertelsmann R. Allogeneic bone marrow transplantation from unrelated donors using in vivo anti-T-cell globulin. Br J Haematol 2000; 111: 303-313.

17 Kroger N, Zabelina T, Kruger W, Renges H, Stute N, Rischewski J, Sonnenberg S, Ayuk F, Togel F, Schade U, Fiegel H, Erttmann R, Loliger C, Zander AR. In vivo T cell depletion with pretransplant anti-thymocyte globulin reduces graft-versus-host disease without increasing relapse in good risk myeloid leukemia patients after stem cell transplantation from matched related donors. Bone Marrow Transplant 2002; 29: 683-689.

18 Finke J, Schmoor C, Lang H, Potthoff K, Bertz H. Matched and mismatched allogeneic stem-cell transplantation from unrelated donors using combined graft-versus-host disease prophylaxis including rabbit anti-T lymphocyte globulin. J Clin Oncol 2003; 21: 506-513.

19 Thomas ED, Storb R, Clift RA, Fefer A, Johnson L, Neiman PE, Lerner KG, Glucksberg H, Buckner CD. Bone-marrow transplantation (second of two parts). N Engl J Med 1975; 292: 895-902.

20 Meijer E, Dekker AW, Rozenberg-Arska M, Weersink AJL, Verdonck LF. Influence of Cytomegalovirus-seropositivity on outcome after T cell depleted bone marrow transplantation: contrasting results between recipients of grafts from related and unrelated donors. Clin Infect Dis 2002; 35: 703-712.

21 Meijer E, Dekker AW, Verdonck LF. Influence of Antithymocyteglobulin dose on outcome in Cytomegalovirus-seropositive recipients of partially T cell depleted stem cell grafts from matched unrelated donors. Br J Haematol 2003; 121: 473-476.

Chapter 4

Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T cell depleted SCT

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Abstract

Reactivation of the Epstein-Barr virus (EBV) after allogeneic stem cell transplantation (SCT) may evoke a protective cellular immune response or may be complicated by the development of EBV-lymphoproliferative disease (EBV-LPD). So far, very little is known about the incidence, recurrence, and sequelae of EBV reactivation following SCT. EBV reactivation was retrospectively monitored in 85 EBV-seropositive recipients of a T cell depleted (TCD) SCT and 65 EBV-seropositive recipients of an unmanipulated SCT. Viral reactivation (more than 50 EBV copies (c) /ml) was monitored frequently by quantitative real-time plasma polymerase chain reaction (PCR) until day 180 after SCT. Probabilities of developing viral reactivation were high after both unmanipulated and TCD allogeneic SCT (31% ± 6% versus 65% ± 7%, respectively). A high CD34+ cell number of the graft appeared as a novel significant predictor ($p=0.001$) for EBV reactivation. Recurrent reactivation was observed more frequently in recipients of a TCD graft, and EBV-LPD occurred only after TCD SCT. High-risk disease status, TCD, and use of Antithymocyteglobulin (ATG) were predictive for developing EBV-LPD. Plasma EBV DNA quantitatively predicted EBV-LPD. The positive and negative predictive values of a viral load of 1000 c/ml were, respectively, 39% and 100% following TCD. Treatment-related mortality did not differ significantly between TCD and non-TCD transplants, but the incidence of chronic graft-versus-host disease was significantly less in TCD patients. It is concluded that EBV reactivation occurs frequently after TCD and unmanipulated SCT, especially in recipients of grafts with high CD34+ cell counts. EBV-LPD, however, occurred only after TCD and EBV viral load quantitatively predicted EBV-LPD in recipients of a TCD graft.

Introduction

Epstein-Barr virus-associated lymphoproliferative disease (EBV-LPD) is a serious complication of allogeneic stem cell transplantation (SCT) and solid organ transplantation[1-3]. Although the incidence of EBV-LPD is generally less than 2% after SCT, it may increase to 20% in patients with established risk factors, such as unrelated donor SCT, the use of T cell depleted (TCD) grafts, use of Antithymocyteglobulin (ATG) and immuno-suppression for prevention and treatment of graft-versus-host disease (GVHD)[4-8]. EBV-LPD is associated with a poor prognosis despite the use of anti B lymphocyte monoclonal antibody therapy, donor lymphocyte infusion (DLI) and infusion of EBV-specific cytotoxic T cells (CTL)[9-15]. Therefore, early diagnosis and preventive measures such as B cell depletion of the donor graft, and pre-emptive therapy may be clinically useful[4,7,16-24].

We developed a real-time polymerase chain reaction (PCR) assay for the quantitative detection of EBV-DNA in plasma[25]. The assay accurately monitors viral load in plasma from patients with infectious mononucleosis and immunocompromised patients at risk of EBV-LPD or with established EBV-LPD[25,26]. In contrast to cytomegalovirus (CMV) antigenemia after SCT and the risk of developing CMV-disease, little is known about reactivation of EBV during the first 3 to 6 months after SCT and the predictive value of EBV reactivation for subsequent EBV-LPD. Although several studies have shown an association of viral load and a diagnosis of EBV-LPD, no study has longitudinally followed a larger cohort of SCT recipients with multiple risk factors[27-43]. We set out to monitor EBV reactivation by real-time PCR at regular time intervals after SCT. Incidences, risk factors and sequelae of EBV reactivation were compared between patients receiving a TCD SCT and patients transplanted with an unmanipulated stem cell graft. We show that subclinical EBV reactivation is a very frequent event after SCT and that quantification of EBV DNA appears useful to identify patients at risk of progression to overt EBV-LPD.

Methods

Patients The study population consisted of 152 consecutive patients treated at 4 transplant centres, who received stem cell transplants between March 1996 and June 1999. Patients underwent allografting at the department of haematology of the university hospitals of Utrecht (TCD SCT) or Rotterdam (TCD SCT), the Netherlands; Essen (non-TCD SCT), Germany; or Genoa (non-TCD SCT), Italy. Transplant protocols were approved by local institutional review boards and all patients provided informed consent. Patient characteristics are presented in Table 1. Eighty-five patients received a TCD SCT and 67 patients received a non-TCD SCT. Median age was 41 years (range, 17-55 years) in the TCD group and 31 years (range, 17-56 years) in the non-TCD group ($p<0.01$). Low-risk patients had a diagnosis of acute lymphoblastic leukaemia (ALL) in first complete remission (CR1), acute myeloid leukaemia (AML) in CR1, chronic myeloid leukaemia (CML) in first chronic phase and untreated (very) severe aplastic anaemia (SAA), all other diagnoses were considered high-risk. The non-TCD group included more patients with CML and fewer patients with lymphoma, multiple myeloma or high-risk disease ($p=0.001$). Unrelated donor grafts were used more frequently in the non-TCD group ($p=0.001$). The use of ATG added to the conditioning regimen for prevention of rejection was confined to patients transplanted with TCD grafts from unrelated donors.

Transplantation The conditioning regimen preceding a TCD SCT consisted of cyclophosphamide (120 mg/kg) and total body irradiation (TBI) (12 Gy in 2 fractions). Rabbit ATG (Thymoglobulin™, Sangstat, Amstelveen, the Netherlands) was given for prevention of rejection prior to SCT in recipients of a TCD unrelated donor graft. If patients had previously been treated with locoregional irradiation, the conditioning regimen consisted of oral busulfan (4 mg/kg on each of 4 successive days) and cyclophosphamide (120 mg/kg). The conditioning regimen in case of an unmanipulated SCT consisted of cyclophosphamide (120 mg/kg) and TBI (10 Gy in 4 fractions or 10 Gy in 3 fractions).

Partial T cell depletion was performed using sheep erythrocyte rosetting (n=53) or CD34 selection (CellPro, Wezembeek, Belgium) (n=32). Median T cell numbers differed more than 2 logs between TCD and unmanipulated grafts ($2.0x10^5$/kg versus $510x10^5$/kg), but numbers of CD34+ cells did not differ significantly between the groups of patients. Peripheral blood-derived stem cells were used relatively more often than bone marrow-derived stem cells in patients receiving a TCD graft as compared with patients receiving an

Table 1 Patient characteristics

Characteristics	TCD SCT (n=85)	nonTCD SCT (n=67)	P
Age, y (range)	41 (17-55)	31 (17-56)	<0.01
Diagnosis			
AML CR1	11	3	
AML >CR1	8	8	
ALL CR1	10	6	
ALL >CR1	7	2	
MDS	3	1	
CML CP1	8	28	
CML >CP1	5	16	
SAA	5	0	
MM	15	1	
NHL	10	2	
Other	3	0	
Risk status: low/high	25/60	37/30	0.001
Donor: MRD/MUD	61/24	30/37	0.001
Graft characteristics			
T cells (range; x 10^5/kg)	2.0 (0.01-9.32)	510 (7.40-2195)	<0.001
CD34+ cells (range; x 10^6/kg)	1.25 (0.06-6.43)	2.2 (0.04-14.10)	
Conditioning regimen			
Cy/TBI	59	67	
Cy/TBI/ATG	23	0	
Other	3	0	
EBV serostatus			
R-/D-	0	2	
R+/D+, R+/D-, R-/D+	85	65	
Stem cell source: BM/PB	66/19	63/4	<0.01

Data are no. of patients, unless otherwise indicated; AML CR1 or >CR1 = acute myeloid leukaemia in first or subsequent complete remission; ALL CR1 or >CR1 = acute lymphoblastic leukaemia in first or subsequent CR; MDS = myelodysplastic syndrome; CML CP1 or >CP1 = chronic myeloid leukaemia in first or subsequent chronic phase; SAA = severe aplastic anaemia; MM = multiple myeloma; NHL = Non-Hodgkin's lymphoma; MRD = matched related donor; MUD = matched unrelated donor; Cy = cyclophosphamide; TBI = total body irradiation; ATG = Antithymocyteglobulin; R/D = recipient/donor; BM = bone marrow; PB = peripheral blood.

unmanipulated graft ($p<0.01$). Graft-versus-host (GVH) prophylaxis was cyclosporin A (3 mg/kg) from day -3 until day +100 after TCD SCT, and the combination methotrexate (15 mg/m^2 on day 1; 10 mg/m^2 on day 3, 6 and 11) and cyclosporin A was used in recipients of an unmanipulated SCT.

All patients received ciprofloxacin and fluconazole for prevention of infection during neutropenia, and cotrimoxazole was given after neutrophil recovery until day 180 to 360 after SCT. Patients transplanted in Utrecht (TCD SCT) and Genoa (non-TCD SCT) received long-term acyclovir prophylaxis from day 0 until day 360. Blood products were leukocyte depleted and subsequently irradiated (25 Gy). Patients were hospitalised in reverse isolation and rooms with high-efficiency particulate-filtered air. All patients received food with a low microbial count until discharge, and parenteral alimentation was given in case of severe mucositis.

Real-time Taqman assay Taqman PCR primers were selected from the EBV-DNA genome encoding for the non-glycosylated membrane protein BNRF1-p143 and generated a DNA product of 74 base-pairs, as described before[25]. A known EBV-DNA copy number based on a reference standard quantified by electron microscopy (ABI Advanced Biotechnologies, Columbia, MA, USA) was used for standardization. Serial dilutions ranging from 10 to 10^7 EBV-DNA c/ml were made to characterize linearity, precision, specificity and sensitivity. The Taqman assay appeared to detect viral DNA in plasma over a linear span between 50 and 10^7 c/ml with an average coefficient of variation of 1.56% (range, 0.7- 7.0%). Test results below 50 c/ml were considered negative. No viral DNA was detected in plasma of healthy EBV-seropositive individuals[25]. EBV reactivation was defined as a plasma EBV-DNA level exceeding 50 c/ml. Recurrent reactivation was defined by a positive PCR (more than 50 c/ml) after (at least) two consecutive negative PCR results following a preceding episode of reactivation. Viral load was monitored in blood samples drawn at 2-week intervals starting at SCT until day 180 after SCT.

EBV-LPD diagnosis A diagnosis of EBV-LPD was preferably based on lymph node histology or cytology and was classified according to the criteria of Knowles et al[44]. Immunohistology included antibody staining with CD19-specific (Becton Dickinson, San José, CA, USA), CD20-specific (DAKO, Glostrup, Denmark) and EBV latent membrane protein-1-specific (DAKO) monoclonal antibodies. Furthermore, clonality was assessed using immunohistochemical staining with monoclonal antibodies to kappa and lambda light chains (DAKO). In situ hybridisation was performed to detect EBV-encoded small RNA mole-

cules (EBV-EBER) using an EBV-EBER probe (DAKO) and PCR for detection of EBV-DNA encoding for the *Bam*HI fragment. EBV-LPD staging included physical examination, whole-body computed tomography scanning (CT) scanning, and flow cytometric detection of monoclonal B lymphocytes in blood, bone marrow and, if indicated, cerebrospinal fluid.

Endpoints and statistical analysis The data were analysed as of January 2000. Patient characteristics of non-TCD patients and TCD patients were compared using Fisher exact test or Pearson chi-square test, whichever was appropriate, in case of discrete variables, or the Wilcoxon rank-sum test in case of continuous variables. End points of the study included time to EBV reactivation, EBV-LPD, acute GVHD grades II to IV, chronic GVHD and treatment-related mortality (TRM). Time to first EBV reactivation was determined from the date of transplantation until day 180 and patients were censored at the date of last serum sample if this sample had been taken before day 180. Time to EBV-LPD was measured from SCT until EBV-LPD. Patients who died without EBV-LPD were censored at the date of death. Patients still alive at the date of analysis were censored at the last follow-up date. Two EBV-seronegative donor-recipient pairs were excluded from the analysis of EBV reactivation and EBV-LPD. GVHD was diagnosed and graded according to consensus criteria[45]. Chronic GVHD was evaluated among patients who survived at least 100 days after transplantation. TRM was defined according to standard criteria[46]. Time to EBV reactivation, EBV-LPD, acute and chronic GVHD, and TRM were estimated by the Kaplan-Meier method, and Kaplan-Meier curves were generated to illustrate differences between subgroups of patients[47]. The following variables were included in the analysis of prognostic factors: sex, male patient and female donor, age, risk status, donor (MRD versus MUD), source of stem cells (bone marrow versus peripheral blood), type of transplant (non-TCD versus TCD without ATG versus TCD with ATG) and graft characteristics (number of mononuclear cells, number of CD34+ cells, number of CD3+ cells and granulocyte-macrophage colony forming units infused). Univariate survival analysis was performed using the log-rank test[48]. The variables that appeared significant in the univariate analysis were included in a multivariate Cox regression[49]. Moreover, Cox regression was performed using EBV reactivation within day 180 as a time-dependent covariate to assess whether EBV reactivation predicted EBV-LPD and TRM. All reported P-values are 2-sided and a significance level of $p=0.05$ was used.

Results

EBV-reactivation The probability of developing EBV reactivation was greater after TCD allogeneic SCT than after non-TCD SCT (Figure 1, Table 2). That difference, however, could be largely attributed to the use of ATG in conjunction with TCD (Figure 1, Table 3). Probabilities of viral reactivation were not different between recipients of a non-TCD SCT and recipients of TCD SCT without concomitant ATG. Median time to first reactivation was 58 days (range, 5-180 days) in the TCD group and 63 days (range, 2-107 days) in the non-TCD group (not significant). Plasma EBV-DNA levels measured at the peak of the first reactivation did not differ between the groups. Recurrent reactivation was significantly more frequent after TCD (Table 2): 14 of 85 patients (16%) experienced multiple episodes of EBV reactivation after TCD SCT, including 8 patients with 2 episodes, 5 patients with 3 episodes, and 1 patient showing 4 distinct periods of reactivation. This is exemplified for a recipient of a TCD donor graft who experienced 3 episodes of EBV reactivation without developing EBV-LPD (Figure 2). In contrast, only 2 of 65 patients (3%) receiving non-TCD grafts had a second period of reactivation. ATG appeared not to be associated with recurrent reactivation, as only 2 out of 14 patients with recurrent reactivation after TCD also received ATG as part of the conditioning regimen.

Table 2 EBV reactivation and EBV-LPD

Parameter	TCD SCT (n=85)	non-TCD SCT (n=65)
No. of patients with EBV reactivation (%)	46 (54)	18 (28)
Days post-transplant to first EBV reactivation	58 (5-180)	63 (2-107)
Maximum VL (c/ml) of first reactivation	535 (50-3200000)	808 (55-540000)
No. of patients with recurrent reactivation (%)	14 (16)	2 (3)
No. of patients with EBV-LPD (%)	10 (12)	0
Days post-transplant to EBV-LPD	87 (50-168)	0
Days from first EBV reactivation to EBV-LPD	22 (13-120)	0
EBV-LPD VL (c/ml)	110000 (1800-790000)	

Data are expressed as median (range), unless otherwise indicated; VL = viral load; other abbreviations: see Table I.

Figure 1

Upper panel: Incidence of EBV-reactivation after TCD allogeneic SCT with ATG (n=24), TCD SCT without ATG (n=61), and non-TCD SCT (n=65). Only TCD combined with ATG significantly increased the risk of EBV reactivation (p<0.001).
Lower panel: Incidence of EBV reactivation by number of CD34+ cells in the graft. The median number of CD34+ cells was 1.35 x 10^6/kg. Patients with grafts containing more than 1.35 x 10^6/kg CD34+ cells were at higher risk (p=0.001) of EBV reactivation.

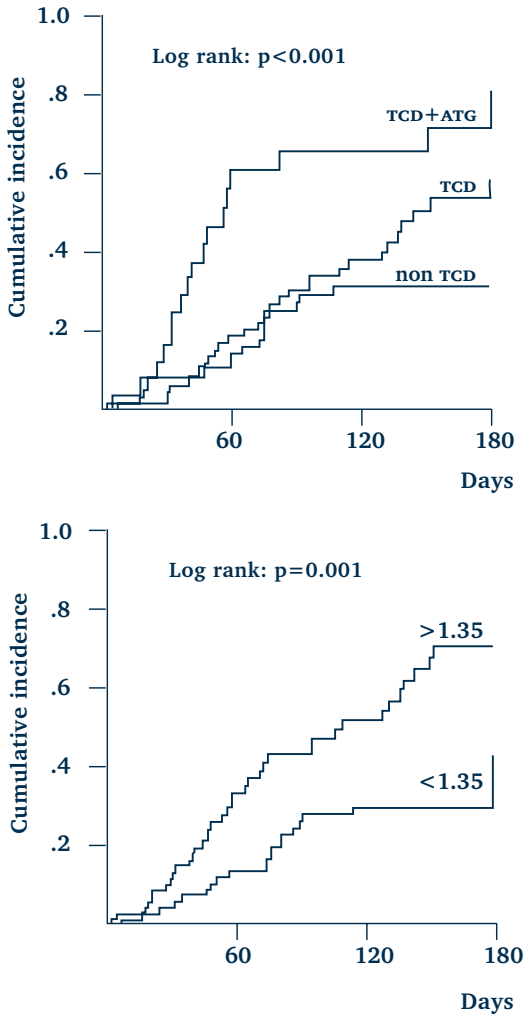
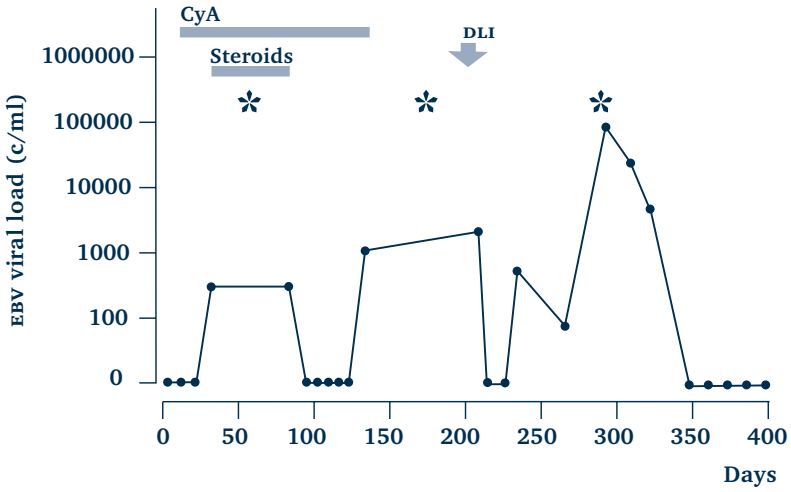


Figure 2

Monitoring of EBV viral load after a matched unrelated SCT. A 16-year-old EBV-seropositive male with a philadelphia chromosome-positive (Ph+) ALL in first complete remission received a TCD matched unrelated donor graft from an EBV-seropositive donor. Multiple EBV reactivations were observed; however, no EBV-LPD ensued. Frequent examination of bone marrow for the presence of monoclonal B cells and whole-body CT to detect lymphadenopathy were negative at various time points (*). At day 211, DLI (1.0 x 10^5 CD3+ T cells/kg) was administered because of molecular relapse of his Ph+ ALL. Currently, the patient is free of disease and well at day 800 after SCT. CyA indicates cyclosporin A.



Several risk factors predicted for first reactivation in univariate analysis (Table 3), including TCD (p=0.02), use of ATG in the conditioning regimen (p<0.001), transplantation of unrelated donor graft (p=0.02), and a high CD34+ cell number of the graft (p=0.001) (Figure 1). Following multivariate analysis, only use of ATG, a high CD34+ cell count (>1.35x10^6/kg) and a high-risk disease status remained independently associated with EBV reactivation (Table 3). Numbers of CD34+ and CD3+ cells were not associated with each other.

EBV-LPD EBV-LPD was only observed following TCD SCT (Table 2, Figure 3). Five patients developed EBV-LPD after HLA identical sibling SCT and 5 after unrelated donor SCT (Table 4). Five of these patients had received ATG before unrelated donor SCT, and 9

Table 3 Univariate and multivariate Cox regression analysis of risk factors for Epstein-Barr virus reactivation

Risk factor	Univariate Analysis			Multivariate analysis		
	RR	95% CI	P	RR	95% CI	P
TCD, no ATG	1.5	0.8-2.7	0.02	1.5	0.8-2.9	0.3
TCD, ATG	3.5	1.8-6.9	< 0.001	3.4	1.6-7.1	0.001
High-risk status	1.6	1.0-2.8	0.07	1.4	0.8-2.6	0.02
MUD	1.8	1.1-2.9	0.02	0.9	0.3-2.9	0.8
CD34+ cell count*	2.4	1.4-4.1	0.001	2.6	1.5-4.6	0.001

* = >1.35 x 10^6/kg.

Figure 3

Incidence of EBV-LPD (n=10) after TCD allogeneic SCT combined with ATG (n=24), TCD SCT without ATG (n=61), and non-TCD SCT (n=65).

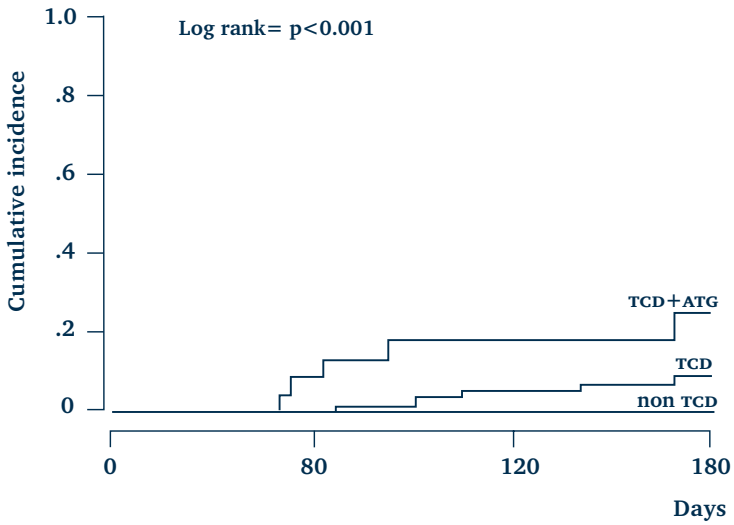


Table 4 Epstein-Barr virus-lymphoproliferative disease following TCD SCT

		EBV-LPD diagnosis			Therapy			Outcome		
Patient no.	Donor	Morpho-logy	Clonality	Viral load	SI	anti CD20	DLI	Response	Survival, d	COD
1	MRD	III	Mono	1800	+	-	+	PD	Dead	LPD
2	MUD	II	Poly	92000	+	+	+	CR	Dead	GVHD
3	MUD	II	Poly	6500	+	+	-	CR	Alive, 620	
4	MRD	III	Mono	790000	+	+	+	PD	Dead	LPD
5	MRD	III	Mono	128000	+	+	-	CR	Alive, 351	
6	MRD	II	Mono	74000	+	+	-	CR	Dead	GVHD
7	MUD	III	Mono	133000	+	+	+	PD	Dead	LPD
8	MRD	ND	Mono	7900	+	-	-	PD	Dead	LPD
9	MUD	ND	ND	310000	-	-	+	CR	Dead	GVHD
10	MUD	III	Mono	206000	-	-	-	PD	Dead	LPD

SCT indicates stem cell transplantation; MRD = matched related donor; MUD = matched unrelated donor; EBV-LPD = Epstein-Barr virus associated lymphoproliferative disease; I = plasmacell hyperplasia; II = polymorphic hyperplasia; III = Non-Hodgkin's lymphoma (criteria according to Knowles et al[45]); ND = not determined; Mono = monoclonal disease; Poly = polyclonal disease; SI = stop immunosuppression; anti-CD20 = monoclonal anti B cell therapy; DLI = donor lymphocyte infusion; PD = progressive disease; CR = complete remission; COD = cause of death; GVHD = graft-versus-host disease.

of them had been treated for high-risk disease. All EBV-LPD donor-recipient pairs were EBV-seropositive. One donor had negative EBV serology before transplantation. Median time from first reactivation to EBV-LPD was 22 days (range, 13-120 days) (Table 2). Median EBV-DNA level at EBV-LPD diagnosis was 110000 c/ml (range 1800-790000). Histological proof of a diagnosis of EBV-LPD and classification according to the criteria of Knowles et al[44] were obtained in 8 patients. Patient 8 (Table 4), who received an HLA-identical sibling SCT for multiple myeloma, was diagnosed with EBV-LPD by the presence of monoclonal B cells in his cerebrospinal fluid and an elevated plasma EBV-DNA level. Patient 9, who received an unrelated donor SCT because of SAA, was diagnosed with EBV-LPD because of massive lymphadenopathy on CT scanning and a highly elevated plasma EBV-DNA level. Six patients received anti B cell monoclonal antibody therapy (rituximab), 5 patients received DLI, and immune suppression was discontinued in 8 patients (Table 4). Five patients obtained a complete remission and 5 other patients died of progressive EBV-LPD. Two responding patients are currently alive with a follow-up of 620 and 351 days. Three responding patients developed severe GVHD, 2 following DLI, and died due to GVHD-related complications. Use of ATG, application of TCD, and high-risk status of underlying disease significantly predicted EBV-LPD in univariate analysis. Multivariate analysis was not performed because the latter 3 variables appeared strongly associated and the small number of events did not allow a reliable multivariate analysis.

Several risk factors occurring after SCT were evaluated for a possible association with EBV-LPD by time-dependent analysis. A lower lymphocyte count at first EBV reactivation appeared not predictive for developing EBV-LPD. In contrast, EBV load significantly predicted EBV-LPD in a quantitative manner. A stepwise increase of EBV DNA by 1 log (Table 5) yielded a hazard ratio (HR) of 2.9 (95% confidence interval [CI], 1.7-4.8) for those patients receiving a TCD graft ($p<0.001$). Numbers of patients with a TCD SCT with plasma levels of EBV DNA exceeding a certain threshold value and the corresponding positive and negative predictive values for EBV-LPD for that subset of patients are shown in Table 5. Although the positive predictive value was 24% for patients with a copy number of 100 c/ml or higher, it rose to 100% at the level of 500000 c/ml. However, only one patient with EBV-LPD reached that high number, and consequently the negative predictive value measured 89%.

Table 5 Incidence of EBV-LPD by viral load

EBV viral load (c/ml)	No. of patients with reactivations	No. of patients with EBV-LPD	Predictive value	
			Positive (%)	Negative (%)
100	41	10	24	100
1000	26	10	39	100
10000	14	7	50	96
100000	7	5	71	94
500000	1	1	100	89

Abbreviations: see Table 1.

Graft-versus-host disease The actuarial probability of acute GVHD II-IV at day 100 was 57% ± 4% for the whole group and was not significantly different for patients receiving a TCD graft as compared with patients following unmanipulated SCT. An unrelated donor graft and a high CD34+ cell count of the graft (independent from the number of CD3+ T cells in the graft) were the only significant risk factors for developing acute GVHD following multivariate analysis. EBV reactivation was not associated with acute GVHD. Actuarial probabilities of chronic limited and extensive GVHD at 12 months post SCT were significantly higher for non-TCD patients (83% ± 5%) than for TCD patients (38% ± 6%) ($p<0.001$).

Treatment-related mortality The actuarial probability of TRM was 29% ± 4% at 1 year for all patients and did not differ between TCD and unmanipulated SCT. Higher age and a higher CD34+ cell count (> 1.35 x 10^6/kg) of the graft predicted higher TRM in multivariate analysis. Following time-dependent analysis, EBV reactivation (HR: 1.9, 95% CI: 1.0-3.3, $p=0.04$) and acute GVHD grade I-IV (HR: 1.8, 95% CI: 1.0-3.3, $p=0.05$) were associated with higher TRM. In addition, a higher lymphocyte count (> 0.6 x 10^9/l) at the time of first EBV reactivation significantly predicted less TRM (HR 0.3; 95% CI, 0.1-0.8; $p=0.02$).

Discussion

This study demonstrates that EBV reactivation is a very frequent event after both TCD and unmanipulated SCT. In particular, recipients of stem cell grafts with high numbers of CD34+ cell counts appeared to be at risk for EBV reactivation. However, patients receiving a TCD SCT were at significantly higher risk for recurrent reactivation and only these patients developed EBV-LPD. The development of impending EBV-LPD in these patients could be predicted quantitatively by monitoring viral load in plasma at regular intervals during the first 6 months after SCT.

EBV reactivation was observed frequently after TCD SCT and after unmanipulated SCT as well. The high incidence of first EBV reactivation after TCD SCT could be largely attributed to the use of ATG and, as a result, TCD per se did not appear to be an independent risk factor for early EBV reactivation. However, patients receiving a TCD SCT showed more recurrence of reactivation and EBV-LPD was observed only after TCD. Because the conditioning regimen has eradicated autologous EBV-specific immunity after both TCD and unmanipulated SCT, early EBV reactivation may occur after both modes of SCT[50,51]. However, the significantly higher risks for recurrent EBV reactivation and EBV-LPD in TCD SCT as compared with unmanipulated SCT may be explained by the impaired capacity of patients receiving TCD grafts to mount an effective immune response to the reactivating virus. The strongly reduced numbers of EBV-specific memory T cells in TCD as compared with unmanipulated grafts may play a major role in this respect[52,53]. Apart from the use of ATG as part of the conditioning regimen, we identified the number of CD34+ cells in the graft as a novel independent risk factor for developing EBV reactivation (Table 3, Figure 1), and also for acute GVHD and TRM. Przepiorka et al[54] recently reported that recipients of peripheral blood stem cell grafts with high CD34+ cell counts were at higher risk for acute GVHD, an effect that appeared independent of the number of CD3+ T cells[54]. They suggested that GVHD at high CD34+ cell doses may be exacerbated by cytokines released by the markedly expanding myeloid population at the time of engraftment. This explanation is supported by high levels of pro-inflammatory cytokines in patients with severe GVHD[55-57]. In the present study, acute GVHD significantly predicted TRM in a time-dependent analysis. Therefore, the association of CD34+ cell dose and TRM might be explained by an increased incidence of GVHD. The association of CD34+ cell dose and EBV reactivation is, however, less likely to be explained by more GVHD, as EBV reactivation preceded the onset of acute GVHD in a significant num-

ber of patients. Alternative explanations may include infusion of a higher number of EBV-infected B cells together with larger stem cell grafts, or stimulation of B-cell proliferation by cytokines produced by the higher number of rapidly maturing myeloid progenitors. The latter explanation is supported by a number of preclinical as well as clinical studies showing that proinflammatory cytokines, such as interleukin-1 (IL-1), tumor necrosis factor *α* and *-β*, and IL-6, may very effectively stimulate the growth of EBV-infected B cells[58]. In particular, IL-6 may play an important role as a growth factor, promoting the progression toward overt EBV-LPD[59-62]. Apart from monocyte-macrophages and endothelial cells as an established source of proinflammatory cytokines, the rapid proliferating myeloid population of grafts containing high CD34+ cell doses may add to cytokine release and thus contribute to viral reactivation.

A number of studies have demonstrated a correlation between high levels of viral load and a diagnosis of EBV-LPD after both SCT and solid-organ transplantation[26-43]. No study, however, has longitudinally followed SCT recipients with multiple risk factors from day 0 until day 180 and reported positive and negative predictive values. Lucas et al[41] evaluated the predictive value of a quantitative PCR using DNA extracted from peripheral blood MNCs in a cohort of 195 patients receiving a solid-organ transplantation[41]. Although the negative predictive value appeared very high (100%), the positive predictive value was 38%. Our results observed in recipients of an SCT are in line with these findings. Considering both TCD and non-TCD transplants, the negative and positive predictive values of a copy number of 1000 c/ml were, respectively, 100% and 28%. Higher predictive values were obtained when the analysis was restricted to patients receiving a TCD SCT. The positive predictive value of a high EBV-DNA level of more than 1000 c/ml and more than 10000 c/ml for patients receiving a TCD SCT were 39% and 50%, respectively (Table 5).

Although highly significant, these predictive values also indicate that most patients (even recipients of TCD grafts) were able to mount an effective immune response and clear their viral reactivation. Monitoring of the reconstitution of HLA-specific T lymphocytes may add to the predictive value of viral load quantification. For this purpose, rapid assays are now available, such as the enumeration of EBV-specific T lymphocytes by tetramer binding or the induction of intracellular interferon-γ in T cells after specific stimulation[63]. The accurate prediction of impending EBV-LPD in patients at risk is important because pre-emptive therapy might be more effective than therapy of established EBV-LPD. Despite the application of new treatment modalities such as DLI and anti B cell immunotherapy, the mortality of patients

with established EBV-LPD is still high. Ten patients developed EBV-LPD in the present study: 5 died due to progressive EBV-LPD and 3 patients secondary to GVHD following DLI, resulting in a 80% (8 of 10) mortality. Pre-emptive infusion of EBV-specific cytotoxic T cells has been shown to reduce viral load and may prevent the evolution toward EBV-LPD[20]. However, the preparation and use of such EBV-specific T cells is expensive and difficult to implement on a wide scale. B cell depletion of the donor graft has been shown to effectively reduce the incidence of EBV-LPD[7,16]. Therefore, anti B cell immunotherapy aimed at in vivo B cell depletion after SCT in patients at high risk of EBV-LPD might be a promising new means of pre-emptive therapy. A prospective phase II study with that specific aim is currently being performed[64]. Because the depletion of B cells may add to the impaired immune status of these patients, one may argue to restrict pre-emptive therapy to those patients at highest risk. A threshold of 1000 c/ml, as observed in our patient population, may thereby serve as a critical level of viral load to start pre-emptive therapy. Thus, pre-emptive therapy may be administered selectively to high-risk patients to prevent EBV-LPD and to avoid treatment of patients who have recovered their EBV-specific immunity to protective levels. The frequent monitoring of EBV load after SCT may therefore be considered for patients with a high-risk profile for EBV-LPD and may preferably be combined with close monitoring of the reconstitution of EBV-specific T lymphocytes.

References

1 Shapiro RS, McClain K, Frizzera G, Gajl-Peczalska KJ, Kersey JH, Blazar BR, Arthur DC, Patton DF, Greenberg JS, Burke B, Ramsay NKC, Filipovich AH. Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. Blood 1988; 71: 1234-1243.

2 Zutter MM, Martin PJ, Sale GE, Shulman HM, Fisher L, Thomas ED, Durnam DM. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. Blood 1988; 72: 520-529.

3 Hanto DW, Frizzera G, Gajl-Peczalska KJ, Simmons RL. Epstein-Barr virus, immunodeficiency, and B-cell lymphoproliferation. Transplantation 1985; 39: 461-472.

4 Gross TG, Steinbusch M, DeFor T, Shapiro RS, McGlave P, Ramsay NKC, Wagner JE, Filipovich AH. B cell lymphoproliferative disorders following hematopoetic stem cell transplantation: risk factors, treatment and outcome. Bone Marrow Transplant 1999; 23: 251-258.

5 Micallef INM, Chhanabhai M, Gascoyne RD, Shepherd JD, Fung HC, Nantel SH, Toze CL, Klingemann H-G, Sutherland HJ, Hogge DE, Nevill TJ, Le A, Barnett MJ. Lymphoproliferative disorders following allogeneic bone marrow transplantation: the Vancouver experience. Bone Marrow Transplant 1998; 22: 981-987.

6 Curtis RE, Travis LB, Rowlings PA, Socié G, Kingma DW, Banks PM, Jaffe ES, Sale GE, Horowitz MM, Witherspoon RP, Shriner DA, Weisdorf DJ, Kolb H-J, Sullivan KM, Sobocinski KA, Gale RP, Hoover RN, Fraumeni JF, Deeg HJ. Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. Blood 1999; 94: 2208-2216.

7 Hale G, Waldmann H for CAMPATH Users. Risks of developing Epstein-Barr virus-related lymphoproliferative disorders after T-cell-depleted marrow transplants. Blood 1998; 91: 3079-3080.

8 Walker RC, Marshall WF, Strickler JG, Wiesner RH, Velosa JA, Habermann TM, McGregor CGA, Paya CV. Pretransplantation assessment of the risk of lymphoproliferative disorder. Clin Infect Dis 1995; 20: 1346-1353.

9 Fischer A, Blanche S, Le Bidois J, Bordigoni P, Garnier JL, Niaudet P, Morinet F, Le Deist F, Fischer A. Anti-B-cell monoclonal antibodies in the treatment of severe B-cell lymphoproliferative syndrome following bone marrow and organ transplantation. N Engl J Med 1991; 324: 1451-1456.

10 Faye A, Van den Abeele T, Peuchmaur M, Mathieu-Boue A, Vilmer E. Anti-CD20 monoclonal antibody for post-transplant lymphoproliferative disorders. Lancet 1998; 352: 1285.

11 Milpied N, Vasseur B, Parquet N, Garnier JL, Antoine C, Quartier P, Carret AS, Bouscary D, Faye A, Bourbigot B, Reguerre Y, Stoppa AM, Bourquard P, Hurault de Ligny B, Dubief F, Mathieu-Boue A, Leblond V. Humanized anti-CD20 monoclonal antibody (Rituximab) in post transplant B-lymphoproliferative disorder: a retrospective analysis on 32 patients. Ann Oncol 2000; 11: S113-S116.

12 Kuehnle J, Huls MH, Liu Z, Semmelmann M, Krance RA, Brenner MK, Rooney CM, Heslop HE. CD20 monoclonal antibody (Rituximab) for therapy of Epstein-Barr virus lymphoma after hemopoetic stem-cell transplantation. Blood 2000; 95: 1502-1505.

13 Papadopoulos E, Ladanyi M, Emmanuel D, Mackinnon S, Boulad F, Carabashi MH, Castro-Malaspina H, Childs BH, Gillio AP, Small TN, Young JW, Kernan NA, O'Reilly RJ. Infusions of donor lymphocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. N Engl J Med 1994; 330: 1185-1191.

14 Heslop HE, Ng CYC, Li C, Smith CA, Loftin SK, Krance RA, Brenner MK, Rooney CM. Long term restauration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. Nat Med 1996; 2: 551-555.

15 Rooney CM, Smith CA, Ng CYC, Loftin S, Li C, Krance RA, Brenner MK, Heslop HE. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. Lancet 1995; 345: 9-13.

16 Cavazzana-Calvo M, Bensoussan D, Jabado J, Haddad E, Yvon E, Moskwa M, Tachet des Combes A, Buisson M, Morand P, Virion JM, Le Deist F, Fischer A. Prevention of EBV-induced B-lymphoproliferative disorder by ex vivo marrow B-cell depletion in HLA-phenoidentical or non-identical T-depleted bone marrow transplantation. Br J Haematol 1998; 103: 543-551.

17 McDiarmid SV, Jordan S, Lee GS, Toyoda M, Goss JA, Vargas JH, Martin MG, Bahar R, Maxfield AL, Ament ME, Busuttil RW. Prevention and pre-emptive therapy of posttransplant lymphoproliferative disease in pediatric liver recipients. Transplantation 1998; 66: 1604-1611.

18 Green M, Kaufmann M, Wilson J, Reyes J. Comparison of intravenous ganciclovir followed by oral acyclovir with intravenous ganciclovir alone for prevention of cytomegalovirus and Epstein-Barr virus disease after liver transplantation in children. Clin Inf Dis 1997; 25: 1344-1349.

19 Kuo PC, Dafoe DC, Alfrey EJ, Sibley RK, Scandling JD. Posttransplant lymphoproliferative disorders and Epstein-Barr virus prophylaxis. Transplantation 1995; 59: 135-138.

20 Gustafsson A, Levitsky V, Zou JZ, Frisan T, Dalianis T, Ljungman P, Ringden O, Winiarski J, Ernberg I, Masucci MG. Epstein-Barr (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. Blood 2000; 95: 807-814.

21 Davis CL, Harrison KL, McVicar JP, Forg PJ, Bronner MP, Marsh CL. Antiviral prophylaxis and the Epstein-Barr virus-related post-transplant lymphoproliferative disorder. Clin Transplant 1995; 9: 53-59.

22 Trigg ME, Finlay JL, Sondel PM. Prophylactic acyclovir in patients receiving bone marrow transplants [letter]. N Engl J Med 1985: 26: 1708-1709.

23 Darenkov IA, Marcarelli MA, Basadonna GP, Friedman AL, Lorber KM, Howe JG, Crouch J, Bia MJ, Kliger AS, Lorber MI. Reduced incidence of Epstein-Barr virus-associated posttransplant lymphoproliferative disorder using pre-emptive antiviral therapy. Transplantation 1997; 64: 848-852.

24 Green M, Reyes T, Jabbour N, Yunis E, Putnam P, Todo S, Rowe D. Use of quantitative PCR to predict onset of Epstein-Barr viral infection and post-transplant lymphoproliferative disease after intestinal transplantation in children. Transplant Pro 1996; 28: 2759-2760.

25 Niesters HGM, Van Esser J, Fries E, Wolthers KC, Cornelissen JJ, Osterhaus ADME. Development of a real-time quantitative assay for detection of Epstein-Barr virus. J Clin Microbiol 2000; 38: 712-715.

26 Van Esser JWJ, Niesters HGM, Thijsen SFT, Meijer E, Osterhaus ADME, Wolthers KC, Boucher ChAB, Gratama JW, Budel LM, Van Der Holt B, Van Loon AM, Löwenberg B, Verdonck LF, Cornelissen JJ. Molecular quantification of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus-associated lymphoproliferative disease after allogeneic stem cell transplantation. Br J Haematol 2001; 113: 814-821.

27 Lucas KG, Burton RL, Zimmerman SE, Wang J, Cornetta KG, Robertson KA, Lee CH, Emanuel DJ. Semiquantitative Epstein-Barr virus (EBV) polymerase chain reaction for the determination of patients at risk for EBV-induced lymphoproliferative disease after stem cell transplantation. Blood 1998; 91: 3654-3661.

28 Savoie A, Perpête C, Carpentier L, Joncas J, Alfieri C. Direct correlation between the load of Epstein-Barr virus-infected lymphocytes in the peripheral blood of pediatric transplant patients and risk of lymphoproliferative disease. Blood 1994; 83: 2715-2722.

29 Beck R, Westdörp I, Jahn G, Schäfer H, Kanz L, Einsele H. Detection of Epstein-Barr virus DNA in plasma from patients with lymphoproliferative disease after allogeneic bone marrow transplantation or peripheral blood stem cell transplantation [letter]. J Clin Microbiol 1999; 37: 3430-3431.

30 Limaye AP, Huang M-L, Atienza EE, Ferrenberg JM, Corey L. Detection of Epstein-Barr virus DNA in sera from transplant recipients with lymphoproliferative disorders. J Clin Microbiol 1999; 37: 1113-1116.

31 Rooney CM, Loftin SK, Holladay MS, Brenner MK, Krance RA, Heslop HE. Early identification of Epstein-Barr virus-associated post-transplantation lymphoproliferative disease. Br J Haematol 1995; 89: 98-103.

32 Hoshino Y, Kimura H, Kuzushima, Tsurumi T, Nemoto K, Kikuta A, Nishiyama Y, Kojima S, Matsuyama T, Morishima T. Early intervention in post-transplant lymphoproliferative disorders based on Epstein-Barr viral load. Bone Marrow Transplant 2000; 26: 199-201.

33 Rowe DT, Qu L, Reyes N, Jabbour N, Yunis E, Putnam P, Todo S Green M. Use of quantitative competitive PCR to measure Epstein-Barr virus genome load in the peripheral blood of pediatric transplant patients with lymphoproliferative disorders. J Clin Microbiol 1997; 35: 1612-1615.

34 Kenagy DN, Schlesinger Y, Weck K, Ritter JH, Gaudreault-Keener MM, Storch GA. Epstein-Barr virus DNA in peripheral blood leucocytes of patients with posttransplant lymphoproliferative disease. Transplantation 1995; 60: 547-554.

35 Telenti A, Marshall WF, Smith TF. Detection of Epstein-Barr virus by polymerase chain reaction. J Clin Microbiol 1990; 28: 2187-2190.

36 Yamamoto M, Kimura H, Hironaka T, Hirai K, Hasegawa S, Kuzushima K, Shibata M, Morishima T. Detection and quantification of virus DNA in plasma of patients with Epstein-Barr virus associated diseases. J Clin Microbiol 1995; 33: 1765-1768.

37 Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, Matsuyama T, Morishima T. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol 1999; 37: 132-136.

38 Riddler SA, Breinig MC, McKnight LC. Increased levels of circulating Epstein-Barr virus (EBV)-infected lymphocytes and decreased EBV nuclear antigen antibody responses are associated with the development of posttransplant lymphoproliferative disease in solid-organ transplant recipients. Blood 1994; 84: 972-984.

39 Green M, Cacciarelli TV, Mazariegos GV, Sigurdsson L, Qu L, Rowe DT, Reyes J. Serial measurement of Epstein-Barr viral load in peripheral blood in pediatric liver transplant recipients during treatment for posttransplant lymphoproliferative disease. Transplantation 1998; 66: 1641-1644.

40 Barkholt LM, Dahl H, Enbom N, Lindé. Epstein-Barr virus DNA in serum after liver transplantation - surveillance of viral activity during treatment with different immunosuppressive agents. Transpl Int 1996; 9: 439-445.

41 Lucas KG, Filo R, Heilman DK, Lee CH, Emmanuel DJ. Semiquantitative Epstein-Barr virus polymerase chain reaction analysis of peripheral blood from organ transplant patients at risk for the development of lymphoproliferative disease. Blood 1998; 92: 3977-3978. Letter.

42 Krieger NR, Martinez OM, Krams SM, Cox K, So S, Esquivel CO. Significance of detecting Epstein-Barr-specific sequences in the peripheral blood of asymptomatic pediatric liver transplant recipients. Liver Transplantation 2000; 6: 62-66.

43 Baldanti F, Grossi P, Furione M, Simoncini L, Sarasini A, Comoli P, Maccario R, Fiocchi R, Gerna G. High levels of Epstein-Barr virus DNA in blood of solid organ transplant recipients and their value in predicting posttransplant lymphoproliferative disorders. J Clin Microbiol 2000; 38: 613-619.

44 Knowles DM, Cesarman E, Chadburn A, Frizzera G, Chen J, Rose EA, Michler RE. Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of posttransplantation lymphoproliferative disorders. Blood 1995; 85: 552-565.

45 Przepriorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, Thomas ED. 1994 consensus conference on acute GVHD grading. Bone Marrow Transplant 1995; 15: 825-829.

46 Clift R, Goldman J, Gratwohl A, Horowitz M. Proposals for standardized reporting of results of bone marrow transplantation for leukaemia. Bone Marrow Transplant 1989; 4: 445-451.

47 Kaplan EL, Meier P. Nonparametric estimation for incomplete observations. J Am Stat Assoc 1958; 53: 457-481.

48 Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966; 50: 163-170.

49 Cox DR. Regression models and life-tables (with discussion). J R Stat Soc B 1972; 34: 187-220.

50 Gratama JW, Oosterveer MAP, Zwaan FE, Lepoutre J, Klein G, Ernberg I. Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: implications for sites of viral latency. Proc Natl Acad Sci USA 1988; 85: 8693-8696.

51 Gratama JW, Oosterveer MAP, Lepoutre JMM, Van Rood JJ, Zwaan FE, Vossen JMJJ, Kapsenberg JG, Richel D, Klein G, Ernberg I. Serologic and molecular studies of Epstein-Barr virus infection in allogeneic marrow graft recipients. Transplantation 1990; 49: 725-730.

52 Lucas KG, Small TN, Heller G, Dupont B, O'Reilly RJ. The development of cellular immunity to Epstein-Barr virus after allogeneic bone marrow transplantation. Blood 1996; 87: 2594-2603.

53 Rickinson AB, Moss DJ. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. Annu Rev Immunol 1997; 15: 405-431.

54 Przepiorka D, Smith TL, Folloder J, Khouri I, Ueno NT, Mehra R, Körbling M, Huh YO, Giralt S, Gajewski J, Donato M, Cleary K, Claxton D, Braunschweig I, Van Besien K, Andersson BS, Anderlini P, Champlin R. Risk factors for acute graft-versus-host disease after allogeneic blood stem cell transplantation. Blood 1999; 94: 1465-1470.

55 Ferrara JL. Cytokine inhibitors and graft-versus-host disease. Ann N Y Acad Sci 1995; 770: 227-236.

56 Rus V, Svetic A, Nguyen P, Gause WC, Via CS. Kinetics of Th1 and Th2 cytokine production during the early course of acute and chronic murine graft-versus-host disease. Regulatory role of donor CD8+ T cells. J Immunol 1995; 155: 2396-2406.

57 Hill GR, Crawford JM, Cooke JR, Brinson YS, Pan L, Ferrara JLM. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. Blood 1997; 90: 3204-3213.

58 Tosato G, Teruya-Feldstein J, Setsuda J, Pike SE, Jones KD, Jaffe ES. Post-transplant lymphoproliferative disease (PTLD): lymphokine production and PTLD. Springer Semin Immunopathol 1998; 20: 405-423.

59 Tosato G, Tanner J, Jones KD, Revel M, Pike SE. Identification of interleukin-6 as an autocrine growth factor for Epstein-Barr virus-immortalized B-cells. J Virol 1990; 64: 3033-3041.

60 Scala G, Quinto I, Ruocco MR, Arcucci A, Mallardo M, Caretto P, Forni G, Venuta S. Expression of an exogenous interleukin 6 gene in human Epstein Barr virus B cells confers growth advantage and in vivo tumorigenicity. J Exp Med 1990; 172: 61-68.

61 Tosato G, Jones KD, Breinig MK, McWilliams HP, McKnight JLC. Interleukin-6 production in posttransplant lymphoproliferative disease. J Clin Invest 1993; 91: 2806-2814.

62 Jones K, Rivera C, Sgadari C, Franklin J, Max EE, Bhatia K, Tosato G. Infection of human endothelial cells with Epstein-Barr virus. J Exp Med 1995; 182: 1213-1221.

63 Marshall NA, Howe JG, Formica R, Krause D, Wagner JE, Berliner N, Crouch J, Pilip I, Cooper D, Blazar BR, Seropian S, Pamer EG. Rapid reconstitution of Epstein-Barr virus-specific T lymphocytes following allogeneic stem cell transplantation. Blood 2000; 96: 2814-2821.

64 Cornelissen JJ, Van Esser JWJ, Van Der Holt B, Gratama JW, Löwenberg B, Trenschel R, Schaefer UW, Frassoni F, Bacigalupo A, Meijer E, Verdonck LF, Osterhaus ADME, Niesters HGM. Pre-emptive anti B-cell immunotherapy guided by quantitative PCR effectively reduces the incidence and mortality of Epstein-Barr virus (EBV) lymphoproliferative disease (LPD) after allogeneic T-cell depleted (TCD) stem cell transplantation (SCT). Blood 2000; 96: S507a. Abstract.

Chapter 5

EBV reactivations in recipients of allogeneic stem cell transplants are frequently caused by re-infections with exogenous EBV strains

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Abstract

Recipients of allogeneic stem cell transplants often have Epstein-Barr virus (EBV) reactivations which may progress to EBV-associated lymphoproliferative disorders. It is not known whether these EBV reactivations are true reactivations of the endogenous EBV strain or re-infections with an exogenous EBV strain. Fifty-three recipients of matched related or matched unrelated donor grafts were studied. EBV monitoring was based on a realtime TaqMan™ EBV DNA PCR assay in plasma. In 17 patients EBV DNA PCR monitoring was performed in peripheral blood mononuclear cells (PBMCs) as well. From all patients and family donors mouth washes (MWs) were collected pre-transplant. Both pre-transplant EBV DNA from MWs and post-transplant EBV DNA from plasma or PBMCs was successfully obtained in 6 patients. A nested PCR targeting the EBV LMP-1 C-terminus gene was used to determine sequence variations enabling EBV strain typing. In 3 of 6 patients the post-transplant EBV sequence pattern differed from the pre-transplant pattern, indicating a re-infection post-transplant with an exogenous strain instead of a reactivation of the original endogenous EBV strain. In the other 3 patients the endogenous strain was identified. EBV reactivations frequently result from a re-infection with an exogenous EBV strain instead of a true reactivation of the endogenous strain.

Introduction

Recipients of allogeneic stem cell transplants (SCT) often have Epstein-Barr virus (EBV) reactivations[1]. Patients with EBV reactivations are at risk for developing EBV-associated lymphoproliferative disorders (EBV-LPD)[2], especially when other risk factors are present like: 1) T cell depletion (TCD) of stem cells using monoclonal antibodies (Moabs) directed at T cells or T and NK cells or using E-rosetting, 2) use of unrelated or ≥ 2 HLA antigen mismatched related donors, 3) use of Antithymocyteglobulin for prophylaxis or treatment of acute graft-versus-host disease (aGVHD) and 4) treatment of aGVHD with anti CD3 Moabs[1,3].
Currently it is not known whether these EBV reactivations are true reactivations of the endogenous EBV strain or re-infections with an exogenous EBV strain. EBV strains can be identified by examining size variation of the EBV nuclear antigen (EBNA) proteins (ebnotyping) or genes[4,5]. Ebnotyping requires the production of EBV transformed lymphoblastoid cell lines (LCLs). This technique is laborious and not all EBV strains are capable of efficient transformation of B cells[4]. Others have used DNA restriction fragment length polymorphisms (RFLP) of EBV DNA[6,7] or sequence analysis of the latent membrane protein (LMP)-1 gene[8-11] to characterise EBV strains. Here we report results of the LMP-1 sequencing approach for EBV strain identification in recipients of partially T cell depleted allogeneic SCT, using a nested PCR and direct sequencing targeting the EBV LMP-1 C-terminus gene to determine sequence variations.

Materials and Methods

Patients For this study data of 53 consecutively treated patients receiving either stem cells from matched related donors (MRD, n=26) or from matched unrelated donors (MUD, n=27) were analysed. EBV monitoring was performed weekly by quantitative EBV DNA detection in plasma samples until day 180 post-transplant. In 17 patients EBV DNA detection was performed in PBMCs as well. From all patients and family donors mouth washes (MWs) were collected pre-transplant to acquire endogenous EBV DNA. In 6 patients pre- and post-transplant EBV DNA could be obtained and analysed.

Patients with acute leukaemia in first complete remission, chronic myeloid leukaemia (CML) in first chronic phase and untreated severe aplastic anaemia (SAA) were considered low-risk regarding transplant related mortality (TRM) and relapse. All patients with other diseases were considered high-risk. Transplantations were performed between April 2001 and May 2002 at the Department of Haematology of the University Medical Centre Utrecht. Patients were treated according to clinical protocols approved by the local investigation review board after informed consent was obtained.

Transplantation procedures, pre-emptive treatment of CMV reactivations and treatment of CMV disease were performed as described[12].

Conditioning regimen The conditioning regimen consisted of cyclophosphamide (60 mg/kg/day) on each of two successive days, followed by total body irradiation (TBI) (600 cGy/day) on each of 2 successive days, with partial shielding of the lungs (total lung dose 850 cGy). The graft was infused after the second TBI fraction (day 0). Antithymocyte-globulin (ATG) (Thymoglobulin™, Sangstat, Amstelveen, the Netherlands) was given to MUD patients before cyclophosphamide was infused, in a dose of 2 mg/kg/day for 4 days.

TCD In vitro TCD of bone marrow (BM) from unrelated donors was performed using the immunorosette depletion technique as described[12]. MRD patients received a peripheral blood stem cell (PBSC) graft. TCD of these G-CSF stimulated PBSC grafts was performed by positive selection of CD34+ cells (CliniMacs™, Miltenyi Biotec, Bergisch Gladbach, Germany). After these maximal TCD procedures the residual number of T cells was counted and nonmanipulated T cells (from a small BM/PBSC fraction that was set apart) were added to obtain the desired fixed low number of T cells (1-2 x 10^5 T cells/kg recipient weight).

HLA-matching In MRD recipient/donor pairs HLA-A, B and Cw matching was based on serological typing and HLA-DRB1 and DQB1 matching on low resolution molecular typing with sequence specific primers (SSP). In MUD recipient/donor pairs HLA-A, B and DRB1 matching was based on high resolution sequence based typing and HLA-Cw and DQB1 matching on low resolution molecular typing with SSP.

CMV monitoring CMV monitoring was based on a realtime TaqMan™ CMV DNA PCR assay in EDTA-plasma[13] and was performed weekly until day 180 post-transplant in CMV-seropositive recipient/donor pairs. In patients with active GVHD or with CMV reactivation, defined as a viral load (VL) > 400 copies (c) /ml, monitoring was performed twice a week. Pre-emptive antiviral treatment of CMV reactivations was instituted when VL was > 1000 c/ml.

EBV serostatus Serum samples were tested for IgG antibodies to Epstein-Barr viral capsid antigen (VCA) by an indirect immunofluorescence assay using slides coated with EBV-infected P3HR-1 cells. Individuals were considered positive when reactivity against EBV VCA could be detected at a serum dilution of 1:10 or higher.

EBV monitoring EBV monitoring was based on a realtime TaqMan™ EBV DNA PCR assay in plasma and was performed weekly until day 180 post-transplant in all patients. In 17 patients EBV DNA PCR monitoring was performed in PBMCs as well. In patients with active GVHD or with EBV reactivation, defined as a VL > 50 c/ml plasma, monitoring was performed twice a week. Pre-emptive treatment of EBV reactivations was instituted when the EBV VL was > 1000 c/ml and consisted of rituximab (Mabthera™, Roche, Basel, Switzerland), an anti CD20 monoclonal antibody, given as a single infusion (375 mg/m^2) as described[1,14]. Immunosuppressive medication was decreased or stopped if possible. Furthermore, valacyclovir, given to all patients prophylactically in a dose of 500 mg twice a day, was increased to 1 gram 3 times a day.

Mouth washes From all patients and family donors MWs were collected pre-transplant by gargling the mouth with 15 ml phosphate buffered salt (PBS) during 1 minute. After centrifugation of the MWs for 10 minutes at 900g, the cell pellet (resuspended in 400 μL PBS) and the supernatant were stored separately at -70° C until use. The cell pellet was used to detect EBV by realtime Taqman™ EBV DNA PCR assay. When EBV was detected in these MWs, also post-transplant MWs were collected.

Peripheral blood mononuclear cells In 17 patients EBV DNA PCR monitoring post-transplant was performed in PBMCs as well. EBV reactivation in PBMCs was defined as a VL > 50 c/10^6 cells. PBMCs were isolated from 9 ml of EDTA-anticoagulated peripheral blood by Ficoll-Paque density centrifugation (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at 1000g for 10 minutes, without brake. PBMCs were washed twice with PBS and stored at -70° C.

DNA isolation DNA from plasma was isolated with the MagNA Pure LC™ total nucleic acid isolation kit - large volume (Roche Diagnostics, Almere, the Netherlands). DNA from MWs-cell pellets or PBMCs was isolated with the MagNA Pure LC™ DNA isolation kit I by an automated nucleic acid extractor (MagNA Pure extractor, Roche Diagnostics, Almere, the Netherlands).

Realtime TaqMan™ EBV DNA PCR A quantitative realtime EBV DNA PCR assay was performed as described by Niesters et al[15]. Test results were quantified by comparison with a standardised control (EBV B95-8, Advanced Biotechnologies Incorporated, Columbia, Md.). As an internal control a standardised quantity of phocine herpes virus was added to each sample. EBV test results < 50 c/ml were considered negative. In order to quantify EBV DNA in MWs-cell pellets and PBMCs an albumin realtime TaqMan™ PCR was performed in these samples as well. The primers used for the albumin PCR were 5'-TGA.AAC.ATA.CGT.TCC.CAA.AGA.GTT.T-3' (forward primer) and 5'-TCT.CTC.CTT.CTC.AGA.AAG.TGT.GCA.TA-3' (reverse primer). A fluorogenic probe (5'-TGC.TGA.AAC.ATT.CAC.CTT.CCA.TGC.AGA-3') was synthesised by PE Biosystems (Nieuwerkerk ad IJssel, the Netherlands) with a FAM reporter molecule attached to the 5' end and a TAMRA quencher linked at the 3' end. VL in PBMCs is depicted as $c/10^6$ cells.

EBV strain typing Because EBV DNA encoding the EBV LMP-1 gene is most variable, a nested PCR targeting the EBV LMP-1 C-terminus gene was used to determine sequence variations. The PCR was performed using a modified primer scheme according to Knecht et al[8]. Sequences of the outer primers 5 and 12 (primer 12 was modified) were: 5'-CTA.CAA.CAA.AAC.TGG.TGG.ACT-3', position (168.843-168.823) and 5'-AGA.CAG.TGT.GGC.TAA.GGG.AGT-3', position (168.059-168.039), respectively. Sequences of the inner primers 8' and 11 were: 5'-TGC.TCT.CAA.AAC.CTA.GGC.GCA-3', position (168.609-168.589) and 5'-TGA.TTA.GCT.AAG.GCA.TTC.CCA-3', position (168.075-168.095), respectively. The nested PCR product of the LMP-1 C-terminus PCR was used for sequencing as described[11]. Positions of the nucleotides were compared to the reference strain B95-8[16].

Statistical analysis Differences between groups were compared using Fisher's exact test or Pearson chi-square analyses, whichever was appropriate, in case of discrete variables. In case of continuous variables a Students t-test or Mann Whitney U test, whichever was appropriate, was used. Probability of EBV reactivation was calculated by the cumulative incidence procedure, death without EBV reactivation being the competing risk. The following variables were included in the analysis of prognostic factors: age, risk status (low vs high), recipient CMV serostatus (negative vs positive), donor type (related vs unrelated), graft characteristics (CD34+ cell count, T/B cell ratio), CMV reactivation (no vs yes), aGVHD (No-I vs II-IV). Univariate analyses were performed using the log rank test. Variables which showed to influence EBV reactivation at a level of $p<0.1$ were used in a multivariate Cox regression analysis. P values from regression models were calculated with the Wald test. The

post-transplant variables 'CMV reactivation' and 'aGVHD' were as well analysed as time-dependent covariates. Calculations were performed using SPSS/PC+ 10.0 (SPSS Inc, Chicago Il, USA).

Results

Patients (Table 1). Fifty-three patients were monitored weekly for EBV reactivation by quantitative EBV DNA detection in plasma. Twenty-six patients received grafts from MRDs, 27 from MUDs. MRD recipients were significantly older and were more often diagnosed with aGVHD grade II-IV compared to MUD recipients (44 vs 35 year and 50% vs 22%, respectively). The incidence of CMV reactivations was 4% in the MRD group compared to 22% in the MUD group, however, this difference was not significant.

EBV reactivations in plasma samples (Table 2). Among MRD recipients 7 (27%) patients showed an EBV VL > 50 c/ml. All 7 patients were EBV-seropositive and had a seropositive donor. Two of these 7 had a VL > 1000 c/ml and received anti B cell therapy with rituximab which resulted in clearance of plasma EBV DNA in one patient. The second patient suffered from aGVHD grade III and was given rituximab 3 times with intervals of one week. EBV VL was not responding. She finally died from pulmonary bleeding during severe aGVHD, without symptoms of EBV-LPD.

Among MUD recipients 16 (59%) patients showed an EBV VL > 50 c/ml. Twelve patient/donor pairs were EBV-seropositive (R+/D+), 2 patients were seropositive while their donor serostatus was unknown, one patient was seropositive and had a negative donor, one patient/donor pair was seronegative (R-/D-). Nine of these 16 had a VL > 1000 c/ml and received anti B cell therapy with rituximab which resulted in clearance of plasma EBV DNA in 6 patients. One of these 6 patients had lymphadenopathy and an EBV VL > 1000 c/ml at the same day. Pathologic and immunohistochemical examination of a lymph node was consistent with EBV-LPD. The seventh patient most probable was suffering from EBV-LPD with central nervous system (CNS) involvement. This patient received intensive immunosuppression because of aGVHD grade III and was treated with rituximab once, thereafter further treatment was refused and death resulted from rapid neurological deterioration. The two other patients showed increasing VL after treatment with rituximab given

Table 1 Patient characteristics

	MRD (%)	MUD (%)	P-value
No. of patients	26	27	
Age (range)	44 (21-53)	35 (17-55)	0.002
Diagnosis			ns
AML	4 (15)	8 (30)	
ALL	4 (15)	6 (22)	
CML	3 (12)	6 (22)	
SAA	1 (4)	0	
Other	14 (54)	7 (26)	
Risk status			ns
Low	10 (39)	6 (22)	
High	16 (61)	21 (78)	
EBV serostatus			ns
R+/D+	23 (89)	19 (70)	
R+/D?	0	4 (15)	
R+/D-	1 (4)	3 (11)	
R-/D+	2 (8)	0	
R-/D-	0	1 (4)	
CMV serostatus			ns
R+/D+	10 (39)	8 (30)	
R+/D-	4 (15)	7 (26)	
R-/D+	1 (4)	4 (15)	
R-/D-	11 (42)	8 (30)	
aGVHD			0.035
No-I	13 (50)	21 (78)	
II-IV	13 (50)	6 (22)	
CMV VL>1000 c/ml			ns
Yes	1 (4)	6 (22)	
No	25 (96)	21 (78)	

R/D = recipient/donor.

Table 2 Characteristics of EBV reactivations in plasma samples of MRD and MUD recipients

	MRD (n=26)	MUD (n=27)	P-value
No. of patients with EBV VL>50 c/ml (%)	7 (27)	16 (59)	0.018
No. of patients with EBV VL>1000 c/ml (%)	2 (8)	9 (33)	0.021
Median time to EBV reactivation (d; range)	109 (24-172)	57 (17-180)	ns
Median maximum viral load (c/ml; range)	190 (135-9958)	1788 (85-139.495)	ns
Median time to EBV DNA clearance after rituximab in complete responders (d; range)	10 (7-34)		

d = days.

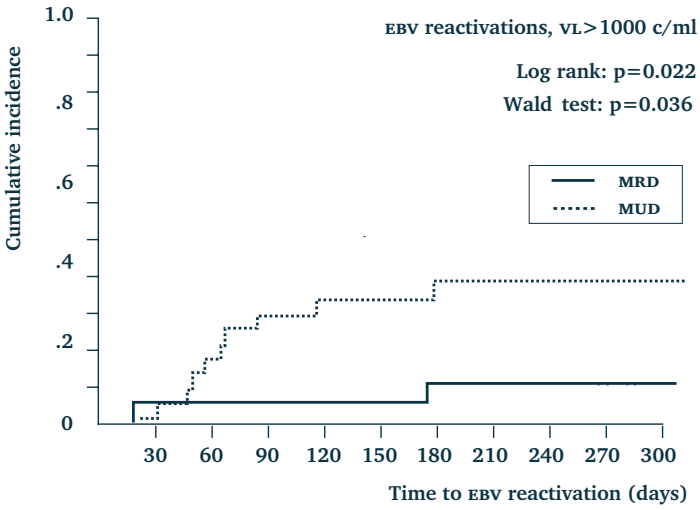
twice. Donor lymphocytes were infused (1 x 10^5 T cells/kg), resulting in a clearance of plasma EBV DNA in both patients.

Median time to EBV reactivation and median maximum VL showed a trend to be shorter and higher, respectively, in MUD compared to MRD recipients (57 vs 109 days and 1788 vs 190 c/ml, respectively, ns). Seven patients had complete responses after rituximab therapy. In 6 patients rituximab was given once, in the seventh patient twice. Median time to clearance of plasma EBV DNA after rituximab infusion was 10 days (range: 7-34). No recurrent EBV reactivations were diagnosed.

After multivariate analysis of prognostic factors for EBV reactivation (VL > 1000 c/ml) only CMV reactivation and a lower CD34+ cell count in the graft affected the incidence of EBV reactivation ($p<0.001$ and $p=0.072$, respectively). Multivariate analysis of only pre-transplant factors revealed that 'donor type' was the most important factor influencing the incidence of EBV reactivation ($p=0.036$, Figure 1). Since 6 of 7 CMV reactivations occurred in MUD recipients (see Table I), it is obvious there is an interaction between 'CMV reactivation' and 'donor type'.

Figure 1

EBV reactivations in recipients of MUD or MRD grafts.



EBV reactivations in plasma and PBMC samples (Table 3). In 17 patients (MRD: n=9; MUD: n=8) EBV monitoring was performed in plasma and PBMCs as well. Only 4 of 17 (23%) patients showed a reactivation in plasma samples compared to 15 of 17 (88%) in PBMC samples ($p<0.001$). Median time to EBV reactivation and median maximum VL showed a trend to be shorter and higher, respectively, in PBMC samples compared to plasma samples (49 vs 95 days and 5568 vs 2470 c/ml, respectively, ns). None of the patients with a reactivation in PBMCs only received rituximab and none progressed to EBV-LPD.

LMP-1 sequences of EBV in mouth washes and plasma or PBMC samples (Table 4)**.** In six patients pre-transplant EBV DNA from mouth washes and post-transplant EBV-DNA from plasma or PBMCs were successfully obtained and could be used for sequence analysis. In two of these, patient 5 and 6, also post-transplant mouth washes were analysed. Patient 1 and 2 were recipients of related grafts, only from donor 1 an EBV DNA positive mouth wash was obtained. Patients 3-6 were recipients of unrelated grafts, no donor mouth

Table 3 Results of double monitoring in plasma and PBMC samples of 17 patients

	Plasma	PBMC	P-value
No. of patients with EBV VL>50 c/ml (%)	4 (23)	15 (88)	<0.001
Median time to EBV reactivation	95	49	0.079
(d; range)	(47-159)	(17-131)	
Median maximum viral load	2470	5568	ns
(c/ml; range)	(135-12.175)	(190-937.500)	

washes were available. In 5 of 6 patients EBV serostatus was positive in both patients and donors. Patient 5 had a positive EBV serostatus, whereas donor serostatus was unknown.
In all 15 samples tested, a sequence pattern could be determined. Eight of these 15 patterns were unique. In 3 of 6 patients (patient 2, 3, and 5) the post-transplant EBV sequence pattern differed from the pre-transplant pattern, indicating a re-infection post-transplant with an exogenous strain rather than a reactivation of the original endogenous EBV strain. In patient 1 it is not possible to differentiate since pre-transplant, post-transplant and donor sequence patterns were identical. In patient 4 pre- and post-transplant sequence patterns were identical too, which indicates that a reactivation of the endogenous EBV strain is likely in this patient. Post-transplant EBV strains of patient 6 differed by only one nucleotide from the pre-transplant strain. This might have been the result of a point mutation during viral replication, making a definite conclusion about reactivation or re-infection impossible although it is more likely that this patient suffered a reactivation.
Finally, 3 of 53 recipients were EBV-seronegative pre-transplant. In 2 of these patients (1 MRD recipient with an EBV-seropositive donor; 1 MUD recipient with an EBV-seronegative donor) an EBV primo-infection was documented post-transplant.

Table 4 LMP-1 sequences of EBV in mouth washes and plasma or PBMC samples

Position	B95-8	Pat.1 MW Pre-SCT	Donor 1 MW	Pat.1 PBMC Post-SCT	Pat.2 MW Pre-SCT	Pat.2 PBMC Post-SCT	Pat.3 MW Pre-SCT	Pat.3 Plasma Post-SCT	Pat.4 MW Pre-SCT	Pat.4 PBMC Post-SCT	Pat.5 MW Pre-SCT	Pat.5 MW Post-SCT	Pat.5 PBMC Post-SCT	Pat.6 MW Pre-SCT	Pat.6 MW Post-SCT	Pat.6 PBMC Post-SCT
168357	C				G		A	G	A	A	G	G	G	A	A	A
168356	A						C					G	G			
168355	A								T	T				T	T	T
168352	G										T					
168341	T							A								
168339	G	C	C	C		C										
168337	A														C	C
168330	G						C									
168329	G						A									
168320	A				G			G	G	G	G	G	G	G	G	G
168309	T						C									
168308	T				C		C	C	C	C	C	C	C	C	C	C
168295	A				T		T	T	T	T	T	T	T	T	T	T
168293	G				A											
168279	C								-	-		A	A	-	-	-
168275	A								-	-	C			-	-	-
168269	G								-	-		C	C	-	-	-
168267	C						A		-	-				-	-	-
168266	A				G			G	-	-	G	G	G	-	-	-
168257	G				A				-	-				-	-	-
168253	T	C	C	C		C										
168248	A						C									
168238	G				A			A			A	A	A			
No. of differences		0			10		11		0		5			1		
Donor type		MRD			MRD		MUD		MUD		MUD			MUD		
SS R/D		+/+			+/+		+/+		+/+		+/?			+/+		

SS= EBV serostatus; R/D= recipient/donor.

Discussion

Our study shows that EBV reactivations in recipients of partially TCD SCT occur very frequently, especially among recipients of MUD transplants. When EBV DNA was monitored in PBMCs, significantly more patients showed EBV reactivations compared to plasma samples. In all patients with a PBMC EBV reactivation only, a spontaneous clearance of EBV DNA was observed and none progressed to EBV-LPD. Of 11 patients with a plasma EBV reactivation and a VL > 1000 c/ml, two developed EBV-LPD (18%) despite pre-emptive therapy with anti CD20 Mabs. In 6 patients, LMP-1 sequences could be determined in pre- and post-transplant samples, enabling EBV strain identification. In 3 of 6 patients the post-transplant EBV strain differed from the pre-transplant strain, indicating a re-infection with an exogenous strain.

In patient 1, who received a graft from a matched related donor, the pre-transplant, post-transplant and donor strains were identical. It is known from other studies[6,17] that intrafamilial spread does play an important role in EBV transmission. In these two studies, EBV DNA RFLP and size variation of the EBNA proteins expressed in EBV transformed lymphoblastoid cell lines (ebnotyping) were used to distinguish EBV strains. It was found that different members of the same family could carry the same EBV strain and that a given EBV strain was observed only within a single family. All unrelated individuals (apart from one husband and wife) did carry individually distinct viral strains[17]. In the setting of allogeneic SCT, in 4 of 6 related patient/donor pairs similar strains were isolated pre-transplant in patients and donors[18]. Therefore, our finding of identical recipient and sibling donor strains is not unusual and does not allow differentiation between reactivation and re-infection. Identical strains in pre- and post-transplant samples were also found in patient 4, a MUD recipient. These results also indicate the feasibility of LMP-1 sequence analysis for strain identification.

The technique we used to characterise EBV strains has also been used by others[9-11]. In those studies and the present report the EBV strain found in patient 1 and 4 is relatively common, making a definite exclusion of a re-infection impossible. All other identified strains showed unique sequences. The finding that a specific EBV strain was carried by several unrelated individuals, is different from data published by Gratama et al[17] and Katz et al[6]. These authors showed that all unrelated individuals carried individually distinct viral strains, when strain identification was performed by ebnotyping or RFLP.

Table 5 Results of studies performing EBV strain typing in patient/donor pairs

Study	No. of patients	Origin of EBV strain post-transplant	
		Endogenous	Exogenous
Gratama[4]*	2	0	2 (1 donor, 1 husband)
Gratama[18]*	1#	1	0
Van Kooij[11]**	1	1	0
Present study **	5#	2$	3

* = EBV strain typing with "Ebnotyping"; ** = EBV strain typing using LMP-1 sequence analysis; # = In both studies more patient/donor pairs (n=4 in Gratama et al; n=1 in the present study) were analysed. These pairs were related and pre-, post-transplant and donor strains were identical. Therefore, no differentiation was possible between endogenous reactivation or exogenous re-infection; $ = Exogenous re-infection is unlikely, however, has not been excluded.

The post-transplant EBV strains of patient 6 differed by only one nucleotide from the pre-transplant strain. This might have been the result of a point mutation acquired during viral replication, making it more likely that this patient suffered a reactivation instead of a re-infection.

In combination with previously published data, the present data show that the original EBV strain could no longer be detected post-transplant in 5 of 9 patients (see Table 5). We conclude that in these patients a re-infection with an exogenous strain has occurred. It is well known that EBV persistence requires the establishment of a latent infection in circulating B cells. As EBV replicates poorly in B cells in vitro, oropharyngeal epithelial cells have long been considered to be the site of EBV replication[19,20,21]. If the oropharyngeal epithelium is a permanent EBV reservoir, one would expect the pre-transplant EBV strain of the recipient to persist after SCT. The absence of the pre-transplant EBV strain in 5 of 9 patients, however, is inconsistent with that assumption. Furthermore, in patients with X-linked agammaglobulinemia, who are deficient in mature B cells, no evidence of past or present EBV infection in mouth washings and peripheral blood samples could be found[19,20]. However, our findings can not prove the eradication of endogenous strains, since multiple infections do occur in immunocompromised patients[22-25] and the original endogenous strain might have become non-dominant.

In 20 healthy asymptomatic EBV carriers, EBV strains present in mouth washes and peripheral blood were recently found to differ very often[26]. These authors used a very sensitive heteroduplex tracking assay. Their findings are not supported by other studies among a total of 267 healthy individuals[23,27]. We were able to compare EBV strains from mouth washes with strains from PBMCs in two patients. In both patients, EBV strains from the two compartments were identical. Therefore, current knowledge does not support the hypothesis of compartmentalisation of EBV infections.

Analysis of two solid organ transplant recipients with EBV-LPD, showed that EBV transmission from donor cells to host cells is possible. The EBV strain identified was of donor origin, while in both patients the lymphoma resulted from proliferation of B lymphocytes of recipient origin[7]. This finding suggests that, apart from EBV transmission from transplant B cells to host cells, other routes of transmission (oral or by transfusions) are possible as well, which is supported by the documented primo-infection of our EBV-seronegative (Recipient-/Donor-) patient.

In conclusion, EBV reactivation is a very frequent event, especially in recipients of TCD grafts from matched unrelated donors and it frequently results from a re-infection with an exogenous EBV strain instead of a true reactivation of the endogenous strain.

References

1 van Esser JWJ, van der Holt B, Meijer E et al. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-depleted SCT. Blood 2001; 98: 972-978.

2 Meijer E, Dekker AW, Weersink AYL, Rozenberg-Arska M, Verdonck LF. Prevention and treatment of Epstein-Barr virus associated lymphoproliferative disorders in recipients of bone marrow and solid organ transplants: a review. Br J Haematol; 2002; 119: 596-607.

3 Curtis RE, Travis LB, Rowlings PA et al. Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. Blood 1999; 94: 2208-2216.

4 Gratama JW, Oosterveer MAP, Zwaan FE et al. Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: implications for sites of viral latency. Proc Natl Acad Sci 1988; 85: 8693-8696.

5 Haque T, Thomas JA, Falk KI, Parratt R, Hunt BJ, Yacoub M, Crawford DH. Transmission of donor Epstein-Barr virus (EBV) in transplanted organs causes lymphoproliferative disease in EBV-seronegative recipients. J Gen Virol 1996; 77: 1169-72.

6 Katz BZ, Niederman JC, Olson BA, Miller G. Fragment length polymorphisms among independent isolates of Epstein-Barr virus from immunocompromised and normal hosts. J Infect Dis 1988; 157: 299-308.

7 Cen H, Breinig MC, Atchison RW, Ho M, McKnight JL. Epstein-Barr virus transmission via the donor organs in solid organ transplantation: polymerase chain reaction and restriction fragment length polymorphism analysis of IR2, IR3, and IR4. J Virol 1991; 65: 976-80.

8 Knecht H, Bachmann E, Joske DJ, Sahli R, Emery-Goodman A, Casanova JL, Zilic M, Bachmann F, Odermatt BF. Molecular analysis of the LMP (latent membrane protein) oncogene in Hodgkin's disease. Leukemia 1993; 7: 580-585.

9 Schafer H, Berger C, Aepinus C et al. Molecular pathogenesis of Epstein-Barr virus associated posttransplant lymphomas: new insights through latent membrane protein 1 fingerprinting. Transplantation 2001; 72: 492-496.

10 Berger C, van Baarle D, Kersten MJ, Klein MR, Al-Homsi AS, Dunn B, McQuain C, van Oers R, Knecht H. Carboxy terminal variants of Epstein-Barr virus-encoded latent membrane protein 1 during long-term human immunodeficiency virus infection: reliable markers for individual strain identification. J Infect Dis 1999; 179: 240-244.

11 Van Kooij B, Thijssen SFT, Meijer E, Niesters HGM, van Esser JWJ, Cornelissen JJ, Verdonck LF, van Loon AM. Sequence analysis of EBV DNA isolated from mouth washings and PBMCs of healthy individuals and blood of EBV-LPD patients. J Clin Virol 2003; in press. Available online.

12 Meijer E, Dekker AW, Rozenberg-Arska M, Weersink AJL, Verdonck LF. Influence of Cytomegalovirus-seropositivity on outcome after T cell depleted bone marrow transplantation: contrasting results between recipients of grafts from related and unrelated donors. Clin Infect Dis 2002; 35: 703-712.

13 Van Doornum GJJ, Guldemeester J, Osterhaus ADME, Niesters HGM. Diagnosing herpes virus infections by real-time amplification and rapid culture. J Clin Microbiol 2003; 41: 576-580.

14 van Esser JWJ, Niesters HGM, van der Holt B et al. Prevention of Epstein-Barr virus-lymphoproliferative disease by molecular monitoring and pre-emptive rituximab in high-risk patients after allogeneic stem cell transplantation. Blood 2002; 99: 4364-4369.

15 Niesters HG, van Esser J, Fries E, Wolthers KC, Cornelissen J, Osterhaus AD. Development of a real-time quantitative assay for detection of Epstein-Barr virus. J Clin Microbiol 2000; 38: 712-715.

16 Hudson GS, Farrell PJ, Barrell BG. Two related but differentially expressed potential membrane proteins encoded by the EcoRI Dhet region of Epstein-Barr virus B95-8. J Virol 1985; 53: 528-535.

17 Gratama JW, Oosterveer MA, Klein G, Ernberg I. EBNA size polymorphism can be used to trace Epstein-Barr virus spread within families. J Virol 1990; 64: 4703-4708.

18 Gratama JW, Oosterveer MA, Lepoutre JM, van Rood JJ, Zwaan FE, Vossen JM, Kapsenberg JG, Richel D, Klein G, Ernberg I. Serological and molecular studies of Epstein-Barr virus infection in allogeneic marrow graft recipients.Transplantation 1990; 49: 725-730.

19 Faulkner GC, Krajewski AS, Crawford DH.The ins and outs of EBV infection. Trends Microbiol 2000; 8: 185-189. Review.

20 Faulkner GC, Burrows SR, Khanna R, Moss DJ, Bird AG, Crawford DH. X-Linked agammaglobulinemia patients are not infected with Epstein-Barr virus: implications for the biology of the virus. J Virol 1999; 73: 1555-1564.

21 Kieff E, Rickinson AB. Epstein-Barr virus and its replication. In: Fields Virology (ed. by D.M. Knipe & P.M. Howley) 2001: pp. 2511-2627. Lippincott Williams & Wilkins, Philadelphia.

22 Gratama JW, Lennette ET, Lonnqvist B, Oosterveer MA, Klein G, Ringden O, Ernberg I. Detection of multiple Epstein-Barr viral strains in allogeneic bone marrow transplant recipients. J Med Virol 1992; 37: 39-47.

23 Gratama JW, Oosterveer MA, Weimar W, Sintnicolaas K, Sizoo W, Bolhuis RL, Ernberg I. Detection of multiple 'Ebnotypes' in individual Epstein-Barr virus carriers following lymphocyte transformation by virus derived from peripheral blood and oropharynx. J Gen Virol 1994; 75: 85-94.

24 Yao QY, Tierney RJ, Croom-Carter D, Dukers D, Cooper GM, Ellis CJ, Rowe M, Rickinson AB. Frequency of multiple Epstein-Barr virus infections in T-cell-immunocompromised individuals. J Virol 1996; 70: 4884-4894.

25 Yao QY, Croom-Carter DS, Tierney RJ, Habeshaw G, Wilde JT, Hill FG, Conlon C, Rickinson AB. Epidemiology of infection with Epstein-Barr virus types 1 and 2: lessons from the study of a T-cell-immunocompromised hemophilic cohort. J Virol 1998; 72: 4352-4363.

26 Sitki-Green D, Covington M, Raab-Traub N. Compartmentalization and transmission of multiple Epstein-Barr virus strains in asymptomatic carriers. J Virol 2003; 77: 1840-1847.

27 Yao QY, Rowe M, Martin B, Young LS, Rickinson AB. The Epstein-Barr virus carrier state: dominance of a single growth-transforming isolate in the blood and in the oropharynx of healthy virus carriers. J Gen Virol 1991; 72: 1579-1590.

Chapter 6

Increased incidence of EBV-associated lymphoproliferative disorders after allogeneic stem cell transplantation from matched unrelated donors due to a change of T cell depletion technique

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Abstract

In this report the influence of T vs T and B cell depletion of grafts on the incidence of EBV-associated lymphoproliferative disorders (EBV-LPD) after bone marrow transplantation (BMT) from matched unrelated donors (MUD) is analysed. From 1982 to 1997 the Soy Bean Agglutinin/Sheep Red Blood Cell (SBA/SRBC) method, which has a risk of transmitting prions or viruses, was used for T cell depletion. Because of this risk a new T cell depletion method was introduced, using CD2 and CD3 monoclonal antibodies (CD2/3 method), which led to an unexpected increase in EBV-LPDs in MUD recipients. SBA depletion was reintroduced and combined with the CD2/3 method (SBA/CD2/3) in this patient population, later replaced by B cell specific (CD19 and CD22) antibodies (CD3/19/22 method). The number of T ($x10^5$/kg) and B ($x10^5$/kg) cells in the graft was 1.5 ± 0.8 and 2 ± 1 (T/B ratio 0.75), 2.2 ± 2.0 and 41 ± 21 (ratio 0.055), 5.0 ± 0.0 and 2 ± 1 (ratio 2.5), 2.5 ± 1.2 and 10 ± 6 (ratio 0.25) using the SBA/SRBC, CD2/3, SBA/CD2/3 and CD3/19/22 techniques, respectively. When B cell depletion was performed 4 out of 31 (13%) MUD recipients developed an EBV-LPD. Without B cell depletion (CD2/3 method) this occurred in 5 out of 7 patients (71%) ($p<0.05$). A T/B cell ratio in the graft of ≥ 0.25 seems sufficient to reduce significantly the incidence of EBV-LPD after BMT from MUDs.

Introduction

After bone marrow transplantation (BMT) from a matched unrelated donor (MUD) transplant related morbidity and mortality are increased compared to BMT from a matched related donor (MRD). This is largely due to an increased incidence of graft-versus-host disease (GVHD) and infectious complications[1,2]. T cell depletion reduces the incidence and severity of GVHD but has also important side-effects[3]. Curtis et al[4] showed that the risk of Epstein-Barr-virus-associated lymphoproliferative disorders (EBV-LPDs) was strongly associated with T cell depletion of donor marrow. Use of Antithymocyteglobulin (ATG) and of grafts from unrelated or HLA-mismatched related donors were other adverse risk factors for the development of EBV-LPD. The risk for EBV-LPD varied according to the techniques used for T cell depletion, being lowest when the Campath-1 method was used which, in contrast to T cell specific monoclonal antibodies (Moabs), removed both T and B cells[4].
In this report the influence of the T vs T and B cell depletion method on the incidence of EBV-LPD among MUD transplant recipients is described, as was observed at our institute. From 1982 to 1997 the Soy Bean Agglutinin/Sheep Red Blood Cell (SBA/SRBC) method was used for T cell depletion. This technique has been well established, but the use of SRBC has a risk of transmitting prions or viruses. Therefore, a new T cell depletion method was introduced, the immunorosette (IR) technique, using tetrameric complexes with T cell specific CD2 and CD3 Moabs instead of sheep red blood cells. Unfortunately, this led to an unexpected high number of EBV-LPDs in patients receiving transplants from MUDs. Since it was suspected that this was caused by the relatively high number of B cells in the graft, SBA depletion -which results in B cell depletion of 1 to 1.5 log- was reintroduced and combined with CD2/3 depletion from May 1998 (SBA/CD2/3 method). In March 1999 SBA was replaced by B cell specific (CD19 and CD22) antibodies (CD3/19/22 method).

Methods

Patients From November 1985 to April 2000 a total of 313 patients received allogeneic BMT, 49 from a MUD and 264 from a Matched Related Donor (MRD). Eleven MUD and 63 MRD transplant recipients died within 6 months after BMT from relapse or transplant

related toxicity, apart from EBV-LPD and were therefore not evaluable for this study. The interval of 6 months was chosen because most EBV-LPDs develop during the first 6 months post-transplant[4]. The distribution of patients according to T cell depletion used is shown in Table 1. Characteristics of recipients of MUD transplants are described in Table 2. All patients were treated with clinical protocols approved by the local investigation review board and gave informed consent.

Transplantation procedure Conditioning regimens consisted of cyclophosphamide (60 mg/kg/day) on each of two successive days, followed by total body irradiation (600 cGray/day) on each 2 successive days. The graft was infused after the second TBI fraction (day 0). ATG (Thymoglobulin™, Sangstat, Amstelveen, the Netherlands) was given to MUD patients before cyclophosphamide was started, at a total dose of 20 mg/kg intravenously. It was lowered to a total dose of 8 mg/kg since April 1999. All patients received cyclosporin from day -2 in a dose of 3 mg/kg/day by continuous infusion for 4 weeks, thereafter it was given orally for 4-6 weeks in a dose that gave comparable through levels, followed by tapering. Cyclosporin was discontinued within 3 months after transplantation if no active GVHD was observed. Infection prevention for all patients consisted of ciprofloxacin, fluconazole and amphotericin B given orally until granulocyte counts exceeded 500 cells/mm^3. Cephalothin was given intravenously for 10 days from day +3. Furthermore co-trimoxazole and valacyclovir were given orally from day +1 until 12 months post-BMT. GVHD was diagnosed according to the Seattle criteria[5]. Acute GVHD grade I was treated with topical corticosteroids; grade II or higher was treated with systemic corticosteroids as described[6].

Table 1 Number of BMTs according to depletion technique

Depletion technique	MUD patients	NE	MRD patients	NE
SBA/SRBC	19	5	202	54
CD2/3	11	4	62	9
SBA/CD2/3	6	0		
CD3/19/22	13	2		

SBA/SRBC = soy bean agglutinin/sheep red blood cell; SBA/CD2/3 = SBA agglutination followed by depletion with anti CD2 and CD3 Moabs; CD2/3 = depletion with anti CD2 and CD3 Moabs; CD3/19/22 = depletion with anti CD3, CD19 and CD22 Moabs; NE = not evaluable because of early death.

Table 2 Characteristics of recipients of MUD transplants

	SBA/SRBC	CD2/3	SBA/CD2/3	CD3/19/22
No. of patients	14	7	6	11
Age, year (range)	29 (17-47)	28 (18-37)	31 (22-47)	32 (18-48)
Diagnosis				
ALL	5	1	1	2
AML	0	3	0	4
CML	6	1	2	4
MDS	1	0	1	1
SAA	2	2	2	0
Sex				
F/F	3	0	0	3
M/M	5	3	3	4
M/F	5	1	2	3
F/M	1	3	1	1
CMV serostatus R/D				
-/-	2	2	3	3
+/+	1	2	1	2
-/+	5	1	1	4
+/-	6	2	1	2
GVHD				
Acute				
I/II	10	4	2	5
III/IV	1	1	0	2
None	3	2	4	4
Chronic				
L	3	1	0	0
E	6	0	1	0
NE	0	3	0	1
None	5	3	5	10

R/D = recipient/donor; NE = not evaluable; L = limited; E = extensive.

Extensive chronic GVHD was treated with systemic corticosteroids, sometimes combined with cyclosporin. During the first 4 months post-transplant, CMV-seropositive patients who demonstrated reactivation of CMV infection or those who were treated with high-dose corti-

costeroids received pre-emptive or prophylactic therapy, respectively, with ganciclovir in a dose of 2.5 mg/kg intravenously twice a day for 14 days[7].

HLA-matching In all MRD patient/donor pairs, class I antigens (A, B and Cw) were analysed by serological typing, in case of doubt low resolution molecular typing was performed. Class II antigens (DRB1, DRB3, DRB4, DRB5 and DQB1) were analysed by serological typing until 1993 and since 1993 by low resolution molecular typing with sequence specific primers. In MUD patient/donor pairs HLA analysis was performed as in MRD recipients until 1993, thereafter class I antigens (A, B) were analysed by serological typing, in case of doubt low resolution molecular typing was performed. Class I Cw and class II antigens (DRB1, DRB3, DRB4, DRB5 and DQB1) were analysed by low resolution molecular typing with sequence specific primers and DRB1, B3, B4 and B5 antigens were as well defined by high resolution typing since January 1999.

T and B cell depletion SBA/SRBC T cell depletion was performed as described[8]. The immunorosette depletion technique (CD2/3) was performed as described by Slaper-Cortenbach et al[9]. In short, tetrameric complexes (CLB, Amsterdam, the Netherlands) were formed by addition of cross-linking RaMIgG1 Moabs to a mixture of murine IgG1 Moabs, one directed against glycophorin A in the membrane of human erythrocytes and another against T cell specific antigens (CD2 or CD3). These complexes were then bound to donor erythrocytes (in case of a MUD transplant obtained from a healthy O-rhesus negative donor from the blood bank) and the coated erythrocytes were washed. After addition of the coated erythrocytes to the bone marrow buffycoat cells, prepared using the COBE 2991, immunorosettes formed. These immunorosettes were removed using Ficoll density separation ($d=1.077$ g/cm^3). In the SBA/CD2/3 method Ficoll density separation was used to prepare mononuclear cells, subsequently SBA was used with the CD2 and CD3 tetrameric complexes instead of SRBC. The CD3/19/22 method used tetrameric complexes with CD3, CD19 and CD22 Moabs for depletion of T and B cells. All monoclonal antibodies were tested for viral and bacterial contamination and, therefore, biosafe. In all T/B cell depletion procedures the residual number of T cells was counted and nonmanipulated T cells (from a small BM fraction that was set apart before the stem cell manipulation started) were added to obtain a low fixed number of T cells[6]. This fixed number of T cells in the graft differed per depletion technique (SBA/SRBC: 1 x 10^5 T cells/kg; CD2/3: 1 x 10^5 T cells/kg; SBA/CD2/3: 5 x 10^5 T cells/kg; CD3/19/22: 2 x 10^5 T cells/kg). In some grafts the residual number of T cells after depletion was above the fixed number, no T cell add-back was performed in these

grafts. T/B cell depletion was evaluated by Facs analysis (FACScan, Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA) on unmanipulated bone marrow and depleted marrow using monoclonal antibodies (CD2-FITC, CD3-PE, CD19-PE, CD20-FITC, CD45-PERCP (Becton Dickinson)).

EBV-LPD EBV-LPD was diagnosed by standard histological criteria[10]. CD20 antibodies were used to assess the B cell origin of the LPD. The presence of EBV infection was determined immunohistochemically by detection of EBNA-2 and LMP-1 proteins and with in-situ hybridisation to detect EBV-encoded RNA (EBER).

Statistics Data are expressed as mean ± SD. Mean differences between groups were assessed by the Mann Whitney U test or Chi square analysis. Calculations were performed using SPSS/PC+ 8.0 (SPSS Inc, Chicago Il, USA).

Results

Incidence of EBV-LPD The incidence of EBV-LPD is summarized in Table 3. When B cell depletion was performed (SBA/SRBC, SBA/CD2/3, CD3/19/22) 4 out of 31 patients (13%) receiving BMT from a MUD developed an EBV-LPD, being the cause of death in 3 patients. Without B cell depletion (CD2/3) 5 out of 7 patients (71%) receiving BMT from a MUD developed an EBV-LPD and two died of this disease. This resulted in a significant difference ($p<0.05$) between B cell depleting techniques and a non-B cell depleting technique concerning the incidence of EBV-LPDs. In contrast to MUD recipients, among patients receiving BMT from an HLA-identical sibling donor the incidence of EBV-LPD was similar when a T and B cell depletion method was used (SBA/SRBC) compared to a T without B cell depletion method (CD2/3). The incidence was 5 and 4%, respectively.

T and B cell numbers in the graft T and B cell counts in the MUD grafts 'according to depletion technique' are shown in Table 4. The SBA/CD2/3 group received significantly more T cells ($p<0.05$), compared to all other groups. This was not due to depletion failure as can be seen from the log depletion reached. In fact, the SBA/CD2/3 was the most efficient T cell depletion method. As has been described earlier, after T cell depletion the residual number of T cells was counted and nonmanipulated T cells were added to obtain a low fixed number of T cells (1-5 x 10^5 T cells/kg recipient weight). In the SBA/CD2/3 group this

number was set at 5 x 10^5/kg. In the SBA/SRBC depleted grafts, B cell numbers were not measured. We can assume, however, that the SBA/SRBC method should yield a B cell depletion comparable to the SBA/CD2/3 method (confirmed by experiments in the laboratory). B cell numbers were not measured in CD2/3 depleted grafts of unrelated donors as well. When the incidence of EBV-LPD increased dramatically in MUD recipients and it was suspected to be due to the relatively high number of B cells in the graft, SBA agglutination was performed prior to CD2/3 depletion. Since then B cells were measured in all grafts (CD2/3 depleted grafts from MRDs, SBA/CD2/3 and CD3/19/22 depleted grafts from MUDs). The CD2/3 group (data derived from MRD transplants) received significantly more B cells compared to all other groups: 41 ± 21 x 10^5/kg vs 2 ± 1 (SBA/CD2/3) and 10 ± 6 (CD3/19/22); $p<0.001$. The T/B cell ratio in the graft according to depletion method was 0.75 for the SBA/SRBC group (ratio was calculated using the B cell count from the SBA/CD2/3 method), 0.055 for the CD2/3 method, 2.5 for the SBA/CD2/3 group and 0.25 for the CD3/19/22 technique. It should be noted that in this last group ATG dose was reduced. Therefore in vivo T cell depletion due to ATG in this group is expected to be less than in the other three groups[11].

Table 3 EBV-LPD according to depletion technique

Depletion technique	MUD patients	EBV-LPD (%)	DOD	MRD patients	EBV-LPD (%)	DOD
SBA/SRBC	14	3 (21)	2	148	7 (5)	5
CD2/3	7	5 (71)	2	53	2 (4)	0
SBA/CD2/3	6	0 (0)	0			
CD3/19/22	11	1 (9)	1			

EBV-LPD = EBV-associated lymphoproliferative disease; DOD = died of EBV-LPD.

Table 4 Depletion of T and B cells from marrow grafts of matched unrelated donors

Technique	T cell count in graft (x10^5/kg)	Log depletion T cells	B cell count in graft (x10^5/kg)	Log depletion B cells	ATG dose	Ratio T/B
SBA/SRBC[a]	1.5 ± 0.8	2.3 ± 0.6	nd		HD	0.75
CD2/3[b]	2.2 ± 2.0	2.3 ± 0.4	41 ± 21	0.2 ± 0.1	HD	0.055
SBA/CD2/3	5.0 ± 0.0	3.2 ± 0.5	2 ± 1	2.0 ± 0.2	HD	2.5
CD3/19/22	2.5 ± 1.2	3.0 ± 0.7	10 ± 6	1.1 ± 0.2	LD	0.25

[a] = to calculate the T/B cell ratio, B cell count from the SBA/CD2/3 group was taken; [b] = B cell count was measured in a group of 24 patients receiving a transplant from a MRD; HD = high dose (20 mg/kg); LD = low dose (8 mg/kg).

Discussion

The most important observation of this report is the significantly increased incidence of EBV-LPD in patients receiving BMT from an unrelated donor which was T but not B cell depleted with an immunorosette technique, using CD2 and CD3 Moabs. Among MRD patients the incidence of EBV-LPD was not influenced by B cell depletion. The SBA/SRBC depletion method was abandoned in 1997 because the use of SRBC has a risk of transmitting prions or viruses. The Moabs used in the immunorosette technique were biosafe (screened for viral and bacterial contamination). The CD2/3 depletion method had proven to result in a similar T cell depletion (Table 4). Engraftment and haematopoietic recovery were comparable for the two techniques (data not shown). Until 1998 there were no reports showing the importance of B cell depletion for prevention of EBV-LPD. Resting memory B cells are thought to be the natural reservoir of EBV within the body[12]. The B cell load of patient origin has been largely destroyed due to the pre-transplant myeloablative conditioning regimen. Gratama et al[13,14] showed that also latently EBV-infected host cells can be eliminated after BMT. Therefore, theoretically, B cell depletion of the graft along with T cell depletion might improve immunological control of EBV infection post-transplant. Indeed, Cavazzana et al[15] showed that none of 19 patients receiving transplants from a partially matched related donor (PMRD) developed EBV-LPD when ex vivo T and B cell depletion was performed,

whereas 7 out of 19 historical controls developed EBV-LPD when only T cell depletion was performed. Two other studies showed that B cell depletion might be of benefit for decreasing the incidence of EBV-LPD[16,17]. Our report emphasizes the importance of B cell depletion, in patients receiving T cell depleted grafts from matched unrelated donors. In MRD recipients T cell depletion without B cell depletion did not result in an increased incidence of EBV-LPD. Therefore, next to ex vivo T cell depletion, there have to be other factors which impair immune surveillance of the Epstein-Barr virus in MUD recipients. Recently it was shown that TCD together with the use of ATG were important factors influencing EBV reactivation post-transplant[18]. All our MUD patients received ATG, giving in vivo T cell depletion as well[11]. In MUD recipients the ratio of T and B cells in the graft seems to be very important for surveillance of EBV. When high dose ATG was used pre-transplant, a T/B cell ratio of 2.5 was sufficient to prevent EBV-LPD in 6 patients (SBA/CD2/3 group). When low dose ATG was used, a ratio of 0.25 did not prevent EBV-LPD totally: 1 out of 11 patients developed EBV-LPD (CD3/19/22 group). The actual T/B cell ratio in vivo might have been higher in this group due to a less severe in vivo T cell depletion. The optimal T/B cell ratio is not known at the moment and will be dependent on several factors such as use and dosage of ATG. The number of patients in our study is limited, so further studies are necessary to establish the degree of B cell depletion needed for efficient prevention of EBV-LPD in patients receiving T cell depleted grafts from donors other than HLA-matched siblings. In the study of Cavazzana et al[15] B and T cell counts in the grafts were $5 \pm 8.5 \times 10^5$/kg and $1.5 \pm 1.7 \times 10^5$/kg, respectively. This gave a ratio of 0.3 and no EBV-LPD was observed post-transplant. These and our data suggest that a ratio ≥ 0.25 can markedly reduce EBV-LPD in such patients.

In conclusion, our data show that the incidence of EBV-associated lymphoproliferative disorders in recipients of allogeneic bone marrow transplants from matched unrelated donors is increased when T cell depletion of the graft is performed, without B cell depletion. We replaced the SBA/SRBC technique for a new technique, based on the use of Moabs instead of SRBC. This resulted in a disproportional higher number of residual B cells in the graft and, consequently, in a dramatic increase in EBV-LPD in recipients of T cell depleted stem cell transplant from matched unrelated donors.

References

1 Kernan NA, Bartsch G, Ash RC et al. Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. N Engl J Med 1993; 328: 593-602.

2 Arnold R, de Witte T, van Biezen A et al. Unrelated bone marrow transplantation in patients with myelodysplastic syndromes and secondary acute myeloid leukaemia: an EBMT study. Bone Marrow Transplant 1998; 21: 1213-1216.

3 Ferrara JLM, Deeg HJ. Graft-versus-host disease. N Eng J Med 1991; 324: 667-674.

4 Curtis RE, Travis LB, Rowlings PA et al. Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. Blood 1999; 94: 2208-2216.

5 Thomas ED, Storb R, Clift RA et al. Bone marrow transplantation. N Engl J Med 1975; 292: 832-843.

6 Verdonck LF, Dekker AW, de Gast GC et al. Allogeneic bone marrow transplantation with a fixed low number of T cells in the marrow graft. Blood 1994; 83: 3090-3096.

7 Verdonck LF, Dekker AW, Rozenberg-Arska M, van den Hoek MR. A risk adapted approach with a short course of ganciclovir to prevent cytomegalovirus (CMV) pneumonia in CMV-seropositive recipients of allogeneic bone marrow transplantation. Clin Infect Dis 1997; 24: 901-907.

8 Reisner Y, Kirkpatrick D, Dupont B et al. Transplantation for acute leukaemia with HLA-A and B nonidentical parental marrow cells fractionated with soybean agglutinin and sheep red blood cells. Lancet 1981; 15: 327-331.

9 Slaper-Cortenbach ICM, Wijngaarden-du Bois MJGJ, de Vries-van Rossen A et al. The depletion of T cells from haematopoietic stem cell transplants. Rheumatology 1999; 38: 751-754.

10 Knowles DM, Cesarman E, Chadburn A et al. Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of posttransplantation lymphoproliferative disorders. Blood 1995; 85: 552-565.

11 Baurmann H, Revillard JP, Bonnefoy-Berard N, Schwerdtfeger R. Potent effects of ATG used as part of the conditioning in matched unrelated donor transplantation. Blood 1998; 92: S290a. Abstract.

12 Cohen JI. Epstein-Barr Virus infection. N Engl J Med 2000; 343: 481-492.

13 Gratama JW, Oosterveer MAP, Zwaan FE et al. Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: Implications for sites of viral latency. Proc Natl Acad Sci 1988; 8: 8693-8696.

14 Gratama JW, Oosterveer MAP, Lepoutre J et al. Serological and molecular studies of Epstein-Barr virus infection in allogeneic marrow graft recipients. Transplantation 1990; 49: 725-730.

15 Cavazzana M, Bensoussan D, Jabado N et al. Prevention of EBV-induced Blymphoproliferative disorder by ex vivo marrow B cell depletion in HLA-phenoidentical or non-identical Tdepleted bone marrow transplantation. Br J Haematol 1998; 103: 543-551.

16 Liu Z, Wilson JM, Jones MC et al. Addition of B cell depletion of donor marrow with anti-CD20 antibody to a T cell depletion regimen for prevention of EBV lymphoma after bone marrow transplantation. Blood 1999; 94: S638a. Abstract.

17. Hale G, Waldmann H. Risks of developing Epstein-Barr virus-related lymphoproliferative disorders after T cell-depleted marrow transplants. Blood 1998; 91: 3079-3083.

18 van Esser JWJ, van der Holt B, Meijer E et al. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T cell depleted SCT. Blood 2001; 98: 972-978.

Chapter 7

Prevention and treatment of Epstein-Barr virus-associated lymphoproliferative disorders in recipients of bone marrow and solid organ transplants: a review

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Abstract

Reactivations of the Epstein-Barr virus (EBV), which may progress to EBV-associated lymphoproliferative disorders (EBV-LPD), are a major threat in recipients of allogeneic bone marrow and solid organs. An overview is given of the monitoring and pre-emptive treatment of EBV reactivations and the incidence, prevention and therapy of EBV-LPD.
Several risk factors for the development of EBV-LPD after solid organ transplantation (SOT) and bone marrow transplantation (BMT), respectively, have been identified: 1) primary EBV infection in EBV-seronegative patients, 2) type of transplanted allograft, 3) cytomegalovirus (CMV) serostatus mismatch (R-/D+), 4) CMV disease 5) use of T cell antibodies in SOT recipients and 1) T cell depletion (TCD) of grafts, 2) use of unrelated or ≥ 2 HLA antigen mismatched related donors, 3) use of Antithymocyteglobulin or 4) anti CD3 monoclonal antibodies (Moabs) in BMT recipients. In high-risk BMT recipients, monitoring of EBV viral load (VL) in preferably cell free plasma should be performed once a week until 6 months post-transplant. No strict guidelines for frequency and duration of monitoring in SOT recipients can be given, largely due to the variable time period in which post-transplant EBV-LPDs can occur in this patient group. However, in high-risk SOT recipients monitoring may be performed fortnightly or at every outpatient visit until 1 year post-transplant. When EBV reactivation is diagnosed, pre-emptive therapy with anti B cell Moabs is advised in BMT as well as SOT recipients. In BMT recipients receiving T cell depleted grafts from unrelated donors, additional B cell depletion can reduce the incidence of EBV-LPD dramatically. Treatment of EBV-LPD should start with withdrawal of or decreasing immunosuppression together with anti B cell Moabs. Donor lymphocyte infusion should be reserved for BMT recipients not responding to anti B cell therapy or with central nervous system (CNS) localisation. SOT recipients with CNS localisation might receive additional radiotherapy and/or chemotherapy as well. The efficacy of antiviral therapy in preventing or treating EBV-LPD, if there is any, is very low. Chemotherapy or IFN might be given to SOT recipients when other treatment options have failed or are not available. Localised disease in this patient group can be cured with surgery or radiotherapy. When available, EBV-specific cytotoxic T lymphocytes (EBVs-CTLs) from HLA-identical donors or autologous EBVs-CTLs can be used as (pre-emptive) treatment of EBV-LPD in BMT or SOT recipients, respectively. Further studies will be necessary to evaluate the safety and effectiveness of EBVs-CTLs obtained from (partially) HLA-matched related and unrelated blood donors in both BMT and SOT recipients, which will make this approach more accessible.

Introduction

The link between severe immunosuppression of transplant recipients and increased incidence of lymphoma has long been apparent and the association with EBV is widely recognized[1]. EBV is the prototype of the gamma subfamily of potentially oncogenic herpes viruses. Taxonomists have renamed EBV as human herpes virus 4 (HHV4). Two EBV types (type 1 and 2) circulate in most populations, of which type 1 is far more common in most populations[2]. There are various isolates of type 1 and 2 EBV. Persistent infection with more than one EBV isolate is not unusual, particularly for immunocompromised patients[3]. In vitro, efficient EBV infection of cells is restricted to mature human B lymphocytes. This results in a latent infection in 10% of cells which subsequently proliferate as immortalised lymphoblastoid cell lines (LCLs). Latently infected B-cells can be induced to become permissive for lytic viral replication, while in some viral replication occurs spontaneously[2]. The presence of latent virus in an infected cell can be readily detected using antibodies to any of the eight different virus proteins that are characteristically expressed in LCLs. These viral proteins include 6 nuclear proteins (EBNA's) and two integral membrane proteins (LMPs). In LCLs also two small nonpolyadenylated RNA's (EBERs) and highly spliced BAMH1 A rightward frame (BARF) transcripts are expressed. This type of latency is termed latency III[2,4]. EBV lymphoproliferative lesions are considered to result from proliferating latently infected B cells, expressing latency type III genes, in the absence of EBV-specific cytotoxic CD8+ T cell surveillance[4,5]. However, also more restricted patterns of EBV latency are observed in EBV lymphoproliferative disorders (EBV-LPD) and cases with latency type I (only expressing EBNA1, BARF transcripts and EBERs) have been described[6]. In addition to the expression of latent EBV genes, viral gene products associated with replicative or lytic infection have been detected in LPDs[4,6,7]. At the moment it is unclear whether EBV replication or lytic infection is of significance in the pathogenesis of EBV-LPD. However, the detection of cell free EBV-DNA and the high sensitivity and specificity of this test in diagnosing EBV-LPD suggests that lytic EBV infection might be more than a bystander in EBV-LPD.

An excellent review of the 4 different histo-pathological classifications of post-transplant LPDs is published by Nalesnik[1]. Most LPDs are of B cell origin, although T cell LPDs sometimes (12%) occur in recipients of solid organ transplants (SOT)[8]. Van Gorp et al[9] report on 3 SOT patients with EBV negative T cell LPDs. A literature search performed by these authors resulted in 22 transplant (SOT: n=19) recipients diagnosed with T cell LPD. A sum-

mary of these patients was given and in only 5 of them an association with EBV was established. Most of these T cell LPDs were occurring late (>1 year post-transplant, while prognosis was variable. LeBlond et al[8] diagnosed 34 cases with LPD after SOT. Four of 34 LPDs were of T cell origin and three of these four were EBV negative. The other 30 cases were of B cell origin and 8 of them were EBV negative. EBV negative LPDs more often occurred late after transplantation (> 2 years), while survival time after diagnosing LPD was significantly shorter compared to patients with EBV-associated LPDs. All EBV negative B cell LPDs were monomorphic, meeting the criteria of diffuse large B cell lymphoma according to the Revised European-American Lymphoma classification. The findings of LeBlond et al[8] were largely supported by other reports[10,11]. No data are available for T cell LPDs or EBV negative LPDs after bone marrow transplantation (BMT).

Some studies were undertaken to analyse whether EBV-LPD is derived from donor or host lymphoid tissue. In both BMT[12-14] and SOT[15-17] recipients post-transplant lymphomas of recipient origin as well as donor origin were found. The origin of the EBV strain infecting these lymphoma (B) cells is unknown. Gratama et al[18] showed that conditioning regimen pre-BMT and/or graft-versus-host disease (GVHD) was able to eliminate the EBV strain of the host. In this study, one patient became infected with a strain indistinguishable from the virus isolated from her husband and another with the donor strain. In 2 EBV-seronegative and 2 EBV-seropositive SOT recipients with EBV-LPD, donor strains and non-donor strains, respectively, were identified[19]. At this moment it is unknown how often EBV strains of donor origin cause re-infection in BMT recipients or primo-infections in seronegative SOT recipients. Oral transmission or transmission through transfusion of blood products might be other possibilities, although after BMT irradiated blood products are used which may prevent transfusion related transmission.

Incidence of EBV-LPD

EBV reactivations or EBV primo-infections in severely immunocompromised patients may result in the development of EBV-LPD, which is associated with a mortality of 80%[20-22].The reported incidence of EBV-LPD varies, but is generally higher in recipients of solid organ transplants (2-8%;[20,23-27]) compared to BMT recipients. In SOT recipients EBV-LPDs usually

develop during the first year post-transplant, however, they continue to occur thereafter[1,20,27-30]. Primary EBV infection in EBV-seronegative SOT recipients, the type of transplanted allograft, cytomegalovirus (CMV) serostatus mismatch (R-/D+), CMV disease and the type (T cell antibodies) and intensity of immunosuppression are important risk factors for the development of EBV-LPD in SOT recipients[29,31-33]. Buda et al[34] showed that in heart transplant recipients hepatitis C virus infection probably is a risk factor as well. Primary EBV infection and EBV-LPD is of greater concern in paediatric SOT recipients: in children with small-intestine transplants the incidence of EBV-LPD was 32%[35]. After BMT the overall cumulative incidence is 1% in 10 year, with most EBV-LPDs occurring within the first 6 months post-transplant[37]. Four major risk factors for early EBV-LPD (< 1 yr) after BMT have been identified[36]: 1) T cell depletion (TCD) using monoclonal antibodies (Moabs) directed at T cells or T and NK cells or TCD using E-rosetting, 2) use of unrelated or ≥ 2 HLA antigen mismatched related donors, 3) use of Antithymocyteglobulin (ATG) for prophylaxis or treatment of acute GVHD and 4) treatment of acute GVHD with anti CD3 Moabs. In patients with 3 or more risk factors the incidence of EBV-LPD was 22%. Other studies reported EBV-LPD incidences in recipients of matched unrelated donor (MUD) grafts from 4.3-24%[12,37,38], in recipients of matched related donor (MRD) grafts from 0-0.7%[12,13,37,38] and in recipients of unrelated umbilical cord transplants of 2%[39].

Monitoring of EBV reactivations

Since 1994 many studies have been performed to analyse the value of EBV-DNA detection in diagnosing EBV-LPD or other EBV-associated diseases. EBV-DNA detection by polymerase chain reaction (PCR) techniques can be performed in peripheral blood mononuclear cells (PBMC), whole blood or cell free plasma. Results of studies performed among SOT and BMT recipients are summarized in Table 1 and 2. In 5 of 13 studies measuring viral load (VL) in PBMC the sensitivity for diagnosing EBV-LPD was less than 100%[28,40-51]. VL detection in cell free plasma seems to be more accurate: in 5 of 6 studies 100% sensitivity was obtained[22,30,48,52-54]. Limaye et al[53] report the only patient with a negative PCR result in cell free plasma, while EVB-LPD was diagnosed. However, the only manifestation of EBV-LPD in this patient was a skin nodule. Specificity varied from 73-100% and was generally higher

Table 1 EBV DNA detection by PCR in PBMC: sensitivity and specificity for diagnosing EBV-LPD

Study	Method	Target gene	Tx	Sensitivity	Specificity	Viral Load cut-off
Riddler[28]	Semiquant. (c/10^5 PBMC)	EBNA1	SOT	88%	100%	20.000
Rooney[40]	Semiquant. (c/μg dna)	BAMH1H	BMT	80%	100%	20.000
George*[41]	Semiquant. (c/μg dna)	BMLF1	BMT	100%	95%	2.000
Lucas*[42,43]	Semiquant. (c/μg dna)	BAMH1H	BMT	71%	94%	40.000
			SOT	100%	97%	40.000
Kimura[44]	Realtime (c/μg dna)	BALF5	LTx	100%	100%	320
Baldanti[45]	Semiquant. (c/0.5μg dna)	EBNA1	SOT	73%	94%	1.000
Stevens[46]	Quantitative (c/ml blood)	EBNA1	LuTx	100%	75%	2.000
Hoshino*[47]	Realtime (c/μg dna)	BALF5	BMT	100%	73%	320
Wagner[48]	Realtime (c/μg dna)	BAMH1K BAMH1W	RTx	100%	89%	5.000
Wagner*[49]	Realtime (c/μg dna)	EBER1	SOT	100%	77%	4000
			BMT	100%	89%	4000
Gartner[50]	Quantitative (c/μg dna)	nd	BMT	87%	91%	100.000
Sirvent*[51]	Semiquant. (c/μg dna)	BAMH1C	BMT	100%	81%	300

PBMC = peripheral blood mononuclear cells; BMT = bone marrow transplantation; SOT = solid organ transplantation; LTx = liver transplantation; LuTx = lung transplantation; RTx = renal transplantation, nd = not described; * = prospective study.

when VL detection was performed in cell free plasma. Wagner et al[48] performed realtime quantitative EBV-DNA detection in PBMC as well as cell free plasma in recipients of renal transplants and healthy volunteers. Sensitivity of both methods was 100%, while specificity of EBV-DNA detection in PBMC was 89% and in cell free plasma 100%. When remission of EBV-LPD was accomplished, EBV DNA was more effectively cleared in plasma compared to

Table 2 EBV DNA detection by PCR in plasma: sensitivity and specificity for diagnosing EBV-LPD

Study	Method	Target gene	Tx	Sensitivity	Specificity	Viral Load cut-off
Fontan[30]	Qualitative	BAMH1W	BMT	100%	100%	Qualitative
			RTx	100%	100%	
			LTx	100%	100%	
Beck[52]	Qualitative	EBNA1	BMT	100%	85%	Qualitative
Limaye[53]	Qualitative	EBER1	SOT	83%	100%	Qualitative
Esser[22]	Realtime (c/ml)	BNRF1/ p143	BMT	100%	89%	1.000
Wagner[48]	Realtime (c/0.1 ml)	BAMH1K BAMH1W	RTx	100%	100%	1.000
Ohga[54]	Realtime (c/ml)	nd	BMT	100%	100%	40.000

BMT = bone marrow transplantation; SOT = solid organ transplantation; LTx = liver transplantation; RTx = renal transplantation; nd = not described.

PBMC, which might suggest that cell free EBV DNA better reflects response to therapy. Contradictory, Stevens et al[46] were not able to detect EBV-DNA in serum of recipients of lung transplants with EBV-LPD, while 68% of all samples of these 6 patients tested positive in the PBMC fraction. However, since this is very different from all other reports, these results might be doubted. It has to be stressed that studies summarized in Table 1 and 2 are hard to compare since different viral load detection techniques were used. Furthermore, most were retrospectively performed in selected patients with and without EBV-LPD, while some were prospectively undertaken. Despite this drawback, in the majority of cases, EBV viral load was increased in patients with EBV-LPD. Overall, according to sensitivity and specificity, cell free EBV-DNA detection seems the most accurate technique to predict the presence or development of EBV-LPD. An increase in EBV VL often preceded the development of EBV-LPD in BMT recipients by several weeks[40,42,51,52]. In SOT recipients the time period for EBV-DNA detection prior to the development of EBV-LPD was more variable and ranged from 0 to >10 months[28,46].

Van Esser et al[55] monitored plasma VL in 14 BMT recipients with EBV-LPD. In patients with response to treatment the VL decreased at least 50% within 72 hours after treatment was started. Patients with progressive disease showed an increase in viral load. VL measurements might therefore also be used to monitor response to therapy.
EBV-DNA detection by PCR techniques has also proven to be useful in some other EBV related diseases such as infectious mononucleosis[30,44,48,56-58], chronic active EBV disorder (CAEBV)[44,59], nasopharyngeal carcinoma[60-62] and HIV-associated central nervous system lymphomas[63-65].

Pre-emptive therapy of EBV reactivations

Van Esser et al[66] performed a prospective study in recipients of T cell depleted (TCD) BMT. EBV-DNA in cell free plasma was monitored weekly in 49 patients. Pre-emptive therapy, consisting of a single infusion of rituximab (anti CD20 monoclonal antibody) was given to patients with a VL ≥ 1000 c/ml. Seventeen patients showed EBV reactivation of which 15 received pre-emptive therapy. Only one progressed to EBV-LPD, responding completely after two infusions of rituximab and donor lymphocyte infusion (DLI). In two patients EBV-LPD and EBV VL ≥ 1000 c/ml was diagnosed at the same day. These patients achieved complete remission (CR) after 2 rituximab infusions. In a historical control group of 85 recipients of TCD-BMT 26 patients showed EBV reactivations, of which 10 developed EBV-LPD (38%). In the prospective study 3 of 17 patients with VL ≥ 1000 c/ml developed EBV-LPD (18%). Mortality in the historical group was 80% compared to 0% in the prospective study. This study highlights the importance of monitoring high-risk patients and the effectiveness of pre-emptive therapy with anti CD20 therapy. Currently no other study has been published that prospectively analyses the value of pre-emptive therapy to prevent EBV-LPD, apart from two small studies where 3 and 5 patients were treated pre-emptively with rituximab and EBV-specific cytotoxic T cells (EBVs-CTL), respectively, for rising EBV VL. One of 5 and none of 3 patients progressed to EBV-LPD[67-68].

Prevention and treatment of EBV-LPD

Engineering of marrow grafts Development of EBV-LPD is strongly associated with T cell depletion of donor marrow. The risk for EBV-LPD varied according to the techniques used for T cell depletion, being lowest (<2%) when the Campath-1 or counterflow elutriation methods were used which, in contrast to T cell specific Moabs, removed both T and B cells[36,69-70]. Cavazzana et al[71] observed that none of 19 patients receiving transplants from a partially matched related donor (PMRD) developed EBV-LPD when ex vivo T and B cell depletion was performed, whereas 7 out of 19 historical controls developed EBV-LPD when only T cell depletion was performed. One other study showed that B cell depletion might be of benefit for decreasing the incidence of EBV-LPD[72]. When in our institute grafts from MUDs were depleted both from T and B cells, 4 out of 31 patients (13%) developed EBV-LPD. Without B cell depletion this occurred in 5 out of 7 patients (71%)[73]. In summary, B cell depletion of grafts is efficacious in preventing EBV-LPD in recipients of T cell depleted grafts from MUDs. The degree of B cell depletion needed is still uncertain but is clearly closely related to the degree of TCD[73]. A mechanism explaining the importance of B cell depletion might be a reduction of the EBV viral load transmitted by the marrow graft. This is probably more important than a reduction of the amount of B cells itself, since EBV-LPDs not always consist of donor lymphoid tissue (see introduction).

Antiviral therapy Most studies using antiviral drugs have been performed with acyclovir and ganciclovir, which are both nucleoside analogues. The nucleosides first have to be converted to monophosphate by a viral enzyme (which is thymidine kinase (TK) in case of EBV). Second and third phosphorylations are performed by cellular kinases. Acyclovir or ganciclovir triphosphate is then preferentially incorporated in DNA by viral DNA polymerase and acts as an obligate chain terminator[74]. The effectiveness of newer agents like cidofovir and foscarnet for prevention or treatment of EBV-LPD has not been studied. Cidofovir is a nucleotide analogue of deoxycytidine monophosphate, while foscarnet is a pyrophosphate analogue forming a complex with the pyrophosphate binding site of viral DNA polymerase. Similar to acyclovir and ganciclovir, both drugs are dependent on viral DNA polymerase expression to be functional. Thymidine kinase and viral DNA polymerase are enzymes expressed only during lytic infection, while EBV-LPD is considered to result from latently infected proliferating B cells. Therefore, theoretically, no effect of these drugs can be expected with respect to prevention and treatment of EBV-LPD. However, as is described in

the introduction, some results suggest lytic infection might have a role in the pathogenesis of EBV-LPD[4,6,7].

Prevention Several studies have shown that treatment with acyclovir results in transient inhibition of EBV shedding in the oropharynx in patients with acute IM and also in long term carriers. However, the frequency of circulating EBV infected B cells remained completely unchanged[75-78]. EBV is also able to transform human lymphocytes despite the presence of 500 μM acyclovir[79], while a 2 week exposure of B lymphoblastoid cell lines to 100 μM acyclovir did not prevent release of infectious EBV virus after irradiation to 75 Gray[80]. Many non-randomised studies have been published describing a decrease in incidence of EBV-LPD among SOT and BMT recipients treated prophylactically with acyclovir or ganciclovir, however, an equal amount of studies observed no effect at all of antiviral prophylaxis[81]. In paediatric liver transplant recipients[82] prophylaxis with a short course (2 weeks) ganciclovir (intravenously) followed by long-term oral high-dose acyclovir resulted in EBV disease in 33% of the recipients compared to 21% in recipients receiving the short course ganciclovir alone. In other randomised trials among SOT recipients using acyclovir or ganciclovir prophylaxis, just a trend towards a lower incidence of EBV-LPD was seen[81]. Mc Diarmid et al[83] treated high-risk (EBV serostatus recipient/donor:-/+) paediatric liver transplant recipients prophylactically with intravenously administered ganciclovir for at least a 100 days. In low-risk patients ganciclovir was replaced by oral acyclovir at discharge. Semiquantitative EBV-DNA monitoring was performed and immunosuppression was decreased when VL increased. The overall incidence of EBV-LPD decreased from 10% (historical) to 5%. This study however, does not yield any evidence for effectiveness of ganciclovir. The decreased incidence of EBV-LPD might very well be attributed to the EBV-DNA based reduction of immunosuppressive therapy.

Therapy According to Cohen[84] acyclovir therapy generally has not been effective for SOT and BMT patients with EBV-LPD. The reduction in immunosuppression that often accompanied acyclovir therapy made it difficult to assess the real effectiveness of acyclovir. Nevertheless, since toxicity of acyclovir therapy is low, treatment with acyclovir is often instituted when EBV-LPD has been diagnosed. Two case reports describe the achievement of CR of EBV disease after treatment with ganciclovir or foscarnet[85,86]. Little information is available on the effectiveness of newer antiviral agents regarding prevention or treatment of EBV-LPD.

Withdrawal of immunosuppressive therapy Withdrawal of or decreasing immunosuppressive therapy has proven to be effective in solid organ transplant recipients and is often undertaken as initial strategy[87]. However, this is associated with a risk of graft rejection which can be supported better in renal transplant recipients compared to other SOT recipients. BMT recipients have a far more pronounced immune suppression, which makes withdrawal of immunosuppressive therapy alone usually not sufficient for treating EBV-LPD[88].

Surgery/Radiotherapy Surgical removal or radiotherapy has been effective in patients with localised disease. Survival in SOT recipients with localised EBV-LPD treated with surgical resection alone was 74% compared with 31% in all transplant recipients with EBV-LPD[84].

Chemotherapy Chemotherapy (CT) is generally considered to be a treatment option when other therapies have failed[5,84], although several case reports/small studies are available demonstrating the effectiveness of chemotherapy[89-93]. Cohen[84] did not detect any survival advantage for patients treated with chemotherapy. Results of other larger studies are summarized in Table 3. The only study in which treatment with chemotherapy resulted in a favourable outcome is the one by Fohrer et al[95]. Twenty-seven recipients of SOT with EBV-LPD were treated with chemotherapy consisting of adriamycin, cyclophosfamide, vincristine, bleomycin and steroids. Granulocyte-colony stimulating factor was given and a total of 6 cycles were scheduled every 2-3 weeks. In 19 patients a CR was observed (70%) of which 7 showed an early relapse (within a median time of 3 months). Actuarial survival at 3, 5 and 10 years was 72%, 66% and 49%, respectively. LeBlond et al[97] and Dotti et al[96] found, after univariate analysis, that treatment of EBV-LPD with CT was an adverse risk factor for overall survival in SOT recipients.

Interferon alpha/anti-interleukine 6 Several case reports are published showing the effectiveness of Interferon-alpha (IFN) in the treatment of patients with EBV-LPD after SOT and BMT (summarized in ref.[98]). In total 14 SOT recipients and 4 BMT recipients with EBV-LPD received IFN, of which 12 obtained CR. Davis et al[99] showed that 8 of 14 recipients of SOT with EBV-LPD obtained CR after treatment with IFN. Patients were treated daily ($3x10^6$ U/m^2) for at least 3 weeks and treatment was continued for 6-9 months in responders. Gross et al[21] describe 26 BMT recipients with EBV-LPD. Thirteen patients received therapy for EBV-LPD of whom only 2 patients responded. Both these patients were treated with IFN. It should be noted that all patients described in the varying studies received addi-

Table 3 Results of Chemotherapy

Study	Tx	CT	No. of patients total/treated with CT	CR	OS
Swinnen[24]	HTx	ProMACE-CytaBOM	19/6 early* 19/8 late**	none 75%	44% (2yr)
Gonzalez[94]	SOT	nd	34/20	42%	33% (2yr)
Fohrer[95]	SOT	ACVBP	27/27	70%	72% (3yr) 66% (5yr) 49% (10yr)
Dotti[96]	SOT	P-VABEC	32/19	26%	6 months (median)

SOT = solid organ transplantation; HTx = heart transplantation; nd = not described; CT = chemotherapy; RT = radiotherapy; PTLD = post-transplant lymphoproliferative disorder; *early = EBV-LPD < 6 months; **late = EBV-LPD > 6 months; CR = complete remission; OS = overall survival; ACVBP = adriamycin, cyclophosphamide, vincristine, bleomycin, steroids; P-VABEC = steroids, vincristine, adriamycin, bleomycin, etoposide, cyclophosphamide.

tional therapies. Therefore, it remains unclear whether IFN might be an effective treatment approach for EBV-LPD.

Results of anti-cytokine (anti-interleukine 6) therapy in 12 SOT recipients were promising showing CR in 5 of 12 patients with EBV-LPD, PR in 3 of 12 and stable disease in one. Data were preliminary and larger studies have to be performed to confirm these results[100].

T cell immunotherapy T cell immunotherapy is able to control EBV-LPD in recipients of BMT[101]. O'Reilly reports data on 18 patients with EBV-LPD who were treated with non-specific donor T lymphocyte infusions (DLI). In 16 of 18 patients eradication of EBV-LPD was accomplished. Ten of 18 patients survived in sustained CR, while 3 died from GVHD and 1 from progressive EBV-LPD. This response rate is rather favourable to data from Lucas et al[42], who observed complete response in 4 of 13 patients while a similar proportion experienced GVHD and only 2 of 13 patients survived. A major side-effect of DLI is GVHD. Therefore, Bordignon and Bonini et al[102,103] treated 8 patients with relapse or EBV-LPD with donor T lymphocytes, which were transduced with the herpes simplex virus thymidine kinase (HSV-tk) suicide gene. Three patients developed GVHD that was successfully treated with ganciclovir (CR in two, partial remission in one). This approach, however, is still exper-

imental. A strategy to limit the risk of GVHD is the administration of EBV-specific cytotoxic T lymphocytes (EBVs-CTL). Rooney et al[104] treated 10 BMT recipients of MUD/PMRD grafts with EBVs-CTLs of whom 3 had evidence of uncontrolled EBV reactivation. In all VL fell to normal and symptoms disappeared. In a subsequent study[105] 39 BMT recipients of MUD/PMRD grafts received prophylactic EBVs-CTLs. None developed EBV-LPD in contrast to 7 of 61 controls not receiving prophylactic therapy. Acute GVHD did not develop in any patient receiving EBVs-CTLs. Gustafsson et al[67] describe 9 BMT recipients of whom 5 showed a rapidly rising EBV VL. These patients were pre-emptively treated with EBVs-CTLs, only one progressed to fatal EBV-LPD. This patient received CTLs lacking an EBV-specific component. Altogether, the use of donor derived (HLA-matched) EBV-specific CTLs seems to be very effective, however, is limited by the long time periods required for the generation of these cells. Furthermore, generating these CTLs for every transplant recipient prior to the development of EBV-LPD is very expensive. Therefore, this technique will not be available in every transplantation centre. Another drawback is highlighted by a report of Gottschalk et al[106], in which an EBV deletion mutant was associated with fatal lymphoproliferative disease unresponsive to therapy with EBVs-CTLs.

Donor derived CTLs are generally not used for prevention or treatment of EBV-LPD in SOT recipients, since the donor mostly is not available and donor and recipient generally are not HLA-matched[107]. However, two case reports have been published in which SOT recipients with EBV-LPD were treated successfully with DLI from an HLA-identical sibling donor[108] and with EBVs-CTLs from a partially HLA-matched unrelated blood donor[109]. Several studies described the development and effectiveness of autologous EBVs-CTLs[110-114]. As is the case in BMT, autologous EBVs-CTLs have to be prepared for all SOT recipients prior to the development of EBV-LPD, which (again) is time-consuming and expensive. Therefore, the use of EBVs-CTLs from partially HLA-matched unrelated blood donors, does create new possibilities[109].

Anti B cell therapy Several case reports have been published demonstrating the effectiveness of anti B cell therapy for treatment of EBV-LPD in BMT and SOT recipients[49,115-120]. Fischer et al[121] and Benkerrou et al[122] (see Table 4) treated 58 SOT and BMT recipients with EBV-LPD with anti CD21 plus CD24 antibodies. CR was seen in 61%, while 5 year overall survival (OS) was 46% compared to 29% in historical controls. However, in recipients of BMT, 5 year OS was only 35%. Milpied et al[123] treated 32 SOT and BMT recipients with EBV-LPD with rituximab. After a median follow up of only 8 months, one year OS was 73%.

Table 4 Results of anti B cell therapy

Study	Tx	No. of patients	Moab	CR	OS
Benkerrou[122]	SOT/BMT	58	anti CD21 and 24	61%	46% (5yr)
Milpied[123]	SOT/BMT	32	anti CD20	63%	73% (1yr)
Faye[124]	BMT	12	anti CD20	66%	nd
Kentos[125]	SOT	7	anti CD20	71%	nd

SOT = solid organ transplantation; BMT = bone marrow transplantation; Moab = monoclonal antibody; CR = complete remission; OS = overall survival; nd = not described.

Response (CR and PR) was 65% for SOT recipients and 83% for BMT recipients. Rituximab was also given to twelve paediatric BMT recipients[124] and to 7 adult SOT recipients[125] with EBV-LPD showing CR rates of 66% and 71%, respectively. Thus, anti B cell therapy seems to be very promising, especially when it is started pre-emptively in high-risk patients with increasing EBV VL[66]. It should be noted that EBV-LPD in the central nervous system (CNS) generally does not respond to anti B cell Moabs because of lack of penetration in the CNS[123]. Recently, one patient with EBV-LPD and CNS localisation was treated with rituximab and cidofovir, which resulted in a CR. Plasma and liquor EBV VL became negative during treatment[126].

Future perspective

Marshall et al[127] used HLA class I tetramers complexed with multiple latent and lytic EBV peptides to characterise the dynamics of EBVs T cells in BMT recipients. In recipients of unmanipulated allogeneic BMT from related donors it was demonstrated that expansion of EBVs T cell populations occurred even in the presence of immunosuppressive therapy. The amount of EBVs T cells correlated with EBV VL in PBMC. In contrast, after in vivo TCD or unrelated cord blood transplantation EBVs T cells were undetectable, even in the presence of EBV viremia. Curtis et al[37] already showed that TCD and the use of unrelated donor grafts

were risk factors for EBV-LPD. Nevertheless, the use of these tetramers might enable us to detect transplant recipients without circulating EBVs T cells. These patients have a high risk of developing EBV-LPD and should be monitored intensively to institute pre-emptive therapy (anti CD20 Moabs, EBVs-CTLs) when EBV VL is rising.

Conclusion

In high-risk BMT recipients monitoring of EBV VL in preferably cell free plasma should be performed once a week until 6 months post-transplant. No strict guidelines for frequency and duration of monitoring in SOT recipients can be given, largely due to the variable time period in which post-transplant EBV-LPDs can occur in this patient group. However, in high-risk SOT recipients monitoring may be performed fortnightly or at every outpatient visit until 1 year post-transplant. When EBV reactivation is diagnosed, pre-emptive therapy with anti B cell Moabs is advised in BMT and SOT recipients. In BMT recipients receiving T cell depleted grafts from unrelated donors, additional B cell depletion can reduce the incidence of EBV-LPD dramatically. Treatment of EBV-LPD should start with withdrawal of or decreasing immunosuppression together with anti B cell Moabs. Donor lymphocyte infusion should be reserved for BMT recipients not responding to anti B cell therapy or with central nervous system (CNS) localisation. SOT recipients with CNS localisation might receive additional radiotherapy and/or chemotherapy as well. The efficacy of antiviral therapy in preventing or treating EBV-LPD, if there is any, is very low. Chemotherapy or IFN might be given to SOT recipients when other treatment options have failed or are not available. Localised disease in this patient group can be cured with surgery or radiotherapy. When available, EBV specific cytotoxic T lymphocytes (EBVs-CTLs) from HLA-identical donors or autologous EBVs-CTLs can be used as (pre-emptive) treatment of EBV-LPD in BMT or SOT recipients, respectively. Further studies will be necessary to evaluate the safety and effectiveness of EBVs-CTLs obtained from (partially) HLA-matched related and unrelated blood donors in both BMT and SOT recipients, which will make this approach more accessible.

References

1 Nalesnik MA. Clinical and pathological features of post-transplant lymphoproliferative disorders. Springer Semin Immunopathol 1998; 20: 325-342.

2 Kieff E, Rickinson AB. Epstein-Barr virus and its replication. In: Fields Virology (ed. by D.M. Knipe & P.M. Howley) 2001: pp. 2511-2627. Lippincott Williams & Wilkins, Philadelphia.

3 Gratama JW, Lenette ET, Lonnqvist MAP et al. Detection of multiple Epstein-Barr viral strains in allogeneic bone marrow transplantation. J Med Virol 1992; 37: 39-47.

4 Rowe M, Niedobitek G, Young LS. Epstein-Barr virus gene expression in post-transplant lymphoproliferative disorders. Springer Semin Immunopathol 1998; 20: 389-403.

5 Cohen JI. Epstein-Barr Virus infection. N Eng J Med 2000; 343: 481-492.

6 Rea D, Delecluse HJ, Hamilton-Dutoit SJ et al. Epstein-Barr virus latent and replicative gene expression in post-transplant lymphoproliferative disorders and AIDS-related non-Hodgkin's Lymphomas. Ann Oncol 1994; 5: S113-S116.

7 Montone KT, Hodinka RL, Salhany KE et al. Identification of Epstein-Barr virus lytic activity in posttransplantation lymphoproliferative disease. Mod Pathol 1996; 9: 621-630.

8 Leblond V, Davi F, Charlotte F et al. Posttransplant lymphoproliferative disorders not associated with Epstein-Barr virus: a distinct entity? J Clin Oncol 1998; 16: 2052-2059.

9 van Gorp J, Doornewaard H, Verdonck LF, Klopping C, Vos PF, van den Tweel JG. Posttransplant T cell lymphoma. Cancer 1994; 73: 3064-3072.

10 Nelson BP, Nalesnik MA, Bahler DW et al. Epstein-Barr virus negative post-transplant lymphoproliferative disorders. Am J Surg Pathol 2000; 24: 375-385.

11 Dotti G, Fiocchi R, Motta T et al. Epstein-Barr virus negative lymphoproliferative disorders in long-term survivors after heart, kidney and liver transplant. Transplantation 2000; 69: 827-833.

12 Shapiro RS, McClain K, Frizzera G et al. Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. Blood 1988; 71: 1234-1243.

13 Zutter MM, Martin PJ, Sale GE et al. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. Blood 1988; 72: 520-529.

14 Schafer H, Berger C, Aepinus C et al. Molecular pathogenesis of Epstein-Barr virus associated posttransplant lymphomas: new insights through latent membrane protein 1 fingerprinting. Transplantation 2001; 72: 492-496.

15 Armes JE, Angus P, Southey MC et al. Lymphoproliferative disease of donor origin arising in patients after orthotopic liver transplantation. Cancer 1994; 74: 2436-2441.

16 Larson RS, Scott MA, McCurley TL et al. Microsatellite analysis of posttransplant lymphoproliferative disorders: determination of donor/recipient origin and identification of putative lymphomagenic mechanism. Cancer Res 1996; 56: 378-4381.

17 Cheung ANY, Chan ACL, Lap-Ping Chung Chan TM et al. Post-transplantion lymphoproliferative disorder of donor origin in a sex-mismatched renal allograft as proven by chromosome in situ hybridisation. Mod Pathol 1998; 11: 99-102.

18 Gratama JW, Oosterveer MAP, Zwaan FE et al. Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: implications for sites of viral latency. Proc Natl Acad Sci 1988; 85: 8693-8696.

19 Haque T, Thomas JA, Falk KI et al. Transmission of donor Epstein-Barr virus (EBV) in transplanted organs causes lymphoproliferative disease in EBV-seronegative recipients. J Gen Virol 1996; 77: 1169-1172.

20 Armitage JM, Kormos RL, Stuart RS et al. Posttransplant lymphoproliferative disease in thoracic organ transplant patients: 10 years of cyclosporine-based immunosuppression. J Heart Lung Transplant 1991; 10: 877-887.

21 Gross TG, Steinbuch M, DeFor T et al. B cell lymphoproliferative disorders following hematopoietic stem cell transplantation: risk factors, treatment and outcome. Bone Marrow Transplant 1999; 23: 251-258.

22 van Esser JWJ, van der Holt B, Meijer E et al. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-depleted SCT. Blood 2001; 98: 972-978.

23 Leblond V, Sutton L, Dorent R et al. Lymphoproliferative disorders after organ transplantation: a report of 24 cases observed in a single centre. J Clin Oncol 1995; 13: 961-968.

24 Swinnen LJ, Mullen GM, Carr TJ et al. Aggressive treatment for postcardiac transplant lymphoproliferation. Blood 1995; 86: 3333-3340.

25 Rogers BB, Sommerauer J, Quan A et al. Epstein-Barr virus polymerase chain reaction and serology in pediatric post-transplant lymphoproliferative disorder: three year experience. Pediatr Dev Pathol 1998;1:480-486.

26 Le Meur Y, Potelune N, Jaccard A et al. Lymphoproliferative syndromes after renal transplantation. Nephrologie 1998; 19: 255-261.

27 Ben-Ari Z, Amlot P, Lachmanan SR et al. Posttransplantation lymphoproliferative disorder in liver recipients: characteristics, management and outcome. Liver Transpl Surg 1999; 5: 184-191.

28 Riddler SA, Breinig MC, McKnight JLC. Increased levels of circulating Epstein-Barr virus (EBV)-infected lymphocytes and decreased EBV nuclear antigen antibody responses are associated with the development of posttransplant lymphoproliferative disease in solid organ transplant recipients. Blood 1994; 84: 972-984.

29 Walker RC, Marshall WF, Strickler JG et al. Pretransplantation assessment of the risk of lymphoproliferative disorder. Clin Infect Dis 1995; 20: 1346-1353.

30 Fontan J, Bassignot A, Mougin C et al. Detection of Epstein-Barr virus DNA in serum of transplanted patients: a new diagnostic guide for lymphoproliferative diseases. Leukemia 1998; 12: 772-775.

31 Swinnen LJ, Costanzo-Nordin MK, Fisher SG et al. Increased incidence of lymphoproliferative disorder after immunosuppression with the monoclonal antibody OKT3 in cardiac transplant recipients. N Eng J Med 1990; 323: 1723-1728.

32 Manez R, Breinig MC, Linden P et al. Post-transplant lymphoproliferative disease in primary Epstein-Barr virus infection after liver transplantation: the role of cytomegalovirus disease. J Infect Dis 1997; 176: 1462-1467.

33 Preitsakis JK, Keay S. Diagnosis and management of posttransplant lymphoproliferative disorder in solid organ transplant recipients. Clin Infect Dis 2001; 33: S38-S46.

34 Buda A, Caforio A, Calabrese F et al. Lymphoproliferative disorders in heart transplant recipients: role of hepatitis C virus and Epstein-Barr virus infection. Transplant Int 2000; 13: S402-S405.

35 Finn L, Reyes J, Bueno J et al. Epstein-Barr virus infection in children after transplantation of the small intestine. Am J Surg Pathol 1998; 22: 299-309.

36 Curtis RE, Travis LB, Rowlings PA et al. Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. Blood 1999; 94: 2208-2216.

37 Gerritsen EJ, Hermans J, van den Berg H et al. Risk factors for developing EBV-related B cell lymphoproliferative disorders after non-HLA-identical BMT in children. Bone Marrow Transplant 1996; 18: 377-382.

38 Micallef IN, Chanabhai M, Gascoyne RD et al. Lymphoproliferative disorders following allogeneic bone marrow transplantation: the Vancouver experience. Bone Marrow Transplant 1998; 22: 981-987.

39 Barker JN, Martin PL, Coad JE et al. Low incidence of Epstein-Barr virus-associated posttransplantation lymphoproliferative disorders in 272 unrelated-donor umbilical cord blood transplant recipients. Biol Blood Marrow Transplant 2001; 7: 395-399.

40 Rooney CM, Loftin SK, Holladay MS et al. Early identification of Epstein-Barr virus-associated post-transplantation lymphoproliferative disease. Br J Haematol 1995; 89: 98-103.

41 George D, Barnett L, Boulad F et al. Semi-quantitative PCR analysis of genomic EBV DNA post-BMT allows close surveillance of patients at risk for development of EBV lymphoproliferative disorders allowing prompt intervention. Blood 1998; 92: S 437a. Abstract.

42 Lucas KG, Burton RL, Zimmerman SE et al. Semiquantitative Epstein-Barr virus polymerase chain reaction for the determination of patients at risk for EBV-induced lymphoproliferative disease after stem cell transplantation. Blood 1998; 91: 3654-3661.

43 Lucas KG, Filo R, Heilman DK et al. Semiquantitative Epstein-Barr virus polymerase chain reaction analysis of peripheral blood from organ transplant patients at risk for the development of lymphoproliferative disease. Blood 1998; 92: 3976-3980. Letter.

44 Kimura H, Morita M, Yabuta Y et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol 1999; 37: 132-136.

45 Baldanti F, Grossi P, Furione M et al. High levels of Epstein-Barr virus DNA in blood of solid-organ transplant recipients and their value in predicting posttransplant lymphoproliferative disorders. J Clin Microbiol 2000; 38: 613-619.

46 Stevens SJC, Verschuuren EAM, Pronk I et al. Frequent monitoring of Epstein-Barr virus DNA load in unfractionated whole blood is essential for early detection of posttransplant lymphoproliferative disease in high-risk patients. Blood 2001; 97: 1165-1171.

47 Hoshino Y, Kimura H, Tanaka N et al. Prospective monitoring of the Epstein-Barr virus DNA by a real-time quantitative polymerase chain reaction after allogeneic stem cell transplantation. Br J Haematol 2001; 115: 105-111.

48 Wagner HJ, Wessel M, Jabs W et al. Patients at risk for development of posttransplant lymphoproliferative disorder: plasma versus peripheral blood mononuclear cells as material for quantification of Epstein-Barr viral load by using real-time quantitative polymerase chain reaction. Transplantation 2001; 72: 1012-1019.

49 Wagner JH, Cheng YC, Liu Z et al. Monitoring of transplanted patients at risk of Epstein-Barr virus (EBV)-induced lymphoproliferative disorder (LPD) by real time PCR quantification of EBV DNA in peripheral blood. Blood 2001; 98: S480a. Abstract.

50 Gartner BC, Schafer H, Marggraff K et al. Evaluation of use of Epstein-Barr viral load in patients after allogeneic stem cell transplantation to diagnose and monitor posttransplant lymphoproliferative disease. J Clin Microbiol 2002; 40: 351-358.

51 Sirvent-von Bueltzingsloewen A, Morand P, Buisson M et al. A prospective study of Epstein-Barr virus load in 85 hematopoietic stem cell transplants. Bone Marrow Transplant 2002; 29: 21-28.

52 Beck R, Westdorp I, Jahn G et al. Detection of Epstein-Barr virus DNA in plasma from patients with lymphoproliferative disease after allogeneic bone marrow or peripheral blood stem cell transplantation. J Clin Microbiol 1999; 37: 3430-3431.

53 Limaye AP, Huang ML, Atienza EE et al. Detection of Epstein-Barr virus DNA in sera from transplant recipients with lymphoproliferative disorders. J Clin Microbiol 1999; 37: 1113-1116.

54 Ohga S, Kubo E, Nomura A et al. Quantitative monitoring of circulating Epstein-Barr virus DNA for predicting the development of posttransplantation lymphoproliferative disease. Int J Hematol 2001; 73: 323-326.

55 van Esser JWJ, Niesters HGM, Thijsen SFT et al. Molecular quantification of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus-associated lymphoproliferative disease after allogeneic stem cell transplantation. Br J Haematol 2001; 113: 814-821.

56 Yamamoto M, Kimura H, Hironaka T et al. Detection and quantification of virus DNA in plasma of patients with Epstein-Barr virus-associated diseases. J Clin Microbiol 1995; 33: 1765-1768.

57 Zingg W, Bossart W, Berli E et al. Detection and quantification of cell-free Epstein-Barr virus by polymerase chain reaction and subsequent DNA enzyme immunoassay. J Virol Methods 1999; 79: 141-148.

58 Meerbach A, Gruhn B, Egerer R et al. Semiquantitative PCR analysis of Epstein-Barr virus DNA in clinical samples of patients with EBV-associated diseases. J Med Virol 2001; 65: 348-357.

59 Kanegane H, Wakiguchi H, Kanegane C et al. Increased cell-free viral DNA in fatal cases of chronic active Epstein-Barr virus infection. Clin Infect Dis 1999; 28: 906-909.

60 Lo YMD, Chan LYS, Chan ATC et al. Quantitative and temporal correlation between circulating cell-free Epstein-Barr virus DNA and tumor recurrence in nasopharyngeal carcinoma. Cancer Research 1999; 59: 5452-5455.

61 Lo YMD, Chan ATC, Chan LYS et al. Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. Cancer Research 2000; 60: 6878-6881.

62 Lin JC, Chen KY, Wang WY et al. Detection of Epstein-Barr virus DNA in peripheral-blood cells of patients with nasopharyngeal carcinoma: relationship to distant metastasis and survival. J Clin Oncol 2001; 19: 2607-2615.

63 de Luca A, Antorini A, Cingolani A et al. Evaluation of cerebrospinal fluid EBV-DNA and IL-10 markers for in vivo diagnosis of AIDS-related primary central nervous system lymphoma. Br J Haematol 1995; 90: 844-849.

64 Antorini A, Cingolani A, De Luca A et al. Epstein-Barr virus in monitoring the response to therapy of acquired immunodeficiency syndrome-related primary central nervous system lymphoma. Ann Neurol 1999; 5: 259-261.

65 Portolani M, Cermelli C, Meacci M et al. Epstein-Barr virus DNA in the cerebrospinal fluid of patients with human immunodeficiency virus infection and central nervous system disorders. New Microbiol 1999; 22: 369-374.

66 van Esser JWJ, Niesters HGM, van der Holt B et al. Prevention of Epstein-Barr virus-lymphoproliferative disease by molecular monitoring and pre-emptive rituximab in high-risk patients after allogeneic stem cell transplantation. Blood 2002; 99: 4364-4369.

67 Gustafsson A, Levitsky V, Frisan T et al. Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T-cells. Blood 2000; 95: 807-814.

68 Gruhn B, Meerbach A, Hafer R et al. Early diagnosis and pre-emptive therapy of Epstein-Barr virus-associated lymphoproliferative disease following hematopoietic stem cell transplantation. Blood 2001; 98: S393a. Abstract.

69 Gross TG, Hinrichs SH, Davis JR et al. Depletion of EBV-infected cells in donor marrow by counterflow elutriation. Exp Hematol 1998; 26: 395-399.

70 Hale G, Waldmann H. Risks of developing Epstein-Barr virus-related lymphoproliferative disorders after T-cell-depleted marrow transplants. Blood 1998; 91: 3079-3083.

71 Cavazzana M, Bensoussan D, Jabado N et al. Prevention of EBV-induced B-lymphoproliferative disorder by ex vivo marrow B-cell depletion in HLA-phenoidentical or non-identical T-depleted bone marrow transplantation. Br J Haematol 1998; 103: 543-551.

72 Liu Z, Wilson JM, Jones MC et al. Addition of B cell depletion of donor marrow with anti-CD20 antibody to a T cell depletion regimen for prevention of EBV lymphoma after bone marrow transplantation. Blood 1999; 94: S638a. Abstract.

73 Meijer E, Slaper-Cortenbach ICM, Thijsen SFT et al. Increased incidence of EBV-associated lymphoproliferative disorders after allogeneic stem cell transplantion from matched unrelated donors due to a change of T-cell depletion technique. Bone Marrow Transplant 2002; 29: 335-339.

74 Ljungman P. Prophylaxis against herpesvirus infections in transplant recipients. Drugs 2001; 61: 187-196.

75 Yao QY, Ogan P, Rowe M et al. Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. Int J Cancer 1989; 43: 67-71.

76 Ernberg I, Andersson J. Acyclovir efficiently inhibits oropharyngeal excretion of Epstein-Barr virus in patients with acute infectious mononucleosis. J Gen Virol 1986; 67: 2267-2272.

77 Tynell E, Aurelius E, Brandell A et al. Acyclovir and prednisolone treatment of acute infectious mononucleosis: a multicenter double-blind, placebo-controlled study. J Infect Dis 1996; 174: 324-331.

78 Luxton JC, Williams I, Crawford DH. Epstein-Barr virus infection of HIV-seropositive individuals is transiently suppressed by high-dose acyclovir treatment. AIDS 1993; 7: 1337-1343.

79 Sixbey JW, Pagano JS. Epstein-Barr virus transformation of human B lymphocytes despite inhibition of viral polymerase. J Virol 1985; 53: 299-301.

80 Keever-Taylor CA, Konings S, Orentas RJ et al. Two week exposure of B lymphoblastoid cell lines (BLCL) to 100(M acyclovir does nor prevent release of infectious Epstein-Barr virus after 75 GY irradiation. Blood 2001; 98: S358b. Abstract.

81 Davis CL. The antiviral prophylaxis of post-transplant lymphoproliferative disorder. Springer Sem Immunopathol 1998; 20: 437-453.

82 Green M, Kaufmann M, Wilson J et al. Comparison of intravenous ganciclovir followed by oral acyclovir with intravenous ganciclovir alone for prevention of cytomegalovirus and Epstein-Barr virus disease after liver transplantation in children. Clin Infect Dis 1997; 25: 1344-1349.

83 McDiarmid SV, Jordan S, Lee GS et al. Prevention and preemptive therapy of posttransplant lymphoproliferative disease in pediatric liver recipients. Transplantation 1998; 66: 1604-1611.

84 Cohen JI. Epstein-Barr virus lymphoproliferative disease associated with acquired immunodeficiency. Medicine 1991; 70: 137-160.

85 Gruhn B, Meerbach A, Egerer R et al. Successful treatment of Epstein-Barr virus-induced transverse myelitis with ganciclovir and cytomegalovirus hyperimmune globulin following unrelated bone marrow transplantation. Bone Marrow Transplant 1999; 24: 1355-1358.

86 Schneider U, Ruhnke M, Delecluse HJ et al. Regression of Epstein-Barr virus-associated lymphoproliferative disorders in patients with acquired immunodeficiency syndrome during therapy with foscarnet. Ann Hematol 2000; 79: 214-216.

87 Penn I. The role of immunosuppression in lymphoma formation. Springer Sem Immunopathol 1998; 20: 343-355.

88 Aguilar LK, Rooney CM, Heslop HE. Lymphoproliferative disorders involving Epstein-Barr virus after hemopoietic stem cell transplantation. Curr Opin Oncol 1999; 11: 96-101.

89 Garrett TJ, Chadburn A, Barr ML et al. Posttransplantation lymphoproliferative disorders treated with cyclophosphamide-doxorubicin-vincristine-prednisone chemotherapy. Cancer 1993; 72: 2782-2785.

90 Raymond E, Tricottet V, Samuel D et al. Epstein-Barr virus-related localized hepatic lymphoproliferative disorders after liver transplantation. Cancer 1995; 76: 1344-1351.

91 Balfour IC, Wall D, Luisiri A et al. Cyclophosphamide/Prednisone for combination immunosuppression and therapy of lymphoproliferative disease. J Heart Lung Transplant 1999; 18: 492-495.

92 Smets F, Vajro P, Cornu G et al. Indications and results of chemotherapy in children with posttransplant lymphoproliferative disease after liver transplantation. Transplantation 2000; 69: 982-984.

93 Watts RG, Hilliard LM, Berkow RL. Tailored chemotherapy for non-Hodgkin's lymphoma arising in the setting of post-transplant lymphoproliferative disease following solid organ transplantation. Blood 2001; 98: S253b. Abstract.

94 Gonzalez-Barca E, Domingo-Domenech E, Cabrera J et al. Spanish multicenter study of post-transplant lymphomas: pathology, clinical presentation and outcome. Blood 2001; 98:S338a. Abstract.

95 Fohrer C, Koumarianou A, Caillard S et al. Safety and efficacy of low-dose ACVBP chemotherapy for post-transplant lymphoproliferative disorders (PTLD): a report of 27 cases from a single institution. Blood 2001; 98: S341a. Abstract.

96 Dotti G, Fiocchi R, Motta T et al. High toxicity and low response rate after chemotherapy in patients with advanced stage posttransplant lymphoproliferative disorders. Blood 2001; 98: S343a. Abstract.

97 Leblond V, Dhedin N, Bruneel MFM et al. Identification of prognostic factors in 61 patients with posttransplantation lymphoproliferative disorders. J Clin Oncol 2001; 19: 772-778.

98 Faro A. Interferon-alpha and its effects on post-transplant lymphoproliferative disorders. Springer Sem Immunopathol 1998; 20: 425-436.

99 Davis CL, Wood BL, Sabath DE et al. Interferon-alpha treatment of posttransplant lymphoproliferative disorder in recipients of solid organ transplants. Transplantation 1998; 66: 1770-1779.

100 Durandy A. Anti-B cell and anti-cytokine therapy for the treatment of post-transplant lymphoproliferative disorder: past, present and future. Transplant Infect Dis 2001; 3: 104-107.

101 O'Reilly RJ, Small TN, Papadopoulos E et al. Biology and adoptive cell therapy of Epstein-Barr virus associated lymphoproliferative disorders in recipients of marrow allografts. Immunol Rev 1997; 157: 195-216.

102 Bordignon C, Bonini C, Verzeletti S et al. Transfer of the HSV-tk gene into donor peripheral blood lymphocytes for in vivo modulation of donor anti-tumor immunity after allogeneic bone marrow transplantation. Hum Gene Ther 1995; 6: 813-819.

103 Bonini C, Ferrari G, Verzeletti S et al. HSV-tk gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. Science 1997; 276: 1719-1724.

104 Rooney CM, Smith CA, Ng CYC et al. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. Lancet 1995; 345: 9-13.

105 Rooney CM, Smith CA, Ng CYC et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. Blood 1998; 92: 1549-1555.

106 Gottschalk S, Ng CYC, Perez M et al. An Epstein-Barr virus deletion mutant associated with fatal lymphoproliferative disease unresponsive to therapy with virus-specific CTLs. Blood 2001; 97: 835-843.

107 Haque T, Crawford DH. The role of adoptive immunotherapy in the prevention and treatment of lymphoproliferative disease following transplantation. Br J Haematol 1999; 106: 309-316.

108 Emanuel DJ, Lucas KG, Mallory GB et al. Treatment of posttransplant lymphoproliferative disease in the central nervous system of a lung transplant recipient using allogeneic leukocytes. Transplantation 1997; 63: 1691-1694.

109 Haque T, Taylor C, Wilkie GM et al. Complete regression of posttransplant lymphoproliferative disease using partially HLA-matched Epstein-Barr virus-specific cytotoxic T cells. Transplantation 2001; 72: 1399-1402.

110 Nalesnik MA, Rao AS, Furukawa H et al. Autologous lymphokine-activated killer cell therapy of Epstein-Barr virus-positive and -negative lymphoproliferative disorders arising in organ transplant recipients. Transplantation 1997; 63: 1200-1205.

111 Haque T, Amlot PL, Helling N et al. Reconstitution of EBV-specific T cell immunity in solid organ transplant recipients. J Immunol 1998; 160: 6204-6209.

112 Khanna R, Bell S, Sherritt M et al. Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. Proc Natl Acad Sci 1999; 96: 10391-10396.

113 Savoldo B, Goss J, Liu Z et al. Generation of autologous Epstein-Barr virus-specific cytotoxic T cells for adoptive immunotherapy in solid organ transplant recipients. Transplantation 2001; 72: 1078-1086.

114 Comoli P, Labirio M, Basso S et al. Infusion of autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. Blood 2002; 99: 2592-2598.

115 Lazarovits AI, Tibbles LA, Grant DR et al. Anti-B cell antibodies for the treatment of monoclonal Epstein-Barr virus-induced lymphoproliferative syndrome after multivisceral transplantation. Clin Invest Med 1994; 17: 621-625.

116 Cook RC, Connors JM, Gascoyne RD et al. Treatment of post-transplant lymphoproliferative disease with rituximab monoclonal antibody after lung transplantation. Lancet 1999; 354: 1698-1699.

117 McGuirk JP, Seropian S, Smith B et al. Use of rituximab and irradiated donor-derived lymphocytes to control Epstein-Barr virus-associated lymphoproliferation in patients undergoing related haplo-identical stem cell transplantation. Bone Marrow Transplant 1999; 24: 1253-1258.

118 Zompi S, Tulliez M, Conti F et al. Rituximab for the treatment of patients with clonal lymphoproliferative disorders after orthotopic liver transplantation: a report of three cases. J Hepatol 2000; 32: 521-527.

119 Kuehnle I, Huls MH, Liu Z et al. CD20 monoclonal antibody (rituximab) for therapy of Epstein-Barr virus lymphoma after hemopoietic stem-cell transplantation. Blood 2000; 95: 1502-1505.

120 Zilz ND, Olson LJ, McGregor CG. Treatment of post-transplant lymphoproliferative disorder with monoclonal CD20 antibody (rituximab) after heart transplantation. J Heart Lung Transplant 2001; 20: 770-772.

121 Fischer A, Blanche S, Le Bidois J et al. Anti B-cell monoclonal antibodies in the treatment of severe posttransplant B-cell lymphoproliferative syndrome following bone marrow and organ transplantation. N Eng J Med 1991; 324: 1451-1456.

122 Benkerrou M, Jais JP, Leblond V et al. Anti-B-cell monoclonal antibody treatment of severe posttransplant B-lymphoproliferative disorder: prognostic factors and long-term outcome. Blood 1998; 92: 3137-3147.

123 Milpied N, Vasseur B, Parquet N et al. Humanized anti CD20 monoclonal antibody (rituximab) in post transplant B-lymphoproliferative disorder: a retrospective analysis on 32 patients. Ann Oncol 2000; 11: S113-S116.

124 Faye A, Quartier P, Reguerre Y et al. Chimaeric anti-CD20 monoclonal antibody (rituximab) in post-transplant B-lymphoproliferative disorder following stem cell transplantation in children. Br J Haematol 2001; 115: 112-118.

125 Kentos A, Knoop C, Wissing M et al. Anti-CD20 monoclonal antibody as first-line treatment of post-transplant B-lymphoproliferative disorders in solid organ transplant recipients. A report of 8 cases observed in a single center. Blood 2001; 98: S344a. Abstract.

126 Hanel M, Fiedler F, Thorns C. Anti-CD20 monoclonal antibody (rituximab) and cidofovir as successful treatment of an EBV-associated lymphoma with CNS involvement. Onkologie 2001; 24: 491-494.

127 Marshall NA, Howe JG, Formica R et al. Rapid reconstitution of Epstein-Barr virus-specific T lymphocytes following allogeneic stem cell transplantation. Blood 2000; 96: 2814-2821.

Chapter 8

Influence of Cytomegalovirus-seropositivity on outcome after T cell depleted bone marrow transplantation: contrasting results between recipients of grafts from related and unrelated donors

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Abstract

Whether cytomegalovirus (CMV)-seropositivity still remains a serious adverse risk factor for overall survival (OS) and transplant-related mortality (TRM) in allogeneic bone marrow transplantation (BMT) is under debate. We therefore analysed the effect of CMV serostatus on OS and TRM in 253 consecutively treated patients receiving partial T cell depleted (TCD) bone marrow from either matched related donors (MRD, n=205) or matched unrelated donors (MUD, n=48). All patients were given leukocyte-depleted blood products. CMV monitoring was performed using the pp65 antigenemia assay. Pre-emptive therapy consisted of short-course (2 weeks) low-dose (2.5 mg/kg intravenously b.i.d.) ganciclovir treatment as soon as a positive antigenemia assay was obtained (≥ 1 positive staining granulocyte/150.000 cells). Ganciclovir prophylaxis, identical to pre-emptive therapy, was given to CMV-seropositive patients with acute graft-versus-host disease (aGVHD) grade II-IV who were treated with high-dose corticosteroids. After multivariate analyses, inferior OS and increased TRM were predicted by extensive chronic (c) GVHD ($p<0.001$) in MRD recipients. Furthermore, high-risk disease status and older age adversely influenced OS ($p=0.001$) and TRM ($p=0.002$), respectively, while older age resulted in a trend towards a decreased OS ($p=0.066$). After multivariate analyses in MUD recipients OS and TRM were strongly influenced by patient (but not donor) CMV-seropositivity ($p=0.013$ and 0.007, respectively), while aGVHD also predicted for increased TRM ($p=0.024$). These data show that CMV-seropositivity is not an adverse risk factor for OS and TRM in MRD recipients of partial TCD-BMT. However, in MUD recipients, patient CMV-seropositivity has a high impact on OS and TRM.

Introduction

Cytomegalovirus (CMV) infections in recipients of allogeneic bone marrow transplants (BMTs) historically have been an important cause of morbidity and mortality, primarily due to CMV pneumonia. It did occur mainly in CMV-seropositive recipients by CMV reactivation, but also in CMV-seronegative recipients who acquired primary CMV infection by transfusion of unfiltered blood components or unmanipulated bone marrow from CMV-seropositive donors[1]. In CMV-seronegative recipients of unmanipulated grafts from CMV-seronegative donors or T cell depleted (TCD) grafts from CMV-seropositive donors, primary CMV infection could be prevented by a transfusion policy making use of either CMV-seronegative donors or leukocyte-depleted blood products[2-5]. A major step in preventing the occurrence of CMV pneumonia in CMV-seropositive patients was accomplished by prophylactic long-term (3-4 months) therapy with antiviral drugs like ganciclovir[6-9]. However, in these trials overall mortality was hardly improved because of side effects of long-term ganciclovir prophylaxis such as neutropenia, resulting in bacterial and fungal infections, and more late-onset CMV disease[7-9]. We previously showed that short-course (2 weeks) low-dose (2.5 mg/kg intravenously b.i.d.) ganciclovir therapy initiated either prophylactically, when high-dose corticosteroids were given for acute graft-versus-host disease (aGVHD), or pre-emptively, when CMV-antigenemia was detected, almost completely prevented the occurrence of CMV pneumonia in CMV-seropositive recipients of partial TCD transplants from matched related donors[10]. Furthermore, short-course ganciclovir did not lead to granulocytopenia or late-onset CMV disease. Whether CMV-seropositivity still remains an important risk factor in allogeneic BMT, preventing CMV-disease as described above, is under debate. We therefore analysed the effect of CMV-serostatus on transplant related mortality (TRM) and overall survival (OS) in 253 recipients of partial TCD BMTs of HLA-identical sibling donors or matched unrelated donors.

Methods

Patients For this study data of 253 consecutively treated patients receiving either bone marrow from matched related donors (MRD) (n=205) or from matched unrelated donors (MUD) (n=48) were analysed. Patients with acute leukaemia's in first complete remissions (CR), chronic myeloid leukaemia (CML) in first chronic phase (CP) and untreated severe aplastic anaemia (SAA) were considered low-risk. All patients with other diseases were considered high-risk. TRM was defined as any mortality after transplantation, except relapse. Transplantations were performed between July 1990 and May 2000 at the Department of Haematology of the University Medical Centre Utrecht. Patients were treated according to clinical protocols approved by the local investigation review board after informed consent was obtained.

Transplantation procedure Conditioning regimens consisted of cyclophosphamide (60 mg/kg/day) on each of two successive days, followed by total body irradiation (TBI) (600 cGy/day) on each of 2 successive days, with partial shielding of the lungs (total lung dose 850 cGy). The graft was infused after the second TBI fraction (day 0). Antithymocyteglobulin (ATG) (Thymoglobulin™, Sangstat, Amstelveen, the Netherlands) was given to MUD patients before cyclophosphamide was infused, in a dose of 4 mg/kg/day intravenously for 5 days. Due to a change in national treatment protocols, ATG dose was lowered to 2 mg/kg/day for 4 days from April 1999. All patients received cyclosporin from day -2 in a dose of 3 mg/kg/day by continuous infusion for 3-4 weeks, thereafter it was given orally for 4-6 weeks in a dose that gave comparable through levels, followed by tapering. Cyclosporin was discontinued within 3 months after transplantation, when no active GVHD was present. Infection prevention for all patients consisted of ciprofloxacin, fluconazole and amphotericin B given orally until granulocyte counts exceeded 500 cells/μl. Cephalothin was given intravenously for 10 days from day +3. Furthermore co-trimoxazole and valacyclovir were given orally from day +1 until 12 months post-BMT or longer in case of active GVHD, in a dose of 480 mg b.i.d. and 500 mg b.i.d., respectively. GVHD was classified according to the Seattle criteria[11]. Acute GVHD grade I was treated with topical corticosteroids; grade II or higher was treated with high-dose systemic corticosteroids as described[12]. Limited chronic GVHD was not treated and extensive chronic GVHD was treated with systemic corticosteroids, sometimes combined with cyclosporin[12].

CMV monitoring During the first 4 months post-transplant, all CMV-seropositive patient/donor combinations (R+/D+, R+/D-, R-/D+) were monitored for CMV antigenemia. When patient serostatus was positive (R+/D+, R+/D-) the pp65 assay was performed thrice a week until day 60 after BMT, thereafter twice a week until day 120. In patients with active GVHD monitoring was continued. When patient serostatus was negative (R-/D+) antigenemia was tested twice a week until discharge, thereafter once a week until 5 consecutive negative tests. Seronegative patient/donor combinations were not monitored. In this patient group, CMV-seronegativity was readdressed 3 months after BMT.

CMV pp65 assay This assay was performed as described[13-14]. CMV reactivation was defined as CMV pp65 antigenemia of ≥ 1 positive staining granulocyte/150.000 cells.

CMV disease Patients with symptoms of pneumonia, gastritis or enteritis underwent bronchoscopy, gastroscopy or sigmoidoscopy, respectively. CMV pneumonia/gastritis/enteritis was defined histologically by typical cytopathic effects and immunohistochemically by immunofluorescence with use of monoclonal antibodies to immediate early CMV antigens in biopsy specimens. When cultures of BAL fluid, saliva, urine and buffy coat were performed in case of infectious complications, these included always CMV cultures, irrespective of CMV serostatus.

Ganciclovir therapy CMV-seropositive patients who demonstrated CMV reactivation or who were treated with high-dose corticosteroids for aGVHD grade II-IV received pre-emptive or prophylactic therapy, respectively, with ganciclovir in a dose of 2.5 mg/kg intravenously twice a day for 14 days[10]. When patients were symptomatic (unexplained fever or symptoms compatible with CMV disease), CMV antigenemia was rising or remained positive after 14 days of treatment, ganciclovir dose was doubled or foscarnet treatment was started instead of ganciclovir in a dose of 60 mg/kg twice a day for 14 days. When serum creatinine increased above 200 μmol/l, ganciclovir dose was reduced. When granulocyte count decreased below 500/μl ganciclovir was replaced by foscarnet. CMV disease was treated with ganciclovir 5 mg/kg twice a day for at least 14 days and continued until symptoms resolved and/or antigenemia became negative, whichever was latest. In case of disease progression or rising antigenemia foscarnet treatment was started instead of ganciclovir in a dose of 60 mg/kg twice a day. Furthermore, treatment with CMV specific immunoglobulins was added to antiviral therapy in patients with CMV pneumonia.

HLA-matching In all MRD patient-donor pairs, class I antigens (A, B and Cw) were analysed by serological typing, in case of doubt low resolution molecular typing was performed. Class II antigens (DRB1, DRB3, DRB4, DRB5 and DQB1) were analysed by serological typing until 1993 and since 1993 by low resolution molecular typing with sequence specific primers. In MUD patient-donor pairs HLA analysis was performed as in MRD recipients until 1993, thereafter class I antigens (A, B) were analysed by serological typing, in case of doubt low resolution molecular typing was performed. Class I Cw and class II antigens (DRB1, DRB3, DRB4, DRB5 and DQB1) were analysed by low resolution molecular typing with sequence specific primers. DRB1, B3, B4 and B5 antigens were as well defined by high resolution typing since January 1999.

BMT In vitro partial TCD of the marrow was performed using the Soy Bean Agglutinin/Sheep Red Blood Cell (SBA/SRBC) technique until 1997[15]. Thereafter, the immunorosette (IR) depletion technique was used[16]. After this maximal T cell depletion procedure the residual number of T cells was counted and nonmanipulated T cells (from a small BM fraction that was set apart) were added to obtain the desired fixed low number of T cells (1-5 x 10^5 T cells/kg recipient weight)[12]. Since May 1998 B cell depletion, for prevention of Epstein-Barr virus-associated lymphoma, was added to grafts from MUDs[17].

Statistical analysis OS was estimated by the Kaplan-Meier method. Probabilities of TRM and aGVHD were calculated by the cumulative incidence procedure. For TRM, death without TRM was the competing risk; for aGVHD death without aGVHD was the competing risk. Univariate analyses were performed using the log rank test. Variables which showed to influence OS/TRM at a level of $p<0.1$ were used in a multivariate Cox regression analysis. P values from regression models were calculated with the Wald test. The post-transplant variables 'CMV reactivation', 'aGVHD' and 'cGVHD' were as well analysed as time-dependent covariates. Calculations were performed using SPSS/PC+ 10.0 (SPSS Inc, Chicago Il, USA).

Results

Patient characteristics Characteristics of MRD and MUD recipients are described in Table 1. MRD recipients were significantly older compared to MUD recipients (40 vs 31 yr, respectively, $p<0.001$). In contrast to the MUD group, 35% of MRD recipients were multiple

myeloma or lymphoma patients. Most patients received bone marrow transplants, some MRD patients received peripheral blood stem cell transplants (PBSCT) (MRD: 89% BMT vs 11% PBSCT; MUD: 100% BMT). Only 40% of recipients of matched related donor grafts and 38% of recipients of matched unrelated donor grafts were considered low-risk. Acute GVHD developed in 83% and 70% of MRD and MUD recipients, respectively (p=0.086), and grade II-IV in 50% and 38%, respectively. Chronic GVHD occurred in 56% of evaluable MRD recipients and in 36% of evaluable MUD recipients (p=0.032). The disease was extensive in 27% and 24% of MRD and MUD recipients, respectively. CMV reactivation was observed in 13% of all MRD patients (26% of the CMV-seropositive recipients) and in 25% of all MUD recipients (50% of the CMV-seropositive recipients) (p=0.054). No primary infections were seen in the group with CMV-seronegative patients with seropositive or seronegative donors. Six patients developed CMV disease: pneumonia (n=4), gastritis (n=1) and encephalitis (n=1). All were CMV-seropositive. The disease developed despite pre-emptive treatment with ganciclovir in 3 patients. In the other 3 patients pre-emptive therapy was omitted because of protocol violation (MRD: n=2, MUD: n=1). Four of the 6 patients died from CMV pneumonia, including the 3 not receiving pre-emptive therapy. Two, while successfully treated for CMV disease, died from other causes: varicella-zoster pneumonia -one year after CMV disease- and aspergillus pneumonia -3 months after CMV disease-. Primary graft failure was observed in two patients (1 MRD, 1 MUD), as was secondary graft failure.
In Table 2 causes of mortality are described. “Other causes” of TRM consist of: acute respiratory distress syndrome (ARDS), acute liver failure, pancreatitis with multi-organ failure, cerebral bleeding or infarction, cardiac toxicity, thrombotic or idiopathic thrombocytopenic purpura (ITP or TTP) with bleeding and suicide. Mortality from infections was significantly higher in CMV-seropositive MUD recipients compared to seronegative recipients (38% vs 8%, p=0.016).

Analyses in MRD recipients

Overall survival Median and mean follow up was 20 and 34 (range 1-118) months, respectively. Median survival was 43 months (CI 95%: 14-72 months). Five year overall survival was 47% (CI 95%: 39-55%). From Table 3 and 4 it appears that after univariate and multivariate analyses, high-risk disease status (p<0.001 and 0.001, respectively) and extensive cGVHD (p<0.001) were adverse risk factors for overall survival. After univariate analysis older age significantly affected OS (p=0.003), however, after multivariate analysis a

Table 1 Patient characteristics

	MRD patients (n=205)	MUD patients (n=48)	P
Age, mean years (range)	40 (16-58)	31 (17-48)	<0.001
Diagnosis			
AML cr1	35 (17)	2 (4)	
AML>cr1	7 (3)	6 (13)	
ALL cr1	17 (8)	1 (2)	
ALL>cr1	4 (2)	6 (13)	
CML cp1	32 (16)	8 (17)	
CML>cp1	7 (3)	8 (17)	
SAA	4 (2)	8 (17)	
NHL	27 (13)	0	
MDS	10 (5)	4 (8)	
MM	46 (22)	0	
Other	16 (8)	5 (10)	
CMV serostatus R/D			
+/+	66 (32)	10 (21)	ns
+/-	35 (17)	14 (29)	
-/+	30 (15)	11 (23)	
-/-	74 (36)	13 (27)	
Source			
BM	183 (89)	48 (100)	
PB	22 (11)		
Risk status			
Low	82 (40)	18 (38)	ns
High	123 (60)	30 (62)	
aGVHD			
I	67 (33)	15 (32)	0.086
II-IV	103 (50)	18 (38)	
III-IV	8 (4)	7 (15)	
No	35 (17)	14 (30)	
cGVHD			
Lim	50 (29)	4 (12)	0.032
Ext	47 (27)	8 (24)	
No	78 (45)	22 (65)	
CMV reactivation			
Yes	26 (13)	12 (25)	0.054
No	179 (87)	36 (75)	
CMV disease			
Yes	3 (1.5)	3 (6)	
No	202 (98.5)	45 (94)	

Data are no. (%) of patients, unless otherwise indicated; AML = acute myeloid leukemia; ALL = acute lymphoid leukemia; CML = chronic myeloid leukemia; SAA = severe aplastic anaemia; NHL = non-hodgkin lymphoma; MDS = myelodysplastic syndrome; MM = multiple myeloma; R/D =recipient/donor; BM = bone marrow; PB = peripheral blood; aGVHD = acute graft-versus-host disease; cGVHD = chronic graft-versus-host disease; Lim = limited; Ext = extensive.

Table 2 Causes of Mortality

	CMV serostatus Recipient/Donor							
	+/+		+/-		-/+		-/-	
	MRD	MUD	MRD	MUD	MRD	MUD	MRD	MUD
No. of patients	35	9	23	11	16	6	31	5
Relapse	12	2	10	2	9	4	12	3
TRM	23	7	13	9	7	2	19	2
CMV disease	1	1	1	1	0	0	0	0
EBV-LPD	0	2	2	1	2	2	3	0
Infections (other)	10	1	3	3	3	0	6	0
GVHD	4	1	1	2	0	0	2	1
IP/toxicity	1	0	4	0	1	0	4	1
VOD	3	0	0	0	1	0	0	0
Other	4	2	2	2	0	0	4	0

EBV-LPD = Epstein-Barr virus associated lymphoproliferative disease; IP = interstitial pneumonia; VOD = veno-occlusive disease.

Table 3 Univariate analyses of OS and TRM in MRD recipients

	OS in MRD	TRM in MRD
	P	P
Recipient CMV serostatus (pos vs neg)	ns	ns
Donor CMV serostatus (pos vs neg)	ns	ns
Age (continuous)	0.003	0.002
Risk status (high vs low)	< 0.001	0.040
R/D sexe (male/female vs other)	ns	0.057
Source (pb vs bm)	ns	0.028
T cell (graft) (continuous)	ns	ns
aGVHD (II-IV vs other)	ns	ns
cGVHD (extensive vs other)	< 0.001	< 0.001
CMV reactivation (yes vs no)	ns	0.020

Abbreviations: see Table 1.

trend towards decreased survival was observed (p=0.066). The effect of CMV serostatus was analysed in two ways. First, the 4 groups with all possible patient/donor CMV serostatus combinations were analysed separately (patient/donor: R+/D+, R+/D-, R-/D+, R-/D-). In a second analysis, the group with CMV-seropositive patients (irrespective of donor serology) was compared to the group with CMV-seronegative patients. After both analyses no significant differences were observed. Adjusted probability of OS according to recipient CMV serostatus is displayed in Figure 1.

Furthermore, donor CMV serostatus did not affect outcome. In contrast to the MUD group, 35% of MRD recipients were multiple myeloma or lymphoma patients. Analyses without these patients gave fully comparable results.

Transplant related mortality TRM at one year was 23% (CI 95%: 17-29%). From Table 3 it appears that TRM was determined by extensive cGVHD (p<0.001), older age (p=0.002), CMV reactivation (p=0.020), source (PB) (p=0.028), high-risk disease status (p=0.040) and recipient/donor sexe (R^m/D^f) (p=0.057) after univariate analyses. After multivariate analysis (Table 4) only extensive cGVHD (p<0.001) and age (p=0.002) predicted increased TRM. Adjusted probability of TRM according to recipient CMV serostatus is displayed in Figure 1.

Analyses in MUD recipients

Overall survival Median and mean follow up was 8 and 20 (range 1-102) months, respectively. Median survival was 7 months (CI 95%: 4-10 months). Five year overall survival in MUD recipients was 30% (CI 95%: 14-46%). Overall survival in MUD recipients was

Table 4 Multivariate analyses of OS and TRM in MRD recipients

	Overall Survival			Transplant Related Mortality		
	RR	**95% CI**	**P value**	**RR**	**95% CI**	**P value**
cGVHD (ext. vs other)	2.70	1.68-4.36	< 0.001	3.71	2.01-6.86	< 0.001
Risk status (high vs low)	2.12	1.36-3.28	0.001			ns
Age (continuous)	1.02	1.00-1.04	0.066	1.04	1.02-1.07	0.002

Abbreviations: see Table 1.

Figure 1

Adjusted probability of overall survival and transplant related mortality in MRD recipients according to recipient CMV serostatus.

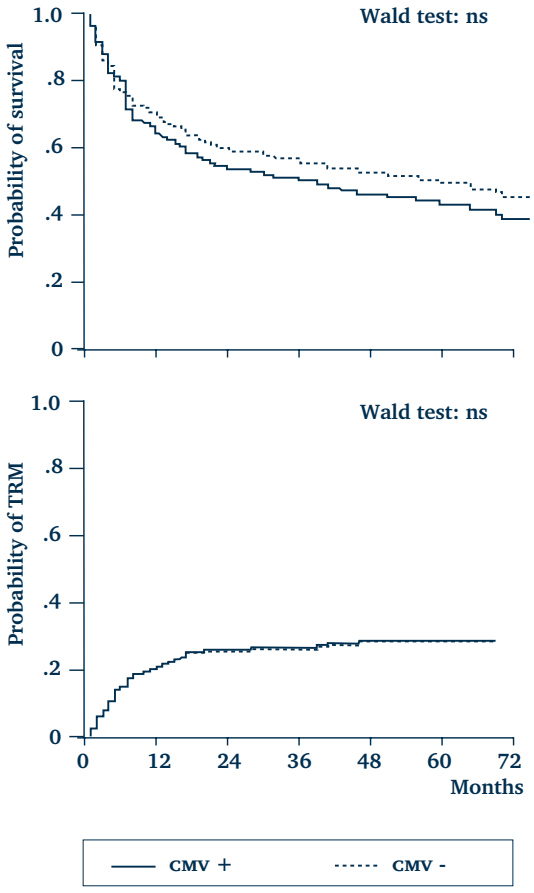


Table 5 Univariate analyses of OS and TRM in MUD recipients

	OS in MRD	TRM in MRD
	P	P
Recipient CMV serostatus (pos vs neg)	0.007	< 0.001
Donor CMV serostatus (pos vs neg)	ns	ns
Age (continuous)	0.051	ns
Risk status (high vs low)	ns	ns
p/d sexe (m/f vs other)	ns	ns
T cell (graft) (continuous)	ns	ns
aGVHD (II-IV vs other)	ns	0.002
cGVHD (extensive vs other)	ns	ns
CMV reactivation (yes vs no)	ns	0.066

Abbreviations: see Table 1.

strongly determined by CMV serostatus (p=0.007) after univariate analyses; age was a less important factor (p=0.051) (Table 5). Again the effect of CMV serostatus was analysed in two ways. First, the 4 groups with all possible patient/donor CMV serostatus combinations were analysed separately (patient/donor: R+/D+, R+/D-, R-/D+, R-/D-). Overall, the groups differed significantly (p=0.027), which could be ascribed to differences between group 1 (R+/D+) and 4 (R-/D-) (p=0.012), group 1 (R+/D+) and 3 (R-/D+) (p=0.062) and group 2 (R+/D-) and 4 (R-/D-) (p=0.068). In a second analysis, the group with CMV-

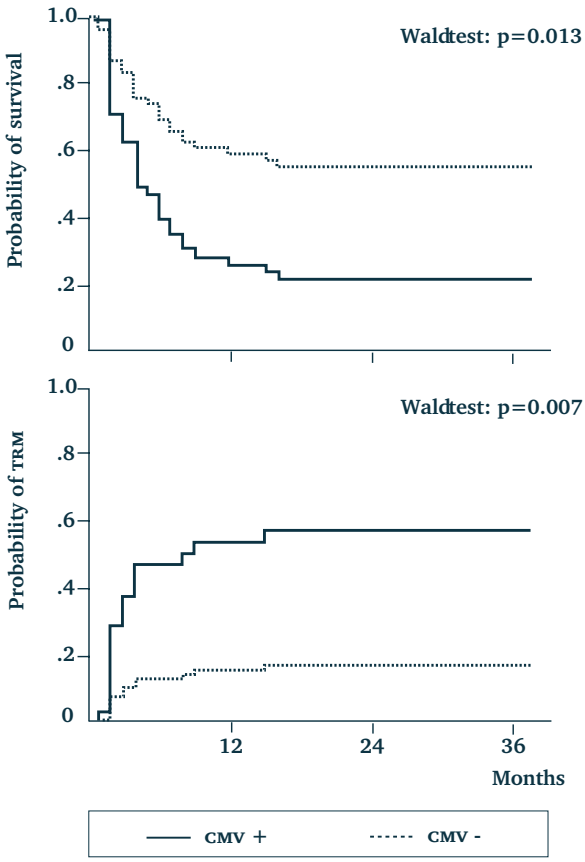
Table 6 Multivariate analyses of OS and TRM in MUD recipients

	Overall Survival			Transplant Related Mortality		
	RR	95% CI	P value	RR	95% CI	P value
Recipient CMV serostatus (pos vs neg)	2.77	1.22-5.40	0.013	4.60	1.51-14.00	0.007
aGVHD (II-IV vs other)			ns	2.94	1.15-7.51	0.024

Abbreviations: see Table 1.

Figure 2

Adjusted probability of overall survival and transplant related mortality in MUD recipients according to recipient CMV serostatus.



seropositive patients (irrespective of donor serology) was compared to the group with CMV-seronegative patients, which revealed a highly significant difference (p=0.007). After multivariate analysis (Table 6 and Figure 2) only a positive recipient CMV serostatus influenced OS (p=0.013), with no effect of donor serostatus.

Transplant related mortality After univariate analysis recipient CMV-seropositivity ($p<0.001$) and aGVHD ($p=0.002$) showed a strong adverse effect on TRM (Table 5), which was also observed after multivariate analysis (recipient CMV-seropositivity: $p=0.007$; aGVHD: $p=0.024$; Table 6 and Figure 2). Cumulative incidence curves for time to aGVHD showed no effect of recipient CMV serostatus on probability of developing aGVHD by day 100 post-transplant. After univariate analysis CMV reactivation also affected TRM. This effect disappeared after multivariate analysis, which is logical given the strong effect of a positive CMV serostatus. TRM at 1 year for all MUD recipients was 37% (CI 95%: 23-51%). In CMV-seropositive recipients one year TRM was 58% (CI 95%: 38-78%) and in CMV-seronegative recipients 17% (CI 95%: 2-32%).

Discussion

Our data show that recipient and/or donor CMV-seropositivity was not predictive for OS and TRM in patients treated with partial T cell depleted bone marrow transplantation from matched related donors, receiving low-dose short-term pre-emptive and prophylactic treatment with ganciclovir. In contrast, in MUD recipients, patient (but not donor) CMV-seropositivity had a great impact on overall survival. In our institution CMV reactivation was monitored by the CMV pp65 antigenemia assay and pre-emptive therapy with ganciclovir was given when antigenemia was demonstrated. Prophylactic therapy was given when CMV-seropositive patients had to be treated with high-dose steroids for aGVHD grade II-IV. A previous study of our group showed that none of the 41 CMV seropositive MRD patients, monitored and treated similar as described above, developed CMV disease[10]. Now we found that 3 of 205 MRD patients (1.5%) and 3 of 48 MUD recipients (6%) developed CMV disease.

Several studies have been published analysing the impact of CMV-seropositivity on OS and TRM in recipients of non-TCD grafts. In three of them outcome was not significantly influenced by patient and/or donor seropositivity[18-20], although in the study of Nichols et al[20] a borderline significant decreased survival was observed in the R-/D+ group compared to the R-/D- group. In MUD recipients only, patient CMV-seropositivity was an adverse risk factor for outcome[21,22], although this was not supported by a subgroup analysis by Nichols et al[20].

Two studies performed analyses among recipients of TCD grafts[23,24]. Broers et al[23] found patient and/or donor seropositivity to be associated with decreased survival and increased TRM in MRD recipients. They instituted pre-emptive treatment when 4 or more cells were positive in the antigenemia assay, used conventional dose ganciclovir, performed no T cell add back and gave no prophylactic therapy to seropositive patients with aGVHD grade II-IV. Results of a study among MUD recipients of TCD grafts[24] were comparable to studies performed among MUD patients receiving non-TCD grafts[21,22]. Overall, when TCD is performed or not, CMV serostatus seems not to influence outcome in MRD recipients in the era of pre-emptive treatment. However, CMV-seropositive MUD recipients were found to have a decreased survival in nearly all studies. In a large EBMT mega file analysis the effect of donor serostatus was analysed in CMV-seropositive recipients. In MRD recipients donor serostatus did not affect outcome. In MUD recipients of non-TCD transplants outcome was improved in those receiving seropositive grafts[25]. We did not observe a positive impact of donor seropositivity in CMV-seropositive recipients, neither in MRD recipients nor in MUD recipients. This is in concordance with the findings of the EBMT study[25], since all our patients received (partial) TCD grafts. In several reports the recovery of CMV-specific (CMVs) immune response was associated with the infusion of bone marrow from seropositive donors[26,27], probably by transfer of CMVs cytotoxic T cells. However, when TCD grafts are infused, CMVs immune recovery is probably not influenced by donor serostatus.

Recently it was demonstrated that CMV infection inhibited maturation and antigen-presenting function of dendritic cells, which can have severe and multiple consequences for T and B cell responses[28] and may contribute to the immunosuppressive effect of CMV infection[29]. It might be hypothesized that many CMV-seropositive transplant recipients suffer from sub clinical CMV infection, which is not detected by the antigenemia assay. Recovery of T cells after T cell depleted BMT is much more impaired in MUD recipients compared to MRD recipients, probably related to the use of ATG in MUD patients[30]. Therefore, considering the immunosuppressive effect of CMV infection, sub clinical CMV infection might be of more importance in MUD recipients compared to MRD recipients. Indeed, mortality from (viral and fungal) infections was higher in the CMV-seropositive MUD group compared to the CMV-seronegative group (38% vs 8%; $p=0.016$).

In conclusion, CMV-seropositivity should not be considered an adverse risk factor for OS and TRM in MRD recipients of partial T cell depleted grafts, when an appropriate prevention of CMV disease is applied. In patients receiving grafts from MUDs, patient (but not donor)

CMV-seropositivity has a high impact on overall survival, may be due to further impairment of immune reconstitution in this already heavily immune suppressed patient population. Approaches that will lead to a better T cell immune reconstitution after TCD stem cell transplantation from unrelated donors are probably necessary to improve outcome in CMV-seropositive MUD recipients.

References

1 Meyers JD, Flournoy N, Thomas ED. Risk factors for cytomegalovirus infection after human marrow transplantation. J Infect Dis 1986; 153: 478-488.

2 Bowden RA, Slichter SJ, Sayers MH et al. Use of leukocyte-depleted platelets and cytomegalovirus-seronegative red blood cells for prevention of primary cytomegalovirus infection after marrow transplant. Blood 1991; 78: 246-250.

3 Verdonck LF, de Graan-Hentzen YCE, Dekker AW et al. Cytomegalovirus seronegative platelets and leukocyte-poor red blood cells from random donors can prevent primary cytomegalovirus infection after bone marrow transplantation. Bone Marrow Transplant 1987; 2: 73-78.

4 van Prooyen HC, Visser JJ, van Oostendorp WR et al. Prevention of primary transfusion-associated cytomegalovirus infection in bone marrow transplant recipients by the removal of white cells from blood components with high-affinity filters. Br J Haematol 1994; 87: 144-147.

5 de Gast GC, Boland GJ, Vlieger AM et al. Abortive human cytomegalovirus infection in patients after allogeneic bone marrow transplantation. Bone Marrow Transplant 1992; 9: 221-225.

6 Schmidt GM, Horak DA, Niland JC et al. A randomized controlled trial of prophylactic ganciclovir for cytomegalovirus pulmonary infection in recipients of allogeneic bone marrow transplants. N Engl J Med 1991; 324: 1005-1011.

7 Goodrich JM, Bowden RA, Fisher L et al. Ganciclovir prophylaxis to prevent cytomegalovirus disease after allogeneic marrow transplant. Ann Intern Med 1993; 118: 173-178.

8 Winston DJ, Ho WG, Bartoni K et al. Ganciclovir prophylaxis of cytomegalovirus infection in allogeneic bone marrow transplant recipients. Ann Intern Med 1993; 118: 179-184.

9 Boeckh M, Gooley TA, Myerson D et al. Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir at engraftment after allogeneic marrow transplantation: a randomized double-blind study. Blood 1996; 88: 4063-4071.

10 Verdonck LF, Dekker AW, Rozenberg-Arska M, van den Hoek MR. A risk adapted approach with a short course of ganciclovir to prevent cytomegalovirus (CMV) pneumonia in CMV-seropositive recipients of allogeneic bone marrow transplantation. Clin Infect Dis 1997; 24: 901-907.

11 Thomas ED, Storb R, Clift RA et al. Bone marrow transplantation. N Eng J Med 1975; 292: 832-843.

12 Verdonck LF, Dekker AW, de Gast GC et al. Allogeneic bone marrow transplantation with a fixed low number of T cells in the marrow graft. Blood 1994; 83: 3090-3096.

13 Schirm J, Timmerije W, van der Bij W et al. Rapid detection of infectious cytomegalovirus in blood with aid of monoclonal antibodies. J Med Virol 1987; 23: 31-40.

14 van der Bij W, Torensma R, van Son WJ et al. Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leukocytes. J Med Virol 1988; 25: 179-188.

15 Reisner Y, Kirkpatrick D, Dupont B et al. Transplantation for acute leukaemia with HLA-A and B nonidentical parental marrow cells fractionated with soybean agglutinin and sheep red blood cells. Lancet 1981; 15: 327-331.

16 Slaper-Cortenbach ICM, Wijngaarden-du Bois MJGJ, de Vries-van Rossen A et al. The depletion of T cells from haematopoietic stem cell transplants. Rheumatology 1999; 38: 751-754.

17 Meijer E, Slaper-Cortenbach ICM, Thijsen SFT, Dekker AW, Verdonck LF. Increased incidence of EBV-associated lymphoproliferative disorders after allogeneic stem cell transplantion from matched unrelated donors due to a change of T-cell depletion technique. Bone Marrow Transplant 2002; 29: 335-339.

18 Humar A, Wood S, Lipton J et al. Effect of cytomegalovirus infection on 1-year mortality rates among recipients of allogeneic bone marrow transplants. Clin Infect Dis 1998; 26: 606-610.

19 Peggs KS, Preiser W, Kottaridis PD et al. Extended routine polymerase chain reaction surveillance and pre-emptive antiviral therapy for cytomegalovirus after allogeneic transplantation. Br J Haematol 2000; 111: 782-790.

20 Nichols WG, Corey L, Gooley T et al. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. J Infect Dis 2002; 185: 273-282.

21 Kröger N, Zabelina T, Krüger W et al. Patient cytomegalovirus seropositivity with or without reactivation is the most important prognostic factor for survival and treatment-related mortality in stem cell transplantation from unrelated donors using pretransplant in vivo T-cell depletion with antithymocyte globulin. Br J Haematol 2001; 113: 1060-1071.

22 Castro-Malaspina H, Harris RE, Gajewski J et al. Unrelated marrow transplantation for myelodysplastic syndromes: outcome analysis in 510 transplants facilitated by the national marrow donor program. Blood 2002; 99: 1943-1951.

23 Broers AEC, van der Holt R, van Esser JWJ et al. Increased transplant-related morbidity and mortality in CMV-seropositive patients despite highly effective prevention of CMV disease after allogeneic T-cell-depleted stem cell transplantation. Blood 2000; 95: 2240-2245.

24 Craddock C, Szydlo RM, Dazzi F et al. Cytomegalovirus seropositivity adversely influences outcome after T-depleted unrelated donor transplant in patients with chronic myeloid leukaemia: the case for tailored graft-versus-host disease prophylaxis. Br J Haematol 2001; 112: 228-236.

25 Ljungman PT, Brand R, Einsele H et al. Donor CMV serostatus influences outcome after unrelated donor stem cell transplantation; an EBMT megafile analysis. Blood 2001; 98: S480a. Abstract.

26 Cwynarski K, Ainsworth J, Cobbold M et al. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. Blood 2001; 97:1232-1240.

27 Li CR, Greenberg PD, Gilbert MJ et al. Recovery of HLA-restricted cytomegalovirus (CVM)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood 1994; 83: 1971-1979.

28 Moutaftsi M, Mehl AM, Borysiewicz LK, Tabi Z. Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells. Blood 2002; 99: 2913-2921.

29 Verdonck LF, de Gast GC. Is cytomegalovirus infection a major cause of T cell alterations after (autologous) bone-marrow transplantation? Lancet 1984; 8383: 932-935.

30 Meijer E, Bloem AC, Dekker AW, Verdonck LF. Effect of Antithymocyteglobulin on quantitative immune recovery and graft-versus-host disease after partially T cell depleted bone marrow transplantation: a comparison between recipients of matched related and matched unrelated donor grafts. Transplantation 2003; 75: 1910-1913.

Chapter 9

Influence of Antithymocyteglobulin dose on outcome in Cytomegalovirus-seropositive recipients of partially T cell depleted stem cell grafts from matched unrelated donors

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Abstract

Recently, several reports stressed the adverse impact of a positive recipient Cytomegalovirus (CMV) serostatus on outcome in recipients of matched unrelated donor (MUD) grafts. In this study we evaluated whether CMV-seropositive MUD recipients transplanted after 1999, still showed inferior outcome compared to CMV-seronegative recipients. In that year two important changes in transplantation procedure were introduced: 1) reduction of Antithymocyteglobulin (ATG) dose (ATG was given prior to the myeloablative conditioning regimen to prevent non-engraftment), 2) introduction of sequence based typing of HLA-DRB1. In total 80 patients received partial T cell depleted grafts, 36 before 1999 and 44 after 1999. CMV-seropositive patients transplanted before 1999 showed a highly significant inferior outcome compared to seronegative recipients. In contrast, in patients transplanted after 1999 no difference in outcome was observed between the two groups.

Introduction

Cytomegalovirus (CMV) infections in recipients of allogeneic stem cell transplants (SCT) historically have been an important cause of morbidity and mortality, primarily due to CMV pneumonia. This very serious complication occurred mainly in CMV-seropositive recipients, with acute graft-versus-host disease (aGVHD) being an important risk factor[1]. Since the introduction of pre-emptive treatment of CMV reactivations, a positive CMV serostatus no longer is an adverse risk factor for outcome in recipients of matched related donor (MRD) grafts[2-5]. However, in recipients of grafts from matched unrelated donors (MUD), recipient CMV-seropositivity has a major negative impact on survival after SCT[5-8]. Most patients in these studies were treated before 1999. In our transplantation centre two important changes in transplantation procedure were introduced in that year. All patients received partial T cell depleted (TCD) grafts and were pre-treated with Antithymocyteglobulin (ATG) prior to the myeloablative conditioning regimen to prevent non-engraftment. In April 1999 ATG dose was lowered from 20 mg/kg to 8 mg/kg. Furthermore, in January 1999 sequence based typing (SBT) of HLA-DRB1 was introduced. Both changes may result in an improved immune reconstitution post-transplant. Low-dose ATG by a direct effect on T lymphocyte counts and high resolution HLA-DRB1 typing by a decreased incidence of GVHD. Considering the immunosuppressive effect of (latent) CMV infection[9-10], this may have a positive impact on outcome in CMV-seropositive SCT recipients, which is analysed in the present study.

Patients and methods

Patients For this study data of 80 consecutively treated patients receiving stem cells from MUDs were analysed. Thirty-six patients were treated from July 1990 until January 1999 and 44 from January 1999 until January 2002. Patients with acute leukaemia's (AL) in first complete remissions (CR), chronic myeloid leukaemia (CML) in first chronic phase (CP) and untreated severe aplastic anaemia (SAA) were considered low-risk. All patients with AL and CML in more advanced stages and other diseases were considered high-risk. TRM was defined as any mortality after transplantation, except relapse. Transplantations were performed at the Department of Haematology of the University Medical Centre Utrecht.

Patients were treated according to clinical protocols approved by the local investigation review board after informed consent was obtained.

Transplantation procedure The conditioning regimen consisted of cyclophosphamide (60 mg/kg/day for 2 days), followed by total body irradiation (600 cGy/day for 2 days) with partial shielding of the lungs (total lung dose 850 cGy). The graft was infused after the second TBI fraction (day 0). Antithymocyteglobulin (Thymoglobulin™, Sangstat, Amstelveen, the Netherlands) was given to MUD patients before cyclophosphamide was infused, in a dose of 4 mg/kg/day intravenously for 5 days. Due to a change in national treatment protocols, ATG dose was lowered to 2 mg/kg/day for 4 days from April 1999. Post-transplant immunosuppression consisted of cyclosporin which was discontinued within 3 months after transplantation, when no active GVHD was present. Infection prevention for all patients consisted of ciprofloxacin, fluconazole and amphotericin B given orally until granulocyte counts exceeded 500 cells/μl. Cephalothin was given intravenously for 10 days from day +3. Furthermore co-trimoxazole and (val)acyclovir were given orally from day +1 until 12 months post-BMT or longer in case of active GVHD, in a dose of 480 mg b.i.d. and 500 mg b.i.d., respectively.

CMV monitoring Until April 2001 CMV monitoring was pp65 based as described[5]. Since then monitoring was performed using a real-time Taqman™ CMV DNA PCR. CMV reactivation was defined as CMV pp65 antigenemia of $\geq$ 1 positive staining granulocyte/150.000 cells or CMV DNA viral load (VL) of > 1000 copies/ml.

CMV disease Patients with symptoms of pneumonia, gastritis or enteritis underwent bronchoscopy, gastroscopy or sigmoidoscopy, respectively. CMV pneumonia/gastritis/enteritis was defined histologically by typical cytopathic effects and immunohistochemically by immunofluorescence with use of monoclonal antibodies to immediate early CMV antigens in biopsy specimens. When cultures of BAL fluid, saliva, urine and buffy coat were performed in case of infectious complications, these included always CMV cultures, irrespective of CMV serostatus.

Ganciclovir therapy CMV-seropositive patients who demonstrated CMV reactivation or who were treated with high-dose corticosteroids for aGVHD grade II-IV received pre-emptive or prophylactic therapy, respectively, with ganciclovir in a dose of 2.5 mg/kg intravenously twice a day for 14 days. When patients were symptomatic (unexplained fever or symptoms compatible with CMV disease), CMV antigenemia/VL was rising or remained positive after 14 days of treatment, ganciclovir dose was doubled or foscarnet treatment was

started instead of ganciclovir in a dose of 60 mg/kg twice a day for 14 days. CMV disease was treated with ganciclovir 5 mg/kg twice a day for at least 14 days and continued until symptoms resolved and antigenemia/VL became negative. In case of disease progression or rising antigenemia/VL foscarnet treatment was started instead of ganciclovir in a dose of 60 mg/kg twice a day. Furthermore, treatment with CMV specific immunoglobulins was added to antiviral therapy in patients with CMV pneumonia.

HLA-matching HLA-A and B matching was based on serological typing and HLA-C, DRB1 and DQB1 matching was based on low resolution molecular typing with sequence specific primers. Since January 1999 and July 2000 SBT of HLA-DRB1 and HLA-A, B and DRB1, respectively, was performed as well.

BMT In vitro partial TCD of the marrow was performed using the Soy Bean Agglutinin/Sheep Red Blood Cell technique until 1997, thereafter, the immunorosette depletion technique was used as described[5]. After this maximal T cell depletion procedure the residual number of T cells was counted and nonmanipulated T cells (from a small BM fraction that was set apart) were added to obtain the desired fixed low number of T cells (1-5 x 10^5 T cells/kg recipient weight).

Statistical analysis Overall survival (OS) was estimated by the Kaplan-Meier method. Probability of transplant related mortality (TRM) was calculated by the cumulative incidence procedure, death from relapse being the competing risk. Univariate analyses were performed using the log rank test. Variables which showed to influence OS/TRM at a level of $p<0.1$ were used in a multivariate Cox regression analysis. P values from regression models were calculated with the Wald test. Pre- and post-transplant variables analysed were: CMV serostatus of recipient (positive vs negative), CMV serostatus of donor (positive vs negative), patient age (continuous), risk status (high vs low), patient/donor sex (m/f vs other), T cell count of the graft ($< 2 \times 10^5$/kg vs $\geq 2 \times 10^5$/kg), ATG dose (high-dose vs low-dose), aGVHD (II-IV vs other), chronic GVHD (extensive vs other), CMV reactivation (yes vs no).The post-transplant variables 'aGVHD' and 'cGVHD', 'CMV reactivation' were as well analysed as time-dependent covariates. Calculations were performed using SPSS/PC+ 10.0 (SPSS Inc, Chicago Il, USA).

Results

Patient characteristics (Table 1). Thirty-six patients were treated before 1999 (historical group) and 44 since 1999 (recent group). In both groups one patient was excluded from analysis because of non-engraftment. Acute GVHD grade II-IV was more often diagnosed in the historical group compared to the recent group (40% vs 23%, respectively, ns) as was extensive cGVHD (17% vs 7%, respectively, ns). Most important is the percentage of CMV reactivations which was only 14% in the recent group compared to 31% in the historical group (p=0.063). Furthermore, most patients in the historical group experienced recurrent CMV reactivations (6 of 11 patients), compared to 1 of 6 patients in the recent group. Two patients in the historical group showed ganciclovir related neutropenia, compared to none in the recent group. Median follow up of survivors was 68 months (range: 45-115) in the historical group and 20 months (range: 6-43) in the recent group.

OS and TRM Figure 1 shows OS and TRM in patients transplanted before and after 1999 according to recipient CMV serostatus. After univariate analyses, the only negative risk factor for OS in the historical group was a positive recipient CMV serostatus (p=0.009), while recipient CMV-seropositivity, CMV reactivation and aGVHD grade II-IV were adverse risk factors for TRM in the historical group. However, after multivariate analysis, only recipient CMV-seropositivity adversely affected TRM (p=0.004). In the historical group 11/17 CMV-seropositive patients died from fatal viral (CMV pneumonia: n=3) and fungal infections, compared to 1/18 (CMV disease: n=0) in the recent group (p=0.005). In the historical group, survival analyses were also performed for all 4 recipient/donor CMV serostatus combinations separately (R+/D+, R+/D-, R-/D+, R-/D-), showing no effect of donor serostatus in the CMV-seropositive recipients. In the group treated since 1999 recipient CMV-seropositivity did not affect outcome anymore.

When analyses were performed among CMV-seropositive patients, only ATG dose was a negative risk factor for outcome (OS: p=0.06; TRM: p=0.007).

Discussion

This study shows that lowering ATG dose and introducing SBT of HLA- DRB1 (and later also HLA-A and B) had an important impact on OS and TRM in CMV-seropositive MUD recipients of partial TCD grafts. HLA-matching based on high resolution DNA typing resulted in a decreased incidence of acute and chronic GVHD (although ns). Since ATG dose was the only risk factor for outcome in seropositive patients and GVHD showed to have no impact, we postulate that lowering the ATG dose has been the most important factor diminishing mortality in CMV-seropositive recipients. Until April 2001 CMV monitoring was performed using

Figure 1

Probability of OS and TRM in MUD recipients treated before and after 1999 according to CMV serostatus.

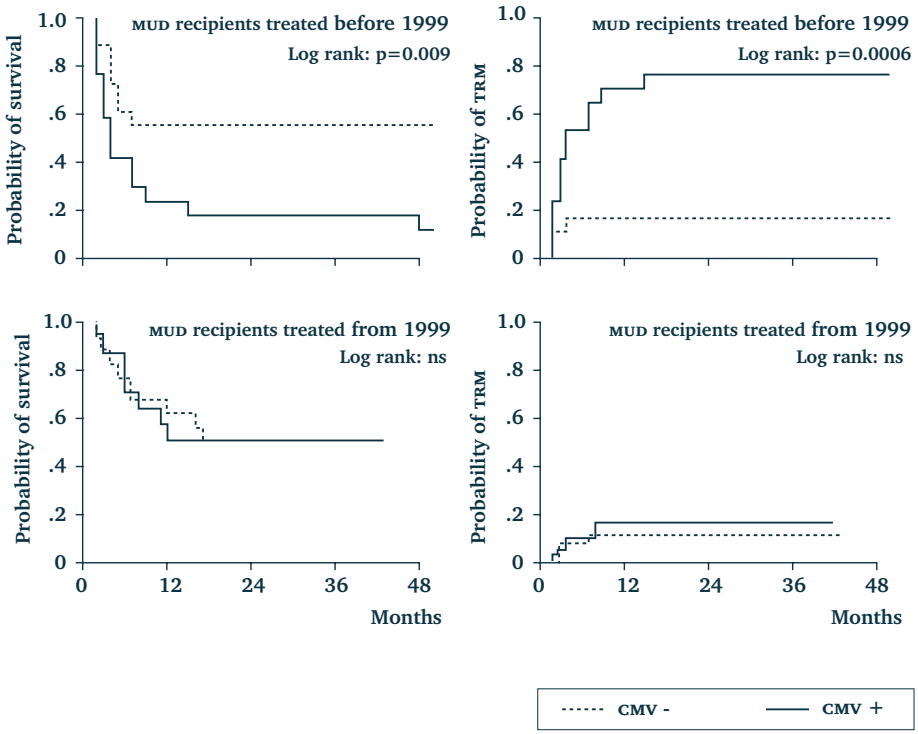

Table 1 Patient characteristics

	Before 1999	After 1999	P
No. of patients	35	43	
Age, yr (range)	31 (17-47)	33 (17-55)	ns
Diagnosis (%)			ns
AML	6 (17)	10 (23)	
ALL	8 (23)	12 (28)	
CML	10 (29)	13 (30)	
SAA	7 (20)	1 (2)	
Other	4 (11)	7 (16)	
CMV serostatus R/D (%)			ns
+/+	6 (17)	9 (21)	
+/-	11 (31)	9 (21)	
-/+	7 (20)	8 (19)	
-/-	11 (31)	17 (40)	
Risk status (%)			ns
Low	13 (37)	9 (21)	
High	22 (63)	34 (79)	
aGVHD (%)			ns
No-I	21 (60)	33 (77)	
II-IV	14 (40)	10 (23)	
cGVHD (%)			ns
No-L	29 (83)	40 (93)	
E	6 (17)	3 (7)	
CMV reactivation (%)			0.06
Yes	11 (31)	6 (14)	
No	24 (69)	37 (86)	
Recurrent CMV reactivations			ns
One	5	5	
Two or more	6	1	
Time to first reactivation			ns
median days (range)	31 (20-52)	50 (11-78)	
Granulocytes >500 x 10^6/L			ns
recovery	100%	100%	
median days (range)	23 (13-48)	19 (12-92)	
Platelets >50 x 10^9/L			ns
recovery	88%	95%	
median days (range)	33 (17-148)	28 (12-208)	

R/D = recipient/donor; L = limited; E = extensive.

the CMV pp65 antigenemia assay, thereafter monitoring was based on a real-time TaqMan™ CMV DNA PCR. This method is known to be more sensitive[11-13] compared to the antigenemia assay, however, the incidence of CMV reactivations was still lower in the recent group compared to the historical group. In conclusion, CMV-seropositive MUD recipients of partial TCD grafts treated since 1999 show survival rates comparable to CMV-seronegative recipients. This effect largely resulted from a decrease in dosage of ATG, although high resolution HLA-antigen typing may have contributed. Low-dose ATG did not result in an increase in non-engraftment, while high-dose ATG is associated with an increase in acute toxicity. Therefore, we recommend when ATG is used in the setting of TCD MUD transplantation, to use the low-dose of 8 mg/kg.

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References

1 Meyers JD, Flournoy N, Thomas ED. Risk factors for cytomegalovirus infection after human marrow transplantation. J Infect Dis 1986; 153: 478-488.

2 Humar A, Wood S, Lipton J et al. Effect of cytomegalovirus infection on 1-year mortality rates among recipients of allogeneic bone marrow transplants. Clin Infect Dis 1998; 26: 606-610.

3 Peggs KS, Preiser W, Kottaridis PD et al. Extended routine polymerase chain reaction surveillance and pre-emptive antiviral therapy for cytomegalovirus after allogeneic transplantation. Br J Haematol 2000; 111: 782-790.

4 Nichols WG, Corey L, Gooley T et al. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. J Infect Dis 2002; 185: 273-282.

5 Meijer E, Dekker AW, Rozenberg-Arska M, Weersink AJL, Verdonck LF. Influence of Cytomegalovirus-seropositivity on outcome after T cell depleted bone marrow transplantation: contrasting results between recipients of grafts from related and unrelated donors. Clin Infect Dis 2002; 35: 703-712.

6 Kröger N, Zabelina T, Krüger W et al. Patient cytomegalovirus seropositivity with or without reactivation is the most important prognostic factor for survival and treatment-related mortality in stem cell transplantation from unrelated donors using pretransplant in vivo T-cell depletion with antithymocyte globulin. Br J Haematol 2001; 113: 1060-1071.

7 Castro-Malaspina H, Harris RE, Gajewski J et al. Unrelated marrow transplantation for myelodysplastic syndromes: outcome analysis in 510 transplants facilitated by the national marrow donor program. Blood 2002; 99: 1943-1951.

8 Craddock C, Szydlo RM, Dazzi F et al. Cytomegalovirus seropositivity adversely influences outcome after T-depleted unrelated donor transplant in patients with chronic myeloid leukaemia: the case for tailored graft-versus-host disease prophylaxis. Br J Haematol 2001; 112: 228-236.

9 Moutaftsi M, Mehl AM, Borysiewicz LK, Tabi Z. Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells. Blood 2002; 99: 2913-2921.

10 Zaia JA. Cytomegalovirus infections. In: Thomas ED, Blume KG, Forman SJ, eds. Hematopoietic cell transplantation. Massachusetts, PA: Blackwell Science 1999: 560-583.

11 Machida U, Kami M, Fukui T et al. Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation. J Clin Microbiol 2000; 38: 2536-2542.

12 Griscelli F, Barrois M, Chauvin S, Lastere S, Bellet D, Bourhis JH. Quantification of human cytomegalovirus DNA in bone marrow transplant recipients by real-time PCR. J Clin Microbiol 2001; 39: 4362-4369.

13 Yakushiji K, Gondo H, Kamezaki K et al. Monitoring of cytomegalovirus reactivation after allogeneic stem cell transplantation: comparison of an antigenemia assay and quantitative real-time polymerase chain reaction. Bone Marrow Transplant 2002; 29: 599-606.

Chapter 10

Prevention of Cytomegalovirus disease in recipients of allogeneic stem cell transplants: a review

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Abstract

The main risk factors for cytomegalovirus (CMV) disease in recipients of allogeneic stem cell transplants (SCT) are recipient CMV-seropositivity and acute graft-versus-host disease. Currently, two antiviral strategies -prophylactic or pre-emptive antiviral treatment- are used for prevention of CMV disease, which is most favourable when short-term (14 days) pre-emptive treatment is applied.

Several methods are available for monitoring of CMV reactivations. PCR based CMV DNA detection assays are the most sensitive methods, however, the clinical benefit of this high sensitivity is unclear. Even more, there is lack of clarity whether PCR tests can better be performed in plasma, whole blood or peripheral blood leukocyte samples.

Recovery of a CMV-specific CD8+ cytotoxic T lymphocyte (CTL) response is necessary for preventing CMV reactivation and disease. Reconstitution of absolute CMV-specific CTL counts to values above 10-20 x 10^6 CTLs/L is associated with protection from CMV disease. In the near future, pre-emptive therapy might be withheld in patients with CMV reactivation who show to have adequate CMV-specific cytotoxic T cell levels.

Antiviral therapy with (val)acyclovir has only been studied as prophylactic treatment modality for prevention of CMV infection. High-dose oral valacyclovir is more effective compared to acyclovir, when used in addition to pre-emptive treatment of CMV reactivations with ganciclovir or foscarnet.

Three antiviral drugs have been tested for pre-emptive therapy of CMV reactivations and/or treatment of CMV disease. Although intravenous ganciclovir is considered the drug of choice, foscarnet has similar efficacy and less, especially haematologic, toxicity. Cidofovir has not been tested extensively, so far results are disappointing. Oral valganciclovir for pre-emptive treatment is currently studied among SCT recipients.

In addition to antiviral therapy, adoptive immunotherapy with CMV-specific cytotoxic T cells as prophylactic or pre-emptive therapy is a very elegant strategy, however, generation of these cells is expensive and time-consuming and therefore not available at every transplantation centre. Magnetic selection of CMV-specific CD8+ T cells from peripheral blood using HLA class I-peptide tetramers may be very promising, making this strategy more accessible.

Introduction

In the era before introduction of ganciclovir (GCV), cytomegalovirus (CMV) infection and pneumonia developed in 38 and 17%, respectively, of recipients of allogeneic stem cell transplants (SCT), while mortality due to CMV pneumonia was 85%[1]. This very serious complication occurred mainly in CMV-seropositive patients, with acute graft-versus-host disease (aGVHD) being the most important risk factor[1]. Treatment of CMV pneumonia with GCV and immunoglobulin decreased mortality to 30-50%[2-3]. In CMV-seronegative recipients, primary CMV infection could be prevented by a transfusion and transplantation policy making use of either CMV-seronegative donors or leukocyte-depleted blood products/grafts[4-7]. Currently, two antiviral strategies, prophylactic or pre-emptive treatment, are used for prevention of CMV disease. Prophylactic treatment usually consists of antiviral therapy started at engraftment until al least day 100 post-transplant. Pre-emptive therapy is defined as antiviral treatment based on the detection of reactivated CMV infection by positive CMV cultures, a positive antigenemia (Ag) assay or positive molecular assays.

In the present report, we review these antiviral strategies. Furthermore, several other aspects of prevention of CMV disease are reviewed, which are: 1) methods available for early detection of CMV reactivations, 2) monitoring of CMV-specific T cell responses, 3) the value of several antiviral drugs and 4) adoptive immunotherapy as prophylaxis or (pre-emptive) treatment of CMV reactivations/CMV disease.

Antiviral strategies: prophylaxis or pre-emptive treatment

In randomised trials[8,9] (Table 1) among CMV-seropositive recipients, long-term (3-4 months) GCV prophylaxis initiated at engraftment showed to be effective in suppressing early CMV disease (<100 days post-transplant). However, mortality was not influenced due to an increased incidence of bacterial and fungal infections and late CMV disease[8-12]. When studying recovery of CMV-specific cytotoxic T lymphocytes (CMVs-CTLs), it appeared that long-term ganciclovir treatment impaired CMVs-CTL reconstitution causing the increase in late CMV infections[13].

Table 1 Ganciclovir prophylaxis of CMV disease

Study	Randomised trial	Donor	Dose of GCV	Early CMV disease Placebo vs GCV	Overall mortality Placebo vs GCV
Goodrich[8]	Yes	MRD/ MUD	10 mg/kg/d (5d) fb 5 mg/kg/d[a]	29% vs 0% (p<0.001)	26% vs 30% (at d 180, ns)
Winston[9]	Yes	(P)MRD/ (P)MUD	6 mg/kg/d[b]	24% vs 10% (p=0.06)	36% vs 30% (at d 120, ns)

GCV = ganciclovir; (P)MRD = (partially) matched related donor; (P)MUD = (partially) matched unrelated donor; d = day; fb = followed by; ns = not significant; [a] from engraftment until day 100; [b] from engraftment until day 120.

Many studies have been performed using pre-emptive therapy in SCT recipients to prevent CMV disease. The paper by Schmidt et al[14] was the first to evaluate this strategy, based on positive CMV cultures of bronchoalveolar lavage (BAL) fluid. Patients underwent routine BAL on day 35. The 40 patients with positive CMV cultures were randomised between pre-emptive GCV treatment or observation. In the GCV group 5 of 20 patients died or had CMV pneumonia before day 120 compared to 14 of 20 in the observation group (p=0.01), while in the group of patients with negative CMV cultures the rate of CMV infection was 12 of 55. Results of reports with a minimum of 30 patients and published after 1995 are summarized in Tables 2-5, according to the CMV monitoring assay used; three of them were randomised trials[10,15,18]. Pre-emptive treatment based on qualitative CMV DNA detection by PCR lowered the incidence of CMV disease and CMV-associated mortality compared with pre-emptive therapy instituted when positive CMV cultures were obtained (Table 5 and 2, respectively; p=0.02)[15]. Boeckh et al[10,11] compared two types of Ag based pre-emptive therapy (Table 4) with prophylactic treatment. Ag based treatment was given for 28 days[10] or until day 100 post-transplant[11]. In both the prophylactically treated group and the group receiving long-term pre-emptive treatment late CMV disease was diagnosed more frequently, while more invasive fungal infections were seen in the prophylactically treated group only. The incidence of CMV disease at day 400 post-transplant and overall survival were similar in the three treatment arms. Humar et al (Table 4 and 3) showed that Ag based pre-emptive treatment reduced the incidence of CMV disease at day 400 post-transplant to 1.7% compared to

Table 2 Pre-emptive therapy of CMV reactivation based on positive CMV cultures

Study	Randomised trial	Donor	No.	Dose of GCV	CMV disease % (at day)	CMV-a-mortality % (at day)	Overall Survival % (at day)
Einsele[15]	Yes, see Table 5	(P)MRD/ (P)MUD	34	5 mg/kg bid (14d) fb 5 mg/kg/d until negative PCR	32 (180)	15 (180)	59 (180)
Ljungman[16]	No	(P)MRD/ MUD	58	5 mg/kg bid (14d)	19 (100)	2 (100)	nd[a]

GCV = ganciclovir; CMV-a = CMV associated; (P)MRD = (partially) matched related donor; (P)MUD = (partially) matched unrelated donor; bid= twice a day; d = day; fb = followed by; nd = not described; [a] overall survival was not specified, however, reported as similar as observed in patients receiving PCR-based pre-emptive treatment, see Table 5.

Table 3 Pre-emptive therapy of CMV reactivation based on CMV positive BAL

Study	Randomised trial	Donor	No.	Dose of GCV	CMV disease % (at day)	CMV-a-mortality % (at day)	Overall Survival % (at day)
Reddy[17]	No	MRD/ MUD	55	5 mg/kg bid (14-28 d) fb maintenance (16 w)	11 (nd)	5 (nd)	57 (3yr)
Humar[18]	Yes, see Table 4	(P)MRD/ MUD	58	5 mg/kg bid (14d) fb maintenance (8 w)	12.1 (400)	nd	72.4% (365)

TCD = T cell depletion; GCV = ganciclovir; CMV-a = CMV associated; (P)MRD = (partially) matched related donor; (P)MUD = (partially) matched unrelated donor; bid= twice a day; d = day; fb = followed by; w= weeks; nd = not described.

Table 4 Pre-emptive therapy of CMV reactivation based on antigenemia

Study	Rando-mised trial	Donor	No.	No. of positive cells	Dose of GCV	CMV disease % (at day)	CMV-a-mortality %	Overall Survival % (at day)
Boeckh[10]	Yes	(P)MRD/ MUD	114	≥ 3 cells/ 2 slides	5 mg/kg bid (7d) fb maintenance (21d)	22 (400)	nd	73 (180) 61 (400)
Verdonck[19]	No	MRD	41	any antigenemia	2.5 mg/kg bid (14d)[a] fb 5 mg/kg bid until negative test	0 (180)	0	76 (180)
Manteiga[20]	No	MRD	52	any antigenemia	5 mg/kg bid (7d) fb maintenance (21d)	13 (100)	2	nd
Boeckh[11]	No	(P)MRD/ MUD	102	any antigenemia	5 mg/kg bid (7d) fb maintenance (100d)	17 (400)	nd	77 (180) 66 (400)
Machado[21,22]	No	MRD	76	≥ 2 cells/ 300.000	5 mg/kg bid (14-21d) or until negative test	4 (200) 14.4 (365)	0 0	nd

Table 4 continued

Study	Randomised trial	Donor	No.	No. of positive cells	Dose of GCV	CMV disease % (at day)	CMV-a-mortality %	Overall Survival % (at day)
Mori[23]	No	MRD/ MUD	50	≥ 10 cells/ 2 slides or [b] any antigenemia	5 mg/kg bid (14d) fb 5 mg/kg/d until negative test	16 (100)	0	nd
Kanda[24]	No	(P)MRD/ (P)MUD	77	≥ 5-10/ 50.000	5 mg/kg bid (7d) fb 5 mg/kg/d until negative test	3 (365)	0	86 (100) 73 (365)
Small[25]	No	MRD	61	≥ 2 cells/ slide	nd	7 (nd)	7	nd
Humar[18]	Yes, see Table 3	(P)MRD/ MUD	60	any antigenemia	5 mg/kg bid (14d) or until negative test	1.7 (400)	0	80 (365)

GCV = ganciclovir; CMV-a = CMV associated; (P)MRD = (partially) matched related donor; (P)MUD = (partially) matched unrelated donor; bid= twice a day; d = day; fb = followed by; nd = not described; [a] patients treated with high-dose corticosteroids for aGVHD grade II-IV received prophylactic GCV similar to the pre-emptive dose; [b] during aGVHD grade II-IV.

Table 5 Pre-emptive therapy of CMV reactivation based on CMV DNA PCR

Study	Rando-mised trial	Donor	No.	Therapy started at	Dose of GCV	CMV disease % (at day)	CMV-a-mortality %	Overall Survival % (at day)
Einsele[15]	Yes, see Table 2	(P)MRD/ (P)MUD	37	2nd positive PCR (Qual)	5 mg/kg bid (14d) fb 5 mg/kg/d until negative PCR	8 (180)	0 (180)	84 (180)
Ljungman[16]	No	(P)MRD/ MUD	58	2nd positive PCR (Semi-Q)	5 mg/kg bid (14d) or until negative PCR	6 (100) 10 (180)	0 (180)	nd[a]
Einsele[12]	No	(P)MRD/ (P)MUD	86	2nd positive PCR (Qual)	5 mg/kg bid (14d) fb foscarnet 60 mg/kg bid until negative PCR	12 (>100)	1 (>100)	nd
Peggs[26]	No	MRD/ MUD	52	1st or 2nd positive PCR (Qual)	5 mg/kg bid (14d) fb foscarnet 60 mg/kg bid until negative PCR	6 (100)	2 (100)	nd

Table 5 continued

Study	Rando-mised trial	Donor	No.	Therapy started at	Dose of GCV	CMV disease % (at day)	CMV-a-mortality %	Overall Survival % (at day)
Hebart[27]	No	(P)MRD/ (P)MUD	52	2nd positive PCR (Qual)	5 mg/kg bid (14d) fb 5 mg/kg/d until negative PCR	12 (?)	6 (?)	nd
Mori[28]	No	MRD/ MUD	39	1st positive PCR (real-time)	5 mg/kg/d (14d) until negative test 10 mg/kg/d when VL increased	8 (120)	0	nd

GCV = ganciclovir; CMV-a = CMV associated; (P)MRD = (partially) matched related donor; (P)MUD = (partially) matched unrelated donor; Qual = qualitative; Semi-Q = semi-quantitative; bid = twice a day; d = day; fb = followed by; nd = not described; VL= viral load; [a] overall survival was similar as observed in a historical control group receiving pre-emptive treatment based on positive conventional cultures.

12.1% when therapy was instituted at the detection of positive CMV cultures of BAL fluid obtained at day 35 post-transplant (p=0.022)[18]. Again overall survival was similar in the two treatment arms.

Prevention of CMV disease with low CMV-associated mortality seemed to be superior in studies using a short-term (14 days) Ag or PCR based pre-emptive GCV treatment[12,15,16,18,19,21,22,24,26,27]. In these studies pre-emptive treatment was only extended when CMV monitoring tests were still positive after the short-term treatment period. When 'overall survival' was considered the endpoint, the varying pre-emptive treatment strategies all were equally effective.

The introduction of pre-emptive therapy among CMV-seropositive patients receiving grafts from matched related donors has resulted in similar transplant-related mortality and survival rates compared to CMV-seronegative recipients[26,29-31]. However, in CMV-seropositive recipients of grafts from matched unrelated donors transplant-related mortality and overall survival rates were still inferior compared to CMV-seronegative recipients, despite pre-emptive antiviral treatment[31-34].

Overall, the introduction of pre-emptive antiviral therapy has greatly reduced the incidence and mortality rate of CMV disease. Prophylactic treatment has no advantage over pre-emptive treatment, in fact it results in an increased incidence of bacterial and fungal infections and late CMV disease. Pre-emptive treatment based on the Ag assay or PCR tests is superior to culture or BAL fluid based strategies. Especially short-term (14 days) antiviral treatment is the most favourable approach for prevention of CMV disease.

Antigenemia vs molecular monitoring

The Ag assay is widely used to monitor SCT recipients for CMV reactivations. This assay has some drawbacks compared to molecular tests: 1) during neutropenia no monitoring can be performed, which is similar for molecular tests performed in leucocytes, 2) the test is laborious and 3) liable to intra/interobserver variability. Furthermore, a false negative Ag test (using C10/C11 antibodies) was reported in a SCT recipient with CMV disease. Re-examination of the Ag negative samples with a different pp65 antibody pool (CINA antibodies) revealed a high level of Ag[35]. Compared to Ag assays the workload per sample has been

reduced from approximately 4 to less than 2 hours when automated DNA isolation and PCR tests are used. The difference in workload is even more obvious when large amounts of samples are processed, since the Ag assay is not automated and every sample has to be processed separately.

With the molecular assays a qualitative or quantitative detection of CMV DNA or RNA is performed in cell-free plasma, peripheral blood leucocytes (PBL) or whole blood (WB). Technical details of several methods for CMV monitoring have been reviewed by Boeckh et al[36].

Cobas Amplicor™ CMV DNA (monitor) test The Cobas Amplicor™ CMV DNA test is a commercially available qualitative CMV DNA PCR assay for plasma, while the quantitative Cobas Amplicor™ CMV DNA monitor test can be performed in cell-free plasma, PBL or WB. Five studies found CMV DNA PCR monitoring (qualitative or quantitative) to be more sensitive compared to Ag, irrespective of performance in plasma, PBL or WB[37-41]. In only one report leucocyte-based assays (Ag and PBL-PCR) were more sensitive compared to a plasma PCR, showing a higher number of patients with a CMV reactivation, earlier positivity and a more rapid decrease of viral load after the start of pre-emptive antiviral therapy[42]. In one other study the gold standard to calculate sensitivity and specificity of the Ag assay and PCR test was defined as "CMV reactivation based on positive results of the Ag assay or PCR test"[37]. This method of calculation is incorrect and results in the exclusion of false positive results, giving a specificity and positive predictive value of 100%. The value of a higher sensitivity of molecular assays in a clinical setting is not clear. Solano et al[40] reported 9 of 43 SCT recipients with positive plasma PCR, while Ag was negative. None of these 9 patients progressed to CMV disease, although they did not receive pre-emptive treatment. Furthermore, none of the patients with an initial positive Ag and PCR result who remained PCR positive after conversion of the Ag assay to a negative result, did develop CMV disease. The authors concluded that the Ag assay appeared to be most suitable for guiding initiation of pre-emptive therapy and monitoring response to antiviral therapy.

Real-time automated CMV DNA PCR test using a TaqMan™ probe With the real-time automated CMV DNA PCR assay using a TaqMan™ probe, a quantitative CMV monitoring in plasma, WB and PBL can be performed. With this method PCR products are detected as they accumulate during the PCR, in contrast to other quantitative PCR techniques such as the Cobas Amplicor™ CMV DNA monitor test. This results in a greater linear dynamic detection range of the real-time TaqMan™ PCR compared to the Cobas Amplicor™ CMV DNA monitor test. Analogous to the Cobas Amplicor™ tests, this assay also proved to

be more sensitive compared to a positive Ag test. CMV DNA detection by real-time PCR often preceded Ag and yielded more positive samples[43-45].

Qualitative and quantitative in house CMV DNA PCR assay In (partly) retrospective studies, CMV DNA monitoring by in house PCR assays in plasma, WB or PBL yielded similar results as described above, with higher sensitivity for molecular tests compared to the Ag assay[38,46,47]. Hebart et al[48] prospectively monitored CMV reactivation by Ag and in house semiquantitative PCR in plasma and WB. WB-PCR showed the lowest sensitivity, however, overall a good correlation was seen between Ag, WB-PCR and plasma PCR. All three assays were negative after 14 days of GCV treatment in 12 of 13 patients. In contrast, 2 studies[49,50] reported the in house plasma PCR to be less sensitive compared to PBL-PCR or Ag.

Murex™ CMV DNA Hybrid Capture assay This assay is a commercially available solution hybridisation antibody capture assay (HCA) for the quantitative detection of CMV DNA in leukocytes. It was less sensitive in diagnosing CMV infection compared to an in house qualitative PCR[38,51]. When CMV disease was used as the gold standard for comparison, however, the positive predictive value of the HCA and PCR assay was 33% and 22%, respectively[51].

CMV mRNA based monitoring The qualitative determination of CMV pp67 mRNA by nucleic acid sequence-based amplification proved to be the least sensitive technique to assess CMV reactivation compared to DNA based assays and the Ag assay[38,52,53]. Detection of immediate-early mRNA[54], the $beta_{2.7}$ transcript[55] or spliced late CMV genes[56] all were shown to be more useful, however, these results have not been validated by other groups.

Summary In Table 6 results of studies comparing commercially available surveillance methods with the Ag assay or in house PCR tests are summarized. Not all studies described above are included, due to varying study designs and endpoints or lack of clinical data[37,41,42,55,56]. In several papers sensitivity, specificity, positive predictive value and negative predictive value of the experimental assay(s) were calculated. In these studies the gold standard to calculate these values was defined as CMV reactivations based on positive results from Ag and/or PCR assays[37,55,56]. In our view, a surveillance method for CMV reactivation should be judged on its clinical merits, the incidence of CMV disease and transplant related mortality being the most significant endpoints.

Table 6 Surveillance methods

Study	CMV reactivations (n)	No. of days of earlier positivity of experimental assay compared to standard assay	CMV disease (no. of patients/total)
Schulenberg[39] Quantitative PCR Cobas (plasma and blood) vs Ag	10 PCR+/Ag+ 7 PCR+/Ag- 0 PCR-/Ag-	11	2/74
Solano[40] Qualitative PCR Cobas (plasma) vs Ag	14 PCR+/Ag+ 18 PCR+/Ag- 5 PCR-/Ag+	7	2/43 (PCR+/Ag-: no disease)
Griscelli[44] Realtime Taqman (PBL) vs Ag	20 PCR+/Ag+ 2 PCR+/Ag- 1 PCR-/Ag-	15	2/16
Yakushiji[45] Realtime Taqman (plasma) vs Ag	39 PCR+/Ag+ 4 PCR+/Ag- 0 PCR-/Ag+ 8 PCR-/Ag-	PCR before Ag: 20 (n) Ag before PCR: 5 (n) Simultaneously:14 (n)	2/51
Hebart[51] Hybrid Capture assay (PBL) vs ih PCR	12 HCA+/PCR+ 6 HCA-/PCR+	HCA after or simultaneously with PCR: 18 (n)	4/18 (HCA-/PCR+: no disease)
Gerna[52] Qualitative pp67 NASBA vs Ag	9 NASBA+/Ag+ 6 NASBA-/Ag+ 9 NASBA-/Ag-	-3	2?/24
Hebart[53] Qualitative pp67 NASBA vs ih PCR	16 NASBA+/PCR+ 1 NASBA+/PCR- 3 NASBA-/PCR+	0	3/33 (NASBA+/PCR-: no disease)
Gerna[54] Qualitative IE NASBA vs Ag	32 NASBA+/Ag+ 6 NASBA+/Ag- 0 NASBA-/Ag+ 13 NASBA-/Ag-	2	0/51 (NASBA+/Ag-: no disease)

PBL = peripheral blood leukocytes; ih = in house.

Overall, one can conclude that PCR based CMV DNA monitoring is more sensitive compared to Ag based monitoring. However, the clinical benefit is unclear. Even more, there is lack of clarity whether PCR tests can better be performed in plasma, WB or PBL samples, although molecular monitoring in plasma has the advantage of performance irrespective of neutropenia. Currently, there is no evidence that qualitative CMV detection assays have a better or worse predictive value for the occurrence of CMV disease after SCT as compared to quantitative assays. To answer this question, randomised controlled trials should be performed, monitoring patients prospectively with either detection assay without applying pre-emptive treatment of CMV reactivation. Such trials will never be done. Theoretically, a quantitative method enables monitoring of response to therapy. In case of an increasing viral load after the start of pre-emptive therapy, a dose or drug modification may be applied. This was implemented by Mori et al[28] but did not significantly change the incidence of CMV disease.

Monitoring of CMV-specific T cell responses

Studies of immune recovery after allogeneic SCT have shown a temporal delay in the recovery of CMVs T cell responses and have identified a decisive role for the recovery of CD8+ CTL responses in preventing the development of CMV disease[57-59]. Generation of CD8+ CMVs-CTLs was associated with recovery of CD4+ CMVs T helper cells[58]. Li et al[13] analysed the kinetics of endogenous reconstitution of CD4+ and CD8+ CMVs T cell responses, by lymphoproliferation and cytotoxicity assays, in 47 allogeneic SCT recipients who were randomised to GCV prophylaxis or placebo after recovery of peripheral neutrophil counts. Between day 40 and 90 post-transplant recovery of CD8+ and CD4+ CMVs T cell responses occurred in the majority of individuals receiving placebo, but in a minority of patients receiving GCV. Thus, long-term prophylactic GCV treatment can delay post-transplant reconstitution of CMVs-CTL responses. Today, several reports have been published using screening assays for CMVs T cell reconstitution to identify patients at risk of developing CMV disease[60-65]. Krause et al[60] performed lymphoproliferation assays, to assess CD4+ CMVs T helper (Th) response, at regular monthly intervals. None of the patients with a CMVs T cell proliferation on day 120 developed CMV disease after day 120. In contrast, of the patients lacking such a response at day 120, 30.8% developed late CMV disease (after day 120).

Hebart et al[65] quantified CD8+ CMVs-CTLs and CD4+ CMVs Th cells by intracellular interferon-γ staining with flow cytometry after CMV-specific stimulation. Reconstitution of both cell types was associated with rapid clearance of CMV infection. Next to cytotoxicity and interferon-γ staining assays, the use of HLA-peptide tetramers to quantify CMVs CD8+ T cell reconstitution might enable prediction of the development of CMV disease[61-63]. Reconstitution of absolute CMVs-CTL counts to values above 10-20 x 10^6/L was associated with protection from CMV disease[62,63]. In contrast, Ozdemir et al[64] recently reported that frequencies and absolute numbers of CMVs CD8+ T cells were greater in subjects who experienced CMV Ag following SCT. They conclude that recovery of CMVs-CTLs, as measured by HLA-peptide tetramer staining, is insufficient to control CMV Ag. However, it is not described whether these patients with Ag did develop CMV disease. This might be important, since only patients with Ag and decreased recovery of CMVs-CTLs, progressed to CMV disease in the study of Gratama et al[61]. Patients with Ag who did not develop CMV disease, demonstrated higher levels of CMVs CD8+ T cells compared to CMV-seropositive recipients without Ag. It should be noted that most CMVs-CTL studies discussed in this review were performed retrospectively and used tetrameric complexes of HLA A*0201 and/or B*0702 molecules. Larger prospective studies have to be performed to evaluate CMVs CD8+ T cell reconstitution after allogeneic SCT. When the abovementioned results will be validated, pre-emptive antiviral therapy might be withheld in patients with CMV reactivation who show to have adequate CMVs-CTL levels. At this moment, several other HLA class I (A*0101, A*0301, A*1101, A*2401, A*6801/2, B*3502, B*3801/2, B*44XX) restricted pp65 and pp150 derived epitopes have been identified[65,66,67] which will make tetramer based or interferon-γ staining based quantification of CMVs CD8+ T cell recovery accessible for more SCT recipients.

Antiviral therapy

Intravenous GCV is generally considered the drug of choice for pre-emptive therapy of CMV reactivations or treatment of CMV disease[68]. Several other antiviral drugs have in vitro or in vivo activity against CMV (acyclovir -ACV-, valacyclovir -VACV-, foscarnet, cidofovir -CDV-, valganciclovir -VGCV-). (V)ACV and (V)GCV are nucleoside analogues. VACV and VGCV are

oral prodrugs of ACV and GCV, respectively, and converted to ACV and GCV, respectively, after cleavage of the valine moiety by the liver and intestine. The nucleosides first have to be converted to monophosphate by a viral protein kinase (which is the gene product of UL97 in case of CMV). Second and third phosphorylations are performed by cellular kinases. ACV or GCV triphosphate is then incorporated in viral DNA and acts as an obligate chain terminator[69]. Furthermore GCV triphosphate is a competitive inhibitor of the CMV DNA polymerase. VGCV has a ten-fold greater bio-availability than oral GCV. Pharmacokinetic studies of VGCV have been performed in HIV infected individuals and recipients of liver transplants[70]. Among SCT recipients a randomised cross-over trial using intravenous GCV or oral VGCV as pre-emptive treatment will be conducted at European Group for Blood and Marrow Transplantion (EBMT) centres[70]. The nucleotide analogue CDV already is a monophosphate, therefore no phosphorylation by viral enzymes is necessary. Foscarnet is a pyrophosphate analogue forming a complex with the pyrophosphate binding site of viral DNA polymerase. This is an essential site during incorporation of nucleotides in DNA in which a pyrophosphate group has to be spliced from the nucleotide. Thereby, foscarnet is inhibiting viral DNA polymerase activity.

Acyclovir/Valacyclovir (V)ACV has only been studied as prophylactic therapy for prevention of CMV reactivations/disease and not as (pre-emptive) treatment. ACV has only limited activity against CMV when tested in vivo. Two studies using ACV prophylaxis were performed in SCT recipients, however, before the strategy of pre-emptive GCV therapy based on Ag or PCR was introduced[71,72]. Intravenous ACV followed by high-dose oral ACV maintenance therapy was not effective as prevention of CMV disease, but resulted in a decreased CMV-associated mortality and increased survival. Vusirikala et al[73] compared data of 31 SCT recipients who were prophylactically treated with VACV 1 gram 3 times a day with a group receiving only low-dose oral ACV. Primary and secondary CMV reactivations were observed in 3/12 and 5/19 VACV treated patients, respectively, compared to 24/31 and 16/24 in the control group, respectively. Since this was a retrospective report with small patient numbers combining primary and secondary reactivations, it just suggested a potential benefit of VACV as prevention for CMV reactivation. In a large randomised multi-centre study oral VACV showed to be more effective in preventing CMV viremia in SCT recipients compared to oral ACV, although overall survival and the incidence of CMV disease did not differ between the two groups (75% vs 76% and 5.5% vs 3.5% for the ACV and VACV groups, respectively, ns). All patients included were initially treated with intravenous acy-

clovir until day 28 after transplantation or until discharge[74]. In these two studies a PCR or Ag based pre-emptive treatment with GCV or foscarnet was used as well[73,74].

Foscarnet Intravenous foscarnet is considered second line therapy for CMV reactivations or disease, however, for patients developing dose-limiting neutropenia or CMV strains resistant to GCV it is the drug of choice[68]. In a survey of herpesvirus resistance to antiviral drugs, GCV was replaced by foscarnet in 15 patients with suspected or proven GCV resistance and this resulted in a better clinical or virological outcome in 13 of these 15 patients[75].

Four non-randomised studies were published using foscarnet prophylactically[76-79]. In all four studies patient numbers were very small (≤ 21), therefore, no firm conclusions can be drawn regarding effectiveness of foscarnet prophylaxis.

Preemptive treatment consisting of foscarnet has also been reported in four studies, showing similar efficacy of foscarnet compared to GCV[80-83]. Only two were randomised trials[82,83], one with a low patient number[82]. Reusser et al[83] treated 110 patients with CMV reactivation (Ag or PCR diagnosed) with foscarnet 60 mg/kg bid and 103 patients with GCV 5 mg/kg bid. When test results were still positive after 14 days of treatment both drugs were continued in a reduced dose (90 mg/kg/day:foscarnet; 6mg/kg/day:GCV) for again 2 weeks (5 days a week). When CMV was still detectable after this second treatment period, treatment was considered a failure, and patients were treated at the discretion of the investigator. Event free survival and overall survival at day 180 were similar in both groups, as was the occurrence of CMV disease and treatment failures. No difference was observed regarding other herpesvirus infections or major nonviral infections. Preemptive treatment with foscarnet did not raise safety concerns (when appropriate hydration was used) and was associated with significantly less serious haematotoxicity than GCV. In the GCV group neutropenia was more often observed, despite the use of growth factors.

Cidofovir Ljungman et al[84] performed a retrospective survey among 17 BMT centres and enrolled 82 patients who were treated with CDV for CMV disease (n=20) or for CMV reactivation (primary pre-emptive treatment n=24; secondary pre-emptive treatment in patients who had failed or relapsed after previous pre-emptive treatment with another antiviral drug n=38). The dosage was 1-5 mg/kg per week followed by maintenance treatment and all patients received probenicid and prehydration. Overall 62% showed a response to CDV therapy; the response being defined as disease regression without addition of other specific therapy or, for pre-emptive therapy, conversion of a positive Ag or PCR test. Twenty-one patients developed renal toxicity, which persisted after cessation of therapy in 9 patients. No

toxicity was seen in 45 patients, while 15 developed other side effects potentially associated with CDV therapy (nausea, vomiting, thrombocytopenia, rash, ophthalmologic and neurologic toxicity). Kiehl et al[85] published prospective data on 21 patients receiving first-line pre-emptive treatment with CDV 5 mg/kg once a week for 2 weeks, thereafter fortnightly. Treatment was Ag and/or DNA PCR based and continued until test results were negative for at least three weeks. Only one patient showed a complete response. In 15 patients PCR became negative, however, 2-3 weeks later a positive PCR was again observed in all 15 patients. In 5 patients CMV reactivation was not cleared. The authors stated that it might be more effective to give CDV once a week for a longer period, which is supported by the low toxicity rate observed in this study: only one patient developed renal toxicity. Chakrabarti et al[86] treated 4 patients pre-emptively with CDV 5 mg/kg/week for 4 weeks. Two responded but developed severe nausea/vomitus and uveitis. Two were non responders of which one died from CMV pneumonia and one developed CMV pneumonia eventually responding to foscarnet. The less favourable outcome of the last two reports are in concordance with recent prospective results from Platzbecker et al[87]. Here, only one of seven SCT recipients showed a transient clearance of pp65 Ag after treatment with CDV 5 mg/kg/week for two weeks, thereafter fortnightly. In contrast, these authors[87] showed that pre-emptive treatment with CDV was very successful in 10 SCT recipients treated with a non-myeloablative conditioning regimen. Toxicity was moderate and consisted of reversible renal impairment (n=4), proteinuria (n=1) and nausea/vomiting (n=3).

Antiviral drug resistance When prolonged (>100 days) antiviral therapy is given, drug resistance may develop[88]. Data about antiviral drug resistance have largely been obtained in AIDS patients and very little information is available about drug resistance in the SCT setting. Resistance of CMV to GCV is associated with lack of therapeutic response and progression of CMV disease[89,90]. The clinical outcome of infections caused by foscarnet and CDV resistant CMV strains is unknown. In 1996 a survey of herpesvirus resistance to antiviral drugs was performed in 68 bone marrow transplantation (BMT) centres. CMV resistance to GCV was proven in 2 patients and suspected in 23 patients[75]. In patients with CMV pneumonia, the virus often persists for a long time despite GCV treatment. GCV resistance was determined in CMV isolates obtained from BAL fluid or from autopsy lung tissue by DNA hybridisation. In only 1 of 12 patients a GCV resistant isolate was detected[91]. In a study among 50 allogeneic SCT recipients, 10 patient exhibited sustained or recurrent antigenemia despite GCV treatment. Samples from these 10 patients were screened for the pres-

ence of the most frequent CMV UL97 mutations by restriction enzyme analysis and none of these mutations were detected[92]. Altogether, antiviral drug resistance has only sporadically been reported in adult SCT recipients[75,91-93]. There is some evidence that it might be more frequent in paediatric SCT recipients, especially in patients with primary immunodeficiencies[90,94,95].

In clinical CMV strains, resistance to antiviral agents has been associated with mutations in the viral protein kinase UL97 (for GCV only) and viral DNA polymerase UL54 (for GCV, foscarnet and CDV) genes[89]. The varying laboratory methods used for drug susceptibility testing of CMV isolates have been reviewed by Erice[89] and may be classified as phenotypic or genotypic. Phenotypic methods generally are culture-based and designed to determine the concentration of an antiviral agent that would inhibit the virus in culture. Genotypic methods are designed to determine known UL97 or UL54 mutations present in the genome of the viruses being studied, using restriction enzyme analysis and/or sequencing, and do not require viral cultures. A drawback of phenotypic methods is the possible in vitro selection of specific virus isolates by several culture passages. This was recently proven by Hamprecht et al[96] who performed only one culture passage of CMV isolates before phenotypic drug susceptibility assays were performed. Virus strains isolated from these cultures were also genotypically analysed by UL97 restriction assays and sequencing and were compared with primary DNA extracts of the same specimens. This resulted in the molecular proof of the in vitro selection of one UL97 mutant strain from 3 viral variants (one wild type and two UL97 mutants) present in vivo.

Summary Overall, in the era before the introduction of pre-emptive antiviral therapy, high-dose prophylactic ACV was shown to be effective in reducing the CMV-associated mortality rate. When pre-emptive treatment with GCV or foscarnet was used, VACV proved to be more effective as prophylaxis against CMV viremia compared to ACV, however, without significantly affecting overall survival and the incidence of CMV disease. Currently it is not clear whether VACV prophylaxis combined with a pre-emptive antiviral strategy is better compared to pre-emptive therapy alone, which has to be tested in a randomised controlled trial. Although intravenous GCV is considered the drug of choice for (pre-emptive) treatment of CMV reactivations or disease, foscarnet has similar efficacy and less haematologic toxicity. The third agent used for pre-emptive treatment, CDV, has prospectively been tested in only a few studies all of them showing disappointing results.

Adoptive immunotherapy with CMVs T cells

Adoptive transfer of CD8+ CMVs-CTLs for prevention of CMV reactivation/disease has been shown effective[59,97]. When no CMVs Th response developed, CMVs-CTLs declined progressively. However, none of the 17 patients treated with CD8+ CMVs-CTLs developed CMV infection[59,97]. Einsele et al[98] treated 8 patients with antiviral resistant CMV reactivation, who had a CMV-seropositive donor, with CMVs T cells (10^7 CMVs T cells/m^2). These cells consisted of CD4+ CMVs Th cells and CD8+ CMVs-CTLs. Only patients lacking a CMVs lymphoproliferative response in vitro, indicating a deficient CMVs Th response, were enrolled. A response was seen in 6 of 7 evaluable patients, in which CMV reactivation was cleared. Once CMVs T cells emerged in the peripheral blood, they persisted at numbers comparable to those in healthy individuals. The authors hypothesize that adoptive immunotherapy is more effective when CD4+ CMVs Th cells are given together with CD8+ CMVs-CTLs, by inducing expansion of CD8+ CMVs-CTLs from precursors that without T cell help would not have been activated. In this study 3 patients subsequently developed invasive aspergillosis or respiratory syncytial virus interstitial pneumonitis. In a commentary it is stated that more efficient culture systems are needed to make this therapy more accessible. Furthermore, since eventually 3 patients died from fungal or viral infections a more comprehensive approach to reconstitute immunity in SCT recipients is required[99]. Peggs et al[100] used monocyte-derived dendritic cells to process and present CMV antigen to generate donor-derived CMVs cell lines containing both CD4+ and CD8+ T cells with a simple and rapid 21 day culture. These cells were administered pre-emptively to 13 allogeneic transplant recipients, of which 10 received a non-myeloablative conditioning regimen. Within 23 days following infusion CD8+ CMVs-CTLs reached absolute counts as high as 540 x 10^6/L and were detectable for up to 6 months post-transfusion. Six patients cleared CMV without antiviral drugs and no cases of CMV disease were diagnosed. Only 1 of 12 evaluable patients showed subsequent CMV reactivation. This patient had CMVs T cells administered at day 14 post-transplant, when Campath-1H (the IgG1 humanized monoclonal antibody against CD52) probably was still circulating, which might have induced lysis of these T cells.

Adoptive immunotherapy with CMV-specific cytotoxic T cells as pre-emptive therapy is a very elegant strategy, however, generation of these cells is expensive and time-consuming and therefore not available at every transplantation centre. Magnetic selection of CMVs CD8+ T cells from peripheral blood of CMV-seropositive donors using HLA class I-peptide

tetramers as decribed by Keenan et al[101] may be very promising, making adoptive immunotherapy more accessible.

Conclusion

This review focused on prevention of CMV disease in recipients of allogeneic SCT. The introduction of pre-emptive antiviral therapy has greatly reduced the incidence and mortality rate of CMV disease, especially when Ag or PCR based CMV monitoring is performed. A lot of questions still remain to be answered. We currently do not know whether the increased sensitivity of PCR based CMV DNA assays has any clinical benefit. Furthermore, it is not clear whether PCR tests can better be performed in plasma, whole blood or peripheral blood leukocyte samples. At which viral load or Ag level should antiviral therapy be instituted and for how long should it be continued? The current conclusion is that prevention of CMV disease with low CMV-associated mortality seems to be superior in studies using a short-term (14 days) Ag or PCR based pre-emptive treatment. In these studies pre-emptive treatment was only extended when CMV monitoring tests were still positive after the short-term treatment period. When overall survival was considered the endpoint, the varying pre-emptive treatment strategies all were equally effective.

In the near future, monitoring CMVs T cell recovery may change our current pre-emptive treatment strategy. The presence of CMVs T cells in patients with a documented CMV reactivation, might protect these patients from developing CMV disease. Prospective studies are needed to confirm the results derived from retrospectively performed analyses.

Lastly, efforts should focus on immune reconstitution. Once adoptive immunotherapy will become more accessible, controlled trials should be designed to study the effectivity of immunotherapy regarding prevention of CMV disease.

References

1 Meyers JD, Flournoy N, Thomas ED. Risk factors for cytomegalovirus infection after human marrow transplantation. J Infect Dis 1986; 153: 478-488.

2 Emanuel D, Cunningham I, Jules-Elysee K et al. Cytomegalovirus pneumonia after bone marrow transplantation successfully treated with the combination of ganciclovir and high-dose intravenous immune globulin. Ann Intern Med 1988; 109: 777-782.

3 Reed EC, Bowden RA, Dandliker PS et al. Treatment of cytomegalovirus pneumonia with ganciclovir and intravenous cytomegalovirus immunoglobulin in patients with bone marrow transplants. Ann Intern Med 1988; 109: 783-788.

4 Bowden RA, Slichter SJ, Sayers MH et al. Use of leukocyte-depleted platelets and cytomegalovirus-seronegative red blood cells for prevention of primary cytomegalovirus infection after marrow transplant. Blood 1991; 78: 246-250.

5 Verdonck LF, de Graan-Hentzen YCE, Dekker AW et al. Cytomegalovirus seronegative platelets and leukocyte-poor red blood cells from random donors can prevent primary cytomegalovirus infection after bone marrow transplantation. Bone Marrow Transplant 1987; 2: 73-78.

6 van Prooyen HC, Visser JJ, van Oostendorp WR et al. Prevention of primary transfusion-associated cytomegalovirus infection in bone marrow transplant recipients by the removal of white cells from blood components with high-affinity filters. Br J Haematol 1994; 87: 144-147.

7 de Gast GC, Boland GJ, Vlieger AM et al. Abortive human cytomegalovirus infection in patients after allogeneic bone marrow transplantation. Bone Marrow Transplant 1992; 9: 221-225.

8 Goodrich JM, Bowden RA, Fisher L et al. Ganciclovir prophylaxis to prevent cytomegalovirus disease after allogeneic marrow transplant. Ann Intern Med 1993; 118: 173-178.

9 Winston DJ, Ho WG, Bartoni K et al. Ganciclovir prophylaxis of cytomegalovirus infection in allogeneic bone marrow transplant recipients. Ann Intern Med 1993; 118: 179-184.

10 Boeckh M, Gooley TA, Myerson D et al. Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir at engraftment after allogeneic marrow transplantation: a randomized double-blind study. Blood 1996; 88: 4063-4071.

11 Boeckh M, Bowden RA, Gooley TA, Myerson D, Corey L. Successful modification of a pp65 antigenemia-based early treatment strategy for prevention of cytomegalovirus disease in allogeneic marrow transplant recipients. Blood 1999; 93: 1781-1782.

12 Einsele H, Hebart H, Kauffman-Schneider C et al. Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. Bone Marrow Transplant 2000; 25: 757-763.

13 Li CR, Greenberg PD, Gilbert MJ et al. Recovery of HLA-restricted cytomegalovirus (CVM)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood 1994; 83: 1971-1979.

14 Schmidt GM, Horak DA, Niland JC, Duncan SR, Forman SJ, Zaia JA. A randomized, controlled trial of prophylactic ganciclovir for cytomegalovirus pulmonary infection in recipients of allogeneic bone marrow transplants; The City of Hope-Stanford-Syntex CMV Study Group. N Engl J Med 1991; 324: 1005-1011.

15 Einsele H, Ehninger G, Hebart H et al. Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. Blood 1995; 86: 2815-2820.

16 Ljungman P, Lore K, Aschan J, Klaesson S, Lewensohn-Fuchs I, Lonnqvist B, Ringden O, Winiarski J, Ehrnst A. Use of a semiquantitative PCR for cytomegalovirus DNA as a basis for pre-emptive antiviral therapy in allogeneic bone marrow transplant recipients. Bone Marrow Transplant 1996; 17: 583-587.

17 Reddy V, Hao Y, Lipton J, Meharchand J, Minden M, Mazzulli T, Chan C, Messner HA. Management of allogeneic bone marrow transplant recipients at risk for cytomegalovirus disease using a surveillance bronchoscopy and prolonged pre-emptive ganciclovir therapy. J Clin Virol 1999;13 : 149-159.

18 Humar A, Lipton J, Welsh S, Moussa G, Messner H, Mazzulli T. A randomised trial comparing cytomegalovirus antigenemia assay vs screening bronchoscopy for the early detection and prevention of disease in allogeneic bone marrow and peripheral blood stem cell transplant recipients. Bone Marrow Transplant 2001; 28: 485-490.

19 Verdonck LF, Dekker AW, Rozenberg-Arska M, van den Hoek MR. A risk adapted approach with a short course of ganciclovir to prevent cytomegalovirus (CMV) pneumonia in CMV-seropositive recipients of allogeneic bone marrow transplantation. Clin Infect Dis 1997; 24: 901-907.

20 Manteiga R, Martino R, Sureda A, Labeaga R, Brunet S, Sierra J, Rabella N. Cytomegalovirus pp65 antigenemia-guided pre-emptive treatment with ganciclovir after allogeneic stem cell transplantation: a single-center experience. Bone Marrow Transplant 1998; 22: 899-904.

21 Machado CM, Dulley FL, Vilas Boas LS, Castelli JB, Macedo MCA, Silva RL, Pallota R, Saboya RS, Pannuti CS. CMV pneumonia in allogeneic recipients undergoing early treatment or pre-emptive ganciclovir therapy. Bone Marrow Transplant 2000; 26: 413-417.

22 Machado CM, Menezes RX, Macedo MCA, Mendes AVA, Vilas Boas LS, Castelli JB, Dulley FL, Pannuti CS. Extended antigenemia surveillance and late cytomegalovirus infection after allogeneic BMT. Bone Marrow Transplant 2001; 28: 1053-1059.

23 Mori T, Okamoto S, Matsuoka S et al. Risk-adapted pre-emptive therapy for cytomegalovirus disease in patients undergoing allogeneic bone marrow transplantation. Bone Marrow Transplant 2000; 25: 765-769.

24 Kanda Y, Mineishi S, Saito T et al. Pre-emptive therapy against cytomegalovirus (CMV) disease guided by CMV antigenemia assay after allogeneic hematopoietic stem cell transplantation: a single center experience in Japan. Bone Marrow Transplant 2001; 27: 437-444.

25. Small TN, Papadopoulos EP, Boulad F, Kiehn T, Stiles J, Sepkowitz K, O'Reilly RJ. Outcome of pre-emptive or prophylactic anti-viral therapy to prevent CMV disease following T cell depleted related or unrelated BMT. Blood 2001; 98: S394a. Abstract 1659.

26 Peggs KS, Preiser W, Kottaridis PD et al. Extended routine polymerase chain reaction surveillance and pre-emptive antiviral therapy for cytomegalovirus after allogeneic transplantation. Br J Haematol 2000; 111: 782-790.

27 Hebart H, Brugger W, Grigoleit U et al. Risk for cytomegalovirus disease in patients receiving polymerase chain reaction-based pre-emptive antiviral therapy after allogeneic stem cell transplantation depends on transplantation modality. Blood 2001; 97: 2183-2185. Letter.

28 Mori T, Okamoto S, Watanabe R, Yajima T, Iwao Y, Yamazaki R, Nakazato T, Sato N, Iguchi T, Nagayama H, Takayama N, Hibi T, Ikeda Y. Dose-adjusted pre-emptive therapy for cytomegalovirus disease based on real-time polymerase chain reaction after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant 2002; 29: 777-782.

29 Humar A, Wood S, Lipton J et al. Effect of cytomegalovirus infection on 1-year mortality rates among recipients of allogeneic bone marrow transplants. Clin Infect Dis 1998; 26: 606-610.

30 Nichols WG, Corey L, Gooley T et al. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. J Infect Dis 2002; 185: 273-282.

31 Meijer E, Dekker AW, Rozenberg-Arska M, Weersink AJL, Verdonck LF. Influence of Cytomegalovirus-seropositivity on outcome after T cell depleted bone marrow transplantation: contrasting results between recipients of grafts from related and unrelated donors. Clin Infect Dis 2002; 35: 703-712.

32 Craddock C, Szydlo RM, Dazzi F et al. Cytomegalovirus seropositivity adversely influences outcome after T-depleted unrelated donor transplant in patients with chronic myeloid leukaemia: the case for tailored graft-versus-host disease prophylaxis. Br J Haematol 2001; 112: 228-236.

33 Kröger N, Zabelina T, Krüger W et al. Patient cytomegalovirus seropositivity with or without reactivation is the most important prognostic factor for survival and treatment-related mortality in stem cell transplantation from unrelated donors using pretransplant in vivo T-cell depletion with antithymocyte globulin. Br J Haematol 2001; 113: 1060-1071.

34 Castro-Malaspina H, Harris RE, Gajewski J et al. Unrelated marrow transplantation for myelodysplastic syndromes: outcome analysis in 510 transplants facilitated by the national marrow donor program. Blood 2002; 99: 1943-1951.

35 Seropian S, Ferguson D, Salloum E, Cooper D, Landry ML. Lack of reactivity to CMV pp65 antigenemia testing in a patient with CMV disease following allogeneic bone marrow transplant. Bone Marrow Transplant 1998; 22: 507-509.

36 Boekch M, Boivan G. Quantitation of cytomegalovirus: methodologic aspects and clinical applications. Clin Microbiol Rev 1998; 11: 533-554.

37 Hiyoshi M, Tagawa S, Takubo T et al. Evaluation of the Amplicor CMV test for direct detection of cytomegalovirus in plasma specimens. J Clin Microbiol 1997; 10: 2692-2694.

38 Preiser W, Brauninger S, Schwerdtfeger R, Ayliffe U, Garson JA, Brink NS, Franck S, Doerr HW, Rabenau HF. Evaluation of diagnostic methods for the detection of cytomegalovirus in recipients of allogeneic stem cell transplants. J Clin Virol 2001; 20: 59-70.

39 Schulenburg A, Watkins-Riedel T, Greinix HT, Rabitsch W, Loidolt H, Keil F, Mitterbauer M, Kalhs P. CMV monitoring after peripheral blood stem cell and bone marrow transplantation by pp65 antigen and quantitative PCR. Bone Marrow Transplant 2001; 28: 765-768.

40 Solano C, Munoz I, Gutierrez A, Farga A, Prosper F, Garcia-Conde J, Navarro D, Gimeno C. Qualitative plasma PCR assay (Amplicor CMV test) versus pp65 antigenemia assay for monitoring cytomegalovirus viremia and guiding pre-emptive ganciclovir therapy in allogeneic stem cell transplantation. J Clin Microbiol 2001; 39: 3938-3941.

41 Flexman J, Kay I, Fonte R, Herrmann R, Gabbay E, Palladino S. Differences between the quantitative antigenemia assay and the Cobas Amplicor monitor quantitative PCR assay for detecting CMV viremia in bone marrow and solid organ recipients. J Med Virol 2001; 64: 275-282.

42. Boivin G, Belanger R, Delage R, Beliveau C, Demers C, Goyette N, Roy J. Quantitative analysis of cytomegalovirus (CMV) viremia using the pp65 antigenemia assay and the Cobas Amplicor CMV monitor test after blood and marrow allogeneic transplantation. J Clin Microbiol 2002; 38: 4356-4360.

43 Machida U, Kami M, Fukui T et al. Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation. J Clin Microbiol 2000; 38: 2536-2542.

44 Griscelli F, Barrois M, Chauvin S, Lastere S, Bellet D, Bourhis JH. Quantification of human cytomegalovirus DNA in bone marrow transplant recipients by real-time PCR. J Clin Microbiol 2001; 39: 4362-4369.

45 Yakushiji K, Gondo H, Kamezaki K et al. Monitoring of cytomegalovirus reactivation after allogeneic stem cell transplantation: comparison of an antigenemia assay and quantitative real-time polymerase chain reaction. Bone Marrow Transplant 2002; 29: 599-606.

46 Gerna G, Furione M, Baldanti F, Percivalle E, Comoli P, Locatelli F. Quantitation of human cytomegalovirus DNA in bone marrow transplant recipients. Br J Haematol 1995; 91: 674-683.

47 Kanda Y, Chiba S, Suzuki T, Kami M, Yazaki Y, Hirai H. Time course analysis of semi-quantitative PCR and antigenemia assay for prevention of cytomegalovirus disease after bone marrow transplantation. Br J Haematol 1998; 100: 222-225.

48. Hebart H, Muller C, Loffler J, Jahn G, Einsele H. Monitoring of CMV infection: a comparison of PCR from whole blood, plasma-PCR, pp65-antigenemia and virus culture in patients after bone marrow transplantation. Bone Marrow Transplant 1996; 17: 861-868.
49 Boeckh M, Gallez-Hawkins GM, Myerson D, Zaia JA, Bowden RA. Plasma polymerase chain reaction for cytomegalovirus DNA after allogeneic marrow transplantation. Transplantation 1997; 64: 108-113.
50 Matsunaga T, Sakamaki S, Ishigaki S et al. Use of PCR serum in diagnosing and monitoring cytomegalovirus reactivation in bone marrow transplant recipients. Int J Hematol 1999; 69: 105-111.
51 Hebart H, Gamer D, Loeffler J et al. Evaluation of Murex CMV DNA hybrid capture assay for detection and quantitation of cytomegalovirus infection in patients following allogeneic stem cell transplantation. J Clin Microbiol 1998; 36: 1333-337.
52 Gerna G, Baldanti F, Middeldorp JM, Furione M, Zavattoni M, Lilleri D, Revello MG. Clinical significance of expression of human cytomegalovirus pp67 late transcript in heart, lung, and bone marrow transplant recipients as determined by nucleic acid sequence-based amplification. J Clin Microbiol 1999; 37: 902-911.
53 Hebart H, Rudolph T, Loeffler J, Middeldorp J, Ljubicic T, Jahn G, Einsele H. Evaluation of the NucliSens CMV pp67 assay for detection and monitoring of human cytomegalovirus infection after allogeneic stem cell transplantation. Bone Marrow Transplant 2002; 30: 181-187.
54 Gerna G, Baldanti F, Lilleri D et al. Human cytomegalovirus immediate-early mRNA detection by nucleic acid sequence-based amplification as a new parameter for pre-emptive therapy in bone marrow transplant recipients. J Clin Microbiol 2000; 38: 1845-1853.
55 Aono T, Kondo K, Miyoshi H, Tanaka-Taya K, Kondo M, Osugi Y, Hara J, Okada S, Yamanishi K. Monitoring of human cytomegalovirus infections in pediatric bone marrow transplant recipients by nucleic acid sequence-based amplification. J Infect Dis 1998; 178: 1244-1249.
56 Boriskin YS, Fuller K, Powles RL, Vipond IB, Rice PS, Booth JC, Caul EO, Butcher PD. Early detection of cytomegalovirus (CMV) infection in bone marrow transplant patients by reverse transcription-PCR for CMV spliced late gene UL21.5: a two site evaluation. J Clin Virol 2002; 24: 13-23.
57 Quinnan GV, Kirmani N, Rook AH et al. Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and Non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone marrow transplant recipients. N Engl J Med 1982; 307: 6-13.
58 Reusser P, Riddell S, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood 1991; 78: 1373-1380.
59 Riddell ST, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science 1992; 257: 238-241.
60 Krause H, Hebart H, Jahn G, Muller CA, Einsele H. Screening for CMV-specific T cell proliferation to identify patients at risk of developing late onset CMV disease. Bone Marrow Transplant 1997; 19: 1111-1116.
61 Gratama JW, van Esser JWJ, Lamers CHJ, Tournay C, Lowenberg B, Bolhuis RLH, Cornelissen JJ. Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. Blood 2001; 98: 1358-1364.
62 Cwynarski K, Ainsworth J, Cobbold M et al. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. Blood 2001; 97: 1232-1240.
63 Aubert G, Hassan-Walker AF, Madrigal JA et al. Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. J Infect Dis 2001; 184: 955-963.

64 Ozdemir E, St. John LS, Gillespie G et al. Cytomegalovirus reactivation following allogeneic stem cell transplantation is associated with the presence of dysfunctional antigen-specific CD8+ T cells. Blood 2002; 100: 3690-3697.

65 Hebart H, Daginik S, Stevanovic S et al. Sensitive detection of human cytomegalovirus peptide-specific cytotoxic T-lymphocyte responses by interferon-gamma-enzyme-linked immunospot assay and flow cytometry in healthy individuals and in patients after allogeneic stem cell transplantation. Blood 2002; 99: 3830-3837.

66 Longmate J, York J, La Rosa C, Krishan R, Zhang M, Senitzer D, Diamond DJ. Population coverage by HLA class-I restricted cytotoxic T-lymphocyte epitopes. Immunogenetics 2001; 52: 165-173.

67 Lim JB, Provenzano M, Bettinotti M, Stroncek DF. Identification of a new candidate HLA-A3 restricted CMV epitope as a target antigen to induce specific CMV cytotoxic lymphocytes. Blood 2001; 98: S780a. Abstract 3244.

68 Nichols WG, Boeckh M. Recent advances in the therapy and prevention of CMV infections. J Clin Virol 2000; 16: 25-40.

69 Ljungman P. Prophylaxis against herpesvirus infections in transplant recipients. Drugs 2001; 61: 187-196.

70 Reusser P. Oral valganciclovir: a new option for treatment of cytomegalovirus infection and disease in immunocompromised hosts. Expert Opin Investig Drugs 2001; 10: 1745-1753.

71 Meyers JD, Reed EC, Shepp DH et al. Acyclovir for prevention of cytomegalovirus infection and disease after allogeneic marrow transplantation. N Engl J Med 1988; 318: 70-75.

72 Prentice HG, Gluckman E, Powles RL et al. Long-term survival in allogeneic bone marrow transplant recipients following acyclovir prophylaxis for CMV infection. Bone Marrow Transplant 1997; 19: 129-133.

73 Vusirikala M, Wolff SN, Stein RS, Brandt SJ, Morgan DS, Greer JP, Schuening FG, Dummer JS, Goodman SA. Valacyclovir for the prevention of cytomegalovirus infection after allogeneic stem cell transplantation: a single institution retrospective cohort analysis. Bone Marrow Transplant 2001; 28: 265-270.

74 Ljungman P, de la Camara R, Milpied N, Volin L, Russell CA, Crisp A, Webster A. Randomized study of valacyclovir as prophylaxis against cytomegalovirus reactivation in recipients of allogeneic bone marrow transplants. Blood 2002; 99: 3050-3056.

75 Reusser P, Cordonnier C, Einsele H, Engelhard D, Link H, Locasciulli A, Ljungman P. European survey of herpesvirus resistance to antiviral drugs in bone marrow transplant recipients. Bone Marrow Transplant 1996; 17: 813-817.

76 Reusser P, Gambertoglio JG, Lilleby K, Meyers JD. Phase I-II trial of foscarnet for prevention of cytomegalovirus infection in autologous and allogeneic marrow transplant recipients. J Infect Dis 1992; 166: 473-479.

77 Bacigalupo A, Tedone E, Van Lint MT et al. CMV prophylaxis with foscarnet in allogeneic bone marrow transplant recipients at high risk of developing CMV infections. Bone Marrow Transplant 1994; 13: 783-788.

78 Bregante S, Bertilson S, Tedone E et al. Foscarnet prophylaxis of cytomegalovirus infections in patients undergoing allogeneic bone marrow transplantation (BMT): a dose-finding study. Bone Marrow Transplant 2000; 26: 23-29.

79 Ordemann R, Naumann R, Geissler G, Kroschinsky F, Bornhauser M, Schwerdtfeger R, Ehninger G. Foscarnet-an alternative for cytomegalovirus prophylaxis after allogeneic stem cell transplantation. Ann Hemat 2000; 79: 432-436.

80 Bacigalupo A, Van Lint MT, Tedone E et al. Early treatment of CMV infections in allogeneic bone marrow transplant recipients with foscarnet or ganciclovir. Bone Marrow Transplant 1994; 13: 753-758.

81 Ljungman P, Oberg G, Aschan J, Ehrnst A, Lonnqvist B, Pauksen K, Sulila P. Foscarnet for pre-emptive therapy of CMV infection detected by a leukocyte-based nested PCR in allogeneic bone marrow transplant patients. Bone Marrow Transplant 1996; 18: 565-568.

82 Moretti S, Zikos P, Van Lint MT et al. Foscarnet vs ganciclovir for cytomegalovirus (CMV) antigenemia after allogeneic hemopoietic stem cell transplantation (HSCT): a randomised study. Bone Marrow Transplant 1998; 22: 175-180.

83 Reusser P, Einsele H, Lee J, Volin L, Rovira M, Engelhard D, Finke J, Cordonnier C, Link H, Ljungman P. Randomized multicenter trial of foscarnet versus ganciclovir for pre-emptive therapy of cytomegalovirus infection after allogeneic stem cell transplantation. Blood 2002; 99: 1159-1164.

84 Ljungman P, Lambertenghi Deliliers G, Platzbecker U et al. Cidofovir for cytomegalovirus infection and disease in allogeneic stem cell transplant recipients. Blood 2001; 97: 388-392.

85 Kiehl MG, Basara N. Cidofovir for cytomegalovirus pre-emptive therapy in stem cell transplant recipients. Blood 2001; 98: 1626. Letter.

86 Chakrabarti S, Collingham KE, Osman H, Fegan CD, Milligan DW. Cidofovir as primary pre-emptive therapy for post-transplant cytomegalovirus infections. Bone Marrow Transplant 2001; 28: 879-881.

87 Platzbecker U, Bandt D, Thiede C et al. Successful pre-emptive cidofovir treatment for CMV antigenemia after dose-reduced conditioning and allogeneic blood stem cell transplantation. Transplantation 2001; 71: 880-885.

88 Emery VC, Griffiths PD. Prediction of cytomegalovirus load and resistance patterns after antiviral chemotherapy. Proc Natl Acad Sci U S A. 2000; 97: 8039-8044.

89 Erice A. Resistance of human cytomegalovirus to antiviral drugs. Clin Microbiol Rev 1999; 12: 286-297.

90 Wolf DG, Yaniv L, Honigman A, Kassis L, Schonfeld T, Ashkenazi S. Early emergence of ganciclovir-resistant human cytomegalovirus strains in children with primary combined immunodeficiency. J Infect Dis 1998; 178: 535-538.

91 Slavin MA, Bindra RR, Gleaves CA, Pettinger MB, Bowden RA. Ganciclovir sensitivity of cytomegalovirus at diagnosis and during treatment of cytomegalovirus pneumonia in marrow transplant recipients. Antimicrob Agents Chemother 1993; 37: 1360-1363.

92 Gilbert C, Roy J, Belanger R, Delage R, Beliveau C, Demers C, Boivin G. Lack of emergence of cytomegalovirus UL97 mutations conferring ganciclovir (GCV) resistance following preemptive GCV therapy in allogeneic stem cell transplant recipients. Antimicrob Agents Chemother 2001; 45: 3669-3671.

93 Erice A, Borrell N, Li W, Miller WJ, Balfour HH. Ganciclovir susceptibilities and analysis of UL97 region in cytomegalovirus (CMV) isolates from bone marrow recipients with CMV disease after antiviral prophylaxis. J Infect Dis 1998; 178: 531-534.

94 Eckle T, Prix L, Jahn G, Klingebiel T, Handgretinger R, Selle B, Hamprecht K. Drug-resistant human cytomegalovirus infection in children after allogeneic stem cell transplantation may have different clinical outcomes. Blood 2000; 96: 3286-3289.

95 Prix L, Hamprecht K, Holzhuter B, Handgretinger R, Klingebiel T, Jahn J. Comprehensive restriction analysis of the UL97 region allows early detection of ganciclovir-resistant human cytomegalovirus in an immunocompromised child. J Infect Dis 1999; 180: 491-495.

96 Hamprecht K, Eckle T, Prix L, Faul C, Einsele H, Jahn G. Ganciclovir-resistant cytomegalovirus disease after allogeneic stem cell transplantation: pitfalls of phenotypic diagnosis by in vitro selection of an UL97 mutant strain. J Infect Dis 2002; 187: 139-143.

97 Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, Riddell SR. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med 1995; 333: 1038-1044.

98 Einsele H, Roosnek E, Rufer N et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. Blood 2002;99:3916-3922.

99 June CH. CD4 T cells to kiss off chemotherapy-resistant CMV? Blood 2002; 99: 3883. Editorial.

100 Peggs K, Verfuerth S, Pizzey A, Khan N, Moss P, Mackinnon S. Cytomegalovirus-specific adoptive cellular therapy results in massive and persistent expansions of CMV-specific T-cells and significantly reduces the incidence of CMV reactivation following allogeneic transplantation. Blood 2001; 98: S779a. Abstract 3240.

101 Keenan RD, Ainsworth J, Khan N, Bruton R, Cobbold M, Assenmacher M, Milligan DW, Moss PA. Purification of cytomegalovirus-specific CD8 T cells from peripheral blood using HLA-peptide tetramers. Br J Haematol 2001; 115: 428-434.

Chapter 11

Summary

Recipients of stem cell transplants (SCT) are severely immunocompromised at least during the first 6 months post-transplant. In the introduction (**chapter 1**) this immunodeficiency is further described. During the neutropenic period patients are at risk for developing bacterial and fungal infections. Once neutropenia has recovered, opportunistic viral infections are a major threat. Both the Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) are herpes viruses and share the phenomenon that primary infection is followed by latent infection. In immunocompetent individuals latent infection of these viruses is controlled by T cell immunity. However, in immunocompromised patients herpes viruses frequently show reactivations, regularly resulting in herpes virus related disease. In this thesis, T cell immunodeficiency and EBV and CMV infections after allogeneic SCT are studied.

The extent of the immunodeficiency is determined by several factors such as patient age, development of graft-versus-host disease (GVHD) and the institution of immunosuppressive therapy. Furthermore, ex vivo T cell depletion (TCD) of grafts, the use of alternative donors (partially matched related donors or matched unrelated donors) and the source of stem cells may play a role as well, although their contribution is less clear. Antithymocyteglobulin (ATG) is generally used in recipients of TCD grafts from unrelated donors to prevent graft failure. In **chapter 2** the effect of ATG on quantitative immune recovery and GVHD was analysed in recipients of MRD grafts compared to MUD recipients. Apart from ATG infusion in MUD recipients treatment of both patient groups was similar. Recovery of T cell subsets, NK cells and B cells was determined at 2, 3, 6 and 8 months post-transplant. In the MRD group markedly higher T cell and subset values were measured during this period. MRD recipients received significantly less T cells and suffered more acute and chronic GVHD compared to MUD recipients. These data suggest that ATG is an important negative factor in the reconstitution of T cells and their subsets in MUD recipients. On the other hand, ATG results in a lower incidence and severity of GVHD.
In **chapter 3** the effect of 3 different ATG doses (8 mg/kg, 6 mg/kg and 4 mg/kg) on graft failure and incidence of GVHD was evaluated. Lowering ATG dose from 8 mg/kg to 6 mg/kg did not influence the occurrence of graft failure nor the incidence of GVHD. However, when ATG dose was decreased to 4 mg/kg the incidence of severe acute GVHD grade III-IV and extensive chronic GVHD rose dramatically, without affecting the occurrence of graft failure.

EBV reactivations or primo/re-infections in severely immunocompromised patients may result in the development of EBV-associated lymphoproliferative disorders (EBV-LPD). In MUD recipients of TCD grafts reactivation of EBV is a frequent event, which is described in **chapter 4**. In this study it was shown that plasma EBV DNA quantitatively predicted EBV-LPD. The negative and positive predictive values of a viral load of 1000 c/ml were 100% and 39%, respectively. EBV-LPD was not diagnosed in recipients of non-TCD grafts, although EBV reactivations did occur in these patients. The use of ATG in MUD recipients, was an important risk factor for the occurrence of EBV reactivations.

Until now it is not known whether EBV reactivations result from a true reactivation of the endogenous patient strain or a re-infection with exogenous strains. In **chapter 5** preliminary data are presented of EBV strain typing in six patients in pre-transplant collected mouth-washes and post-transplant collected plasma samples, peripheral blood mononuclear cells (PBMCs) and mouth washes. Furthermore, prospective data of EBV DNA monitoring performed in PBMC as well as cell free plasma are given. In 3 of 6 patients the post-transplant EBV sequence pattern differed from the pre-transplant pattern, indicating a re-infection post-transplant with an exogenous strain instead of a reactivation of the original endogenous EBV strain. In one MRD patient it was not possible to differentiate since pre-transplant, post-transplant and donor sequence patterns were identical. In a MUD recipient pre- and post-transplant sequence patterns were identical too, which makes a reactivation of the endogenous EBV strain likely in this patient, although a re-infection is not excluded. Post-transplant EBV strains of the sixth patient differed by only one nucleotide from the pre-transplant strain. This might have been the result of a point mutation acquired during viral replication, making it more likely this patient suffered a reactivation instead of a re-infection.

In 17 patients double EBV DNA monitoring was performed in PBMCs and plasma. Only 4 of 17 (23%) patients showed a reactivation in plasma samples compared to 15 of 17 (88%) in PBMC samples ($p<0.001$). None of the patients with a reactivation in PBMCs only progressed to EBV-LPD, compared to 18% of patients with EBV reactivation in plasma. Therefore, it can be concluded that monitoring in PBMC samples is less specific for predicting EBV-LPD compared to plasma samples.

The risk for EBV-LPD varies according to the techniques used for T cell depletion, being lowest (<2%) when methods are used which remove both T and B cells. In **chapter 6** the incidence of EBV-LPD among MRD and MUD recipients of TCD grafts is presented. All MUD

recipients were treated with ATG. When grafts from MUDs were both T and B cell depleted, 4 out of 31 patients (13%) developed EBV-LPD compared to 5 of 7 patients (71%) when no B cell depletion was performed. A T/B cell ratio in the graft of ≥ 0.25 seemed sufficient to significantly reduce the incidence of EBV-LPD after TCD SCT from MUDs. In contrast, among recipients of grafts from an HLA-identical sibling donor the incidence of EBV-LPD was not influenced by B cell depletion in addition to TCD (5% in T and B cell depleted group; 4% in TCD group).
In **chapter 7** an overview is given of the prevention and treatment of EBV-LPD in recipients of stem cell and solid organ transplants. Several risk factors for the development of EBV-LPD after solid organ transplantation (SOT) and SCT, respectively, have been identified: 1) primary EBV infection in EBV-seronegative patients, 2) type of transplanted allograft, 3) CMV serostatus mismatch (seronegative recipient/seropositive donor), 4) CMV disease 5) use of T cell antibodies in SOT recipients and 1) TCD of grafts, 2) use of unrelated or ≥ 2 HLA antigen mismatched related donors, 3) use of ATG or 4) anti CD3 monoclonal antibodies in SCT recipients. Guidelines for monitoring of EBV viral load are given (which patients should be monitored, at what frequency, for how long). Furthermore, the varying treatment modalities are reviewed and treatment recommendations are given.

Due to the high mortality rate of CMV disease, latent CMV infection has long been considered a negative risk factor for overall survival (OS) and TRM. In **chapter 8** the effect of CMV serostatus on OS and TRM in 253 consecutively treated patients receiving partial TCD stem cells from either matched related donors (n=205) or matched unrelated donors (n=48) was analysed. Transplantations were performed between July 1990 and May 2000. All patients were given leukocyte-depleted blood products. CMV monitoring was performed using the pp65 antigenemia assay. Pre-emptive therapy consisted of short-course (2 weeks) low-dose (2.5 mg/kg intravenously b.i.d.) ganciclovir treatment as soon as a positive antigenemia assay was obtained (≥ 1 positive staining granulocyte/150.000 cells). Ganciclovir prophylaxis, identical to pre-emptive therapy, was given to CMV-seropositive patients with acute GVHD grade II-IV who were treated with high-dose corticosteroids. This analysis showed that CMV-seropositivity was not an adverse risk factor for OS and TRM in MRD recipients of partial TCD SCT. However, in MUD recipients, patient CMV-seropositivity still had a high impact on OS and TRM.

The effect of CMV-seropositivity on outcome in MUD recipients is mostly studied in patients treated before 1999. In **chapter 9** it is evaluated whether CMV-seropositive MUD recipients transplanted after 1999, still showed inferior outcome compared to CMV-seronegative recipients. In our transplantation centre two important changes in transplantation procedure were introduced in that year. In April 1999 ATG dose was lowered from 20 mg/kg to 8 mg/kg. Furthermore, in January 1999 sequence based typing (SBT) of HLA-DRB1 was introduced. Both changes may result in an improved immune reconstitution post-transplant. Low-dose ATG by a direct effect on T lymphocyte counts and high resolution HLA-DRB1 typing by a decreased incidence of GVHD. Considering the immunosuppressive effect of (latent) CMV infection this may have a positive impact on outcome in CMV-seropositive SCT recipients. In total 80 patients received a partial TCD graft, 36 before 1999 and 44 after 1999. CMV-seropositive patients transplanted before 1999 showed a highly significant inferior outcome compared to seronegative recipients (see also chapter 8). In contrast, in patients transplanted after 1999 no difference in outcome was observed between the two groups.

In **chapter 10** the prevention of CMV disease in recipients of allogeneic stem cell transplants is reviewed. Before the introduction of ganciclovir, CMV infection and pneumonia developed in 38 and 17%, respectively, of SCT recipients, while mortality due to CMV pneumonia was 85 %. Currently, two antiviral strategies, prophylactic or pre-emptive treatment, are used for prevention of CMV disease in seropositive recipients. Prophylactic treatment usually consists of antiviral therapy started at engraftment until at least day 100 post-transplant. Pre-emptive therapy is defined as antiviral treatment based on the detection of reactivated CMV infection by positive CMV cultures, a positive antigenemia assay or positive molecular assays. In this chapter these antiviral strategies are reviewed and recommendations for prevention of CMV disease are given. Furthermore, several other aspects of prevention of CMV disease are reviewed, which are: 1) methods available for early detection of CMV reactivations, 2) monitoring of CMV-specific T cell responses, 3) the value of several antiviral drugs and 4) adoptive immunotherapy as prophylaxis or (pre-emptive) treatment of CMV reactivations/CMV disease.

In conclusion, recipients of stem cell transplants are severely immunocompromised at least during the first 6 months post-transplant. This is even more true for recipients of grafts from matched unrelated donors. However, several important changes in transplantation proce-

dure have resulted in a more favourable outcome among MUD recipients. B cell depletion - in addition to TCD- of grafts, a reduced dosage of ATG and high resolution DNA typing of HLA-antigens all have contributed to this better outcome. The reduced ATG dose and sequence based typing of HLA-antigens may have improved immune recovery in MUD recipients, thereby eliminating CMV-seropositivity as an adverse risk factor for overall survival and transplant related mortality. In MRD recipients pre-emptive treatment of CMV reactivations alone was sufficient to eliminate CMV-seropositivity as an adverse risk factor for outcome. The optimal dose of ATG is unknown, however, considering the immunosuppressive and direct toxic effects of ATG, the lowest possible dose should be used. In the setting of SCT with (partially) TCD grafts from MUDs, using limited post-transplant immunosuppression with cyclosporine A, a total dose of 6-8 mg/kg is recommended. A further decrease greatly increases the risk of severe GVHD. The future aim is to further improve immune reconstitution post-transplant and to implement new methods for monitoring, prevention and treatment of opportunistic infections. With respect to prevention of EBV-LPDs, it is important to know the most frequent transmission route of EBV re-infections. Further studies using LMP-1 fingerprinting for strain identification of patient, donor and partner samples are necessary.

Samenvatting voor niet-ingewijden

Bij diverse kwaadaardige en enkele niet-kwaadaardige hematologische ziektebeelden biedt een allogene stamceltransplantatie met stamcellen van een donor (allogene SCT) de enige kans op genezing. Een SCT is een zware behandeling met diverse mogelijke complicaties. Eén van die complicaties betreft het optreden van opportunistische infecties, dit zijn infecties die je bij mensen met een normale afweer vrijwel niet ziet. De toegenomen vatbaarheid voor infecties is het gevolg van een sterk verminderde afweer (immuundeficiëntie) tijdens de post-transplantatie fase. Geleidelijk herstelt de afweer (immuniteit) zich, maar met name gedurende de eerste 6 maanden post-transplantatie is deze sterk gestoord. In de introductie (**Hoofdstuk 1**) wordt deze immuundeficiëntie beschreven. Kort na de SCT is juist de vatbaarheid voor bacteriële en schimmel infecties verhoogd, terwijl na enige weken vooral virus infecties een belangrijke bedreiging vormen. Zowel het Epstein-Barr virus (EBV) als het Cytomegalovirus (CMV) behoort tot de groep van herpes virussen. Herpes virussen blijven na een eerste ofwel primo-infectie latent in het lichaam aanwezig. Het merendeel van de mensen raakt tijdens de kinderjaren met deze virussen geïnfecteerd. Bij gezonde mensen worden die virussen onder controle gehouden door specifieke afweer cellen (cytotoxische T cellen). Dit is niet het geval bij immuundeficiënte mensen, zoals patiënten die een SCT hebben ondergaan. Bij deze patiënten vertonen herpes virussen frequent een reactivatie, hetgeen kan leiden tot ernstige en vaak fatale ziektebeelden. In dit proefschrift worden diverse aspecten van de T cel immuundeficiëntie en EBV en CMV infecties na allogene SCT bestudeerd.

De mate van immuundeficiëntie in de post-transplantatie fase wordt door verschillende factoren bepaald, zoals de leeftijd van de patiënt, de ontwikkeling van een omgekeerde afstotingsreactie (“graft-versus-host disease”, GVHD) en het gebruik van afweeronderdrukkende medicijnen (immuunsuppressiva). GVHD is een complicatie die veroorzaakt wordt door afweercellen van de donor in het transplantaat (de “graft”). Deze donor cellen herkennen de patiënt (de “host”) als vreemd en gaan daar een afweerreactie tegen ontwikkelen. Bij het zoeken naar een potentiële donor worden specifieke weefselkenmerken van zowel patiënt als donor in kaart gebracht (HLA-typering). De beoogde donor moet een gelijke HLA-typering hebben als de patiënt. De kans op GVHD is groter als gebruik gemaakt wordt van niet verwante donoren of van familiedonoren die niet volledig passend zijn. Patiënten die een allogene SCT hebben ondergaan, krijgen immuunsuppressiva om de GVHD te voorkomen of te beperken. In sommige instituten worden de transplantaten (gedeeltelijk) ontdaan van T

cellen (T cel depletie) om de kans op het optreden van de omgekeerde afstotingsreactie te verminderen. Deze T cel depletie is mogelijk ook een factor die bijdraagt aan de ernst van de immuundeficiëntie, tezamen met het gebruik van niet verwante donoren of gedeeltelijk passende familiedonoren en met de bron van stam cellen (bloed of beenmerg).
Bij ontvangers van een T cel gedepleteerd transplantaat van een onverwante donor bestaat een verhoogde kans op afstoting van het transplantaat (graft failure). Deze afstoting wordt veroorzaakt door resterende T cellen van de patiënt. Antithymocytenglobuline (ATG) is een middel dat een T cel depletie in het lichaam bewerkstelligt. Toediening van het middel vermindert de kans op afstoting van het transplantaat en tevens vermindert het ook de kans op het optreden van GVHD. In **Hoofdstuk 2** wordt het effect van ATG op het herstel van de immuniteit en op het optreden van GVHD bestudeerd in ontvangers van een T cel gedepleteerd transplantaat van onverwante donoren en vergeleken met ontvangers van een familietransplantaat. Het kwantitatieve herstel van specifieke afweercellen (T cellen, NK cellen en B cellen) werd bepaald 2, 3, 6 en 8 maanden na transplantatie. Bij de ontvangers van een familietransplantaat ("matched related donor", MRD groep) werden significant hogere T cel waarden gemeten, terwijl zij minder T cellen via het transplantaat toegediend kregen en frequenter zowel acute als chronische GVHD ontwikkelden vergeleken met ontvangers van een transplantaat van onverwante donoren ("matched unrelated donor", MUD groep). Deze gegevens suggereren dat ATG een belangrijke beperkende factor is met betrekking tot het herstel van T cel aantallen in MUD patiënten. Echter, ATG resulteert tevens in een lagere incidentie en ernst van GVHD.
In **Hoofdstuk 3** wordt het effect van 3 verschillende ATG doseringen (8 mg/kg, 6 mg/kg en 4 mg/kg) op de incidentie van graft failure en GVHD geëvalueerd. Het optreden van graft failure en GVHD werd niet beïnvloed door een dosisverlaging van 8 mg/kg naar 6 mg/kg. Echter, bij een verlaging naar 4 mg/kg nam de incidentie van ernstige acute GVHD (graad III-IV) en uitgebreide chronische GVHD dramatisch toe, zonder het optreden van graft failure te beïnvloeden.

EBV reactivaties of primo/re-infecties kunnen, wanneer ze optreden bij immuundeficiënte personen, leiden tot een ernstig ziektebeeld: een EBV-geassocieerde lymfoproliferatie (EBV-LPD). Dit betreft een woekering van specifieke afweercellen, B cellen, onder invloed van het EBV virus, wat bij SCT patiënten binnen enkele dagen een fatale afloop kan hebben. Het EBV virus heeft zich na een primo-infectie in latente vorm in B cellen verstopt. Zonder ade-

quate T cel immuniteit kan het virus gaan groeien. Een EBV reactivatie, primo/re-infectie of chronisch actieve infectie kan aangetoond worden middels een EBV DNA bepaling in bloed of plasma. Het totale EBV DNA in bloed is de resultante van een latente infectie en een actieve infectie, terwijl EBV DNA in plasma specifiek een actieve infectie weergeeft. In **Hoofdstuk 4** wordt beschreven dat EBV reactivaties met name frequent voorkomen bij MUD patiënten die een T cel gedepleteerd transplantaat ontvangen en met ATG worden behandeld. In deze studie wordt tevens getoond dat EBV DNA in plasma een voorspellende waarde heeft met betrekking tot het optreden van een EBV-LPD. De negatief en positief voorspellende waarde van een virale load van 1000 copieën (c)/ml was 100% en 39%, respectievelijk. Bij ontvangers van een niet-T cel gedepleteerd transplantaat werd geen EBV-LPD vastgesteld, wel werden EBV reactivaties gezien in deze groep. Het gebruik van ATG was een belangrijke risicofactor voor het optreden van EBV reactivaties.

Tot op heden is niet bekend of een EBV reactivatie werkelijk een reactivatie van de endogeen aanwezige patiënten stam is of dat er een re-infectie met een exogene stam heeft plaatsgevonden. Er zijn aanwijzingen dat de voorbehandeling voor de transplantatie in staat is de eigen stam te vernietigen. In **Hoofdstuk 5** worden preliminaire data gepresenteerd betreffende de EBV stam identificatie in 6 patiënten. Ook tijdens een latente infectie wordt het EBV in mondspeeksel uitgescheiden. Mondspeeksel is daardoor de belangrijkste transmissiebron van het virus. Dit verklaart waarom een primo-infectie met EBV ("Pfeiffer") ook wel de "kissing-disease" wordt genoemd. Mondspeeksel is bij latent geïnfecteerde personen de belangrijkste bron voor virusisolatie. We hebben aldus bij alle patiënten en, indien mogelijk, bij donoren voorafgaande aan de transplantatie mondspoelingen verzameld om het EBV virus te isoleren en te karakteriseren. Na de transplantatie werd het virus geïsoleerd uit plasma, bloedcellen en opnieuw mondspoelingen. Bij 3 van de 6 patiënten verschilde de post-transplantatie stam van de pre-transplantatie stam. Dit geeft aan dat bij deze personen een re-infectie heeft plaatsgevonden in plaats van een reactivatie. Bij één MRD patiënt kon geen onderscheid gemaakt worden, daar de pre-transplantatie, post-transplantatie en donor stammen identiek waren (hetgeen niet ongebruikelijk is binnen families). Bij de laatste twee patiënten is er meest waarschijnlijk sprake geweest van een reactivatie, hoewel een re-infectie bij deze twee niet met 100% zekerheid was uit te sluiten.

De kans een EBV-LPD te ontwikkelen wordt mede bepaald door de techniek die gebruikt wordt om de T cel depletie van het transplantaat te verrichten. In **Hoofdstuk 6** wordt het effect van verschillende T cel depletie technieken geanalyseerd met betrekking tot de inci-

dentie van EBV-LPD in MRD en MUD ontvangers. De MUD patiënten werden tevens behandeld met ATG. Als de MUD transplantaten zowel van T als B cellen ontdaan werden, ontwikkelde 13% (4/31) van de patiënten een EBV-LPD vergeleken met 71% (5/7) van de patiënten indien geen B cel depletie verricht werd. Een T/B cel ratio in het transplantaat van ≥ 0.25 lijkt voldoende om de incidentie van EBV-LPD bij MUD patiënten in belangrijke mate te verminderen. Bij ontvangers van familietransplantaten werd de incidentie van EBV-LPD niet beïnvloed door B cel depletie (5% in de groep waarbij B en T cel depletie werd verricht; 4 % als alleen T cel depletie werd verricht). Dit kan goed verklaard worden door het gebruik van ATG in de MUD ontvangers, leidende tot een nog sterker gestoorde disbalans tussen geïnfecteerde B cellen en beschermende T cellen. Het feit dat B cel depletie van het transplantaat beschermt tegen EBV-LPD, pleit tevens voor de hypothese dat een EBV reactivatie niet werkelijk een reactivatie is, maar eerder een re-infectie vanuit donor B cellen (zie Hoofdstuk 5).
Ter afsluiting van het onderdeel EBV infecties wordt in **Hoofdstuk 7** een overzicht gegeven van de preventie en behandeling van EBV-LPD in ontvangers van SCT en van solide orgaan transplantaties (bijvoorbeeld nier-, hart-, lever- en darmtransplantaties). De risicofactoren voor het ontwikkelen van een EBV-LPD worden beschreven alsook richtlijnen voor het monitoren van de EBV DNA virale load. Tevens worden de verschillende behandelingsmodaliteiten samengevat en aanbevelingen gegeven met betrekking tot behandeling.

Een reactivatie van het CMV virus kan leiden tot CMV ziekte. Deze CMV ziekte uit zich in de vorm van een longontsteking, maag/darm/leverontsteking of netvliesontsteking. De meest ernstige vorm van CMV ziekte is de longontsteking die zonder behandeling bij 80% van de patiënten een fataal beloop heeft. Tegenwoordig zijn er antivirale middelen beschikbaar, echter ook met het gebruik hiervan overlijdt 30-50% van de mensen met CMV longontsteking. Ook CMV reactivaties kunnen in het bloed aangetoond worden middels diverse technieken. Veel centra maken hier gebruik van en starten met antivirale therapie op het moment dat het CMV in bloed aantoonbaar is (pre-emptieve behandeling). Dit heeft geresulteerd in een enorme afname van het optreden van CMV ziekte en overlijden hieraan. Sommige centra kiezen voor een andere optie, namelijk het constante gebruik van antivirale medicatie tot een zeker tijdsstip (profylactische behandeling). In tegenstelling tot het EBV virus, geeft het CMV virus bij SCT patiënten zelden primo- of re-infecties, mits de juiste voorzorgsmaatregelen genomen worden. Dus alleen de CMV-seropositieve patiënten, die

patiënten die in het verleden een CMV infectie hebben doorgemaakt en waarbij het virus nu latent aanwezig is, hebben kans op een reactivatie. Ondanks de pre-emptieve behandelstrategie beschouwen veel transplantatie centra CMV-seropositiviteit nog steeds als een negatieve risicofactor voor het overleven na een SCT.

In **Hoofdstuk 8** wordt het effect van de CMV serostatus op de overleving en transplantatie gerelateerde mortaliteit geanalyseerd in MRD patiënten (n=205) en MUD patiënten (n=48) die tussen Juli 1990 en Mei 2000 in het UMCU werden getransplanteerd. Daar CMV overgedragen kan worden via witte bloedcellen (leucocyten), kregen alle patiënten leucocytenarme bloedprodukten. Er werd een pre-emptieve behandelstrategie gevoerd bij CMV reactivaties. Tevens werd een kortdurende profylactische antivirale behandeling gegeven aan CMV-seropositieve patiënten met een acute GVHD die met prednison in hoge dosering behandeld moesten worden. Deze patiëntengroep heeft een vergroot risico op CMV reactivaties. Bij de MRD patiënten bleek CMV-seropositiviteit geen negatieve risicofactor voor de overleving post-transplantatie te zijn. Bij MUD patiënten, daarentegen, was de overleving van CMV-seropositieve patiënten sterk verlaagd ten opzichte van CMV-seronegatieve patiënten, grotendeels veroorzaakt door fatale infecties.

Deze bevinding in MUD patiënten is ook door anderen gerapporteerd. Echter, het merendeel van de studies is verricht onder patiënten die voor het jaar 1999 behandeld werden. In **Hoofdstuk 9** wordt onderzocht of de overleving van MUD patiënten die getransplanteerd zijn sinds 1999 nog steeds beïnvloed wordt door de CMV serostatus. In ons transplantatiecentrum werden in 1999 twee belangrijke wijzigingen in het transplantatie regime doorgevoerd. Ten eerste werd de ATG dosering verlaagd van 20 mg/kg naar 8 mg/kg. Ten tweede werd een veel gevoeliger techniek voor HLA typering ingevoerd (sequence based typing van HLA-DRB1). Beide veranderingen zouden een beter immuunherstel in de post-transplantatie fase tot gevolg kunnen hebben. De dosis verlaging van ATG door een direct effect op het aantal T cellen en de verbeterde weefseltypering middels een betere donor keuze door een verlaagde incidentie van GVHD. Gezien het immuunsuppressieve effect van (latente) CMV infecties, zouden deze wijzigingen de overleving van CMV-seropositieve MUD patiënten positief kunnen beïnvloeden. In totaal 80 patiënten ontvingen een gedeeltelijk T cel gedepleteerd transplantaat, 36 voor 1999 en 44 na 1999. Bij CMV-seropositieve patiënten die voor 1999 getransplanteerd werden, werd een sterk verlaagde overleving gezien vergeleken met CMV-seronegatieve patiënten (zie ook Hoofdstuk 8). Bij patiënten die na 1999 getrans-

planteerd werden, echter, was geen verschil waarneembaar tussen de twee groepen wat betreft overleving en transplantatie gerelateerde mortaliteit.
Ter afsluiting van het onderdeel CMV infecties wordt in **Hoofdstuk 10** een overzicht gegeven van de preventie van CMV ziekte bij ontvangers van allogene SCT. De twee eerder genoemde antivirale strategieën (pre-emptief en profylactisch) worden besproken en aanbevelingen met betrekking tot de preventie van CMV ziekte worden gegeven. Tevens worden enige andere aspecten van de preventie van CMV ziekte samengevat: 1) beschikbare methoden voor de monitoring van CMV reactivaties, 2) monitoring van CMV-specifieke T cel immuniteit, 3) de waarde van verschillende antivirale middelen en 4) van immunotherapie als profylactische of pre-emptieve behandeling van CMV reactivaties/CMV ziekte.

Concluderend kan gesteld worden dat ontvangers van allogene SCT ernstig immuungecompromiteerd zijn gedurende tenminste de eerste 6 maanden post-transplantatie. Dit geldt vooral voor ontvangers van transplantaten van onverwante donoren. Echter, enkele belangrijke veranderingen in het transplantatie programma hebben de transplantatie gerelateerde mortaliteit onder MUD patiënten in sterke mate doen afnemen. B cel depletie van de transplantaten (naast de T cel depletie), de dosis verlaging van ATG en de verbeterde weefseltypering (sequence based typing van HLA-DRB1) hebben allemaal bijgedragen aan deze verminderde mortaliteit. De dosis verlaging van ATG en de sequence based typing van HLA-DRB1 hebben mogelijk geleid tot een minder ernstige immuundeficiëntie, waardoor het effect van de CMV-seropositiviteit niet meer klinisch relevant is. In MRD patiënten bleek dat met het toepassen van een pre-emptieve behandelstrategie van CMV reactivaties, CMV-seropositiviteit geen risicofactor is voor slechtere overleving danwel toegenomen transplantatie gerelateerde mortaliteit. De optimale ATG dosering is niet bekend, echter, gezien de immuunsuppressieve en direct toxische effecten van ATG moet de laagst mogelijke dosis gebruikt worden. Bij SCT met (gedeeltelijk) T cel gedepleteerde transplantaten van onverwante donoren, waarbij post-transplantatie slechts een beperkte immuunsuppressie toegepast wordt bestaande uit cyclosporine, wordt een totale dosis van 6-8 mg/kg aanbevolen. Een verdere dosisreductie heeft geleid tot een sterk verhoogde incidentie van ernstige (graad III-IV) acute GVHD.
In de toekomst moet gewerkt worden aan een verdere verbetering van het immunologische herstel tijdens de post-transplantatie fase. Nieuwe methoden moeten ontwikkeld en geïmplementeerd worden betreffende de monitoring, preventie en behandeling van opportunisti-

sche infecties. Voor de specifieke preventie van EBV-LPD is het noodzakelijk meer inzicht te verkrijgen in de origine van de EBV infecties in de post-transplantatie fase.

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Publications

1 **Meijer E**, Hoekstra JBL. De keerzijde van scherpe diabetesregulatie. Ned Tijdschr Geneeskd 1992; 35: 1689-1691.

2 **Meijer E**, Hoekstra JBL, Erkelens DW. Hypoglycemia Unawareness. Eur J Med 1994; 23: 623-627.

3 **Meijer E**, Hoekstra JBL. Severe hypoglycaemia during intensive insulin therapy: a reversibel phenomenon? Editorial. Neth J Med 1995; 46: 167-170.

4 **Meijer E**, Westerveld HE, de Ruyter FC, van Greevenbroek MMJ, Rienks R, van Rijn HJM, Erkelens DW, de Bruin TWA. Abnormal postprandial apolipoprotein B48 and triglyceride responses in normolipidemic women with greater than 70 per cent stenotic coronary artery disease: a case-control study. Atherosclerosis 1996; 124: 221-235.

5 Westerveld HE, **Meijer E**, Erkelens DW, de Bruin TWA. Postprandial reduction in high-density lipoprotein cholesterol concentrations in postmenopausal women: improvement by 17ß-estradiol. Metabolism 1996; 45: 827-832.

6 Westerveld HE, **Meijer E**, de Bruin TWA, Erkelens DW. Estrogens and postprandial lipid metabolism. Biochem Soc Trans 1997; 25: 45-49.

7 Cabezas MC, de Bruin TWA, Westerveld HE, **Meijer E**, Erkelens DW. Delayed chylomicron remnant clearance in subjects with heterozygous familial hypercholesterolemia. J Int Med 1998; 244: 299-307.

8 van Esser JWJ, Niesters HGM, Thijsen SFT, **Meijer E**, Osterhaus ADME, Wolthers KC, Boucher ChAB, Gratama JW, Budel LM, van der Holt B, van Loon AM. Lowenberg B, Verdonck LF, Cornelissen JJ. Molecular quantitation of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus associated lymphoproliferative disease after allogeneic stem cell transplantation. Br J Haematol 2001; 113: 814-821.

9. van Esser JWJ, van der Holt B, **Meijer E**, Niesters HGM, Trenschel R, Thijsen SFT, van Loon AM, Frassoni F, Bacigalupo A, Schaefer UW, Osterhaus ADME, Gratama JW, Lowenberg B, Verdonck LF, Cornelissen JJ. Epstein-Barr virus reactivation is a very frequent event after allogeneic stem cell transplantation and quantitatively predicts for EBV-lymphoproliferative disease following T-cell depleted stem cell transplantation. Blood 2001; 98: 972-978.

10 van Esser JWJ, Niesters HGM, van der Holt B, **Meijer E**, Osterhaus ADME, Gratama JW, van Loon AM, Verdonck LF, Lowenberg B, Cornelissen JJ. Prevention of Epstein-Barr virus-lymphoproliferative disease by molecular monitoring and pre-emptive rituximab in high-risk patients after allogeneic stem cell transplantation. Blood 2002; 99: 4364-4369.

11 **Meijer E**, Slaper-Cortenbach ICM, Thijsen SFT, Dekker AW, Verdonck LF. Increased incidence of EBV-associated lymphoproliferative disorder after bone marrow transplantation due to a change of T cell depletion technique. Bone Marrow Transplant 2002; 29: 335-339.

12 **Meijer E**, Dekker AW, Rozenberg-Arska M, Weersink AYL, Verdonck LF. Impact of Cytomegalovirus-seropositivity on mortality in recipients of T-cell depleted bone marrow transplants: contrasting results between recipients of related and unrelated grafts. Clin Inf Dis 2002; 35: 703-712.

13 **Meijer E**, Dekker AW, Weersink AYL, Rozenberg-Arska M, Verdonck LF. Prevention and treatment of Epstein-Barr virus associated lymphoproliferative disorders in recipients of bone marrow and solid organ transplants: a review. Br J Haematol 2002; 119: 596-607.

14 **Meijer E**, Dekker AW, Verdonck LF. Influence of Antithymocyteglobulin dose on outcome in CMV-seropositive recipients of partially T cell depleted stem cell grafts from matched unrelated donors. Br J Haematol 2003; 121: 473-476.

15 **Meijer E**, Bloem AC, Dekker AW, Verdonck LF. Effect of Antithymocyteglobulin on quantitative immune recovery and graft-versus-host disease after partially T cell depleted bone marrow transplantation: a comparison between recipients of matched related and matched unrelated donor grafts. Transplantation 2003; 75: 1910-1913.

16 van Kooij B, Thijsen SFT, **Meijer E**, Niesters HGM, van Esser JWJ, Cornelissen JJ, Verdonck LF, van Loon AM. Sequence analysis of EBV DNA isolated from mouth washings and PBMCs of healthy individuals and blood of EBV-LPD patients. J Clin Virol 2003; in press.

17 **Meijer E**, Boland GJ, Verdonck LF. Prevention of CMV disease in recipients of allogeneic stem cell transplants: a review. Clinical Microbiology Reviews; in press.

18 **Meijer E**, Cornelissen JJ, Lowenberg B, Verdonck LF. Antithymocyteglobulin as prophylaxis of graft failure and graft-versus-host disease in recipients of partially T cell depleted grafts from matched unrelated donors: a dose finding study. Experimental Hematology; in press for publication in November 2003.

19 **Meijer E**, Spijkers S, Moschatsis S, Boland GJ, Thijsen SFT, van Loon AM, Verdonck LF. EBV reactivations in recipients of stem cell transplants are frequently caused by re-infections with exogenous EBV strains. Submitted.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 7-7-1966 te Amerongen. In 1984 startte zij met de studie geneeskunde aan de Universiteit Utrecht. Na het behalen van het artsexamen in 1993 werd zij aangenomen voor de opleiding algemene Interne Geneeskunde in het UMCU. Voorafgaand aan de opleiding deed zij onderzoek naar de chylomicronen remnant klaring bij normolipidemische vrouwen met een coronair stenose, onder leiding van Prof. dr. T.W.A. de Bruin in het UMCU. In oktober 1994 werd de opleiding tot internist aangevangen in het Ziekenhuiscentrum Apeldoorn (opleider Dr. D.W. van Toorn) en in september 1997 voortgezet in het UMCU (opleider Prof. dr. D.W. Erkelens). Sinds september 2000 is zij werkzaam bij de afdeling Hematologie binnen het UMCU, eerst als fellow (opleider Prof. dr. A.H. Hagenbeek) en sinds december 2002 als staflid.