GRAFT-VERSUS-LEUKEMIA: APPLICABLE IN THE TREATMENT OF LEUKEMIA WITH BONE MARROW TRANSPLANTATION?

PRECLINICAL STUDIES IN RAT MODELS RELEVANT FOR HUMAN ACUTE LEUKEMIA

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Transplantaat-tegen-leukemie: toepasbaar in de behandeling van leukemie met beenmerg transplantatie?

Pre-klinische studies in ratte-modellen relevant voor humane acute leukemie

PROEFSCHRIFT

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CHAPTER 1

GENERAL INTRODUCTION

PREFACE

'On the one hand, the dose of X-rays which is sufficiently lethal to normal cells of the bone marrow and lymphatic tissues to cause death of the animal might well be completely lethal to leukaemic cells.....On the other hand, if the dose of X-rays sufficient to kill the animal is not 100% lethal to leukaemic cells, the malignant condition would (following isologous bone marrow transplantation) recur by growth from the surviving cells, since neither host nor graft has the ability to resist; but, if homologous bone marrow from a different strain of mouse were given, the colonizing cells might retain the capacity of the donor to destroy by the reaction of immunity these residual leukaemic cells -and perhaps the host.'

Barnes et al. (1956)[1]

1.1 BONE MARROW TRANSPLANTATION

Bone marrow transplantation (BMT) is the intravenous infusion of hemopoietic stem cells and progenitor cells with the aim to re-establish normal marrow function and immunity in a recipient with damaged or defective bone marrow (BM).[2] Although some have traced the origin of this procedure to the end of the past century, when patients were given bone marrow orally as treatment for hematologic disorders,[3] a more realistic starting point is a 1939 report of a patient who received intravenously 18 ml of marrow from his brother as a treatment for aplastic anemia.[4] The beginning of modern BMT can be traced to experiments showing that rodents could be protected against lethal hemopoietic injury by the intravenous infusion of BM.[5] The extensive studies on BMT in animal models, the subsequent identification of transplantation antigens and the development of techniques for freezing and thawing hemopoietic cells laid the groundwork for the difficult and time-consuming clinical trials that have brought BMT to its present, albeit imperfect, state. Few areas in medicine illustrate so well the development of a new treatment through the close interaction between advances in laboratory and in clinical science.

In the late 1960s, the first successful allogeneic bone marrow transplantations have been performed which led to the gradual acceptance of this therapy.[6,7] Autologous BMT was first successfully employed to cure patients with lymphoma in the late 1970s,[8] and its use became widespread in the 1980s.

Table 1.1 Malignant diseases treated with bone marrow transplantation*

Acute leukemia	Acute myelocytic leukemia (AML)
	Acute lymphocytic leukemia (ALL)
Chronic leukemia	Chronic myelocytic leukemia (CML)
	Chronic lymphocytic leukemia (CLL)
Myelodysplastic syndrome (MDS)	
Multiple myeloma	
Lymphomas	Hodgkin's disease
	Non-Hodgkin's lymphoma
Solid tumors	Neuroblastoma
	Germ cell tumors
	Breast carcinoma

* adapted from reference[9]

BMT is now widely accepted as a method of treatment for patients with life-threatening diseases such as leukemias, aplastic anemia, severe immunodeficiencies and inborn errors of metabolism.[10] The success of BMT for malignancy (Table 1.1) is dependent on the ability of the cytotoxic drugs and/or radiation used to eliminate the malignant cells in the patient and on the ability of the donor cells to mediate immunologic reactions termed graft-versus-tumor effects.[9] The success of BMT to treat immune deficiencies or genetic diseases (Table 1.2) is

based on the replacement of defective host hemopoietic cells with normal hemopoietic stem cells capable of manufacturing the deficient cellular product or cell lineage.[9]

Table 1.2 Nonmalignant diseases treated with bone marrow transplantation*

Aplastic anemia / PNH	
Immunologic disorders	Severe combined immunodeficiency disease (SCID)
	Wiskott-Aldrich syndrome
	Chediak-Higashi syndrome
Metabolic disorders	Gaucher's disease
	Osteopetrosis
Sickle cell anemia	
Thalassemia	

* adapted from reference[9]

1.1.1 Allogeneic and Syngeneic BMT

Allogeneic BMT involves the transfer of marrow from one person to another. In the special case of a donor and a recipient who are genetically identical, that is identical twins, the correct term is syngeneic or isologous transplantation.

For patients without a twin, a major histocompatibility complex (MHC)-matched sibling donor is the best choice for an allogeneic BMT.

An individual's immunologic identity is expressed in cell-surface proteins encoded by the MHC. MHC proteins, termed HLA (human leukocyte antigens) in humans, are critical to the proper function of the immune system. Antigen presentation in the (peptide-presenting) groove of the MHC-complex enables effector cells to identify and destroy foreign invaders while preserving normal, healthy tissues.[11] MHC antigens and non-MHC antigens (also termed minor histocompatibility antigens) expressed on transferred cells can lead to graft rejection by immunocompetent cells of the recipient.[11,12] Since the genes for the MHC antigens in humans are found on chromosome 6, the MHC type follows the rules of simple mendelian genetics. That is, any two siblings have a 25% chance of sharing the same MHC type, except for a 1% chance of crossover (a switch in genetic material between chromosomes during meiosis).[13] These rules form the basis for MHC family typing. Because of the relatively small average family size in the Western countries, only 30-40% of the patients have an MHC identical sibling.

For patients who may benefit from an allogeneic BMT but lack an HLA-matched sibling donor, there are two possible solutions. One is to identify an unrelated but closely HLA-matched person willing to donate BM, and the other is to use marrow from a related donor who is less than perfectly matched. An early report of the use of an HLA-matched, unrelated marrow donor for transplantation in a child with acute leukemia demonstrated that this approach can be successful.[14] Subsequent reports have shown that the results improve when the degree of MHC-compatibility of the unrelated donor increases.[15] Concomitantly it was

shown that the removal of T-cells, which are the major mediators of graft-versus-host disease (GVHD),[16] from the graft may ameliorate the severe manifestations of this disease often seen in patients receiving transplants from unrelated donors.[17] The extreme large number of possible MHC phenotypes makes the search for an unrelated donor difficult. International effort has resulted in a worldwide registry of BM donors which have been HLA-typed, greatly increasing chances to find a suitable HLA-matched unrelated donor. It has been estimated that a registry of 200,000 potential donors of European ancestry would provide a 40-50% chance of finding an HLA-matched donor.[18-20]

An alternative approach is to identify a related person who shares some but not all of the patient's MHC antigens.[21] Successful allogeneic transplantation can be performed with marrow from such donors, although the risk of graft rejection and GVHD appears to be increased compared with MHC-matched BMT.[21]

Once a donor has been identified, the next step is to use high doses of chemotherapy (mostly alkylating agents) and/or total body irradiation to accomplish three goals: provide a sufficient degree of immunosuppression to avoid destruction/rejection of the allograft by residual, immunologically active cells of the host; destroy malignant cells; and provide space for the new marrow to lodge and to proliferate. The combinations of the high dose chemo-radiotherapy regimens used are not always fully myeloablative. With T-cell depleted allogeneic transplants, some patients are eventually found to have mixed chimerism, indicating the survival of host hemopoietic stem cells.[22-24] Although the expected reduction in GVHD has been achieved with T-cell depletion, increased rates of graft failure and leukemia relapse in these patients led to the recognition that allogeneic T-cells, in addition to causing GVHD, also mediate several beneficial effects.[25,26] The increased risk of take failure after T-cell depletion of allogeneic marrow grafts was recognized earlier by van Bekkum and co-workers.[27,28] Applying BMT in dogs and monkeys, they have shown that an intensified pre-BMT conditioning regimen, e.g. an increased (two-fraction) dose of total body irradiation, allows reproducible engraftment of T-cell depleted allogeneic marrow.[27,29] At the same time it appeared that leukemia relapse rates were lower with this intensified conditioning regimen.[30] Thus approaches to control GVHD should be chosen such that increased relapse and engraftment/take failure rates are avoided.

1.1.2 Autologous BMT

Autologous BMT involves the use of the patient's own marrow to re-establish hemopoietic cell function after the administration of high dose chemo-radiotherapy. Autologous BMT differs from allogeneic BMT in various ways (Table 1.3)[13]. Unlike allogeneic transplantation, autologous transplantation can be performed in older patients with relative safety, probably because there is no risk of GVHD as a complication. Until it becomes practical for commonly occurring genetic diseases to remove hemopoietic stem cells from a patient, to replace a

defective gene or to add a missing one, and then transplant the altered cells (gene therapy), autologous BMT is not likely to become a common treatment for genetic disorders. It has been suggested, however, that autologous transplantation may be an effective therapy for certain immune disorders because of an immunomodulating effect.[31]

Table 1.3 Comparison of certain features of allogeneic and autologous bone marrow transplantation*

FEATURE	ALLOGENEIC BMT	AUTOLOGOUS BMT
Age limit for candidates (yr)	50-55	60-70
Primary problem in obtaining transplant	Finding a closely HLA-matched sibling or unrelated donor	Collecting a sufficient number of he-mopoietic progenitor cells uncontami-nated by tumor cells
Most serious complication	Graft-versus-host disease	Relapse of original disease
Antitumor effect of infused cells	Proven and suspected in a number of cases	Not proven, but a possible 'syngeneic anti-tumor effect' (CsA-induced)
Use in nonmalignant disorders	Potentially curative in both genetic and immunologic dis-orders	Not useful until gene therapy becomes practical

* adapted from reference.[13] CsA=cyclosporine

A primary concern with autologous BMT in the treatment of malignancies is to make sure the graft contains no viable tumor cells. Numerous methods, including *in vitro* treatment with chemotherapeutic agents or monoclonal antibodies (MCA's) plus complement, have been developed to remove contaminating tumor cells from the graft[32-36] or to concentrate the hemopoietic stem cells,[37,38] referred to as 'marrow purging' and 'positive selection', respectively. Retrospective analyses have suggested that purging leads to a reduced rate of relapse in patients with certain subtypes of acute myelocytic leukemia.[32] However,tumor cells can be cultured from histologically negative BM in some patients with lymphoma, leukemia or breast cancer,[39-42] and such patients have a higher rate of relapse than those with negative cultures.

Experimental and clinical data have indicated that residual tumor cells in the marrow graft re-infused after the conditioning regimen do contribute to tumor relapse,[43-46] but that the majority of relapsing cells seem to originate from the patient, having survived the remission induction treatment and the pre-BMT conditioning regimen. Today, purging is not commonly used in the majority of patients undergoing autologous transplantation.[13]

1.2 GRAFT-VERSUS-HOST DISEASE

The post BMT period can be complicated by various life-threatening problems (Table 1.4)[9,13] Today, GVHD remains one of the major causes of morbidity and mortality after allogeneic

BMT.[47-49] The first description of GVHD was published in 1954,[50] its nature being unknown. Initially this syndrome was detected in experimental animals and was called secondary disease (opposed to the irradiation induced primary disease aplasia) or runting disease. After total body irradiation (TBI) and allogeneic marrow transplantation in contrast to syngeneic BMT, animals developed fatal diarrhea and skin lesions.[51] Other symptoms of what was later referred to as graft-versus-host disease, were varying degrees of hypoplasia of the lymphatic system, focal necrosis of liver cells and growth retardation. From this experience in animals it was stated that the essential requirements for induction of GVHD were: a) the marrow graft must contain immunocompetent cells; b) the host must express tissue antigens that are lacking in the marrow donor; and c) the host must be incapable of rejecting the marrow graft.[52]

Table 1.4 Possible complications after BMT*

Graft-versus-host disease	causing both severe morbidity and mortality	
Host-versus-graft reaction	could lead to a take failure or graft rejection	
Veno-occlusive disease (VOD) of the liver		
Immunological incompetence	infections	- bacterial
		- fungal
		- viral

* adapted from reference.[13]

In man, as well as in a variety of animal species, lymphoblasts, small and large granular lymphocytes appear in the skin, gut and liver of allogeneic but not syngeneic transplanted animals.[51] The main effector cells of GVHD are thought to be cytotoxic T-cells and early after BMT the invading lymphocytes are mainly CD4-positive (CD4^{+}), although in a later stage CD8^{+} cells predominate.[53] However, acute GVHD may also occur in the absence of detectable cytotoxic T-cells in the affected tissues.[53] In experimental animals and in humans it is well established that the removal of T-cells from the donor marrow may completely prevent acute GVHD.[16,54-57]

GVHD can develop when donor and host are mismatched for MHC antigens, but also when they are fully MHC compatible. It has been suggested that disparity of minor histocompatibility (mH) antigens elicit the GVH reaction in the latter case.[12,58] In mice, in certain donor-recipient combinations mH antigens appeared to be able to induce severe GVHD.[59] It is generally accepted that the incidence and severity of GVHD increases as the difference between donor and recipient is greater as regards both major and minor histocompatibility antigens.[21,60,61]

It has been reported that a GVHD-like syndrome can even occur after syngeneic BMT.[62] In this event, syngeneic GVHD might be the result of inappropriate recognition of self-antigens [11,51,63,64] Studies in rats suggest that the essential component for the induction of syngeneic GVHD is the inhibition of clonal deletion of autoreactive T lymphocytes in the

thymus by cyclosporine A (CsA).[63] It can be questioned whether the term GVHD is correctly used. It has been proposed to use 'autologous BMT-associated immune disease' rather than GVHD.[64]

As regards allogeneic BMT, two distinct syndromes can be distinguished: acute and chronic GVHD. In humans, acute GVHD may appear from 1 week up to 3 months after allogeneic BMT, whereas chronic GVHD by definition develops from 3 months after transplantation.[49] Acute GVHD is graded from I to IV.[65] When GVHD is absent it is called grade 0. Mild GVHD (grade I) is a local skin rash (starting in the palms, soles or face), which in some cases disappears without therapy; grade II or moderate GVHD can be a skin rash affecting most of the body, or a skin rash in association with gut (diarrhea) or liver symptoms; grade III is severe GVHD involving skin, gut and liver and grade IV is life-threatening. The reason why the skin, liver and the intestinal tract are the principal target organs of acute GVHD[66] remains unclear. It has been postulated that primitive cell surface antigens on undifferentiated epithelial cells are the targets.[64] Others hypothesized that these organs are all barriers to microorganisms and that these barriers are damaged by the conditioning regimen. Endotoxins released by the gram-negative organisms present, may induce local cytokine release and subsequently increased expression of MHC antigens and adhesion molecules.[47] The manifestations of chronic GVHD include skin disease, keratoconjunctivitis, generalized sicca syndrome, severe oral mucositis, malabsorption, wasting, liver disease and pulmonary insufficiency. Severe chronic GVHD often leads to a syndrome which has similarities with some autoimmune diseases.[66,67] A serious problem with chronic GVHD is decreased immune function leading to bacterial and viral infections.[68,69] Incidence and severity of acute GVHD is closely related to the number of mature T-cells in the graft.[70] It has been suggested that chronic GVHD in humans, usually following acute GVHD, may be caused by low numbers of long lived lymphocytes of donor origin that have been sensitized to unknown antigens of the host.[71]

It has to be emphasized that the diagnosis of GVHD must be supported by proof of engraftment of donor cells as well as by histological confirmation of the presence of characteristic lesions. Clinical observations alone are not enough, since they can be easily confused with symptoms caused by a variety of infections which occur as a result of the severe immunosuppression or by toxicity from the conditioning regimen which complicates the post BMT course in many cases.[72]

1.2.1 GVHD and lymphocytes essential for allorecognition

The antigen-binding site of most major histocompatibility complex (MHC) class I and II antigens is occupied by peptides that are derived from the proteolysis of endogenous proteins. Together with MHC proteins, these 'self'-peptides define our immunological self and shape the repertoire of both T cells and NK cells that recognize 'nonself'. Self-peptide expression

allows the recognition mechanism of T and NK cells to check the integrity of the cell genome. The distinction between self and nonself permits the rejection of nonself or neoplastic cells. This objective is particularly pertinent to the area of BMT.[73]

1.2.1.a T-cells

Before the discrepancy between T and B-cells was established, it was clear that GVHD was induced by lymphocytes.[70] Subsequently, it has been clearly shown that, more specifically, T-cells play a major role in GVHD[11,55] Initially, T-cells respond to nonself antigens only when presented by antigen-presenting cells (APC), such as dendritic cells and macrophages. The recognition of antigens presented by APC occurs in the context of class I and/or class II MHC antigens, and is referred to as MHC presentation. Foreign antigens are processed intracellularly to small peptides which are bound in the groove of MHC molecules. These MHC-peptide complexes can be recognized by non-activated T-cells.[74] Once evoked, the activated T-cells no longer need APC to perceive these peptides.

Class I and class II molecules have similarities in their structure and function, but differ in their fine structure and in their expression on various cell types. Class I molecules are constitutively expressed on almost all nucleated cells,[75] whereas class II molecules are expressed only on specific cell types such as B-cells, macrophages, monocytes, Langerhans cells and dendritic cells. They can be induced on T-cells, endothelial cells, renal tubular cells, pancreatic and thyroid cells as well as on several other cell types by certain cytokines, especially interferon-gamma (IFNγ).[76,77]

T lymphocytes can be divided into two subclasses, based on their cell surface antigen expression: a CD4$^+$ and a CD8$^+$ T-cell subset. CD4$^+$ T-cells are mainly class II restricted, whereas CD8$^+$ T-cells are mostly MHC class I restricted. Although previously it was accepted that the phenotype of a T-cell correlated with its function, it has now become clear that phenotype and function are not strictly related.[78] In general, helper functions are performed by CD4$^+$ T-cells and cytotoxic/suppressor functions by CD8$^+$ T-cells. But sometimes CD4$^+$ and CD8$^+$ T-cells exert capacities normally ascribed to CD8$^+$ and CD4$^+$ T-cells, respectively.

Based on their cytokine profile T-cells can be further subdivided. In this context, two CD4$^+$ T helper (Th) cell subsets can be recognized: Th1 and Th2. Th1 cells produce interleukin-2 (IL-2) and IFNγ, whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10. Recently, it has been suggested that these Th subsets have a common precursor (Thp), which secretes IL-2 and differentiates into a Th0 cell, which secretes IL-2 and IFNγ as well as IL-4 and IL-5.[79] The cytokine profile of CD8$^+$ T-cells resembles that of the Th1 subset, but their capacity to produce IL-2 is limited.[80] Although these data are obtained from *in vitro* studies, evidence is increasing that certain strong immune responses in mice and humans are preferentially mediated by Th1 or Th2 cells.[81] Preferential upregulation of Th1 and downregulation of Th2 cells has been reported for cytokines such as IL-1, IL-12 and TNF.[82]

Th1 and Th2 cells also crossregulate each other: Th2 cells inhibit the cytokine synthesis of Th1 cells by IL-10 (originally named cytokine synthesis inhibitory factor). On the other hand, Th1 cells inhibit the proliferation of Th2 cells through the production of IFNγ.[83,84] Improving our knowledge of the cytokine network in relation to T-cell activity may reveal new therapeutical approaches to minimize the devastating effects of GVHD.

T lymphocytes use the T-cell receptor (TCR), which consists of two subunits, the α and β chain, to recognize antigens. Other cell surface molecules contribute to the interaction between T-cells and target/stimulator cells: co-receptors such as CD4 and CD8, accessory molecules like CD28, CD44 and CD45, lymphocyte function associated antigens such as LFA-1 (CD11a/CD18), LFA-2 (CD2), LFA-3 (CD58)[76,85-87] and the adhesion molecules ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50) and ELAM-1. These molecules may intensify the binding/contact between T-cells and APC. Some of these molecules may also provide co-stimulatory signals for T-cell activation.

Upon contact of T-cells with multivalent (allo)antigens, TCR signal transduction occurs via the CD3 complex, which consists of transmembrane polypeptide chains that are associated with the TCR. This leads to intracellular activation and a cascade of events that result in the transcription of genes for cytokine production and their receptors. Among the first to be activated are the genes for IL-2 and the IL-2 receptor (IL-2R). IL-2 production is essential for T-cell proliferation. Slightly later, IFNγ, IL-4, IL-5 and IL-6 production starts. As a consequence, T lymphocytes differentiate and thereby gain specialized functions and/or cytokine production profiles.[76]

Both CD4+ and CD8+ T lymphocytes have been shown to play a role in GVHD in mice. It appears that the T-cell subset involved was dependent on the MHC or mH antigen differences between donor and recipient. Highly purified CD4+ T-cells were able to cause lethal GVHD in a class II incompatible mouse strain combination, whereas purified CD8+ T-cells caused GVHD in a class I disparate strain combination.[88] In mH antigen disparate strain combinations, either the CD4+ or the CD8+ T-cell subset can play a dominant role. In some mH antigen disparate strain combinations both subsets appear to be equally involved.[55]

1.2.1.b NK cells

Natural killer cells are generally defined as lymphocytes that spontaneously mediate the non MHC-restricted or MHC-independent lysis of certain transformed or virally infected cells.[89,90] Strictly speaking, NK cells are CD3-negative large granular lymphocytes, which express certain surface markers such as CD16 and CD56.[91] A variable percentage (30-80%) of NK cells also express CD57.[92] NK cells do not express the T-cell receptor or the T-cell receptor associated protein complex, CD3.[93] However, they do express a signal-transducing subunit (CD3ζ) of the CD3 complex on their surface which is associated with their Fc receptor for immunoglobulins (CD16)[94,95]

Murine NK cells generally do not have T-cell markers such as CD4 and CD8,[96,97] or B-lymphocyte markers and have therefore also been called 'null' cells.[96] When activated however, a percentage of murine NK cells express Thy-1, a marker commonly associated with T-cells.[98] A subpopulation of human NK cells do express CD8.[97]

NK cells are also noted for their ability to mediate antibody-dependent cell mediated cytotoxicity (ADCC) through their expression of the Fc receptor (CD16).[99] Murine NK cells express the glycolipid asialo GM1 (ASGM1), which is also found on some macrophages and activated T-cells. Therefore, ASGM1 is not considered to be NK-specific.[96] Unlike resting T-cells, which require both the antigen-MHC complex and cytokines (IL-1/IL-2) for proliferation, NK cells are activated and proliferate directly in response to IL-2, both *in vitro* and *in vivo*.[100,101] In addition, NK cells constitutively express the p75 medium affinity IL-2R.[9297] Moreover, NK cells are activated in response to interferons.[102] When activated, NK cells exhibit a broad lytic spectrum and undergo a morphologic change, i.e. an increase in intracellular granules (containing perforins, a C9 homologue).[93,97] These granules are believed to play a role in the cytolytic process; activated NK cells are known as large granular lymphocytes (LGL). Recently, a novel cytokine called NK cell stimulatory factor (NKSF) or IL-12, produced by peripheral blood mononuclear cells (monocytes and lymphoctes), was described and shown to augment human NK activity, and to have a role in immune and inflammatory cell responses.[103,104]

NK cells produce numerous cytokines, many of which may be mediators for the *in vivo* effects of NK cells. Human NK cells produce granulocyte-macrophage colony-stimulatory factor (GM-CSF),[105] IFNγ,[105] and tumor necrosis factor alpha (TNFα).[106] In one of these studies,[105] IL-2 activated peripheral blood lymphocytes were used, which do not consist solely of NK cells. Purified murine NK cells have been demonstrated to produce GM-CSF, INFγ, G-CSF, IL-1, and transforming growth factor β (TGFβ).[107]

NK cells exert a variety of immunological functions and are considered to be an essential component of 'natural immunity', the immediate immune response system which lacks the capacity of antigen-specific recognition. Unlike T-cells, NK cells have no 'memory' and act spontaneously at first contact to lyse tumor cells both *in vitro* and *in vivo*.[108] Furthermore, NK depletion promotes metastases in experimental animals.[109] This tumor cell-killing capability led to the hypothesis that NK cells play a critical role in tumor surveillance.[110] Abundant *in vivo* data suggest that NK cell do provide significant antitumor effects.[93,97] To date, the mechanism by which NK cells recognize target cells is unknown, although in some models, an inverse correlation has been found between tumor cell MHC expression and their susceptibility to NK lysis.[111]

Mouse and human lymphokine-activated killer (LAK) cells, which comprise activated T-cells and NK cells, have been shown to suppress alloreactivity;[112] this result suggests that LAK cells can suppress the generation of GVHD *in vivo*.[113] Studies on BMT using mice, in

which NK cells were transferred with an allogeneic graft, suggested that donor NK cells suppressed GVHD.[114] Because activated NK cells produce immunosuppressive factors such as TGFβ,[107] the mechanism behind the lack of GVHD in the recipients may be the direct suppression of alloreactive donor T-cells by donor NK cells.

Though these studies suggest that activated donor NK cells may prevent GVHD, controversially, NK cells may be potential effector cells capable of mediating this disease.[115-117] However, many of these studies have used ASGM1 as an NK cell marker. This antigen is also expressed on some activated T-cells.[97] Additionally, NK cells do not recognize classic MHC differences and therefore are unlikely to be the primary mediators of GVHD. Yet, the presence of NK cells in the tissues affected in GVHD[118-121] indicate that NK cells may play a supportive role during the end stage of the disease. While donor T-cells are clearly involved in the primary cause of GVHD in allogeneic BMT, it may be that they can recruit donor NK cells participating in the damage through the production of inflammatory cytokines such as IL-1 and TNFα. The role of NK cells in mediating the pathology of GVHD is still to be resolved.

1.2.2 Acute GVHD as a three step process

It has been well established in experimental and clinical studies that acute GVHD is caused by T-cells in the donor graft.[88,122-126] Efforts to control GVHD have therefore been directed against the donor derived T-cell. Still, the precise mechanisms by which host tissues are damaged remain unclear. Recently, several convergent lines of evidence have suggested that inflammatory cytokines act as mediators of acute GVHD.[127,128] Most of the clinical manifestations of GVHD may in fact be due to significantly increased levels of certain cytokines produced by T cells and other inflammatory cells. Data are accumulating that mechanisms other than direct T-cell mediated cytotoxicity may generate tissue damage associated with GVHD. Recently it has been hypothesized and described by Antin and Ferrara that the activation of T-cells is just one critical step in a three step process of GVHD pathophysiology (Figure 1.1).[47,129] Their hypothesis is described below.

1.2.2.a Step one: The role of host tissue in the development of acute GVHD

First, the expression of cell-surface histocompatibility proteins and adhesion molecules on host target tissues such as skin, intestinal mucosa and liver is increased by inflammatory cytokines,[130] released by endothelial and epithelial cells as a result of conditioning regimen toxicity, infections and possibly the underlying disease.[131-133] For example, endotoxin from the gut may leak through the intestinal wall and stimulate endothelial cells and/or macrophages to produce cytokines such as interleukin-1 (IL-1), TNFα, IL-6, and NKSF, also known as IL-12,[134] even in the absence of clinical sepsis. Recent clinical data indicate that

the kinetics of increased serum levels of TNFα are predictive for transplant related complications and survival.[135,136] Experiments in a murine model showed that the single
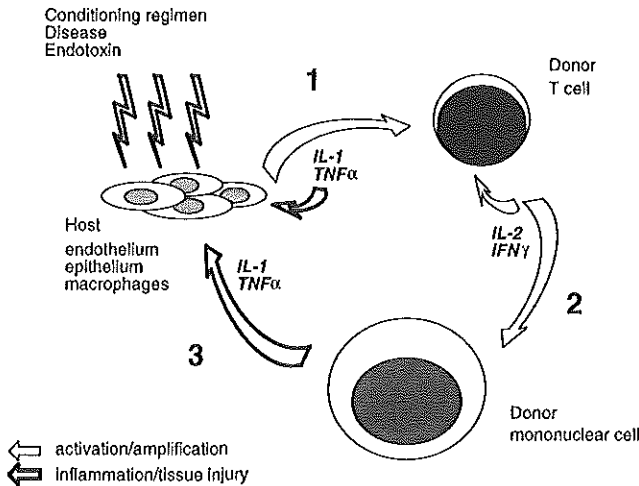
Figure 1.1 Schematic representation of the role of inflammatory cytokines in acute GVHD. In step 1, injury to host tissues, either directly by the conditioning regimen or indirectly through the production of cytokines, results in alterations in the host tissues, such as increased expression of MHC antigens, adhesion molecules, procoagulants etc. In step 2, mature T-cells infused with the bone marrow respond to these changes in the allogeneic host tissues. This response includes the autocrine production of IL-2 and IFNγ, which in turn activate mononuclear cell effectors from donor marrow. In step 3, additional production of inflammatory cytokines by these donor mononuclear cells results in direct tissue injury. Endotoxin released by gram-negative bacteria as well as production of other cytokines such as IL-6, IL-8 and NKSF (IL-12) may further amplify the injury and recruit additional effectors.[47]

injection of an anti-TNFα antibody before recipient irradiation and transplantation, significantly reduced subsequent GVHD mortality.[136]

As opposed to allogeneic BMT with 'full marrow', in syngeneic, autologous, or T-cell depleted allogeneic marrow transplants, the production of inflammatory cytokines by the endothelial and epithelial cells is self-limiting and resolves within 7 to 10 days.[47]

1.2.2.b Step two: The role of T-cells in acute GVHD
In allogeneic BMT, a second step occurs when mature donor T-cells recognize host-MHC antigens and become activated. T-cell recognition of histocompatibility antigens and adhesion to targets is facilitated by consequences of the first step, i.e., the cytokine-induced increase in host cell surface receptors in these tissues and the activation of T-cells by cytokines. The activated donor T-cells then proliferate, express the IL-2R, and secrete IL-2. Nevertheless, evidence that acute GVHD is directly mediated by cytotoxic T lymphocytes is meager. Typically, pathologic specimens of skin, liver or gut involved with acute GVHD are striking for the disproportion between tissue damage and the intensity of the lymphocytic infiltrate in

the organ. In addition, controlled trials of antithymocyte globulin (ATG) and immunotoxins have been disappointing in the therapy of acute GVHD.[137,138]

IL-2 is critical for the activation and proliferation of T-cells. It has been considered, therefore, to play an important role in acute GVHD. However, the role of IL-2 as an effector molecule of GVHD-induced damage has been difficult to establish. Serum IL-2 levels are not increased in acute GVHD, and while increases in IL-2R levels have been observed,[139] the significance of this observation is uncertain because many cell types express IL-2R. Anti IL-2R MCA's provide only transient benefit in steroid resistant GVHD and their mechanism of action is unclear.[140] Furthermore, IL-2 can be administered safely after T-cell depleted allogeneic, or autologous marrow grafting without causing or accelerating GVHD.[141,142] The only setting in which IL-2 can be directly implicated in causing a 'GVH like' syndrome is when high doses of IL-2 are administered together with LAK cells.[143] Even then, much of the IL-2 toxicity is probably mediated through the induction of other inflammatory cytokines.[144] Arguments favoring an indirect mechanism of IL-2 toxicity include: a) the induction of TNFα and IL-1 synthesis by peripheral blood mononuclear cells incubated with IL-2[145]; b) the extensive overlap between IL-2 toxicity and IL-1 and TNFα induced toxicities in both experimental and clinical settings[146,147]; and c) the inability of IL-2 to act directly on a variety of cell types *in vitro*, even when those same cell types are clearly affected by administration of IL-2 *in vivo*.[148] Corticosteroids, which are often an effective therapy for acute GVHD, are typically thought to reduce T-cell numbers, but they also diminish the production of inflammatory cytokines, particularly IL-2 and TNFα, independent of their effect on T-cell viability. Thus, while the importance of T-cells in acute GVHD is unquestioned, their primary role may reflect cytokine production and recruitment of other effectors, rather than direct cell-mediated cytotoxicity.

1.2.2.c Step three: The role of cytokines as effectors
IL-2 activates newly engrafted mononuclear cells from the donated marrow to secrete more inflammatory cytokines, such as IL-2, TNFα and IFNγ. The resulting inflammatory response results in additional release of cytokines and amplifies local organ injury.

Preclinical animal studies have revealed strong evidence of the link between excessive or dysregulated cytokine production and clinical GVHD. TNFα is well established as a cytokine that causes organ damage in experimental acute GVHD. Mice receiving transplants of allogeneic BM plus additional T-cells develop severe skin, gut,[149] and lung lesions that are associated with high levels of TNFα messenger RNA (mRNA) in these tissues.[150] More recent evidence demonstrates TNFα mRNA in the skin of mice developing GVHD after transplants across minor histocompatibility barriers.[151] Studies on TNFα in humans confirm that serum TNFα levels are elevated in patients with GVHD[135] and TNFα message is expressed in blood mononuclear cells.[152] Recent data further implicated inflammatory cytokines in the pathogenesis of GVHD by showing an association between increased TNFα

and IFNγ synthesis in a skin explant model and the development of acute GVHD.[153] Anti-TNFα MCA's are capable of temporarily abrogating steroid resistant acute GVHD in human GVHD, but the transience of the response suggests that the underlying pathophysiology had not been entirely interrupted.[154]

Experimental data demonstrated that IL-1, a central mediator of inflammation, plays an important role in GVHD. IL-1 mRNA increases in the skin of mice after transplant only if the skin is involved with GVHD.[151] A similar increase in mononuclear cell IL-1 mRNA has been observed during clinical acute GVHD.[152] If IL-1 is a central mediator of GVHD, specific inhibition of IL-1 should result in a reduction or elimination of GVHD. IL-1 receptor antagonist (IL-1ra) is a specific, competitive inhibitor of both IL-1α and IL-1β. When administered to mice either for profylaxis[151] or therapy of GVHD,[155] mortality from GVHD was strikingly diminished. The role of IL-1 may also be indirectly inferred from the high dose γ-globulin trials of GVHD prophylaxis. High dose γ-globulin appears to reduce the incidence of acute GVHD.[156] A relevant *in vitro* observation is that IgG bound to mononuclear cell Fc receptors increases IL-1ra production preferentially over IL-1α or IL-1β,[157] suggesting that a similar mechanism might occur *in vivo*. GM-CSF seems to potentiate this preferential production of IL-1ra.[157] Ongoing studies may determine whether GM-CSF can reduce the incidence of clinical GVHD.

At least two mechanisms by which TNFα can cause cell death are known. The first is by activation of phospholipase A_2 (PLA_2). Arachidonic acid metabolites are observed in supernatants of cell cultures exposed to the cytokine before cell death, and quinacrine, a PLA_2 inhibitor, completely protects against cytokine cytotoxicity.[158] A second mechanism by which TNFα can mediate cytotoxicity is the production of intracellular hydroxyl radicals.[159] The sensitivity of several cell lines to cytokine toxicity appears to be inversely related to the radical scavenge capacity of those cells, and compounds that inhibit the production of free radicals can prolong the survival of cells which are exposed to TNFα.[160] It is likely that the production of IL-1 and TNFα can also stimulate the release of cytokines from the IL-8 family, which in turn help to recruit and activate secondary cellular effectors (e.g. neutrophils, NK cells and monocytes) as well as contribute directly to capillary fragility, edema, and cell death. Other cytokines may also be part of this network; e.g. macrophage-derived NKSF may stimulate NK cells to produce INFγ and to display enhanced cytolytic activity against host tissues.[104,161] Recent data indicate that the frequency of lymphocytes producing IFNγ mRNA is dramatically increased in mice with acute GVHD.[162] Such observations suggest that the effects of cytokines such as NKSF (IL-12) may be enhanced if the frequency of responding cells increases.

Thus, multiple inflammatory cytokines may play an important role in augmenting the reactivity of the allogeneic cytotoxic cells, causing direct damage to target organs. But it is conceivable that the role of cytokines as direct mediators of local tissue destruction is very important as well,[47] though cytokines alone have not been shown to induce GVHD-like

symptoms. Further studies will elucidate the value of this hypothesis in the treatment of GVHD.

1.3 GRAFT-VERSUS-LEUKEMIA

BMT is an effective treatment for acute and chronic leukemias. In the management of these disorders, the procedure was originally proposed to allow escalation of myelotoxic chemoradiotherapy to supralethal levels, followed by BMT to rescue hemopoiesis.[65]
The assumption that the (pretransplantation) conditioning regimen is inadequate to completely eradicate leukemia and that the infused cells contribute to a cure was first proposed in 1956.[1] Although studies in rodent models generally confirmed the existence of a graft-versus-leukemia (GVL) effect after allogeneic BMT,[163,164] GVL data in humans used to be entirely indirect (retrospective).

1.3.1 GVL effect in animals

Considerable data from preclinical studies clearly show that an antileukemic effect exists.[165] It was shown that mice which died of GVHD showed a marked reduction in leukemic cell load. By transferring cells from animals that did or did not develop GVHD into secondary recipients, the antileukemic effect of GVHD could be assessed by monitoring (leukemia free) survival.[1,166,167]

In several studies showing antileukemic effects associated with allogeneic BMT, efforts were made to reduce GVHD morbidity and mortality e.g. by using germ-free conditions or reduction of the number of lymphocytes in the marrow inoculum.[167-171] It has been shown that donor T-cells in allogeneic BMT, which are the major contributors to GVHD,[55] are responsible for a reduction in the incidence of leukemia relapse also.[172,173] With a murine T-cell leukemia (spontaneously arising in AKR mice), a potent GVL effect without GVHD was induced by immunizing the MHC-compatible donors with pooled allogeneic lymphocytes.[174] The induction of this GVL effect within the donor required CD8$^+$ T-cells and was activated by non-MHC determinants on the immunizing cells.[175]

The potential of either depletion or administration of donor lymphocyte subsets to improve the outcome of allogeneic BMT has attracted considerable interest. An association between increased NK activity with GVHD has been widely observed,[118,119,121] and donor NK cells or LAK cells have been implicated as effectors of GVHD. Studies involving a variety of GVHD patterns and several strain combinations indicate that the role of NK cells in GVHD is complex and may vary considerably between host-donor combinations.[176] Johnson and Truitt[116] reported a reduction in GVHD, without loss of antileukemic effects or impaired engraftment in MHC-matched, minor antigen mismatched allogeneic BMT when donor NK cells were selectively depleted. However, the lymphocytic atrophy and immunosuppression

resulting from GVHD was not reduced, and this approach was ineffective in a more severe, MHC-mismatched GVHD model.

Studies on the effects of murine T-cell subsets show that CD4+ T-cells contribute to GVL effects,[177] and that predominantly CD8+ cells play a role in causing GVHD in most (4/6) host-donor combinations of MHC-matched, mH antigen mismatched BMT.[59] However, these results can not be extrapolated to the setting of both major and minor histoincompatibilities. The majority of studies evaluating T-cell subsets show that CD4+ T-cells play a critical role in causing GVHD in fully mismatched strain combinations, and that CD8+ T-cells given alone do not cause acute GVHD.[177-179] This observation is surprising, as CD8+ cells can cause GVHD directed against isolated mH antigen or MHC class I disparities.[59,88] Another approach to separating the beneficial from the harmful effects of allogeneic T-cells involve the *in vitro* expansion and cloning of donor T-cells that specifically react to host leukemia associated antigens, but that do not recognize non-malignant recipient cells. The existence of such CTL clones has been reported in mice,[180] but it still needs to be proven that these cells do not lose their specificity and do not cause GVHD after prolonged *in vitro* selection, cloning and expansion.

As described earlier, IL-2 is crucial in T-cell proliferation and has therefore been used in many GVL/GVHD studies. Experimental data on the GVL effect of IL-2 therapy post BMT are controversial. In syngeneic BMT in mice it has been postulated that the marrow graft should be activated with IL-2 (*ex vivo*) and IL-2 therapy should be initiated immediately after BMT to induce a syngeneic GVL effect.[181] The mechanism of this approach is unknown and was observed to be transient.

Following allogeneic BMT of low doses of IL-2 during the first week of GVHD enhanced the severity and mortality seen in class I disparate, but not in class II disparate mouse strain combinations presumably mediated by CD4+ cells.[182] Aggravation of GVHD induced by recombinant IL-2 was observed both in MHC disparate rats[120] and mice mismatched for mH antigens.[183] In contrast, Merluzzi et al. reported not to aggravate GVHD with IL-2 doses able to induce allospecific cytotoxic T lymphocytes (CTLs), using a different murine minor histoincompatibility model.[184] It appears that IL-2 plays a critical role in the early development of acute GVHD, most likely by facilitating the development of donor-anti-host CTLs.[185] Since GVHD and GVL usually coincide, IL-2 may therefore be of importance in the induction of GVL as well.

The effect of IL-2 treatment after allogeneic BMT on GVHD has recently become even more controversial. Sykes et al. found (using the EL4 leukemia growing in B10 recipients and A/J as donor mice) that administration of high doses of IL-2 at the time of infusion of MHC mismatched BM and spleen cells together with T-cell depleted syngeneic BM, to delay GVHD related mortality, further reduces lethal GVHD, promotes alloengraftment and maintains the GVL effect.[171,186] However, initiation of IL-2 administration at later time points, e.g. day 7 post BMT, can exacerbate GVHD.[186,187] Further studies using mice (EL4

and 2B-4-2 leukemia in A/J -> B10 and B10 -> BALB/c BMT, respectively) revealed that in fully MHC mismatched transplantation, early IL-2 treatment selectively inhibits the GVHD producing CD4 activity, but does not inhibit the antileukemic effects of CD4^{+} and CD8^{+} T-cells.[178,188,189] Using allogeneic BMT in several murine donor-host combinations in which both GVL and GVHD were CD4 dependent, IL-2 treatment inhibited GVHD but not the antileukemic effects of CD4^{+} cells.[188] These findings suggest that the inhibitory effect of IL-2 is directed against a limited subset or function of CD4^{+} T-cells and that GVL and GVHD are mediated through different CD4 mechanisms.[188] Finally, it was shown that the effect of CD4^{+} cells on GVHD is determined by the histoincompatibility between donor and host.[179] Unfortunately, to date the clinical use of IL-2 for enhancing the antileukemic effect in allogeneic BMT has been prevented by the property of IL-2 to aggravate GVHD. Though highly interesting, the complicated data of Sykes et al. do not describe the 'separable' CD4-mechanisms of GVL and GVHD, therefore not solving the risk of increased GVHD induced by IL-2 treatment, which leaves this treatment clinically inapplicable.

1.3.2 GVL effect in humans

First clinical evidence for this phenomenon (Table 1.5) was provided by Weiden et al.[190,191] They showed that the likelihood of leukemia relapse was substantially lower in patients with either acute or chronic GVHD compared with unaffected patients. Patients experiencing both acute and chronic GVHD had an even lower rate of leukemia relapse compared to those with either acute or chronic GVHD alone.[192,193] A higher relapse rate after syngeneic transplantation compared with allogeneic transplantation without clinical signs of GVHD was shown retrospectively[194] and confirmed by a larger retrospective study performed by the International Bone Marrow Transplant Registry (IBMTR).[192] These data suggest that the GVL effect can be induced without being accompanied by clinically apparent GVHD. *Ex vivo* T-cell depletion (TCD) of allogeneic donor BM decreases the incidence of both acute- and chronic GVHD but at the same time significantly increases the rate of leukemia relapse.[195]

Table 1.5 Clinical evidence for the existence of a graft-versus-leukemia effect

		INCIDENCE OF LEUKEMIA RELAPSE
Acute and/or chronic GVHD versus no GVHD		reduced
Allogeneic BMT (without GVHD) versus autologous BMT		reduced
GVHD prophylaxis:	T-cell depletion	increased
	post-BMT immunosuppression	increased

Likewise, leukemia relapse was shown to be increased by post-BMT GVHD prophylactic immuno-suppression (cyclosporine, CsA).[196-198] The discontinuation of CsA at first signs of leukemia relapse induced complete hematologic and cytogenetic remission and these patients remain free of disease with a long follow up. Though few cases have been reported,[196,197]

they indicate that GVHD is associated with the GVLR which eradicates the residual leukemic cells.

Several investigators have attempted to deplete T-cells to a lesser dergree prior to BMT, with the aim to reduce GVHD and preserve GVL. Results of these studies suggest that there may be an optimal treatment of the marrow by which the T-cell depletion will decrease the incidence and severity of GVHD while preserving the GVL effect.[16,199,200] An other approach to prevent GVHD may be depletion of a specific subset of lymphocytes (or inhibition of a specific T-cell function), according to the assumption that there is not only a quantitative effect of allogeneic T-cells on GVHD and/or GVL but also a qualitative difference between the T-cells. A promising clinical trial of selective donor CD8 cell depletion (2 log) provided information on T-cell subsets mediating GVHD and GVL in man. This study demonstrated a reduction in, but not absence of, GVHD, whereas leukemic relapse rates were low (Table 1.6).[201,202]

Table 1.6 Allogeneic BMT (HLA-id.sibl.) in CML patients in chronic phase*

MARROW	GVHD (≥ grade II)	RELAPSE RATE	DFS (3yr)
Unmodified (n=31)	58%	0%	45%
CD8 depleted (n=18)	22%	0%	66%
pan T depleted (n=20)	5%	65%	35%

*, deduced from[201]; n, the number of patients; DFS, disease free survival; pan T depleted, all T-cells have been removed (2 logs).

It has been demonstrated that leukemia 'specific' CTL lines and clones could be generated from the peripheral blood of MHC identical siblings of patients with leukemia.[203,204] Both MHC class I as well as class II restricted CTL clones could be generated.[204] To explore the possibility of clinical application of donor-derived CTL lines directed against the leukemic cells from patients who relapsed after allogeneic BMT, a pilot study was performed using eight donor-recipient combinations. In seven of eight combinations donor-derived CTL lines could be generated that showed specific lysis of the leukemic cells from the patient. In five of these cases, the CTL lines showed reactivity with the leukemic cells, but not with the IL-2-stimulated lymphocytes from the same individual. In two cases, the CTL lines were cytotoxic for the IL-2-stimulated lymphoblasts as well as the leukemic cells.[205] It is likely that such leukemia specific T-cells are quite rare in normal peripheral blood. Separation from T-cells that do induce GVHD, prolonged *in vitro* selection, cloning and expansion, (for *in vivo* administration) may be impractical for use in the setting of BMT, in which leukemia reactive cells must eliminate exponentially expanding leukemic cells.[48]

LAK cells and NK cells have also been implied to play a role in the GVL reaction.[192,206,207]These cells are able to lyse, most efficiently in the presence of IL-2, a

variety of tumor cells with limited effect on normal cells. Acute lymphocytic leukemia (ALL) and acute myelocytic leukemia (AML) blasts are reported to be susceptible to the lytic activity of autologous and allogeneic LAK cells *in vitro* as well.[208-212] Clinical studies confirm these preclinical results for acute leukemias.[213,214] Other studies suggest that in AML, but not in ALL, the LAK cell compartment plays a role in the clinical course and overall outcome of the disease.[215]

Results of pilot studies indicate that treatment of acute leukemia of both myelocytic and lymphocytic origin with recombinant IL-2 alone is feasible and may result in the disappearance of chemotherapy-resistant blasts in patients with limited but detectable disease. The IL-2 treatment was shown capable of inducing profound immunophenotypic and functional modifications in PB and in BM lymphocytes, particularly in patients with more limited disease. The evidence of the *in vivo* activation of cytotoxic cells (a.o. LAK cells), particularly in the BM, helps to explain the clinical responses observed in individual acute leukemia patients.[216,217]

Concerns have been raised that IL-2 therapy might accelerate the growth of residual leukemia cells. Human *in vitro* and murine *in vivo* data (human leukemia cells growing in immunosuppressed nude mice) indicated that IL-2 in general does not promote the proliferation and growth of human acute leukemia cells of myelocytic and lymphocytic origin.[218] However, more recently, relapses were reported after IL-2 treatment of AML patients in complete remission, where the IL-2R-α chain was detected on the surface of leukemia cells from such patients during relapse.[219] Similarly, *in vitro* proliferation of leukemic blasts of the monocytic lineage (FAB M4 and M5) from a number of patients, was stimulated by IL-2, which was significantly correlated with the expression of the IL-2R-β chain.[220]

Overall findings suggest that measures that simply reduce GVHD may also impair the GVL effect of allogeneic BMT and/or that the induction of GVL concomitantly leads to GVHD.[221] As calculated for human allogeneic HLA-matched BMT with full marrow, the GVLR induces a 1 log leukemic cell kill together with significant (lethal) GVHD.[30] Therefore in acute leukemia it is strongly advised to apply T-cell depletion which eliminates death from GVHD and seek for other means to achieve additional leukemic cell kill, such as intensification of the conditioning regimen and/or treatment with low dose chemo- or immunotherapy after BMT,[222,223] as is currently being applied in many medical centers.

Nevertheless, the first <u>direct</u> evidence of a GVL effect induced in humans was recently provided by Kolb et al.[224] They used a combination of IFNα and donor buffy coat cells obtained by leukapheresis to induce cytogenetic remission in three patients whose CML had relapsed after allogeneic BMT. Excitement over these observations led to rapid confirmation by several other groups.[225-232] Although details of the protocols differed, these investigators have shown that allogeneic buffy coat mononuclear cells have a striking capacity to identify

and destroy CML *in vivo*, resulting in a clinical response rate of 83%. The potency of this GVL response is very impressive (Table 1.7).

Table 1.7 Summary of reports on donor buffy coat transfusions for relapsed CML after allogeneic BMT*

reference number	n	IFNα	mononuclear cell dose $(x10^8/kg)$	clinical response	bcr/abl⁻ by PCR	cytopenia	acute GVHD	therapy related death
224	3	3/3	4.4, 5.1, 7.4	3/3	ND	0/3	2/3	0
227	2	0/2	1.8, 2.7	2/2	2/2	0/2	1/2	0
229	6	3/6	0.34-5.2	5/6	4/5	2/6	5/6	1
226	8	6/8	2.5 - 5.0	7/8	5/6	4/8	7/8	1
230	3	3/3	3.8, 5.7, 12.3	3/3	2/2	3/3	3/3	1
232	8	8/8	0.9 - 7.9	7/8	5/8	7/8	7/8	1
233	6	6/6	NR	5/6	2/2	6/6	5/6	3
234	10¢	4/7	2-3 infusions	NR	NR	3/10	7/10	3
Summary		33/43 (77%)	0.34-12.3	32/36 (83%)	20/25 (80%)	25/46 (54%)	37/46 (80%)	10/46 (22%)

*, adapted from reference[235]; acute GVHD, grade I-IV GVHD; n, number of patients; NR, not reported; ND, not done;¢, seven of ten were treated for CML.

A retrospective comparison of the disease free survival reported in these studies with the results of a second conditioning regimen and BMT, shows that both the relapse rate and therapy-induced mortality are substantially lower with buffy coat infusions.[236,237]

Interestingly, it appears that the GVL reaction is more effective in chronic-phase CML than in other hematologic malignancies. Retrospective analyses support the presence of a GVL effect in non-Hodgkin's lymphoma[238] and the acute leukemias,[192,228] but further studies will be needed to determine whether it can be induced reliably with buffy coat or T-cell infusions. The ability to treat relapsed CML with reduced morbidity and without mortality of a second transplant would unquestionably be a valuable contribution to the care of these patients.

1.4 OUTLINE OF THIS STUDY: ARE GVL AND GVHD SEPARABLE?

It is not clear whether the processes operating in the GVLR are separable from those operative in GVHD. Evidence has been reported about at least two elements of the GVLR; one that is dependent on the presence and the intensity of GVHD[239] and the other one operating without clinically overt GVHD.[240,241] Thus, the latter may allow enhancing the GVLR. In this way a more safe and complete eradication of leukemic cells still present after the conditioning regimen may be achieved. However, to determine whether this can be

accomplished, a better understanding of the mechanisms of the GVLR and its mediators is warranted.

In some animal models, the GVLR can be separated from the GVHD reactivity[170,242] leading to the speculation that GVL and GVH reactive cells are distinct. These preclinical data support the assumption that specific antigens are present on leukemic cells, not shared by normal cells of the host. However, no leukemia-associated specific surface markers have been identified so far on human leukemia cells. Alternatively, the same cells may mediate both GVL and GVH reactions, but quantitative differences in sensitivity of leukemic and normal cells could account for apparently distinct GVL/GVH reactions, inducing a 'threshold' effect.

Several reports mention the *in vitro* induction of cytotoxic T-lymphocytes specific for MHC-matched allogeneic leukemic cells, nonleukemic cells, or lysing both leukemic and nonleukemic target cells,[203,204,243] and their correlation to the GVLR *in vivo* in a murine model.[180]

The question remains whether it will be possible to optimize the beneficial GVL effects without increasing the risk of lethal GVHD? So far, no convincing clinical evidence has been reported on a GVLR separable from GVHD.

To study GVL in animal models relevant to human leukemia, the studies reported here were performed applying the acute myelocytic leukemia (AML) growing in the inbred Brown Norway rat strain (BNML)[222,244,245] and the L4415 acute lymphocytic leukemia (ALL) growing in the inbred WAG/Rij rat strain.[246,247] Both leukemia models show a reproducible growth pattern upon intravenous (i.v.) transfer. Survival time following i.v. inoculation is inversely related to the logarithm of the number of leukemic cells in the inoculum. This log-linear relationship between leukemic cell load and survival time allows an accurate estimation of the treatment-induced antileukemic effect, expressed in terms of log leukemic cell kill (LCK), by recording prolongation of survival. Thus, these rat models offer the opportunity to compare the antileukemic effects of syngeneic, semi-allogeneic and (fully mismatched) allogeneic BMT and additional treatment regimens in the treatment of minimal residual leukemia, i.e. AML and ALL.

The first aim was to induce, in a reproducible manner, a GVL effect in these rat models. Secondly, when the GVL-inducing treatment of the recipient rats was established, the main purpose of this study was to analyze whether the induction of GVL is feasible in the treatment of leukemia. The question whether the GVL reaction was separable from GVHD was emphasized. If GVL seemed inextricably bound up with GVHD, it would be attempted to define if there is a therapeutical window and how this could be maximally enlarged without evoking irreversible GVHD-induced tissue damage.

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CHAPTER 2

ANIMAL MODELS

CHAPTER 2.1

THE BROWN NORWAY ACUTE MYELOCYTIC LEUKEMIA (BNML);
A SUMMARY*

2.1.1 SUMMARY

The studies in the BNML model have added considerably to the understanding of various processes that occur during the development of leukemia, e.g. the interactions of leukemic cells and normal hemopoiesis. The methodology developed in the BNML model allows the quantification of the relative effectiveness of any given treatment with regard to the antileukemic activity compared with the toxicity for normal host tissues, e.g. the bone marrow. Furthermore, the comparison of various treatment modalities, employed for remission-induction treatment or the conditioning regimen prior to bone marrow transplantation (BMT), made it possible to determine the efficacy of the various approaches. Obviously, there are restrictions with regard to the extrapolation of the rat data to the human disease. Leukemia growth in inbred rats is highly reproducible, while in humans it presents a high degree of individual variation. However, several characteristics are shared and the aim should be to identify and study the similarities as well as the dissimilarities between human and rat leukemia. In that way progress may be envisaged with respect to optimizing the treatment for leukemia and finally reaching the goal of curing human leukemia.

2.1.2 INTRODUCTION

'Minimal residual disease' (MRD) in leukemia is defined as the relatively low number of leukemic cells which have survived successful remission-induction chemotherapy, out of sight below the conventional cytological detection level. Obviously, the presence of MRD predicts a leukemia relapse at a later stage and therefore today's leukemia research and treatment are focused on developing methods to decrease the detection level of residual leukemic cells to immediately continue treatment and on defining strategies to subsequently eradicate MRD.

As much of the questions to be answered are difficult to approach in human leukemia, there is a great need for realistic animal models to translate clinical problems into laboratory experiments and to extrapolate new diagnostic and therapeutic tools from models to man. A particular animal model should be chosen based upon the specific question to be answered. In bone marrow transplantation research various animal species are employed, such as rodents, dogs and subhuman primates. However, if leukemia is the main subject of investigation only rodents remain as there are no reproducibly growing (transplantable) leukemias available in the larger mammals.

Apart from investigations in 'spontaneously' arising rodent leukemias, the majority of experimental studies are performed in transplantable leukemias. Two important prerequisites for evaluating the efficacy of a given treatment are a) a reproducible growth pattern upon injection of leukemic cells; and b) a linear relationship between the number of inoculated leukemic cells and the survival time.

2.1.3 THE BN ACUTE MYELOCYTIC LEUKEMIA (BNML)

In 1974 an acute myelocytic leukemia (AML) transplantable in the inbred Brown Norway rat

Table 2.1.1 Major characteristics of the BNML

induced by 9,10-dimethyl-1,2-benzanthracene (DMBA)
analogy with human acute (pro)myelocytic leukemia:
    1. cytology- cytochemistry
    2. slow growth rate (10^7 cells i.v.: death at day 23)
    3. severe suppression of normal hemopoiesis due to an absolute numerical decrease in normal
       hemopoietic stem cells
    4. diffuse intravascular coagulation (DIC)
    5. response to chemotherapy as in human AML
    6. leukemic clonogenic cells present
    7. low antigenicity
    8. no virus

strain (BNML) was developed. The major characteristics of this model have been described in detail [1-4] and are summarized in Table 2.1.1. In particular as regards its biology of growth

and its sensitivity to chemotherapy, the BNML has now widely been accepted as a relevant model for human AML.
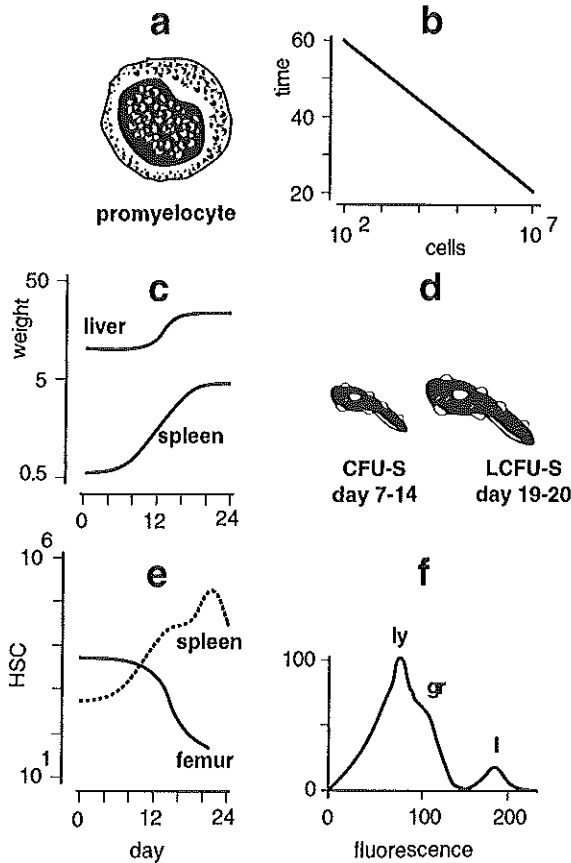
Figure 2.1.1 Main characteristics of the BNML model; **a**, promyelocytic nature; **b**, linear dose-survival relationship; **c**, increase in liver- and spleen weight during leukemia progression; **d**, discriminative spleen colony assays to quantify normal hemopoietic stem cells (CFU-S) and clonogenic leukemic cells (LCFU-S); **e**, normal hemopoietic stem cells (HSC) in bone marrow and spleen during leukemia progression; **f**, detection of leukemic cells with a monoclonal antibody and flow cytometry (ly, lymphocytes; gr, granulocytes; l, leukemic cells).

The Brown Norway acute myelocytic leukemia (BNML) was classified as an acute pro-myelocytic leukemia (Figure 2.1.1.a), with signs of diffuse intravascular coagulation as the leukemia progresses. The leukemia can be transferred by intraperitoneal (i.p.), subcutaneous (s.c.), intrathecal (i.t.) or intravenous (i.v.), injection of single cell suspensions. The i.p. injection of leukemic cells results in the formation of greenish colored colonies of leukemic cells in the mesenterium (chloromas), followed eventually by disseminated leukemia growth. No ascites fluid is formed. The s.c. transfer of leukemic cells results in the slow development of a subcutaneous tumor, followed by scattered outgrowth of the leukemia. The i.p. and s.c. transfer of leukemia cells were not investigated in depth, in contrast to the i.t. transfer of

leukemic cells [5] which was studied extensively with the purpose of the development of a model for central nervous system (CNS) leukemia.

Most studies, however, were done after intravenous transfer of leukemia. The survival time after i.v. injection of leukemic cells is linearly correlated with the injected cell number (Figure 2.1.1.b). After injection, leukemic cells spread to all organs, but they predominantly home to the bone marrow, liver and spleen where they start to proliferate.[6] The ED50, which represents the number of cells that is required to induce leukemia in 50% of the animals, was found to be 24.7 cells (as deduced from the sigmoid curve, representing the number leukemic cells inoculated versus the percentage of rats killed). The leukemic cells are transferred in a syngeneic system, i.e. the inbred BN rat strain, and were found not to be immunogenic. The growth pattern of the leukemia appeared to be highly reproducible.

The liver and spleen increase in size and weight during the progression of the disease (Figure 2.1.1.c). During the development of leukemia the bone marrow is rapidly replaced by leukemic blast cells. At this stage leukemic cells are appearing in the peripheral blood leading to increased numbers of peripheral white blood cells, predominantly leukemic cells.

The fate of the pluripotent hemopoietic stem cells during this process has been studied. Hemopoietic stem cells are detected by a spleen-colony-assay (SCA) (Figure 2.1.1.d). Normal stem cells form colonies on the surface of the spleen and are therefore named colony forming units-spleen (CFU-S). Lethally irradiated mice or rats are inoculated with bone marrow cells; the number of cells is chosen such that 5-30 colonies are expected to appear on the surface of the recipient spleen 7-14 days after inoculation. Each colony is derived from one clonogenic stem cell. The fraction of inoculated hemopoietic stem cells entering the spleen was found to be 0.007 (=f-factor).[7] By counting the number of CFU-S and correcting for the f-factor, the number of clonogenic stem cells in a cell suspension can be calculated. Concurrently, with the replacement of the normal bone marrow, the number of hemopoietic stem cells decreases and reaches very low levels during the terminal stage of the disease. Simultaneously, the number of stem cells in the spleen and in the peripheral blood increases (Figure 2.1.1.e).

Another important characteristic of the BNML is the clonogenic property of the leukemic cells. The i.v. injection of low leukemic cell numbers (less than 10^5) into (unirradiated) normal recipient rats results in the development of leukemic colonies in the spleen, defined as LCFU-S (leukemic-colony-forming-unit-spleen). The colonies are clearly visible on the spleen surface 19-20 days after injection (Figure 2.1.1.d). This indicates that normal hemopoietic stem cells proliferate approximately twice as fast as clonogenic leukemic cells. In analogy to the spleen colony formation of normal stem cells, it is assumed that each colony is derived from one clonogenic leukemic cell. The fraction of inoculated leukemic cells entering the spleen, the f-factor, was found to be 0.002.[7] By multiplying the number of LCFU-S with the reciprocal of the f-factor, the number of clonogenic (leukemic) cells in the inoculated cell suspension is derived. Every viable leukemic cell has clonogenic potential,[8]

however, after the i.v. injection, a certain proportion of the cells home to places which are unfavorable for leukemic cell growth. This explains the discrepancy between an ED_{50} value of 24.7 cells and a clonogenic potential of close to 1.

Finally, the BNML has the advantage that the leukemic cells can be discriminated from normal bone marrow cells by the use of a mouse anti-BNML monoclonal antibody (MCA). The MCA reacts with an antigen, present in a low density on normal granulocytes and in a high density on leukemic blast cells, but which is absent on normal lymphocytes and normal blast cells. Flow cytometry enables the specific detection of leukemic cells (Figure 2.1.1.f).

2.1.4 DETECTION OF BNML

2.1.4.a Cytology/cytochemistry
Simple total nucleated cell counting in combination with May-Grünwald-Giemsa staining for differential cell counting is sufficient to determine leukemic cell numbers when they are present in frequencies between 5 % and 100 %. Cytochemical characterization revealed that the leukemic cells were promyelocytes.

2.1.4.b The organ weight parameter
The growth of leukemic cells in liver and spleen results, during the later stages of leukemia development, in an increase in the weight of both organs. It was found that 1 gram increase in weight corresponds to an increase of 10^9 cells (schematically shown in Figure 2.1.2).

Once the organ weight is significantly increased, the leukemic cell number can be deduced from it. The spleen increases in weight from about 350 mg to 3-4 g towards the terminal stages of leukemia. The liver increases in weight from 6-8 to 20-25 g. To become significantly different from the normal background organ weight, the spleen has to be increased more than 200 mg (corresponding to 2×10^8 cells). For the liver the increase has to
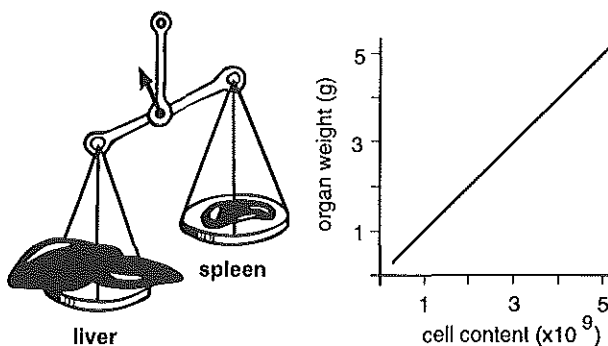

Figure 2.1.2 Measuring the leukemic cell load by increase in spleen and liver weights (one gram equals 10^9 cells).

be at least 1 gram to become evident, corresponding with 1×10^9 cells. By weighing the spleen and the liver the effectiveness of a treatment can be quantified. The method is valuable for studying advanced disease. Changes in the tumor load in the range of 5×10^8 to $1-2 \times 10^{10}$ can be studied corresponding to one and a half decade (expressed as 1.5 log).

2.1.4.c The survival time assay

A more sensitive method is based on the relation between the survival time and the number of injected cells. This is schematically illustrated in Figure 2.1.3. A single cell suspension can be prepared from the total content of a femoral bone by flushing the marrow cavity with a
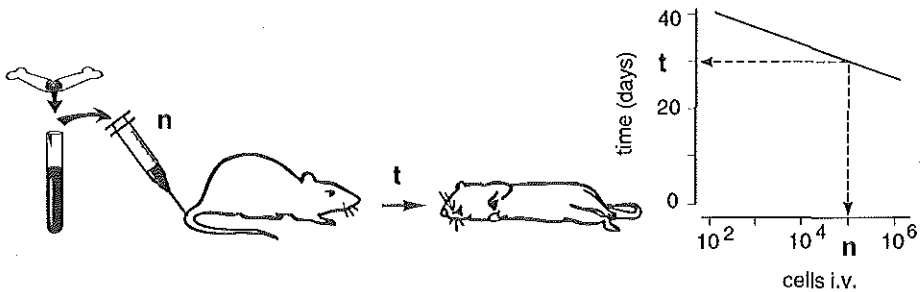
Figure 2.1.3 The dose-survival assay for measuring small numbers of leukemic cells.

physiological fluid (e.g. phosphate buffered saline). The total volume of this suspension is measured as well as its cell content. A fraction of the total volume is injected i.v. into previously untreated recipient rats. If leukemic cells are present in the cell suspension, the animals will eventually die from leukemia. The survival time is recorded. Based on the known relation between the number of leukemic cells injected and the survival time, the originally injected leukemic cell content can be deduced. To determine the leukemic cell content of any organ, the survival time assay can be used which implies that the total leukemic cell content for each organ of choice can be determined at any time. For very low numbers of leukemic cells (i.e. below 100 cells) the survival times deviate from the straight line: i.e., the survival times increase disproportionally.[2] However, by using the results from the dose-survival curve, the leukemic cell number can be deduced from the percentage of animals that develop leukemia. The limits of detection of this dose-survival bioassay spans 8-9 logs.

2.1.4.d Immunophenotyping

A monoclonal antibody (MCA) was developed against BNML cells, i.e. Rm124 (Dr. R.J. Johnson, Johns Hopkins University, Baltimore, Md, USA), which detects an antigen that is present in a high density on leukemic cells, in a low density on normal granulocytes and absent on lymphocytes and normal blast cells.[9,10] After labeling of cells with the FITC

(fluorescein isothiocyanate) conjugated MCA under standard conditions it is possible to discriminate between normal and leukemic cells on the basis of fluorescence intensity using a flow cytometer.
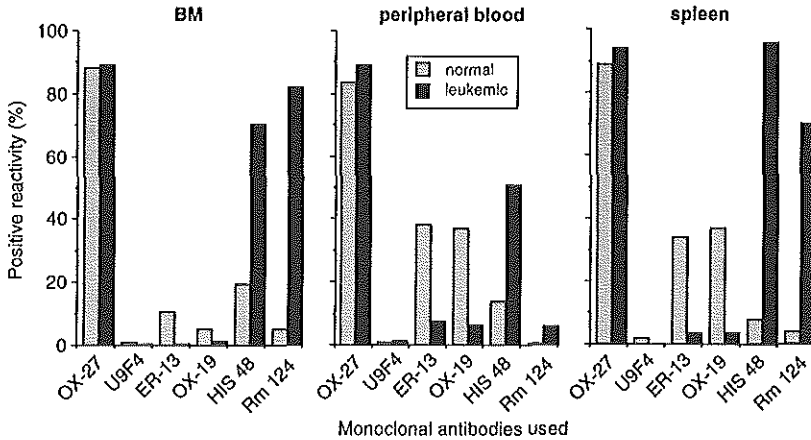
Figure 2.1.4 Comparison of antigen expression in BM-, peripheral blood- and spleen cells of healthy and terminal BNML leukemic rats. Monoclonal antibodies used were specific for: BN class I, RT-1.A^n (OX-27), WAG/Rij class I, RT-1.A^u (U9F4), class II [I$_a$], RT-1.B (ER-13), CD5 (OX-19), erythroid cells and granulocytes (HIS 48), and BNML (Rm124).

When bone marrow (BM), splenocytes and peripheral blood samples were taken from healthy and terminal stage leukemic animals for immunophenotypic analysis, significant changes in percentages of cells reacting with the various antibodies were found. This is a result of changes in the cell population-composition, because of BNML infiltration, rather than changes in antigen expression itself (Figure 2.1.4). Taking into account that the BM and the spleen are highly infiltrated with leukemic cells, figure 2.1.4 indicates that the BNML not only specifically binds the MCA Rm124,[9] but also attaches to the MCA's specific for BN MHC class I (OX-27; RT-1.A^n)[11] and erythroid cells/granulocytes (HIS 48).[12] Concomitantly, it shows that BNML is negative for WAG/Rij-specific MHC class I (U9F4; RT-1.A^u),[13] class II (ER-13)[14] and CD5 (OX-19).[15,16]

A number of different techniques to detect MRD have been studied in the BNML model as well, such as the leukemic colony forming unit assay (LCFU-S), flow karyotyping and genetic marking of leukemic cells. These studies will not be discussed here.

2.1.5 TREATMENT OF MINIMAL RESIDUAL DISEASE

One of the major problems in today's leukemia treatment employing either allogeneic or autologous bone marrow transplantation (BMT) is leukemia relapse. Apparently, leukemic

cells have survived high dose conditioning treatment prior to BMT. In case of autologous BMT, leukemic cells reinfused with the graft might in addition contribute to leukemia relapse after BMT.

Table 2.1.2 Efficacy of ablative conditioning regimens prior to BMT in AML

	A log leukemia cell kill (BNML)	B % relapse after allo-BMT in first remission human AML
Bu + TBI	5*	-
TBI + Bu	6	-
Cy + TBI	8 - 9	25
TBI + Cy	9 - 10**	10
Cy + Bu	10	-
HDAC + Cy + TBI	>10***	5 - 10
Bu + Cy	>10	8

A, rat BNML; B, human AML; Cy, cyclophosphamide; TBI, total body irradiation; Bu, busulphan; HDAC, high-dose cytosine arabinoside.
*70%; **20%; ***25% treatment-related mortality

Studies in the BNML have contributed to the development of more effective remission-induction and maintenance regimens, including anthracyclines, [17,18] AMSA [19] and high-dose cytosine arabinoside timed sequential treatment. [20,21]

In addition, in the BNML a variety of high-dose marrow ablative conditioning regimens has been studied prior to BMT. [22,23] With methods described before, e.g. the survival time assay, their efficacy could be evaluated in terms of 'log leukemic cell kill' (LCK). A summary of the data obtained so far is given in Table 2.1.2. It is of interest to see that the efficacy of the various regimens in the BNML shows an inverse relationship with relapse rates in human acute myelocytic leukemia (AML) treated with similar conditioning regimens. Obviously, this points at the relevance of the BNML in predicting the outcome of treatment in man.

Furthermore, the BNML has served as the preclinical model to develop the concept of eliminating leukemic cells from autologous marrow grafts with *in vitro* chemotherapy. [24,25]

A major drawback in allogeneic BMT is graft-versus-host disease (GVHD). [26] Clinical and preclinical data indicate however, that GVHD is associated with a significant graft-versus-leukemia (GVL) effect which may eradicate the residual leukemic cells. [27] The intriguing question remains whether it will be possible to optimize the beneficial GVL effects without increasing the risk of lethal GVHD.

What is the optimal treatment after patients enter a phase of complete remission (CR; number of leukemic cells below detection limits) induced by the remission-induction treatment (Figure 2.1.5)? Though cures are induced by the subsequent conditioning regimen and BMT, eradicating the minimal residual disease (MRD), 50-60% of patients with AML
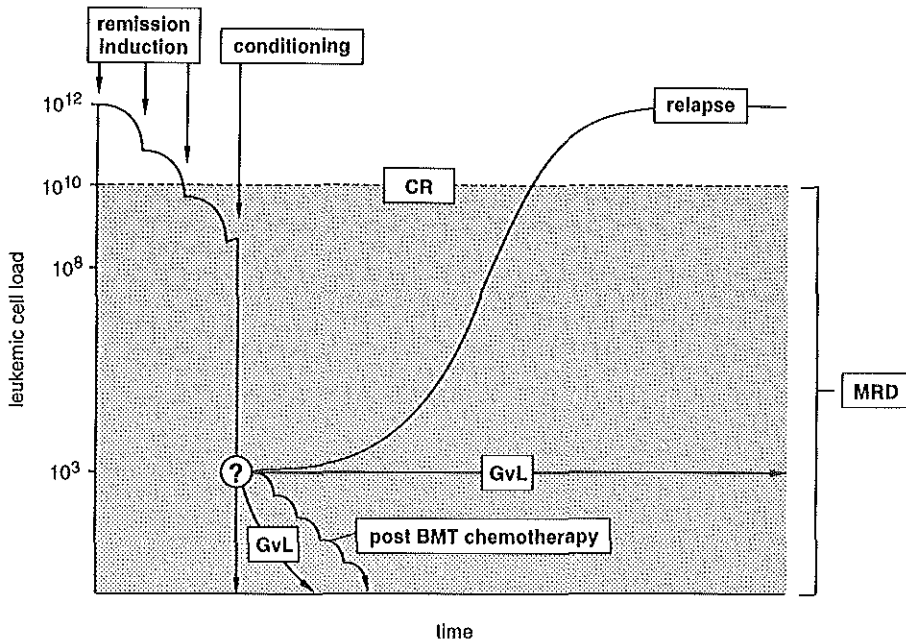
Figure 2.1.5 Cure after allogeneic bone marrow transplantation: Chemo-radiotherapy and/or Graft-versus-Leukemia? GVL, Graft-versus-Leukemia; BMT, bone marrow transplantation; CR, complete remission; MRD, minimal residual disease.

receiving an autologous graft in first CR will relapse.[28,29] The probability of leukemia relapse after allogeneic, HLA-identical sibling transplants for AML in first remission was reported to be 7-27%, depending on the occurrence and severity of GVHD.[29] Post-BMT chemotherapy could lead to a further reduction in leukemia relapse rates but may also jeopardize the marrow graft. After allogeneic BMT, the GVL effect can contribute to an increase in cure rates by eradication of the residual leukemic cells or may suppress their growth and keep the leukemic cells in a so called 'dormant state' (Figure 2.1.5). The BNML offers the opportunity to study the GVL effect (and GVHD) after syngeneic, allogeneic and semi-allogeneic BMT in a well characterized model relevant for human AML.

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CHAPTER 2.2

L4415: FURTHER CHARACTERIZATION OF THE RAT MODEL FOR HUMAN ACUTE LYMPHOCYTIC LEUKEMIA*

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2.2.1 SUMMARY

The biological properties of a transplantable lymphocytic leukemia, L4415 in the WAG/Rij rat, are described. The radiation-induced L4415 leukemia is characterized as a relatively slowly growing, non-immunogenic, immature T-cell leukemia which shows a reproducible growth pattern upon intravenous (i.v.) transfer. Survival time following i.v. inoculation is inversely related to the number of leukemic cells in the inoculum, which allows a quantitative estimate in terms of log leukemic cell kill of the effect of treatment. The first signs of leukemic growth are found in the bone marrow, the spleen and the liver. Leukemic cells can be detected in the peripheral blood 13 days after inoculation. Due to replacement of normal hemopoietic tissue by leukemic cells and their number increasing exponentially thereafter, normal hemopoiesis is inhibited in the later stages of the disease as indicated by severe thrombocytopenia and anemia. Death is caused by a combination of splenic rupture, gastrointestinal- and pulmonary hemorrhage and impaired functions of heavily infiltrated organs. Hepatosplenomegaly and lymphadenopathy are prominent features at autopsy. Cyclophosphamide- and radiosensitivity of the clonogenic leukemic cells have been determined, a 2.9 log cell kill could be induced by single dose cyclophosphamide inoculation and a dosage giving a surviving fraction of 0.37 (D_0) of 0.99 Gy with an extrapolation number (N) of 8.5 were calculated. Based on these data the L4415 rat leukemia may be regarded as a relevant model for human acute lymphocytic leukemia and may thus serve to explore new treatment strategies.

2.2.2 INTRODUCTION

The understanding of normal and pathological processes in the biology of man has benefited a great deal from studies of animal models, with the common goal of exploiting the results obtained in solving clinical problems. Similarities in characteristics between animal tumors and human tumors should serve as selection criteria for an animal model. The majority of transplantable leukemias in mice and rats show marked discrepancies with human leukemias. It should be noted that passage of a transplantable animal leukemia can be compared with the sequence of remissions and relapses in a single patient. In contrast to human leukemia, most transplantable leukemias in rodents show a very high proliferation rate.[1-5] As a consequence, many models do not show the degree of failure of normal hemopoiesis which is usually observed in late stages of human acute leukemia.[6,7]

An animal model for human acute myelocytic leukemia (AML), the transplantable leukemia in the Brown Norway rat (BNML), has been extensively studied in our institute for many years. This leukemia has proven to share a great number of characteristics with those found in human AML so that its response to treatment can be employed in testing a variety of anti-tumor agents and various treatment modalities for clinical application.[1,8-11]

The L4415 WAG/Rij rat leukemia was introduced as a transplantable lymphocytic leukemia in our institute in 1976.[12,13] It was shown that L4415 leukemic cells distribute randomly in the femur marrow cavity after intravenous transfer. This is in contrast to myelocytic BNML cells which home preferentially near the endosteum of femoral bone marrow (BM) before they start dividing.[12] Intrathecally inoculated lymphocytic L4415 leukemic cells show in comparison to the myelocytic BNML cells, a more pronounced leptomeningeal infiltration, mimicking the pattern of leptomeningeal infiltration observed in the respective human leukemias.[13] This paper describes the cell population growth kinetics, immunophenotyping and the use of the model for chemo- and radiotherapy studies.

2.2.3 MATERIALS AND METHODS

Animals
Inbred male WAG/Rij (Wistar Albino Glaxo / Rijswijk) rats at 10-14 weeks of age were used (body weight, 200 to 250 g). The rats were bred at the SPF animal breeding facility of our institute and housed in plastic cages with water and food pellets *ad libitum.*

The L4415 rat leukemia
The L4415 lymphocytic leukemia was induced in our laboratory by total body irradiation of a female WAG/Rij rat with 0.5 MeV neutrons (dose; 5 cGy). Cytologically this leukemia is classified as a lymphocytic leukemia with accumulation of lymphoblasts in the Giemsa stain,

which are negative in the Graham Knoll, Sudan Black and Esterase stains. At autopsy, leukemic animals show enlarged lymph nodes (LN), thymus, spleen and liver and massively infiltrated BM. The major cause of death after inoculation of leukemic cells is significant enlargement and rupture of the spleen. The leukemia is *in vivo* passaged through intravenous (i.v.) inoculation. A large stock of the third passage has been prepared and stored in liquid nitrogen.

Preparation of leukemic spleen cell suspensions
Monocellular suspensions were obtained by mincing the enlarged spleens of end-stage leukemic animals and gently squeezing through a nylon gauze. The cells were washed and suspended in Hanks' buffered balanced salt solution (HBSS; Gibco Paisley, Scotland). The number of nucleated cells was counted, adjusted to the final cell concentration required and inoculated i.v. in a volume of 1 ml.

Blood- and bone marrow suspensions
For hematological follow-up during the experiments, peripheral blood samples were obtained by orbital punctures. The blood was collected in small plastic tubes containing ethane-diamino-tetra-acetic acid (EDTA; British Drugs Houses Ltd., England) to prevent coagulation. When white blood cells (WBC) were prepared for immunophenotypic analysis the erythrocytes were lysed first (lysing buffer: 0.15 M NH$_4$Cl, Merck, Darmstadt; 0.01 M NaHCO$_3$, Merck; 0.1 mM EDTA, Merck). Subsequently the suspension was centrifuged, the pellet was resuspended in HBSS and the WBC were counted.

BM cells were isolated by repeated flushing of the cavity of tibiae and femora with HBSS. A single cell suspension was obtained by sieving the bone marrow through a nylon gauze.

Cell counting
BM- and peripheral WBC were counted in Türks' solution and erythrocytes in phosphate buffered saline (PBS; NPBI, Amsterdam, The Netherlands) in a Bürker hemocytometer. For platelet counting, peripheral blood samples were diluted in 'Diluid azide free' (7.83 g/l NaCl, 0.36 g/l Na$_2$EDTA, 0.28 g/l KCl, 0.26 g/l K$_2$HPO$_4$, 2.35 g/l NaH$_2$PO$_4$; J. T. Baker, Deventer, The Netherlands) and counted in a Baker Diagnostics platelet analyzer (#810).

Immunophenotyping
Single cell suspensions of BM, splenocytes or peripheral WBC in PBS/BSA (PBS, 1% BSA, Sigma, St. Louis, MO, U.S.A.; 0.01% NaN$_3$, Merck, Darmstadt) were labeled with murine monoclonal antibodies (MCA's) directed to specific rat cell surface antigens (Table 2.2.1). Subsequently the cells were incubated with FITC-conjugated rat-anti-mouse Ig (Jackson Laboratories, Maine, U.S.A.) and analyzed on a FACScan flow cytometer operating with a single argon (488 nm.) laser (Becton Dickinson, Mountain View, CA, USA). For data

analysis, the FACScan research software program Consort 30 (Becton Dickinson) was used. Specificity, origin and reference number of the MCA's used are listed in Table 2.2.1. The OX-8[14,15], OX-19[16,17] and OX-27[18] hybridoma cell lines were kindly

Table 2.2.1 Specificity of the Monoclonal Antibodies Used.

MCA	Specificity	References
OX-27	class I, BN rat (RT-1.A^n)	Jefferies *et al.*[18]
U9F4	class I, WAG/Rij rat (RT-1.A^u)	Stet *et al.*[21]
ER-13	class II, [I$_a$] (RT-1.B)	Stet *et al.*[20]
OX-19	CD5	Mason *et al.*[16]; Dallman *et al.*[17]
ER-2	CD4	Thomas *et al.*[19]
OX-8	CD8	Barclay *et al.*[14]; Brideau *et al*[15]

donated by Dr. A. Williams (Oxford, U.K.). Hybridoma cell lines secreting MCA's ER-2[19], ER-13[20] and U9F4[21] were a generous gift of Dr. J. Rozing (IVVO-TNO, Leiden, The Netherlands). The Mark-1 hybridoma was obtained from the American Type Culture Collection (Rockville, M.D.). All antibodies were produced intraperitoneally (i.p.) in BALB/c mice and purified over a protein A column (Dr. P. H. van der Meide, Dept. of Chronic Diseases, MBL-TNO, Rijswijk, The Netherlands).

Growth characteristics

Rats were inoculated with graded numbers of L4415 cells (i.v.) to define the correlation between leukemic cell dose and survival time. In treatment-efficacy experiments rats were given a fixed dose of leukemic cells, each group consisted of 8 rats. Every two or three days after injecting 10^6 L4415 cells, blood samples were taken and three rats were sacrificed and examined. Changes in peripheral blood cell counts, spleen- and liver weight and the size of other organs were recorded.

Chemo- and radiotherapy

Graded doses of cyclophosphamide (Cy; ASTA, Weesp, The Netherlands) were given 10 days after inoculation of 10^6 L4415 cells. Cy was dissolved in 0.9% NaCl (NPBI, The Netherlands) and injected i.p. in a volume of about 1 ml.

Total body irradiation (TBI), doses ranging from 0.0 Gy to 8.0 Gy, followed by syngeneic bone marrow transplantation (BMT) were given 10 days after injection of 10^6 L4415 leukemia cells. The rats were irradiated unilateral in plastic cages, containing 8 animals at the same time, using a Philips X-ray generator (300 kV, 10 mA) at a dose rate of 48 cGy.min^{-1}. Subsequent inoculation of 10^8 syngeneic BM cells i.v. was done on the same day.

Survival time assay

This assay is based on the linear correlation between the number of leukemic cells inoculated and the survival time of the rats.[9-11] The anti-leukemic effect of the treatment used can be deduced from the increase of life span (ILS) compared to untreated leukemic control animals. The treatment inducing an ILS of 3.6 days corresponds with the survival time of animals given a 10-fold lower tumor load and is referred to as a 1 log cell kill (LCK).

2.2.4 RESULTS

2.2.4.a Growth characteristics

After inoculation of graded numbers (10^2 - 10^6) of L4415 cells in WAG/Rij recipient rats, an inverse linear relationship was observed between the number of injected leukemic cells and the survival time (Figure 2.2.1). One log cell number corresponds with 3.6 days survival time. The median survival time in WAG/Rij rats after 10^6 L4415 cell inoculation (i.v.) was 18 days. All animals died of splenic rupture or severe pulmonary hemorrhage. At autopsy grossly enlarged liver, spleen, thymus and lymph nodes (LN) were found. Femoral bone marrow, normally dark red, turned yellowish-pink as a result of massive infiltration by leukemic lymphoblasts.
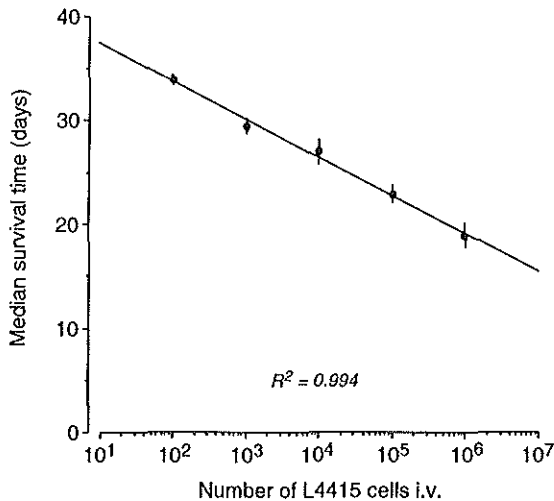
Figure 2.2.1 Correlation between L4415 leukemic cell dose (i.v.) and survival time (n=8 rats per point). Logarithmic regression analysis results in a correlation coefficient of variation of 0.994 (R^2). Vertical bars indicate S.E.

Thirteen days after inoculation of 10^6 L4415 cells in male rats, the first changes in the recipient caused by leukemic cell proliferation were noticed. Thymus-, LN- (data not shown) and more conspicuously, liver- and spleen size increase. At the terminal stage, the spleen

even reaches 10 fold its original weight; liver weight increases from 10 to 20 grams (Figure 2.2.2).
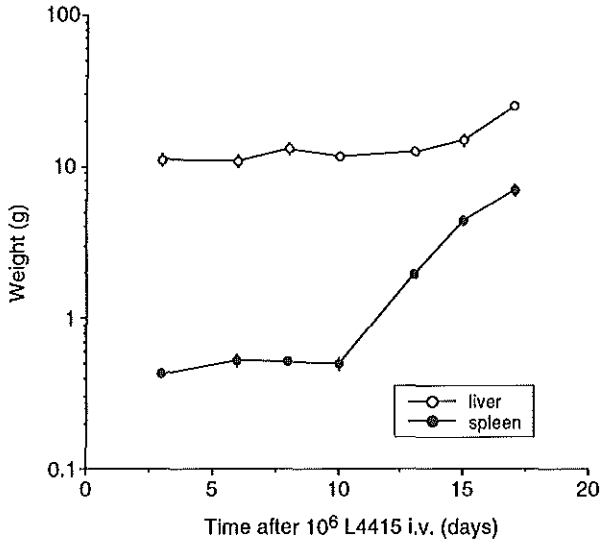
Figure 2.2.2 Increase in spleen and liver weight at various time points after inoculation of 10^6 L4415 cells i.v. (n=3 rats per point). Vertical bars indicate S.E.

Reduced normal hemopoiesis caused by infiltration and proliferation of leukemic cells in the BM cavity, results in severe thrombocytopenia from day 13 (Figure 2.2.3). As a consequence, pulmonary- and gastrointestinal hemorrhage are frequently found
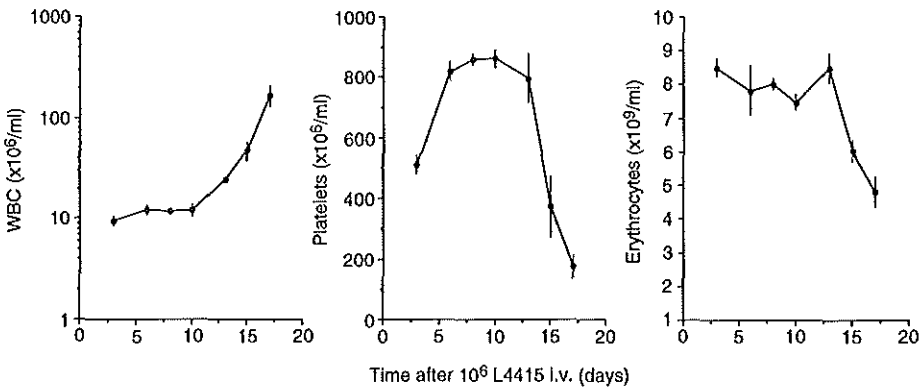
Figure 2.2.3 Changes in peripheral WBC, platelet and erythrocyte counts at various time points after inoculation of 10^6 L4415 cells i.v. (n=3 rats per point). Vertical bars indicate S.E.

and the animals suffer from severe anemia. At the same time a sharp increase in the number of peripheral WBC is observed, mainly consisting of leukemic lymphoblasts.

2.2.4.b Immunophenotyping

When BM, splenocytes and peripheral blood samples were taken from healthy and terminal stage leukemic animals for immunophenotypic analysis, remarkable changes in percentages of cells reacting with the various antibodies were found. This is a result of changes in the cell population-composition, because of L4415 infiltration, rather than changes in antigen expression itself. In the BM a shift of CD5 positive cells from 7.7 % in normal rats to 72.0 % in full blown leukemic animals was striking (Table 2.2.1; reacting with antibody OX-19[16,17]). Splenocytes and WBC of leukemic animals show a strong decrease in cells expressing class II, CD4 and CD8 antigens (MCA ER-13[20], ER-2[19] and OX-8[14,15], respectively) whereas an increase in CD5 positive cells was observed (Figure 2.2.4). These three compartments are apparently heavily infiltrated with L4415 cells. Additionally, leukemic cells show a high expression of WAG/Rij rat-specific MHC class I (RT-1.A^u, MCA U9F4 [21]) and, as a control, no expression of BN rat-specific MHC class I (RT-1.A^n, MCA OX-27[18]) antigens. Based on these data the L4415 can be classified as an acute leukemia originating from immature T-cells.
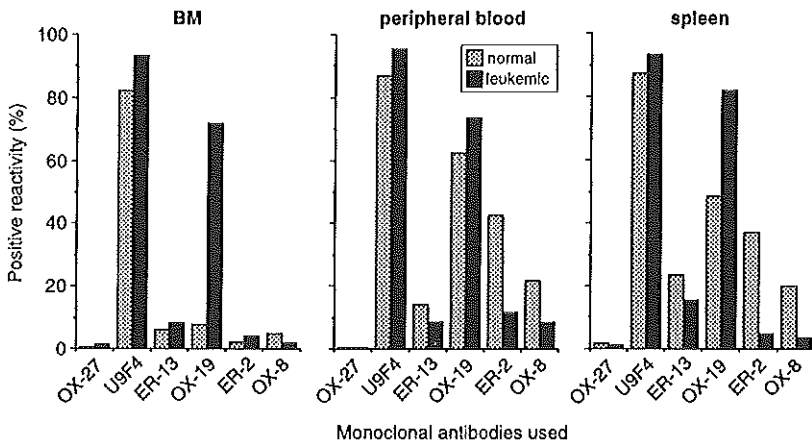
Figure 2.2.4 Monoclonal antibody (MCA) reactivity in BM-, peripheral blood- and spleen cells of healthy and terminal L4415 leukemic rats. MCA's used were specific for: BN class I, RT-1.A^n (OX-27), WAG/Rij class I, RT-1.A^u (U9F4), class II [I$_a$], RT-1.B (ER-13), CD5 (OX-19), CD4 (ER-2) and CD8 (OX-8; see Table 2.2.1).

2.2.4.c Chemo- and radiotherapy

Ten days after inoculation of 10^6 L4415 cells, rats were treated either with graded doses of Cy or with increasing doses of TBI followed by syngeneic BMT to investigate the *in vivo* chemo- and radiosensitivity of this lymphocytic leukemia.
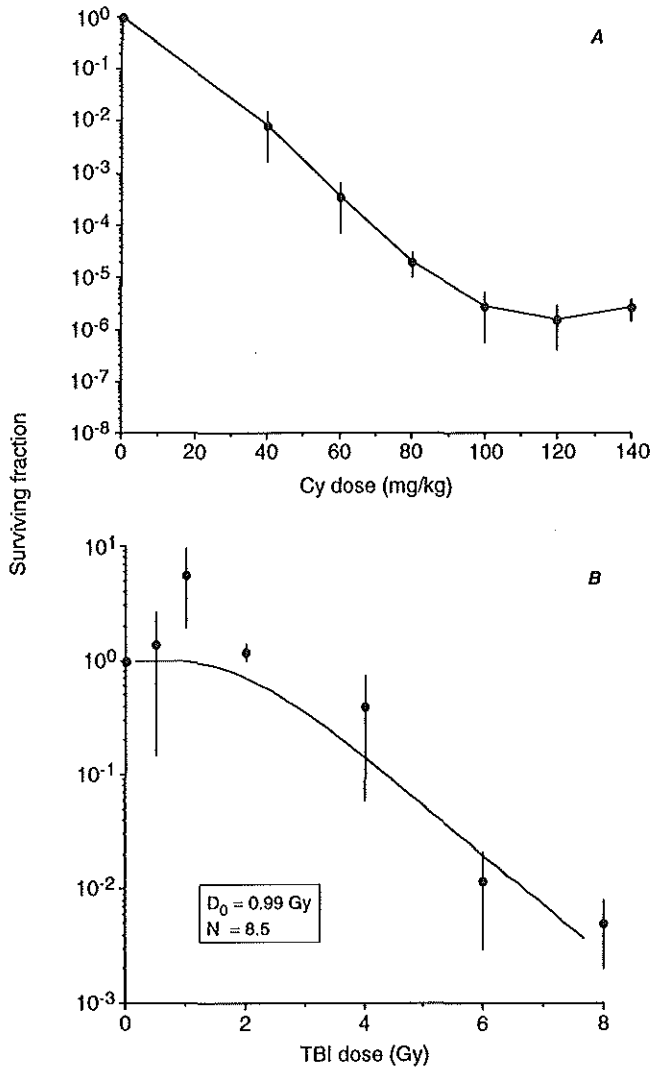
Figure 2.2.5 *In vivo* sensitivity of L4415 leukemia to increasing doses of A: cyclophosphamide (i.p.) and B: X-rays (TBI) followed by syngeneic BMT (10^8 cells i.v.). n=16 rats per point. D_0 is the dosage resulting in a surviving fraction of 0.37, N is the extrapolation number of the linear curve with the Y-axis. Single dose treatment was given 10 days after inoculation of 10^6 L4415 (i.v.). Survival time was translated into the surviving fraction of L4415 cells (see text). Vertical bars indicate S.E.

At relatively low dose Cy therapy, a strong anti-L4415 effect was observed which increased up to a dose of 100 mg Cy per kg body weight where a plateau was reached (Figure 2.2.5A). From the increase of life span (ILS), it could be calculated that up to 6 logs of leukemic cells could be killed with a single injection of Cy.

TBI in the low dose range ($\leq$ 2.0 Gy) did not result in an ILS compared to unirradiated leukemic animals. Higher doses of X-rays (> 2.0 Gy) resulted in an antileukemic effect; a 2.9 LCK was found when the leukemic recipients were exposed to 8.0 Gy (Figure 2.2.5B). As characteristics of the radiosensitivity of the L4415 leukemia, the dosage giving a surviving fraction of 0.37 (D_0) was calculated to be 0.99 Gy with an extrapolation number (N) of 8.5. The single therapy regimens used did not result in treatment-related mortality.

2.2.5 DISCUSSION

A transplantable rat leukemia is presented which may serve as a relevant model for human ALL.

Immunophenotyping of the BM, spleen and peripheral WBC of terminal leukemic WAG/Rij rats shows that, taken into account that the BM and the spleen of the full blown leukemic animals consist of very few cells other than leukemic cells, L4415 leukemia is characterized phenotypically as $CD5^+$, $CD4^-$ and $CD8^-$. Leukemic blast cells in the Giemsa stain are negative in the Graham Knoll, Sudan Black and Esterase stains.[12] Taken together, the L4415 leukemia can be characterized as an acute lymphocytic leukemia of the immature T-cell type.

After inoculation, the first signs of leukemia are found in the BM and the spleen which presumably are the organs where the L4415 cells lodge initially. Ten days after injection, changes in the spleen- and liver weight are observed. The enlargement of the spleen is often leading to spleen rupture. Assuming that the number of normal spleen and liver cells does not change significantly, this increase in weight is due to infiltration and rapid proliferation of leukemic cells.[10,11] Leukemic cells rapidly proliferate and accumulate in the BM and as a consequence normal hemopoiesis is suppressed leading to severe thrombocytopenia and anemia. At the same time, drastic changes are observed in the peripheral blood, i.e. a rapid increase in peripheral WBC numbers. Most LN and the thymus are also enlarged to a great extend by massive infiltration with leukemic cells (data not shown).

During the experiments reported here, no clinical signs of central nervous system (CNS) involvement are observed. However, from previous studies it appeared that after i.v. injection of L4415, clusters of leukemic cells were seen in the ventricles, Virchow-Robin spaces and pia mater in 23, 69 and 92 percent of the rats, respectively. In the spinal cord, epidural and subarachnoidal infiltration occurred in 100 and 36 percent of the animals respectively.[13] Apparently the animals die from systemic leukemia prior to the time that the deposits of the leukemia in the CNS cause neurological symptoms.

Death is due to a combination of several factors. Severely depressed hemopoiesis quickly leads to severe thrombocytopenia and as a consequence bleeding occurs at various sites (gastrointestinal tract, respiratory tract, etc.). The infiltrated lungs often present serious

problems with respect to cardiovascular function and the massively infiltrated liver quite likely has an impaired function. Thus bleeding, spleen rupture and severely impaired organ functions are the main causes of death of the leukemic animals.

After repeated inoculations of *in vitro* lethally irradiated (20 Gy) L4415 cells into normal WAG/Rij rats (i.p.), no beneficial effect is observed when these rats are challenged thereafter with viable leukemic cells compared to previously untreated control animals (data not shown). Thus unlike many virally induced leukemias the L4415 is non-immunogenic as is the case for most human leukemias.

In this study an inverse linear relationship was found between the log number of L4415 leukemia cells inoculated and the survival time of the WAG/Rij recipients. A reduction of 1 log in leukemic cell dose results in a 3.6 days ILS. This relationship forms the basis for evaluating the efficacy of a variety of anti-ALL therapies to be tested in this model.

Cyclophosphamide and total body irradiation are included in most conditioning regimens for BMT in the treatment of leukemia. To study the chemo- and radiosensitivity of L4415 *in vivo*, Cy or TBI were given to animals ten days after inoculation of 10^6 L4415 cells. Increasing doses of Cy result in increasing antileukemic effects with a maximum of 6 LCK when 100 mg per kg body weight is given. At this dose a plateau phase is reached which is uncommon for alkylating drugs. So far, an explanation is lacking. Chemotherapeutical sanctuaries (e.g. the central nervous system) may contain leukemic cells which subsequently cause a relapse. TBI was followed by syngeneic BMT to prevent marrow aplasia. Low doses of X-rays (≤ 2.0 Gy) seem to have no anti-L4415 effect, the survival time of the recipients remaining the same. The higher dose range of TBI (> 2.0 Gy) results in a decreased surviving fraction of leukemic cells with a 2.9 LCK after the highest TBI dose studied, i.e. 8.0 Gy. As characteristics of the radiosensitivity of the L4415 leukemia, a D_0 of 0.99 Gy (dosage resulting in a surviving fraction of 0.37) with an extrapolation number of 8.5 were calculated.

Based on the data reported on L4415 lymphocytic leukemia in this article (hepatosplenomegaly, lymphadenopathy, significantly reduced normal hemopoiesis, non-immunogenicity and the cyclophosphamide- and radiosensitivity) and the previous findings on the distribution of the L4415 cells in the femoral bone marrow[12] and the pattern of leptomeningeal infiltration[13], the L4415 acute lymphocytic leukemia growing in the WAG/Rij rat has a number of characteristics in common with human ALL and may be regarded as a relevant pre-clinical model. Studies on different treatment modalities (e.g. chemo- and radiotherapy, interleukin-2 treatment and BMT, including 'graft-versus-leukemia') are currently being performed.[22]

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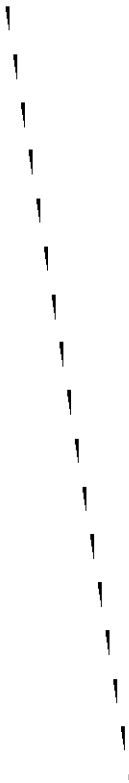
QUANTITATIVE STUDIES ON GRAFT-VERSUS-LEUKEMIA AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION IN RAT MODELS FOR ACUTE MYELOCYTIC AND LYMPHOCYTIC LEUKEMIA*

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3.1 SUMMARY

The major shortcoming of present day treatment of in leukemia treatment with bone marrow transplantation (BMT) remains the relapse of leukemia. It has become clear that a graft-versus-host reaction (GVHR) is accompanied by a graft-versus-leukemia reaction (GVLR) which may prevent a leukemia relapse. In two non-immunogenic rat leukemia models, i.e. the Brown Norway acute myelocytic leukemia (BNML) and the WAG/Rij acute lymphocytic leukemia L4415, total body irradiation (TBI) was given to induce 'minimal residual disease' (MRD). Subsequently, it was attempted to evoke a GVLR by using syngeneic- or allogeneic BMT, with or without addition of graded numbers of lymphocytes. In both leukemia models the addition of high numbers of syngeneic lymphocytes to the syngeneic graft had no antileukemic effect. Allogeneic marrow grafts, which contain at the most 8% lymphocytes, only resulted in a GVLR when splenocytes were added. The therapeutic window was found to be narrow, i.e. in fully mismatched BMT the number of allogeneic splenocytes resulting in a significant GVLR (2-3 log leukemic cell kill) without inducing (lethal) acute GVHD was critical. Increasing the number of allogeneic spleen cells added to the allogeneic BM-graft induces lethal acute GVHD. Depletion of lymphocyte subsets showed that both CD4 and CD8 positive cells are involved in the induction of GVHD. To date, our data indicate that the GVLR is an allogeneic effect, inseparable from GVHD.

3.2 INTRODUCTION

Allogeneic BMT is being used with increasing frequency in the treatment of patients with hematological disorders such as leukemia.[1] The success of BMT is limited by post-transplantation complications, including GVHD and leukemia relapse. The intensity and incidence of GVHD is dependent on the extend of genetic disparity (major and minor histocompatibility complex antigens) between the donor and host[2,3] as well as the quantity and quality of the lymphocytes present in the bone marrow (BM) graft.[4]

Studies using animal models have shown that allogeneic T-cells in allogeneic BMT, which are the major contributors to GVHD,[5] are held responsible for a reduction in the incidence of leukemia relapse compared with isologous/autologous BMT by inducing a graft-versus-leukemia reaction (GVLR).[6,7] Clinical data show that T-cell depletion (TCD) of allogeneic donor BM decreases the incidence of both acute and chronic GVHD but at the same time significantly increases the rate of leukemia relapse.[8,9] Likewise, in a few cases the GVLR was shown to be abrogated by post-BMT GVHD prophylactic immuno-suppression (CsA).[10,11] The discontinuation of CsA at first signs of leukemia relapse induced complete hematologic and cytogenetic remission and these patients remain free of disease with a long follow up. Clearly, GVHD seems to be associated with the GVLR which eradicates the residual leukemic cells. Whether it will be possible to optimize the beneficial GVLR without increasing the risk of lethal GVHD remains to be seen.

In some animal models, the GVLR can be separated from the GVHD reactivity[12,13] leading to the speculation that GVL and GVH reactive cells are distinct. These preclinical data support the assumption that specific antigens are present on leukemic cells not shared by normal cells of the host. However, no leukemia-associated specific surface markers have been identified so far on human leukemic cells. Alternatively, the same cells may mediate both GVL and GVH reactions but quantitative differences in sensitivity of leukemic and normal cells could account for apparently distinct GVL/GVH reactions, inducing a 'threshold' effect. So far, no convincing clinical evidence has been reported on a GVLR separable from GVHD.

To study GVL in animal models relevant to human leukemia, the experiments reported here were performed using the acute myelocytic leukemia (AML) growing in the inbred Brown Norway rat strain (BNML)[14-16] and the L4415 acute lymphocytic leukemia (ALL) growing in the inbred WAG/Rij rat strain.[17,18] Both leukemia models show a reproducible growth pattern after intravenous (i.v.) transfer. Survival time following i.v. inoculation is inversely related to the logarithm of the number of leukemic cells in the inoculum. This log-linear relationship between leukemic cell load and survival time allows an estimation of the treatment-induced antileukemic effect, in terms of log leukemic cell kill (LCK), by recording prolongation of survival. Thus, these rat models offer the opportunity to compare the antileukemic effects of syngeneic and (fully mismatched) allogeneic BMT in the treatment of AML and ALL. Through the addition of graded numbers of spleen cells to the BM graft, the

induction of various degrees of GVLR and/or GVHD and the feasibility of their separation were studied.

3.3 MATERIALS AND METHODS

Animals

Inbred male BN, WAG/Rij (Wistar Albino Glaxo / Rijswijk) and PVG rats at 10-14 weeks of age (body weight: 200 - 250 g) were used in the experiments described. The rats were bred at the SPF animal breeding facility of our institute and housed in plastic cages with water and food pellets *ad libitum.*

The BNML model

The non-immunogenic BNML leukemia was induced in a female BN rat by 9,10-dimethyl-1,2-benzanthracene (DMBA). It shows a slow, reproducible growth pattern upon i.v. cellular transfer within the BN strain and shares many characteristics with human acute (pro-) myelocytic leukemia: severe suppression of normal hemopoiesis, no specific leukemia-associated antigens present, diffuse intravascular coagulation, response to chemotherapy, presence of clonogenic leukemic cells (*in vitro* and *in vivo*; ED_{50} = 25 cells) and no evidence for a virus as an etiological agent. The leukemia is passaged by intravenous (i.v.) cellular transplantation. The rat leukemia model for human AML has been described extensively.[14-16]

The L4415 model

The L4415 lymphocytic leukemia was induced in a female WAG/Rij rat that received 5 cGy total body neutron irradiation (0.5 MeV). Immunophenotyping has shown that the relatively slowly growing, non-immunogenic L4415 is an immature T-cell leukemia (CD 4-, CD 8-, CD 5+). The L4415 leukemia is passaged *in vivo* through (i.v.) cellular transfer, showing a reproducible growth pattern. L4415 leukemia has been described in detail before.[17,18]

Preparation of leukemia or normal spleen cell suspensions

Monocellular suspensions were obtained by mincing the spleens of end-stage leukemic or untreated animals and gently squeezing the suspension through nylon gauze. The cells were washed and suspended in Hanks' balanced salt solution (HBSS; Gibco Paisley, Scotland). The number of nucleated cells was counted and adjusted to the required cell concentration.

Blood- and bone marrow cell suspensions

Peripheral blood (PB) samples were obtained by orbital punctures. The blood was collected in small plastic tubes containing ethane-diamino-tetra-acetic acid (EDTA; British Drugs Houses Ltd., UK) to prevent coagulation. When white blood cells (WBC) were prepared for

immunophenotypic analysis the erythrocytes were lysed first (lysing buffer: 0.15 M NH_4Cl, Merck, Darmstadt; 0.01 M $NaHCO_3$, Merck; 0.1 mM EDTA, Merck). The suspension was then centrifuged, the pellet resuspended in HBSS and WBC counted. BM cells were isolated by repeated flushing of the cavity of tibiae and femora with HBSS. A single cell suspension was obtained by sieving the BM through nylon gauze. Subsequently the cells were washed, counted and the cell concentration adjusted.

Cell counting

Nucleated cells were counted in Türks' solution and erythrocytes in phosphate buffered saline (PBS; NPBI, Amsterdam, The Netherlands) in a Bürker hemocytometer. For platelet counting, peripheral blood samples were diluted in 'Diluid azide free' (7.83 g/l NaCl, 0.36 g/l Na_2EDTA, 0.28 g/l KCl, 0.26 g/l K_2HPO_4, 2.35 g/l NaH_2PO_4; J. T. Baker, Deventer, The Netherlands) and counted in a Baker Diagnostics platelet analyzer.

Total body irradiation

Lethal total body irradiation (TBI, 8.5 Gy) was given 7 days after injection of 10^5 leukemia cells. The rats were irradiated unilaterally in plastic cages, containing eight animals at a time, using a Philips X-ray generator (300 kV, 10 mA) at a dose rate of 48 cGy.min^{-1}. Inoculation of 10^8 BM cells i.v. (BMT) +/- spleen cells was done the next day.

Immunophenotyping

Single cell suspensions of splenocytes, BM cells or peripheral WBC in PBS/BSA (PBS, NPBI, Amsterdam, The Netherlands; 1% BSA, Sigma, St. Louis, MO, U.S.A.; 0.01% NaN_3, Merck, Darmstadt) were labeled with murine monoclonal antibodies (MCA's) directed to specific rat cell surface antigens (Table 3.1), or incubated with PBS/BSA serving as negative controls. Subsequently, the cells were incubated with FITC-conjugated rat anti-mouse Ig (Jackson Laboratories, Maine, U.S.A.) and analyzed on a FACScan flow cytometer operating with a single argon (488 nm) laser (Becton Dickinson, Mountain View, CA, USA). For data analysis, the FACScan research software program Consort 30 (Becton Dickinson) was used. The U9F4 and all OX hybridoma cell lines were kindly donated by Dr. D. Mason (Oxford, UK). Hybridoma cell lines secreting MCA's ER-2 and ER-13 were a generous gift of Dr. J. Rozing (IVVO-TNO, Leiden, The Netherlands). All antibodies were produced intraperitoneally (i.p.) in BALB/c mice and purified on a protein A column (Dr. P. H. van der Meide, Department of Chronic Diseases, MBL-TNO, Rijswijk, The Netherlands).

Survival time assay

This assay is based on the log-linear relation between the number of leukemia cells inoculated and the survival time of the rats.[15,16,18] The anti-leukemic effect of the treatment used can be calculated from the increase of life span (ILS) compared to untreated leukemic control

animals. An ILS of 4.0 days for the BNML and of 3.6 days for the L4415 model, equals a 1 log leukemic cell kill (LCK).

Bioassay for residual leukemia

To detect residual leukemic cells in animals dying of acute GVHD, BM and/or spleen cell suspensions were prepared and 10^8 cells were inoculated in the tail vein (i.v.) of normal, syngeneic recipient rats. The proportion of leukemic cells in the inoculum can be derived from the survival time of the secondary recipients. As shown before, this assay allows the detection of very low numbers of residual leukemia cells (10-100) in the inoculum.[15,16]

Lymphocyte subset depletion

To study the role of CD4 and CD8 positive lymphocytes in the development of GVL and/or GVHD, these lymphocyte subsets were depleted from the spleen cell suspension added to the allogeneic marrow graft using MCA's (OX-19, ER-2 and OX-18; Table 3.1, section 3.4.5) and magnetic beads (Dynal®, Oslo, Norway). The Dynabeads® (M-450 sheep anti-mouse IgG) were coated with purified sheep polyclonal immunoglobulins (Ig) binding to all mouse immunoglobulins of the G subclasses (IgG), thus binding to the murine (IgG) MCA's used.

Before use, the beads were washed in a large volume of PBS/BSA (PBS, NPBI; 1% BSA, Sigma; 0.01% NaN_3, Merck) and isolated using the magnetic particle concentrator (Dynal MPC®-6; 6 strong magnets). Subsequently, the beads were coated with the lymphocyte subset-specific MCA by adding 20 µg MCA to 1 mg of beads and incubated overnight at 4°C on a rotating device. After two wash steps, removing the free MCA, the beads were added to single cell suspensions of splenocytes in a bead to $CD4^+$ or $CD8^+$ cell ratio of 10:1. The bead-cell mix was incubated rotating for 1 h at 4°C. The CD4 or CD8 negative splenocytes were isolated using the magnet and by removing the 'supernatant'. The depletion efficacy was verified by double staining and FACS analysis.

Statistical Analysis

Survival time analysis of rats from the different experimental groups was performed according to Peto *et al.*[19,20]

3.4 RESULTS

3.4.1 Syngeneic BMT in the BNML model

Seven days after inoculation of 10^5 BNML cells (i.v.), BN recipients were subjected to lethal total body irradiation (TBI: 8.5 Gy, X-rays), and subsequently rescued with syngeneic BM 24 hours later. The resulting increase in life span (ILS) compared with the leukemia-control group reflects the significant ($p = 0.001$) anti-leukemic efficacy of the irradiation. TBI

induced a median ILS of 10 days reflecting a 2.5 log leukemic cell kill (LCK; Figure 3.1), with all rats finally dying from leukemia relapse. To estimate the residual leukemic cell load at the time of BMT, the survival time after the irradiation was monitored. The syngeneic BMT group survived 38.5 days post-TBI, indicating 10^3 leukemic cells survived (according to standard BNML growth curves), meeting the criteria for 'minimal residual disease' (MRD).[15,16]
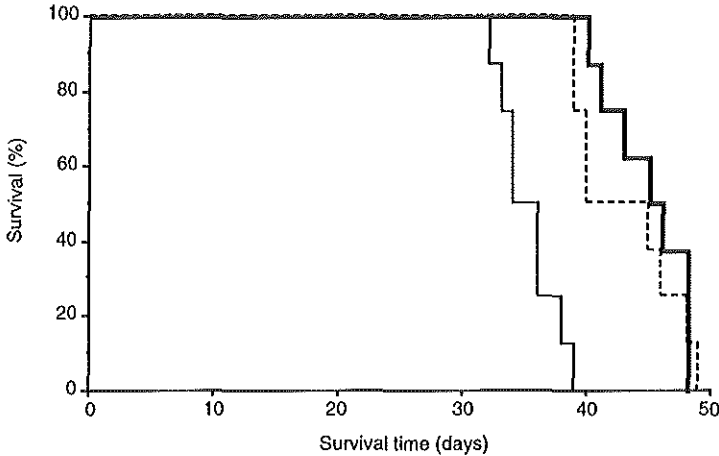
Figure 3.1 BNML model studies: Survival curves of leukemic animals treated with syngeneic BMT +/- splenocytes *versus* the leukemia control group (*n* =8 rats per group). The rats were inoculated with 10^5 BNML cells (i.v.) on day 0, subjected to TBI (8.5 Gy X-rays) on day 7 and given syngeneic marrow ± splenocytes on day 8. All transplanted animals died of leukemia relapse; (—————) 10^5 BNML i.v.; (══════) + syngeneic BMT; (---------) + syngeneic BMT + 10^8 splenocytes.

To evaluate whether syngeneic lymphocytes exert a possible anti-leukemic effect, 10^8 syngeneic splenocytes were added to the syngeneic bone marrow graft since rat BM contains few T lymphocytes (5-8% positive staining on incubation with a CD 5 specific monoclonal antibody; Table 3.1) compared with human-BM (20-30%). This high number of lymphocytes did not result in differences in survival (or: time of death from leukemia relapse; Figure 3.1; *p* = not significant).

3.4.2 Syngeneic BMT in the L4415 model

L4415 cells (10^5) were inoculated at day 0 and subsequently TBI (8.5 Gy, X-rays) and syngeneic BMT (10^8 BM cells i.v.) were performed at day 7 and 8, respectively. TBI yielded a significant ILS (*p* = 0.001) correlating with 4.4 LCK (16 days median ILS: Figure 3.2). The median survival time of the rats receiving a syngeneic graft was 27 days post-TBI, indicating a residual leukemic cell burden of 10^3 cells (MRD).

Addition of 10^8 WAG/Rij splenocytes to the syngeneic BM inoculum did not influence the survival time compared with the transplantation of syngeneic BM alone (data not shown).

Thus, similar to the observation in the BNML model, no GVLR was observed in the rat ALL model in the syngeneic situation.

Figure 3.2 L4415 model studies: Survival curves of leukemic animals treated with allogeneic BMT +/- splenocytes *versus* the syngeneic BMT- and leukemia control groups (n =8 rats per group). The rats were inoculated with 10^5 L4415 cells (i.v.) on day 0, subjected to TBI (8.5 Gy X-rays) on day 7 and given syngeneic or allogeneic marrow ± splenocytes on day 8. All transplanted animals died of leukemia relapse but for the groups receiving 10^7 and 10^8 splenocytes; +10^7 : Three rats died of leukemia relapse, the five recipients with prolonged survival showed severe GVHD morbidity combined with hepato-splenomegaly; +10^8 : All rats died of acute GVHD; (—————) 10^5 L4415 i.v.; (▬▬▬▬) + syngeneic BMT; (··········) + allogeneic BMT; (— — — — —) + allogeneic BMT + 10^6 splenocytes; (–·–·–·–·–·) + allogeneic BMT + 10^7 splenocytes; (•••••••••) + allogeneic BMT + 10^8 splenocytes.

3.4.3 Allogeneic BMT in the BNML model

The WAG/Rij rat (RT-1.A^u) was used for allogeneic BMT in the BNML model as a fully mismatched donor for the BN rat (RT-1.A^n). Recipient rats were injected with 10^5 BNML cells and irradiated at day 7 to induce a state of MRD. At day 8, syngeneic BMT or allogeneic BMT with or without graded numbers of allogeneic splenocytes was given. Increasing numbers of allogeneic spleen cells were added to the allogeneic marrow graft to simulate the higher lymphocyte content in human, non T-cell-depleted, allogeneic marrow. The survival times of the syngeneic and allogeneic BMT groups were similar, indicating no additional antileukemic effect of the allogeneic BM graft (Figure 3.3). All rats died from leukemia relapse in the syngeneic BMT group. After allogeneic BMT one rat (out of eight) died from treatment-induced toxicity.

Addition of 10^5 allogeneic splenocytes to the allogeneic BM graft resulted in an increase in the median survival time of the recipients of 11 days (2.8 LCK) compared with the syngeneic and allogeneic marrow only transplanted groups. All rats died from leukemia relapse indicating a significant (p = 0.05) GVLR without the induction of acute lethal GVHD.

When higher numbers of allogeneic splenocytes were added to the allogeneic BM graft, i.e. 10^6 splenocytes, this led to a reduction in the median survival time compared with the group receiving 10^5 allogeneic splenocytes and severe GVH-morbidity in five out of eight rats (Figure 3.3). At autopsy, these five animals showed signs of leukemia (hepato-splenomegaly) and of GVHD (erythema, hair loss and diarrhea) both of which may have contributed to their death. Three rats died of leukemia without macroscopic signs of GVHD. The increase in survival compared with the syngeneic and allogeneic marrow only transplanted groups was not significant (NS).
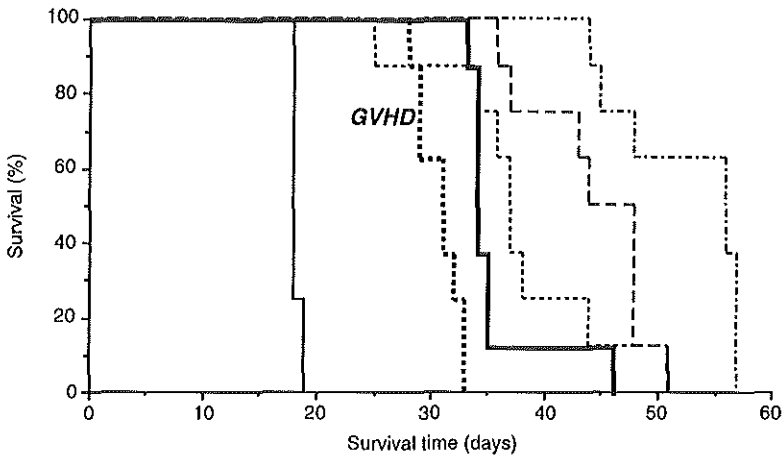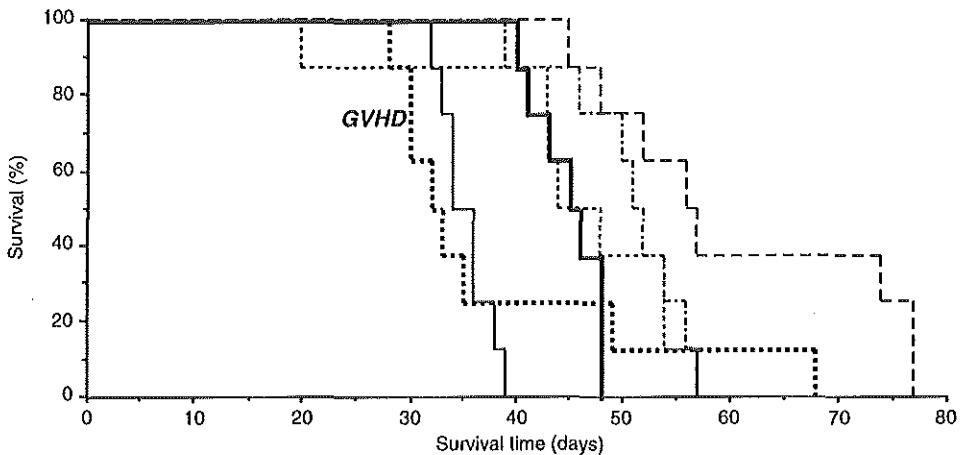

Figure 3.3 BNML model studies: Survival curves of leukemic animals treated with allogeneic BMT +/- splenocytes *versus* the syngeneic BMT and leukemia control groups (n =8 rats per group). The rats were inoculated with 10^5 BNML cells (i.v.) on day 0, subjected to TBI (8.5 Gy X-rays) on day 7 and given syngeneic or allogeneic marrow ± splenocytes on day 8. All transplanted animals died of leukemia relapse but for the group receiving 10^7 splenocytes, all these rats died of acute GVHD; (_____) 10^5 BNML i.v.; (━━━━) + syngeneic BMT; (··········) + allogeneic BMT; (– – – –) + allogeneic BMT + 10^5 splenocytes; (–··–··–··) + allogeneic BMT + 10^6 splenocytes; (•••••••••) + allogeneic BMT + 10^7 splenocytes.

When 10^7 allogeneic splenocytes were added to the allogeneic marrow, all animals died of acute GVHD (Figure 3.3) without any macroscopic evidence of leukemia at autopsy. Six (of eight) rats died from acute GVHD whereas two animals showed a prolonged survival (death at days 49 and 67). They died from protracted GVHD.

3.4.4 Allogeneic BMT in the L4415 model

After an initial inoculum of 10^5 L4415 cells, TBI (8.5 Gy) at day 7 induced MRD. Subsequent inoculation of allogeneic PVG rat (RT-1.A^c) BM (10^8 BM cells i.v. at day 8) caused a small ILS (3 days=0.8 LCK; p = NS) compared with syngeneic BMT. The addition of increasing numbers of PVG-splenocytes resulted in increasing antileukemic effects (Figure 3.2). The 'maximum' GVLR (3.3 LCK, i.e. 12 days median ILS compared with the syngeneic BMT group) was induced with 10^6 allogeneic spleen cells, leukemia relapse was the only

obvious cause of death. Therefore, as in the BNML model, a significant ($p = 0.003$) GVLR could be induced without causing obvious GVHD.

Addition of 10^7 splenocytes caused a more prolonged median survival time (22 days median ILS compared with syngeneic BMT=6.1 LCK ($p = 0.002$)), but at the same time severe GVH-morbidity in the five rats dying at days 56 and 57 (Figure 3.2). At autopsy, these animals showed signs of both leukemia (hepatosplenomegaly) and GVHD (erythema, hair loss and diarrhea). The three rats dying at days 44, 45 and 48 showed no signs of GVHD and died of leukemia relapse.

Finally, 10^8 PVG-splenocytes were added to the allogeneic BM graft resulting in a 100% incidence of lethal acute GVHD with a median survival time of 23 days after the time allogeneic BMT was applied (Figure 3.2). At autopsy there were no signs of recurrent leukemia.

3.4.5 Immunophenotyping

To study the expression of class I and class II antigens and T-cell (sub)populations in normal rats, MCA's OX-27 (class I: RT-1.A^n),[21] U9F4 (class I: RT-1.A^u),[22] ER-13 (class II: RT-1.B),[23] OX-19 (CD5),[24] ER-2 (CD4)[25] and OX-8 (CD8)[26] were used (Table 3.1).

Table 3.1 Specificity of the monoclonal antibodies (MCA's) used and antigen expression of splenocytes, BM cells and peripheral WBC of the recipient rat strains*

MCA's	specificity	BN			WAG/Rij		
		spleen	BM	PB	spleen	BM	PB
OX-27	class I BN (RT-1.A^n)[21]	87.3	86.7	82.1	1.6	0.2	0.4
U9F4	class I WAG/Rij (RT-1.A^u)[22]	1.5	0.7	0.7	77.3	72.0	86.6
ER-13	class II [Ia] (RT-1.B)[23]	33.1	10.5	37.8	23.2	6.0	14.2
OX-19	CD5; pan T-cells[24]	36.1	5.1	36.5	48.3	7.7	62.2
ER-2	CD4; T$_{helper/inducer}$[25]	34.4	12.9	37.5	36.9	12.1	42.7
OX-8	CD8; T$_{suppressor/cytotox}$[26]	10.8	1.8	7.7	22.0	3.9	22.4

* Values are mean of 8 measurements and represent cell percentages positively reacting with the MCA's indicated

Table 3.1 shows data of spleen, BM and peripheral blood nucleated cells of the two rat strains labeled with these MCA's. As can be expected, most cells from the BN rat react with OX-27 and most cells from the WAG/Rij rat with U9F4. Both these MCA's crossreact with PVG class-I antigens (RT-1.A^c; data not shown).

To establish the degree of chimerism in recipients of allogeneic marrow, peripheral blood samples were drawn at various time points. WBC were immunophenotyped for class I antigens using the MCA's OX-27 and U9F4. The mean (+/- S.D.) percentage positive cells of

the control rats *versus* recipients of allogeneic BM cells alone, clearly prove full donor chimerism at each time point investigated after allogeneic BMT (Figure 3.4).
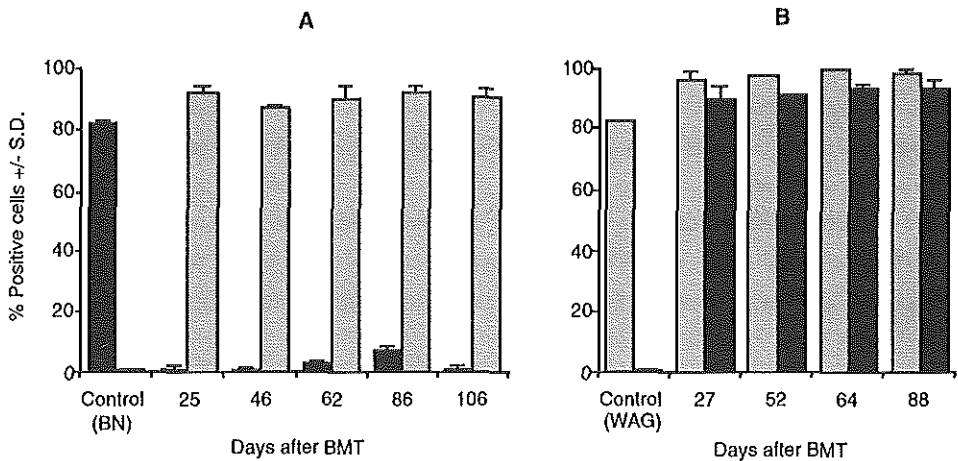
Figure 3.4 Immunophenotyping peripheral WBC of allogeneic transplanted rats (BM alone) at various time points post-BMT *versus* untreated animals. Mean percentages positively reacting cells from 4 animals +/- S.D. are represented: (A) WAG/Rij to BN BMT; all WBC show a high allogeneic and low ('background-level') recipient-class I expression. (B) PVG to WAG/Rij BMT; all WBC show a high expression of both types class I (like normal PVG cells); (■) OX-27 (class I, BN); (□) U9F4 (class I, WAG/Rij).

3.4.6 *Spleen and liver weights at autopsy*

Among the most characteristic symptoms of leukemia in both models, significantly increased spleen (> 8x) and liver weights (> 4x) are observed in rats during the terminal stages of leukemia development. In rats dying from acute lethal GVHD after allogeneic BMT, addition of the highest number of allogeneic splenocytes, i.e. 10^7 WAG/Rij splenocytes in the BNML model and 10^8 PVG splenocytes in the L4415 model, leads to normal or even reduced spleen and liver weights as observed at autopsy, i.e. 24 days after conditioning with TBI. Thus, in 24 days the residual leukemia cells were not able to grow out and cause spleen- and liver enlargement, indicating a significant anti-leukemic effect in conjunction with acute GVHD.

3.4.7 *Residual leukemic cells in acute GVHD*

To detect the presence of small numbers of surviving leukemia cells in the BM or the spleen of recipient rats suffering from acute GVHD, the animals were sacrificed 24 hours before death was expected (as was observed in previous experiments). Spleen and BM cells were isolated and 10^8 cells were inoculated into untreated rats, i.e. in BN and WAG/Rij rats to assay for residual BNML and L4415 cells, respectively. Subsequently, in both models the survival time assay revealed that leukemia cells were not present as none of the secondary recipients died of leukemia (data not shown). This suggests that acute (lethal) GVHD is accompanied by a significant GVLR, eradicating residual leukemia.

3.4.8 *Depletion of lymphocyte subsets*

Allogeneic BMT in the BNML model was used to study the depletion of CD4 and CD8 positive lymphocytes from the added WAG/Rij spleen cells and the effect on GVL and/or GVHD induction. The BMT 'setting' (leukemia dose, TBI and BMT) was similar to the one described earlier.

After the 'one step' lymphocyte depletion procedure, double staining and FACS analysis showed that about a two log depletion of both the CD4$^+$ and CD8$^+$ subsets of the splenocytes was achieved (CD4: from 38.1% to 0.1%; CD8: from 22.7% to 0.5%). Assuming that 40% of the unseparated WAG/Rij spleen cells are CD4$^+$ and 20% CD8$^+$ (Table 3.1), the number of CD4$^-$ or CD8$^-$ splenocytes added to the allogeneic BM graft were adjusted, i.e. 60% and 80% of the number of unseparated lymphocytes, respectively. Unfortunately, in this experiment we did not observe a GVLR when either 10^5 unseparated, 6x10^4 CD4 depleted or 8x10^4 CD8 depleted WAG/Rij lymphocytes were added to the allogeneic marrow graft. All these recipient rats died of leukemia relapse with similar survival times as the rats receiving allogeneic marrow only (median survival 42-45 days; data not shown). Therefore, we can not draw any conclusions from the depletion of CD4 or CD8 positive lymphocytes as regards the GVLR.
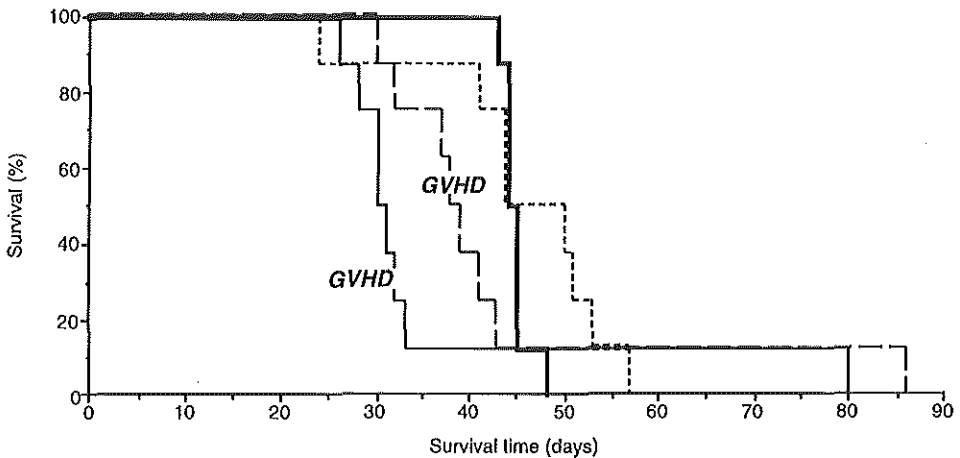
Figure 3.5 Selective depletion of allogeneic spleen cell populations in the BNML model. Survival curves of leukemic animals treated with allogeneic BMT + allogeneic splenocytes *versus* the allogeneic marrow only transplanted group (*n* =8 rats per group). The rats were inoculated with 10^5 BNML cells (i.v.) on day 0, subjected to TBI (8.5 Gy X-rays) on day 7 and given allogeneic marrow ± splenocytes on day 8. All rats transplanted with allogeneic marrow only or allogeneic marrow + 6x10^6 CD4-depleted splenocytes died of leukemia relapse. The groups receiving 10^7 unseparated splenocytes or 8x10^6 CD8-depleted splenocytes died of acute graft-versus-host disease (GVHD); (——————) + allogeneic BMT; (——————) + allogeneic BMT + 10^7 unseparated splenocytes; (- - - - - - - -) + allogeneic BMT + 6x10^6 CD4-depleted splenocytes; (— — —) + allogeneic BMT + 8x10^6 CD8-depleted splenocytes.

Adding 10^7 unseparated allogeneic spleen cells to the allogeneic marrow graft induced acute lethal GVHD in seven out of eight recipient rats with a median survival time of 30.5 days. One animal unexpectedly survived till day 80 and died of an unknown cause (Figure 3.5). The addition of 6×10^6 CD4 depleted lymphocytes did not induce evident GVHD. Seven out of eight rats died from leukemia relapse with a median survival time of 50 days, similar to the rats transplanted with allogeneic marrow only. One rat died at day 24 from BMT-related complications. However, 8×10^6 CD8 depleted lymphocytes did induce lethal acute GVHD in seven out of eight rats, but with an eight days prolongation of median survival time (38.5 days). One rat died at day 86 of an unknown cause (Figure 3.5). These data indicate that both CD4 and CD8 positive allogeneic lymphocytes are involved in the induction of GVHD, the CD4$^+$ lymphocytes being the major contributors and the CD8$^+$ cells responsible for an additional effect.

3.5 DISCUSSION

The involvement of immunocompetent donor T-cells in the GVLR is well established both in animal models[6,7] and in man.[8,9] It is not clear whether the mechanisms of the GVLR are separable from those operative in GVHD. Evidence has been reported for at least two elements of the GVLR; one that is dependent on the presence and the intensity of GVHD[27] and the other operating without clinically overt GVHD.[28,29] Thus, the latter would allow enhancing the GVLR while minimizing GVHD. In this way a more safe and complete eradication of leukemia cells still present after conditioning might be achieved. However, to determine whether this can be accomplished, a better understanding of the mechanisms of the GVLR and its mediators is warranted. The present paper describes our studies on the GVLR in the BNML and the L4415 rat leukemia models using syngeneic and allogeneic BMT with and without the addition of graded numbers of splenocytes.

In both models, the survival times of the animals receiving syngeneic BMT indicated a residual leukemic cell load of 10^3 cells. Addition of high numbers of mature syngeneic lymphocytes to the marrow inoculum did not change the leukemia growth pattern or the survival of the recipient rats. These data indicate that high numbers of syngeneic lymphocytes do not induce a GVLR.

To study GVL after allogeneic BMT in the BNML model, the WAG/Rij rat was applied as a fully mismatched donor. Transplantation in MRD of allogeneic BM alone did not induce a GVLR. Addition of graded numbers of WAG/Rij splenocytes to the inoculum induced an increase in the survival time of the recipient rats which was 'maximal' (without inducing severe GVHD) with 10^5 spleen cells. This significant increase in survival time reflects a 2.8 log leukemia cell kill. Higher numbers of allogeneic splenocytes caused the induction of acute lethal GVHD.

For GVL studies in the ALL rat leukemia model, the PVG rat was used as a mismatched donor. Transplantation of allogeneic BM without splenocytes added to the graft induced a small GVLR (0.8 LCK), which may be caused by different alloreactivity (donor-host combination) as observed in the BNML model where no GVLR was observed after marrow only grafting. The survival time was further increased when 10^6 PVG spleen cells were added to the BM graft with a significant GVLR (3.3 LCK) without clinically evident GVHD. Addition of 10^7 allogeneic lymphocytes induced severe GVHD, but a significantly prolonged survival time corresponding with a 6.1 log leukemic cell kill. The GVHD induced 2.8 extra logs of leukemia cell kill compared with 10^6 additional splenocytes (6.1 *versus* 3.3 LCK, respectively). Thus, in this situation, the GVLR induced a significantly stronger antileukemic effect compared with TBI (4.4 LCK). Further increased numbers of allogeneic splenocytes in the graft induced acute lethal GVHD and significantly reduced life span.

Immunophenotyping the WBC of the recipient rats given an allogeneic BMT, clearly showed that they were full donor chimeras. Autopsy revealed that acute lethal GVHD was accompanied by a significant GVLR. To study the possible presence of residual leukemia cells in the animals suffering from acute GVHD, BM and spleen cells were isolated just before their expected death and subsequently transferred in high numbers to untreated recipients. As these rats did not die from leukemia, apparently no leukemic cells were present in the inoculum. Thus, the acute lethal GVHD at the same time exerts a strong anti-leukemia activity.

The median survival time after (syngeneic) BMT indicates a residual leukemia cell load of 10^3 BNML or L4415 cells, respectively. In both leukemia models a significant GVLR (approximately 3 log leukemia cell kill) without signs of GVHD can be induced using allogeneic BMT, with a critical number of additional allogeneic splenocytes. Nevertheless these animals all die from leukemia relapse; no cures were observed. As the first symptoms of GVHD become evident 8-10 days after allogeneic BMT, the GVLR is probably not operational instantly after BMT. Once evoked, the GVLR may have a continuing antileukemic effect resulting in an increase in the survival time which, using our criteria to calculate antileukemic effects of 'instant-effect' treatment, reflects a 3 log leukemia cell kill. Apparently, the leukemia cell elimination by the GVLR could not keep up with the rate of leukemia cell proliferation, ultimately leading to leukemia relapse.

The data show that syngeneic BMT serves as a model for human autologous BMT. Transplantation of allogeneic rat BM alone induces neither a significant GVLR nor GVHD and therefore resembles human allogeneic (pan-) T-cell depleted BMT, the addition of critical numbers of allogeneic lymphocytes to the marrow graft evokes a significant GVLR and/or acute lethal GVHD, serving as a model for full marrow grafting in humans. To date, we have not been able to separate the GVLR from GVHD. In the experiments discussed here, unseparated T-cell populations (splenocytes) have been added to the marrow graft. Several reports mention the *in vitro* induction of cytotoxic T lymphocytes specific for MHC-matched

allogeneic leukemia or nonleukemia cells or lysing both leukemic and non-leukemic target cells,[30,31] and their correlation to the GVLR *in vivo* in a murine model.[32]

In our first attempt to deplete lymphocyte subsets from the added allogeneic splenocytes, we were unable to induce an evident GVL effect without GVHD. Nevertheless, our preliminary data on the 'two log' CD4^{+} and CD8^{+} lymphocyte depletion in the BNML model indicate that the depletion of CD4^{+} cells completely eradicated the induction of GVHD. When CD8^{+} lymphocytes were depleted it was observed that the incidence of GVHD was 100%, but that death due to GVHD was postponed in all recipient rats. These results are in agreement with experimental data reported by Truitt et al. and Sykes et al. They have shown that CD4^{+} T-cells play a critical role in causing GVHD in fully mismatched murine strain combinations, and that CD8^{+} T-cells given alone do not cause acute GVHD.[33-35] Moreover, it was proven in these murine models that CD4^{+} cells can contribute to GVL effects.[33] A promising clinical trial of selective donor CD8^{+} cell depletion (HLA-identical sibling BMT for CML in chronic phase) demonstrated a reduction in, but not absence of, GVHD, whereas leukemic relapse rates were low.[36,37] Further studies using the rat models will reveal whether the addition of graded numbers of certain (MHC-mismatched) lymphocyte subpopulations to the marrow graft, e.g. CD4^{+} or CD8^{+} cells, may induce a wider therapeutic window.

How do the GVL-results relate to the current clinical experience? Mathematical model computations based on the relapse rate of leukemia patients with or without GVHD suggest that the GVLR associated with GVHD produces one extra log leukemic cell kill which increases the cure rate from 40 to 90% or, in other words, decreases the leukemia relapse rate from 60 to 10%.[38] The higher LCK induced by the GVLR in the rat models may be caused by the fact that donor and recipient are fully mismatched *versus* the MHC matched transplantations performed in patients. As calculated in human HLA-matched allogeneic BMT with full marrow, the GVLR only induces a one log leukemia cell kill together with significant (lethal) GVHD. Therefore in acute leukemia it is advisable to use T-cell depletion which eliminates death from GVHD[16] and seek other means to achieve additional leukemia cell kill, such as intensification of the conditioning regimen or treatment with low-dose chemotherapy after BMT.[39]

Furthermore, transfusions of donor leukocytes in patients showing leukemia relapse (chronic myelocytic leukemia) after allogeneic BMT to induce a GVLR have recently been reported. The majority of patients had complete hematologic and cytogenetic remission persisting 28-91 weeks.[40,41] The value of alternative strategies reported are currently being explored and include; (1) the infusion of interleukin-2 (IL-2) post-BMT to induce a GVLR,[42,43] (2) an improved GVL effect of *in vitro* IL-2 activated syngeneic BM prior to BMT and followed by IL-2 treatment,[43] and (3) combination of allogeneic lymphocyte infusion and IL-2 administration post-BMT.[44] Studies of the nature of lymphocyte (sub) populations and cytokine involvement in GVLR and GVHD in response to major and/or minor histocompatibility complex antigens in allogeneic BMT are essential to develop

therapeutic approaches further. Controversial reports on the application of IL-2 therapy in cancer treatment promoted us to initiate IL-2 therapy studies post allogeneic BMT in the rat models. Experiments in the BNML model clearly show a GVL effect (without GVHD) of low-dose IL-2 administration and induction of acute lethal GVHD when high dose IL-2 therapy is used after allogeneic BMT.[45] These data show that the rat leukemia models are suitable tools for further GVLR/GVHD studies. Currently, several possible treatment strategies to optimize the GVLR without the induction of severe GVHD are under investigation.

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CHAPTER 4

IN VITRO RESISTANCE OF THE BROWN NORWAY RAT ACUTE MYELOCYTIC LEUKEMIA (BNML) TO LYMPHOKINE-ACTIVATED KILLER ACTIVITY*

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4.1 SUMMARY

In *in vivo* allogeneic bone marrow transplantation studies with the Brown Norway (BN) rat as recipient and the WAG/Rij rat as allogeneic donor a significant graft-versus-leukemia (GVL) effect is observed. Studies were performed to investigate whether lymphokine activated killer (LAK) cells play a role in this GVL effect. Splenocytes from WAG/Rij and BN rats were activated *in vitro* by recombinant human interleukin-2 (rhIL-2) for 5-6 days. The cytolytic activity of these LAK cells was tested on four rat solid tumor cell lines, i.e. an urethra carcinoma, a rhabdomyosarcoma and two lung tumors and on leukemic cells derived from the BN rat acute myelocytic leukemia (BNML) and the WAG/Rij acute lymphocytic leukemia (L4415). The panel of target cells also included the murine cell lines P815 and YAC. Both WAG/Rij and BN LAK cells were not capable of lysing the leukemic cells in contrast to significant cytolytic activity on the rat solid tumor cell lines, P815 and YAC. BNML cells showed to be resistant to lysis by human NK cells. Phenotypical analysis of the rat LAK population revealed a decrease in the CD4/CD8 ratio compared to the unstimulated splenocyte population. Rat LAK cells displayed no antibody-dependent cellular cytotoxicity (ADCC) on the leukemic cells, whereas IL-2 stimulated human peripheral blood cells showed moderate ADCC activity on the leukemic cells. To investigate whether cytokines play a role in lysis of leukemic target cells, graded numbers of LAK cells and leukemic cells were co-cultivated for seven days in an agar-based colony culture system. This resulted in moderate suppression of leukemic colony formation. From the current *in vitro* studies it appears that the graft-versus-leukemia observed in *in vivo* allogeneic bone marrow transplantation studies is probably not due to a direct leukemic cell kill by LAK cells.

4.2 INTRODUCTION

To prevent severe graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation, as part of the therapy in hematologic malignancies, T-cell depletion of the marrow graft has proved to be effective.[1] However, the successful prevention of GVHD is accompanied by an increased risk of graft failure and leukemia relapse: i.e. the graft-versus-leukemia (GVL) effect is reduced.[2-4] There is controversy on whether the GVL effect can be separated from the GVHD effect. T-cells are considered major effectors in both reactions,[5-7] although LAK cells and NK cells may also have a role in the GVL reaction.[8,9]

Interleukin-2, a 15-kDa glycoprotein, is a major growth factor for T-cells. It also is required to generate lymphokine-activated killer (LAK) activity.[10] These cells are able to lyse a variety of tumor cells with limited effect on normal cells. This lytic activity is not restricted to solid tumors, but also acute lymphocytic leukemia (ALL) and acute myelocytic leukemia (AML) blasts are reported to be susceptible to the lytic activity of autologous and allogeneic LAK cells *in vitro* .[11-15] Clinical studies confirm these preclinical results for solid tumors[16,17] and acute leukemias.[18,19]

The rat leukemia model (Brown Norway rat acute myelocytic leukemia, BNML) used as a relevant model for human acute myelocytic leukemia in this study, has been described in detail elsewhere (origin, classification, transplantation procedure, growth characteristics, etc.).[20,21] In the past years this experimental leukemia has served as a model to study the detection and treatment of 'minimal residual disease'.[22-24] *In vivo* results showed a GVL effect after allogeneic bone marrow transplantation (BMT) with 10^5 allogeneic spleen cells added to the graft.[25]

The aim of the experiments reported here was to investigate whether GVL activity could be induced *in vitro* by LAK cells and, if so, to establish the phenotype of these effector cells.

4.3 MATERIALS AND METHODS

BNML cells
BNML cell suspensions were made from spleens of full-blown leukemic rats. Spleens were minced with scissors and suspended through a nylon sieve in Hanks buffered salt solution (HBSS) to obtain monocellular suspensions.

The LT12 in vitro BNML cell line
An *in vitro* growing cell line of the BNML was established,[26] based on culturing BNML cells in the presence of a stromal layer. Ultimately BNML cells were capable of autonomous growth. The promyelocytic nature of the BNML cells was retained. Most of the *in vitro* assays presented here are performed with this *in vitro* growing cell line.

Splenocyte isolation

The spleens from WAG/Rij and BN rats (age 12-20 weeks) were removed aseptically, minced with scissors and passed through a nylon sieve. Mononuclear cells were obtained after centrifugation on lymphocyte separation medium (Organon Technika Corporation, Durham, NC, USA). Cells recovered from the interface were washed twice in HBSS and counted prior to use.

Generation of LAK cells

To remove monocytes and macrophages, fresh splenocytes were incubated in plastic culture flasks for 1 hour at 37°C in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Seromed, Biochrom Berlin, Germany). Plastic-non-adherent cells were collected and cultured for five to seven days in RPMI-1640 medium containing 1000 U/ml recombinant human interleukin-2 (rhIL-2; Eurocetus, Amsterdam), 0.5 µM 2-mercapto-ethanol, 0.5 µM indomethacin (Sigma, St.Louis, USA) and 100 U/ml of penicillin at an optimal cell density of 2-3 x 10^6 cells/ml.

Target cells

A panel of target cells was used to determine lytic activity of the effector cells. R1, a WAG/Rij rat rhabdomyosarcoma; RUC-II, a BN rat urethra carcinoma; L37, a moderately differentiated squamous cell lung carcinoma (WAG/Rij) and L44, an anaplastic lung carcinoma (BN). These WAG/Rij and BN tumor cell lines were selected to circumvent differences in species and strain between target and effector cells as is the case with P815 and YAC. As leukemic cell targets the BN acute myelocytic leukemia (BNML), its *in vitro* growing subline LT12 and the WAG/Rij acute lymphocytic leukemia (L4415) were used.[27] The widely used P815, an NK-resistant mouse mastocytoma and YAC, an NK-sensitive Moloney virus induced lymphoma, were used as controls.

Assay for cell-mediated cytotoxicity

Cytotoxicity was determined in a standard 4 hour ^{51}Cr-release assay. For the labeling 1 x 10^6 target cells were incubated with 100 µCi of Na$_2$^{51}CrO$_4$ (Amersham, UK) for 60 minutes at 37°C. After washing, the target cells were added at a concentration of 2 x 10^3 cells/well to various numbers of effector cells. Effector to target ratios were 50:1, 25:1, 12:1 and 6:1. All experiments were performed in triplicate in 96-well round-bottomed microtiter plates.

Incubation was then performed at 37°C for 4 hours. Supernatants were harvested with a Skatron harvesting system and counted in a gamma counter. Spontaneous and maximal release of chromium were determined by incubating target cells in medium alone or with 10% detergent, respectively.

The percentage of specific lysis was calculated by the following equation:

$$\text{Lysis } (\%) = \frac{\text{Experimental release - Spontaneous release}}{\text{Maximal release - Spontaneous release}} \times 100$$

Antibody-dependent cellular cytotoxicity (ADCC)

Human peripheral blood lymphocytes (PBL) were cultured for 7 days in the presence of Epstein-Barr virus (EBV)-transformed B-cell lines, allogeneic PBL and 25 U/ml rhIL-2 yielding a population of cells comprising 56% $CD3^+$, 42% $CD16^+$ and 34% $\gamma\delta^+$ cells. Also a human NK clone was used as effector cell. ADCC was determined in a 4h ^{51}Cr-release assay using P815 target cells. Before the 4 h incubation, rabbit anti-P815 serum (1:1000) was added. Further procedure for determining cytotoxicity was identical as described under cell-mediated cytotoxicity.

Proliferation of LT12 cells

LT12 cells (1.5 x 10^6 per flask) were incubated for 48 h in medium containing 0, 10 or 20 % conditioned supernatant. Conditioned medium was obtained from cultures of WAG/Rij splenocytes in the presence of 25 Gy γ-rays irradiated BN splenocytes. Likewise BN splenocytes were cultured with irradiated WAG/Rij splenocytes. Conditioned supernatants of both cultures were harvested after 72 h. For the proliferation assay, cells were incubated in microtiter plate wells in triplicate at a cell concentration of 4 x 10^5 cells/well in the presence or absence of conditioned medium. Each well was pulsed with 9.25 kBq of ^{3}H-thymidine for 4 h. The wells were harvested on to filter paper and thymidine incorporation was measured by liquid scintillation counting.

Clonogenic assay

The activity of LAK cells on the *in vitro* colony formation of LT12 cells was determined by cocultivating LAK cells with LT12 cells in agar at varying effector/target ratios for 7 days. Plating efficiency was calculated as (the number of LT12 colonies counted/total number of LT12 cells plated) x 100%.

The surviving fraction was calculated by the following equation: (plating efficiency in the presence of LAK cells)/(plating efficiency in the absence of LAK cells) x 100.

Antibodies for phenotyping of LAK cells

Fresh spleen cell suspensions and cultured IL-2 stimulated splenocytes were immuno-phenotyped using a panel of monoclonal antibodies (MCA's): ER13 (anti-class II [Ia], RT-1.B), OX19 (anti-CD5), ER2 (anti-CD4), OX8 (anti-CD8), OX39 (anti-IL-2 receptor), ER15 (macrophages/monocytes).

All OX hybridoma cell lines were kindly donated by Dr. A. Williams (Oxford, UK). Hybridoma cell lines secreting MCA's ER-2, ER-13 and ER-15 were a generous gift of Dr. J.

Rozing (IVVO-TNO, Leiden, The Netherlands). All antibodies were produced intraperitoneally (i.p.) in BALB/c mice and purified using a protein A column (Dr. P.H. van der Meide, ITRI-TNO, Rijswijk).

Cell suspensions in PBS/BSA (PBS; 1% BSA, Sigma, St.Louis, MO, USA; 0.01% NaN_3, Merck, Darmstadt) were incubated for 45 min. at 4°C with optimal concentrations of the specific MCA, then washed twice in PBS/BSA and incubated with fluorescein-isothiocyanate (FITC)-conjugated rat-anti-mouse immunoglobulin (Ig) antibody (Jackson Laboratories, Maine, USA).

Analysis was performed on a FACScan flowcytometer with a single argon (488 nm) laser (Becton Dickinson, Mountain View, CA, USA).

Table 4.1 Lytic activity of WAG/Rij LAK cells and BN LAK cells on a panel of target cells

a. WAG/Rij LAK cells

E/T ratio	P815 L4415	YAC	RIM	RUC-II	LT12	L37	L44	BNML	
6	6.0±0.9	7.3±1.9	14.0±3.3	4.6±0.7	0.2±0.1	1.0	0.0	0.0	0.0
12	9.3±1.4	11.0±2.5	24.6±4.5	6.4±0.8	0.6±0.3	0.0	1.0	0.0	0.0
25	17.9±2.3	18.4±3.3	37.3±5.5	10.4±1.0	1.8±0.6	17.0	8.0	2.0	0.0
50	29.7±3.7	27.9±4.5	47.7±5.7	16.1±1.5	3.1±0.7	38.0	31.0	4.0	0.0
n	*11*	*7*	*7*	*7*	*10*	*1*	*1*	*1*	*4*

b. BN LAK cells

E/T ratio	P815	YAC	RIM	RUC-II	LT12	BNML	L4415
6	12.3±1.4	14.7±5.3	2.5±1.5	2.5±1.5	0.0	0.0	0.0
12	21.3±2.2	21.7±6.0	5.0±2.0	5.0±0.0	0.0	1.0	0.0
25	33.3±3.5	31.0±7.1	9.0±2.0	8.5±0.5	0.0	1.0	0.0
50	44.7±4.6	43.7±7.4	16.5±3.5	13.5±0.5	0.0	1.0	0.0
n	*6*	*3*	*2*	*2*	*4*	*1*	*2*

Lytic activity is expressed as percentage ^{51}Cr-release (mean ± standard error of the mean); E/T ratio, effector to target cell ratio; *n*, number of experiments.

4.4 RESULTS

4.4.1 Cell mediated cytotoxicity

Percentage of specific lysis of the different targets by LAK cells are shown in Table 4.1. LAK cells from both WAG/Rij and BN rats showed very low or no cytotoxicity to LT12 cells in a 4h ^{51}Cr-release assay. Even an overnight incubation did not result in any lysis of LT12 cells. Results obtained with fresh BNML and L4415 cells are identical to that obtained with LT12 cells. All other non-leukemic target cells were lysed to various degrees by these LAK cells. Thus, the lack of cytolytic activity on the leukemic cells is not due to defective activity of the LAK cells, but due to insusceptibility of the leukemic cells.

4.4.2 Phenotypical analysis

To study the phenotype of the cytolytic lymphocytes, FACScan analysis was performed. Results of these assays are showed in Table 4.2. In the WAG/Rij LAK cell populations, a shift in the CD4/CD8 ratio from 1.96 (in the unstimulated splenocytes) to 0.37 (in the LAK cells) is observed. For BN LAK cells, this ratio shifted from 3.09 to 1.54. The percentage of cells that express the IL-2 receptor increased from 2.7 to 22.6% and from 5.1 to 15.6% for the WAG/Rij and BN LAK populations, respectively. The percentage of macrophages in these populations was reduced, as could be expected since the LAK cells are generated from non-adherent cells. No remarkable changes in the percentages of the other cell subpopulations were observed.

Table 4.2 Immunophenotype of WAG/Rij and BN normal spleen and LAK population

MCA's	Target	WAG/Rij		BN	
		Spleen	LAK	Spleen	LAK
ER-13	class II [Ia]	18.9	19.7	33.9	39.9
OX-19	CD5 (pan T)	53.3	53.7	36.1	37.3
ER-2	CD4 (T_h)	40.1	18.4	34.4	27.1
OX-8	CD8 (T_s / T_c)	26.7	54.2	12.0	19.8
ER-15	macr / mono	14.7	7.9	10.5	ND
OX-39	IL-2 receptor	2.7	22.6	5.1	15.6
CD4/CD8 ratio		1.96	0.37	3.09	1.54

Data are expressed as mean percentage of the total cell population; ND, not determined; macr, macrophages; mono, monocytes.

4.4.3 Antibody dependent cellular cytotoxicity (ADCC)

The susceptibility of BNML and LT12 cells to NK-mediated cytotoxicity and ADCC was investigated. Cells of the human NK clone 472 do not lyse P815, BNML and LT12 cells. In an ADCC assay, only the P815 cells were lysed. IL-2 stimulated human PBL (yielding 56% CD3^{+}, 42% CD16^{+}, 34% $\gamma\delta^+$ cells) do not lyse BNML or LT12 cells, whereas 55% lysis of P815 cells was achieved. Up to 30-36% lysis of the leukemic cells is obtained in an ADCC assay with these human PBL as effector cells (Table 4.3), which was clearly effector-cell dose dependent. The results of the ^{51}Cr release assay showed no cytotoxic activity of the rat LAK cells on the leukemic cells. The ability of WAG/Rij and BN LAK cells to perform ADCC on LT12 cells has also been tested in a ^{51}Cr release assay. There was no substantial lysis of the LT12 cells.

Table 4.3 ADCC activity of IL-2 stimulated human PBL, human NK clone 472 and rat LAK cells

Effector	E/T ratio	Specific Lysis (%)					
		P815		LT12		BNML	
		medium	anti-P815	medium	anti-P815	medium	anti-P815
human PBL	1	2	73	0	2	0	7
	3	10	79	2	8	0	10
	9	32	82	5	19	5	21
	27	55	88	4	36	1	30
human NK	1	0	42	0	0		
clone 472	3	0	49	0	0		
	9	0	61	0	0		
	27	1	69	0	0		
WAG	6			0	0		
LAK cells	12			0	0		
	25		29	1	1		
	50	42	68	2	3		
BN	6	6	12	1	0		
LAK cells	12	14	21	0	0		
	25	23	39	0	0		
	50	41	60	1	1		

anti-P815, polyclonal rabbit anti-P815 serum; PBL, peripheral blood lymphocytes; E/T ratio, effector to target cell ratio.

4.4.4 Effect of WAG/Rij or BN supernatant on the growth of LT12 cells

In stead of leukemia cell kill, leukemia growth suppression by soluble growth inhibiting factors could be involved in the GVL effect observed after allogeneic BMT. This situation was simulated *in vitro* by co-cultivation of either WAG/Rij splenocytes with irradiated (25

Table 4.4 Influence of various supernatants on the growth of LT12 cells

Supernatant		Growth	
Type	% (v/v)	Cells/flask (x 10^6)	cpm
Control	0	7.5	37036
	10	6.5	38953
	20	9.5	41748
BN[a]	10	7.0	46214
	20	7.5	44682
WAG/Rij[b]	10	5.0	46174
	20	7.5	48288

1.5×10^6 cells/flask were incubated and cultured for 2 days. ^{3}H-Thymidine incorporation in 4×10^5 cells during 4 h was determined. Supernatants were obtained from 72 h cocultivations of BN splenocytes[a] with irradiated WAG/Rij splenocytes, or the other way around[b].

Gy) BN spleen cells or BN spleen cells together with irradiated WAG/Rij splenocytes. As a result of the allogeneic stimulation, the supernatants of these cultures might contain cytokines with growth inhibiting activity. Addition of up to 20% supernatant to the cell cultures did not result in growth inhibition of LT12 cells as is indicated by equal cell numbers and ^{3}H-thymidine incorporation in control and test groups (Table 4.4).
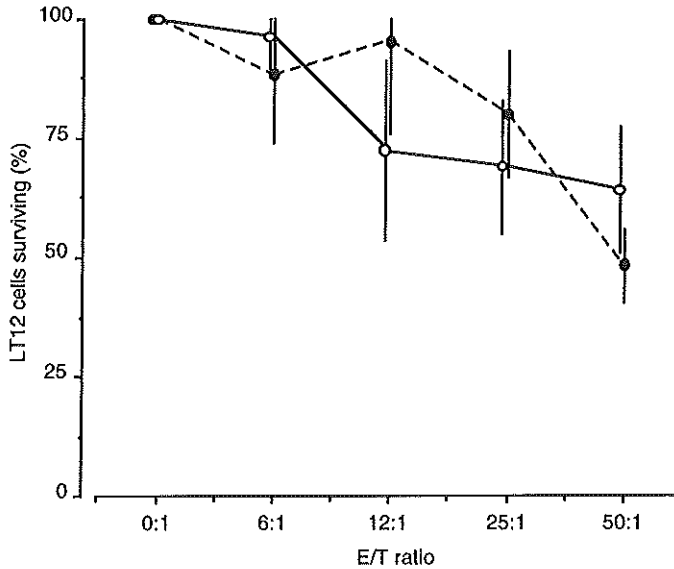
Figure 4.1 Effect of LAK cells on survival of LT12 cells after cocultivation for 7 days in agar. Each data point represents the mean of three experiments. The bar indicates the standard error of the mean. Each data point is calculated relative to the corresponding control group. E/T ratio; effector to target cell ratio; ———O———, 200 LT12 cells/dish; – – ● – –, 2000 LT12 cells/dish.

4.4.5 Agar colony assay

Since no cell kill could be measured in a 4 h ^{51}Cr-release assay and no leukemia growth suppression was induced by (20% v/v) supernatants of allo-stimulated splenocytes, LAK and LT12 cells were cocultured in agar for 7 days. This agar colony assay would allow the detection of an indirect cell kill or a leukemia growth suppressing effect of cytokines produced by the LAK cells. Increasing E/T ratios induce a decrease in LT12 colony formation with a maximal reduction of 50% at a ratio of 50:1 (Figure 4.1).

4.5 DISCUSSION

In several *in vitro* studies it has been demonstrated that some but not all human acute myelocytic and lymphocytic leukemias are susceptible to lysis by autologous and/or

allogeneic LAK cells.[11-15] These results have been confirmed in clinical studies.[18,19] Other reports suggest that LAK cells can mediate a GVL reaction.[8,9]

Since a GVL reaction could be induced in the BNML model after allogeneic BMT and the BNML model is a relevant model for human AML, experiments were performed using this model to study the role of LAK cells in GVL.

As indicated by the 4h ^{51}Cr-release results, fresh BNML cells and the *in vitro* growing subline LT12 are not susceptible to lysis by syngeneic or allogeneic LAK cells, while, in contrast, WAG/Rij and BN solid tumor cells and P815 and YAC are lysed. Although WAG/Rij and BN LAK cells displayed a different pattern of cytotoxic activity on the various target cells, no allo-effect could be distinguished i.e. WAG/Rij LAK cells were not in all cases more cytotoxic on BN target cells than were BN LAK cells. Neither were BN LAK cells more cytotoxic on all WAG/Rij target cells than were WAG/Rij LAK cells. Human NK cells were not capable of killing BNML and LT12 cells either. Only moderate lysis of BNML and LT12 cells is performed by stimulated human PBL in an ADCC assay.

Immunophenotypic analysis of the normal spleen and IL-2 activated spleen populations showed a preferential presence of CD8-positive cells over CD4-positive cells in the activated population. The percentage of cells expressing the IL-2 receptor was also increased. These phenomena occurred in both BN and WAG/Rij populations.

Little is known about the mechanism of LAK-mediated cytotoxicity. Adhesion of effectors to targets may be critically important,[28] although the nature of the target antigens needed for lysis by LAK cells still remains to be elucidated. Apparently, lack of expression of adhesion molecules on BNML and LT12 cells may be the cause of their resistance to lysis. LT12 and BNML cells were shown to be negative for expression of ICAM-1 (Unpublished data of K. Smits et al., Dept. of Pathology, Leiden University). So far, it is not clear whether the *in vivo* GVL effect observed in the BNML model after allogeneic BMT is the result of direct cell killing by NK/LAK cells. Lymphokines produced by these cells could also be involved. To study this phenomenon, LT12 cells were cultured in the presence of supernatant of LAK cultures. Addition of up to 20% supernatant did not result in any growth inhibition (data not shown). Due to differences in medium requirements of LT12 and LAK cells, it was impossible to increase the percentage of supernatant above 20%. This might be the reason why we could not demonstrate any growth inhibition.

In vitro IL-2 stimulation of peripheral blood lymphocytes results in the production of lymphokines like tumor necrosis factor alpha. This or other lymphokines could be responsible for the decreased survival of LT12 cells after co-cultivation in agar for 7 days with LAK cells.

In summary, in the current study it was investigated whether GVL activity could be induced *in vitro* by LAK cells. No direct cell killing was observed, it was therefore concluded that the BNML and LT12 cells are LAK-resistant. Yet a significant GVL effect is observed in

in vivo BMT studies when 10^5 WAG/Rij splenocytes are added to the allogeneic marrow graft in BN rats suffering from minimal residual leukemia.[25]

The results of these *in vitro* studies suggest that if LAK cells do play a role in the *in vivo* observed GVL reaction, this effect is most probably not due to direct leukemia cell killing by LAK cells. Another lymphocyte subset that can be involved in the observed GVL effect is the cytotoxic T-lymphocyte subset. Modulation of this subset might enable us to control and improve the GVL effect.

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CHAPTER 5

INTERLEUKIN-2 THERAPY AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION FOR ACUTE MYELOCYTIC LEUKEMIA*

Studies in a relevant Rat Model for AML

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5.1 SUMMARY

One of the major problems in the treatment of leukemia with BMT remains leukemia relapse. It has generally been established that allogeneic BMT, compared with autologous BMT, gives rise to a graft-versus-leukemia reaction (GVLR), usually associated with GVHD. To explore a possible role for post BMT immunotherapy, recombinant human IL-2 therapy has been studied in the Brown Norway acute myelocytic leukemia (BNML), a rat leukemia model relevant for human AML. The antileukemic efficacy of rhIL-2 therapy is studied applying different doses of rhIL-2 after syngeneic or allogeneic BMT. RhIL-2 treatment post syngeneic BMT, showed a small, borderline significant GVLR. Repeated rhIL-2 treatment after allogeneic BMT resulted either in no significant antileukemic effect or in lethal GVHD when 'low' or 'high' doses were administered, respectively. An intermediate dose, however, induced a significant GVLR without the induction of (lethal) GVHD. Transplantation of allogeneic rat BM, which contains only a few lymphocytes, does not result in a significant GVLR or GVHD and thus resembles human HLA matched allogeneic T-cell depleted (TCD) BMT. In conclusion, from the rat studies presented it appears that the GVLR lost by TCD of the allogeneic graft, may be more than fully compensated by IL-2 treatment post allogeneic TCD BMT.

5.2 INTRODUCTION

One of the major problems of present leukemia treatment remains the incidence of leukemia relapse, even after high dose marrow ablative chemoradiotherapy and BMT. A possible approach to improve leukemia free survival is the application of immunotherapy to eradicate MRD.

To augment the immune response against MRD, IL-2 treatment has been introduced after intensive chemotherapy or following autologous BMT. To date, IL-2 has only rarely been given to humans following allogeneic transplantation because of fear of accelerating GVHD. Although GVHD is accompanied by a profound antileukemic effect (graft-versus-leukemia; GVL), it bears the risk of significant morbidity and even mortality.

IL-2 is critical for the activation and proliferation of T lymphocytes. It has been considered, therefore, to play an important role in acute GVHD. However, the role of IL-2 as an effector molecule of GVHD induced damage has been difficult to establish. Serum IL-2 levels are not increased in acute GVHD, whereas increases in IL-2 receptor (IL-2R) levels have been observed.[1] The significance of this observation is doubtful because many cell types have IL-2 receptors and anti IL-2R MCA therapy provides only transient benefit in steroid resistant GVHD.[2] IL-2 can be administered safely after T-cell depleted allogeneic or autologous marrow grafting without causing or accelerating GVHD.[3] In these settings, IL-2 can only be directly implicated in causing a 'GVH like' syndrome when high doses of IL-2 are administered simultaneous with syngeneic or allogeneic lymphokine activated killer (LAK) cells.[4] Even then, much of the IL-2 toxicity is probably mediated through the induction of other inflammatory cytokines such as IL-1 and TNF.[5]

There are a number of reports of phase I/II studies indicating that IL-2 is tolerated following chemotherapy or autografting in doses which produce substantial immunomodulation.[6-8] IL-2 infusions increase both CD3$^+$ and CD3$^-$ LAK cell function and the effector lymphocytes generated can destroy clonogenic leukemia precursor cells *in vitro*. There is also a considerable increase in LAK cell secretion of cytokines such as IFNγ and TNFα which may have additional antileukemic activity.[9]

The first clinical trials applying IL-2 after autologous BMT in the treatment of AML are encouraging. Various trials employing IL-2 after autologous BMT have been reported to assess definitively the effect, if any, on the relapse rate and the biological mechanisms that might be involved.[10,11]

Case reports give evidence on the existence of GVL in man post allogeneic BMT, which could be enhanced by IL-2 treatment without augmenting GVHD.[12] In addition, GVL was induced by inoculation of allogeneic lymphocytes following initial transplantation with T lymphocyte depleted allografts. The GVL effect could be significantly augmented by concomitant administration of a short course of low dose IL-2 in a murine leukemia model.[13] Further studies on IL-2 administration after allogeneic BMT in patients at high risk of relapse

seem warranted, especially because IL-2 production is very low during the first 2 years post BMT.[14]

Here we report on IL-2 therapy in an animal model relevant to human leukemia, i.e. the Brown Norway acute myelocytic leukemia (BNML) growing in the inbred BN rat strain.[15-17] This leukemia model shows a reproducible growth pattern upon i.v. cellular transfer. It has been used extensively for optimizing BMT conditioning treatment regimens and was found to have predictive value for the clinical outcome of new therapeutic approaches.[15,17] Survival time following i.v. inoculation is inversely related to the number of leukemia cells in the inoculum. This log-linear relationship between leukemia cell load and survival time allows an accurate calculation of the therapy induced antileukemic effect, in terms of LCK, by monitoring the prolongation of the survival time. The BNML model, in combination with the WAG/Rij rat as a fully mismatched donor, has proven to be highly informative in GVL/GVHD studies.[18] We have shown that syngeneic BMT serves as a model for autologous BMT. The addition of critical numbers of allogeneic lymphocytes to an allogeneic marrow graft induced significant GVL and/or acute lethal GVHD, appearing to be a realistic model for HLA matched full marrow grafting in humans. However, transplantation of allogeneic rat BM alone, which contains only 5-8% lymphocytes, induces neither GVL nor GVHD and therefore resembles human HLA matched allogeneic T-cell depleted BMT.

Thus, this rat model offers the opportunity to quantitatively estimate the antileukemic effect of IL-2 therapy in minimal residual leukemia, both after syngeneic and allogeneic BMT.

5.3 MATERIALS AND METHODS

Animals
Inbred male BN recipient rats and WAG/Rij (Wistar Albino Glaxo / Rijswijk) donor rats at 10-14 weeks of age (body weight 200 - 250 g) were used in the experiments described. The rats were bred at and supplied by the SPF animal breeding facility of the Medical Biology Laboratory (MBL)-TNO (Rijswijk, The Netherlands) and housed in plastic cages with water and food pellets *ad libitum.*

BNML model
The BNML leukemia was induced in a female BN rat by 9,10-dimethyl-1,2-benzanthracene (DMBA). It shows a slow, reproducible growth pattern upon i.v. cellular transfer within the BN strain and has many characteristics in common with human acute (pro-) myelocytic leukemia: severe suppression of normal hemopoiesis, diffuse intravascular coagulation, response to chemotherapy as in human AML and the presence of clonogenic leukemia cells (*in vitro* and *in vivo*; $ED_{50} = 25$ cells). The leukemia is passaged by i.v. cellular

transplantation. The rat leukemia model for human AML has been described extensively.[15-17] To study allogeneic BMT, the WAG/Rij rat was used as a fully mismatched donor.

Preparation of cell suspensions

BNML (spleen) cells. Monocellular suspensions were obtained by mincing the enlarged spleens of end stage leukemic animals and gently squeezing the suspension through nylon gauze. The cells were washed and suspended in HBSS (Gibco, Paisley, UK). The number of nucleated cells was counted in Türks' solution using a Bürker-type hemocytometer and adjusted to the final cell concentration required for i.v. transfer.

Peripheral blood. PB samples were obtained by orbital punctures. The blood was collected in small plastic tubes containing EDTA (Sigma, St. Louis, MO, USA) to prevent coagulation. When PBL were prepared for immunophenotypic analysis the erythrocytes were lysed first (lysing buffer: 0.15 M NH4Cl, Merck, Darmstadt, Germany; 0.01 M NaHCO3, Merck; 0.1 mM EDTA, Sigma). The suspension was then centrifuged, the pellet resuspended in HBSS and PBL counted.

Bone marrow. BM cells were isolated by repeated flushing of the cavity of tibiae and femora with HBSS. A single cell suspension was obtained by sieving the BM through nylon gauze. Subsequently the cells were washed, counted and the cell concentration adjusted.

High dose chemoradiotherapy

Eleven days after i.v. inoculation of 10^7 BNML cells, leukemic animals carrying approximately 10^9 BNML cells were subjected to CY treatment (ASTA, Weesp, The Netherlands) and 7.0 Gy X-rays TBI the next day (day 12). CY was dissolved in 0.9% NaCl (Merck) and injected i.p. in a volume of approximately 1 ml. The rats were irradiated unilaterally in plastic cages, containing eight animals at a time, using a Philips X-ray generator (300 kV, 10 mA) at a dose rate of 48 cGy/min. This high dose chemoradiotherapy conditioning regimen induces a state of minimal residual disease (MRD) in which few BNML cells reside in the recipient rats. Subsequently, the rats were rescued with syngeneic or allogeneic BM (10^8 BM cells i.v.) after TBI (day 12).

Recombinant human IL-2

IL-2 (rhIL-2; Proleukin® recombinant human des-alanyl-1, serine-125 human interleukin-2) was supplied by EuroCetus (Amsterdam, The Netherlands). The doses applied are expressed in international units (IU). Each vial of lyophilized Proleukin was reconstituted with 1.2 ml MilliQ-water, the resulting clear solution contained 18×10^6 IU (1 mg) Proleukin per ml. Dilutions were prepared using 5% D-glucose (Merck), 1% rat serum. IL-2 was inoculated s.c. which prolongs the serum activity compared with i.v. administration.[19] The post BMT rhIL-2 immunotherapy was applied on days 15-19 and on days 22-26, twice daily, according to

different dose regimens. Thus, each animal received two separate courses of rhIL-2 for 5 days, each given the indicated dose (e.g. 130,000 IU) twice daily (2x130,000 = 260,000 IU rhIL-2/day).

Survival time assay

This assay is based on the log-linear relation between the number of leukemia cells inoculated and the survival time of the rats.[15,17] The antileukemic effect of the treatment used can be deduced from the increase of life span (ILS) of the treated group compared with the untreated leukemia control group (*n*=10 rats/group). A treatment inducing an ILS of 4.0 days corresponds with the extra survival time of animals with a 10-fold lower leukemia cell load and is referred to as a 1 LCK. In addition, gross pathology (e.g. spleen and liver weight, BM smears) will confirm the cause of death (GVHD, take failures, leukemia).

Immunophenotyping of peripheral blood lymphocytes (PBL) post BMT

Chimerism. Single cell suspensions of PBL in PBS/BSA (PBS (NPBI, Amsterdam, The Netherlands); 1% BSA (Sigma); 0.01% NaN_3 (Merck)) were labeled with murine MCA's directed to the BN (OX-27 [20]) or the WAG/Rij inbred rat strain (U9F4 [21]) specific class I Ag (RT-1.A^n or RT-1.A^u, respectively), or incubated with PBS/BSA serving as negative controls. Subsequently, the cells were incubated with FITC-conjugated rat anti-mouse Ig (Jackson Laboratories, Maine, USA) and analyzed on a FACScan flow cytometer operating with a single argon (488 nm) laser (Becton & Dickinson, Mountain View, CA, USA). For data analysis, the FACScan research software program Consort 30 (Becton & Dickinson) was used.

Lymphocyte subsets. Single cell suspensions of PBL were labeled and subsequently analyzed as described above. The results obtained were compared with values observed in untreated control rats. The murine MCA's used were ER-2 [22], OX-19 [23], OX-8 [24] and OX-39 [25] specifically recognizing CD4, CD5, CD8 and CD25, respectively. Other MCA's used were specific for MHC class II Ag ([Ia]: RT-1.B; ER-1)[26], NK cells (anti-NKR-P1; 3.2.3.)[27], macrophages and monocytes (ED1)[28] and the BNML (Rm124)[29].

The OX hybridomas and the U9F4 hybridoma were kindly donated by Dr. D. Mason (Oxford, UK), the hybridoma cell lines secreting MCA's ER-2 and ER-13 were generous gifts from Dr. J. Rozing (IVVO-TNO, Leiden, The Netherlands). The ED-1 and Rm124 hybridomas were obtained from Dr. C.D. Dijkstra (Department of Cell Biology and Immunology, Medical Faculty, Free University, Amsterdam, The Netherlands) and Dr. R.J. Johnson (John Hopkins University, Baltimore, MD), respectively. The antibodies were produced i.p. in BALB/c mice and purified on a protein A column (Dr. P.H. van der Meide, Immunosciences, BPRC-TNO, Rijswijk, The Netherlands). The 3.2.3 Ab was a generous gift from Dr. W. Chambers (Pittsburgh Cancer Institute, Pittsburgh, PA).

Statistical Analysis

Survival time analysis of rats from the different experimental groups was performed according to Peto *et al.*[30] Animals dying from other causes than leukemia were censored in the statistical analysis.

5.4 RESULTS

5.4.1 IL-2 therapy post syngeneic BMT

The increase of life span (ILS) of the animals receiving syngeneic BMT compared with the leukemia control group reflects the antileukemic effect of the conditioning regimen; 60 mg/kg CY i.p. and 7.0 Gy TBI (X-rays) induced a median ILS of 14.5 days, representing a 3.6 LCK, with all rats dying from leukemia relapse as judged by gross pathology (e.g. significantly enlarged spleen and liver; Figure 5.1). The syngeneic BMT group showed a median survival time (MdST) of 30.0 days post BMT, indicating a residual leukemia cell load after high dose chemoradiotherapy of approximately 2×10^5 cells (deduced from standard BNML growth curves).
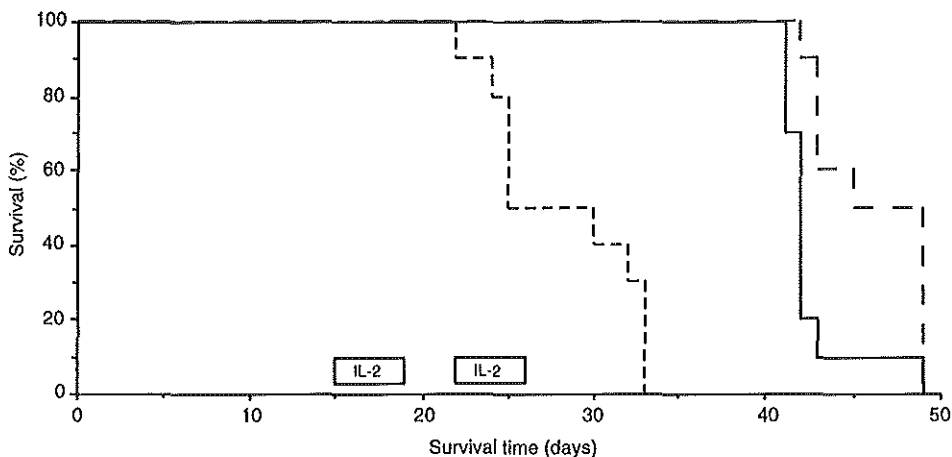
Figure 5.1 rhIL-2 treatment post syngeneic BMT. Survival curves of rats treated with syngeneic BMT ± rhIL-2 therapy (2x130,000 IU/day s.c. for 2x5 days) *versus* the leukemia control group (*n* =10 rats per group). The rats were inoculated with 10^7 BNML cells (i.v.) on day 0 and subjected to CY (i.p.; 60 mg/kg) and subsequently to TBI (7 Gy X-rays) and BMT (10^8 BM cells i.v.) on days 11 and 12, respectively. All animals died of leukemia relapse; (_ _ _ _ _ _) 10^7 BNML i.v.; (_____) + syngeneic BMT; (_ _ _) + syngeneic BMT + 2x130,000 IU IL-2 daily.

To study the antileukemic effect of rhIL-2 in animals with residual leukemia after syngeneic BMT, high dose rhIL-2 therapy (2x130,000 IU rhIL-2/day) was applied. This immunotherapy regimen induced an ILS of 7 days compared with animals receiving syngeneic BM only, with all rats dying from leukemia relapse (Figure 5.1). Since the mechanism(s) of the rhIL-2 induced antileukemic effect remains unclear, the term log

leukemic cell *kill* (LCK) should not be applied. Thus, high dose Proleukin treatment (2x130,000 IU/day for 10 days) induced a borderline significant antileukemic effect (*p*=0.03), although the survival range of both groups totally overlapped.

In a second experiment, the ILS induced by the same high dose IL-2 therapy after syngeneic BMT was not significant (NS; 1 day) and all recipient rats died of leukemia relapse (data not shown). Thus, due to for example minor biological variations between the rats, high dose rhIL-2 therapy after syngeneic BMT induces either a borderline significant or no antileukemic effect.

5.4.2 IL-2 therapy post allogeneic BMT

The lack of alloreactivity after transplantation of allogeneic BM alone, often observed in rodent models, can be explained by the presence of very low numbers of lymphocytes in the marrow (5-8%) compared with human BM (20-30%). We therefore consider the transplantation of allogeneic rat marrow alone as a model for HLA matched, T-cell depleted allogeneic BMT in humans and the transplantation of a graft with additional numbers of splenocytes to induce GVL and/or GVHD as a model for full marrow grafting.[18]

5.4.2.a Dose-finding

Two (of ten) recipients of allogeneic BM died during the aplastic phase (day 32) caused by transplantation related complications (sepsis). The remaining eight animals in this allogeneic

Figure 5.2A Dose-finding rhIL-2 treatment post allogeneic BMT. Overall survival curves of leukemic rats treated with allogeneic BMT ± rhIL-2 therapy (2x26,000, 2x52,000 or 2x104,000 IU/day s.c. for 2x5 days) *versus* the leukemia control group and syngeneic transplanted animals (*n* =10 rats per group). The rats were inoculated with 10^7 BNML cells (i.v.) on day 0 and subjected to CY (i.p.; 60 mg/kg) and subsequently to TBI (7 Gy X-rays) and BMT (10^8 BM cells i.v.) on days 11 and 12, respectively; (_ _ _ _ _) 10^7 BNML i.v.; (_____) + syngeneic BMT; (━━━━━) + allogeneic BMT; (_._._._._.) + allogeneic BMT + 2x26,000 IU IL-2 daily; (_ _ _ _ _ _ _) + allogeneic BMT + 2x52,000 IU IL-2 daily; (_ _ _ _) + allogeneic BMT + 2x104,000 IU IL-2 daily.

BMT group died from leukemia relapse with a MdST of 52 days, compared with the syngeneic BMT group, with an ILS of 10 days (Figure 5.2). Thus, in this particular experiment, allogeneic BM alone induced a significant (p=0.02) GVL effect correlating with 2.5 LCK without the induction of overt GVHD.

After allogeneic BMT, different doses of rhIL-2 were administered to study whether the antileukemic activity could be increased without the induction of severe GVHD. In the group receiving 2x26,000 IU rhIL-2/day, all animals died of leukemia relapse with a MdST of 55.0 days. Thus the ILS compared with the group transplanted with allogeneic BM only, is NS (Figure 5.2A). When 2x52,000 IU rhIL-2 were administered, one rat died of BMT related complications (day 29), two rats died of GVHD (alopecia, erythema, diarrhea, hunched posture and severe cachexia: days 39 and 41; Figure 5.2A) and seven rats died of leukemia relapse with a MdST of 57.0 days (ILS was NS; Figure 5.2B). The addition of 2x104,000 IU rhIL-2 led to death due to GVHD in six rats (days 30, 45, 46, 53, 59 and 61; Figure 5.2A) without signs of leukemia at autopsy. Four rats in this group died of leukemia relapse with a MdST of 69.5 days (Figure 5.2B). Compared with the recipients of allogeneic BMT only, an additional ILS of 17.5 days was induced. Thus, rhIL-2 post allogeneic BMT up to 2x52,000 IU/day does not significantly change the life span. When 2x104,000 IU/day are administered, a high incidence of GVHD mortality is observed (60%). The rats dying of leukemia relapse however, show a significant prolongation in survival compared with allogeneic BMT alone (p=0.0001).
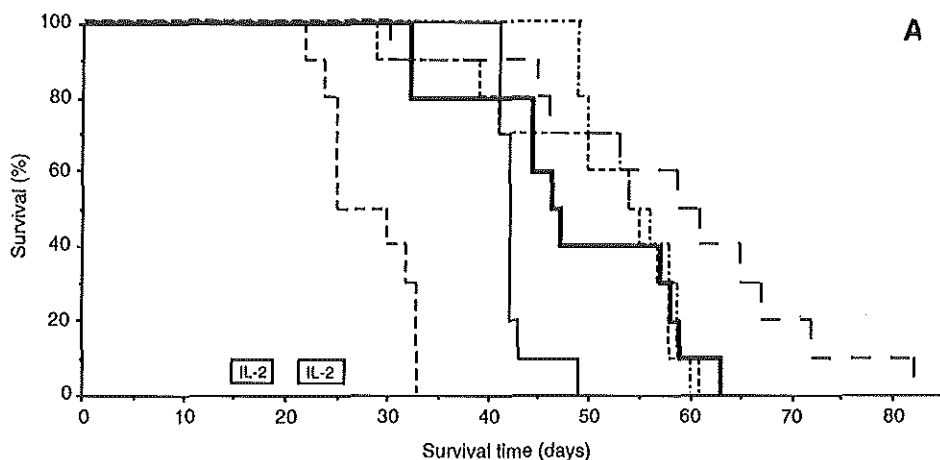

Figure 5.2B Dose-finding rhIL-2 treatment post allogeneic BMT. Leukemia-free survival curves of leukemic rats treated with allogeneic BMT ± rhIL-2 therapy (2x26,000, 2x52,000 or 2x104,000 IU/day s.c. for 2x5 days) *versus* the leukemia control group and syngeneic transplanted animals (n =10 rats per group). Animals dying from transplantation related complications or GVHD are excluded from the graph. The rats were inoculated with 10^7 BNML cells (i.v.) on day 0 and subjected to CY (i.p.; 60 mg/kg) and subsequently to TBI (7 Gy X-rays) and BMT (10^8 BM cells i.v.) on days 11 and 12, respectively; (_ _ _ _ _) 10^7 BNML i.v.; (_____) + syngeneic BMT; (▬▬▬▬) + allogeneic BMT; (_._._._._.) + allogeneic BMT + 2x26,000 IU IL-2 daily; (_ _ _ _ _ _ _ _) + allogeneic BMT + 2x52,000 IU IL-2 daily; (_ _   _ _   _) + allogeneic BMT + 2x104,000 IU IL-2 daily.

5.4.2.b Optimal dose

As is usually seen after BMT in the BNML model, in this experiment, the allogeneic (WAG/Rij to BN) BM alone does not induce a significant antileukemic effect compared with syngeneic marrow (ILS=2 days) and all rats died from leukemia relapse (MdST=44; Figure 5.3).

To study the antileukemic efficacy and/or GVHD induction of an intermediate dose of rhIL-2 derived from the dose-finding study, 2x78,000 IU/day were administered post allogeneic BMT in the same schedule as was mentioned earlier. Two (of ten) rats died of transplantation related complications (days 23 and 26; aplasia). None of the animals showed signs of acute GVHD, one animal survived longer than 150 days and was considered to be cured of leukemia. As regards the animals evaluable for dying from leukemia (8 of 10), this rhIL-2 immunotherapy regimen induced a MdST of 69.5 days and an ILS of 25.5 days

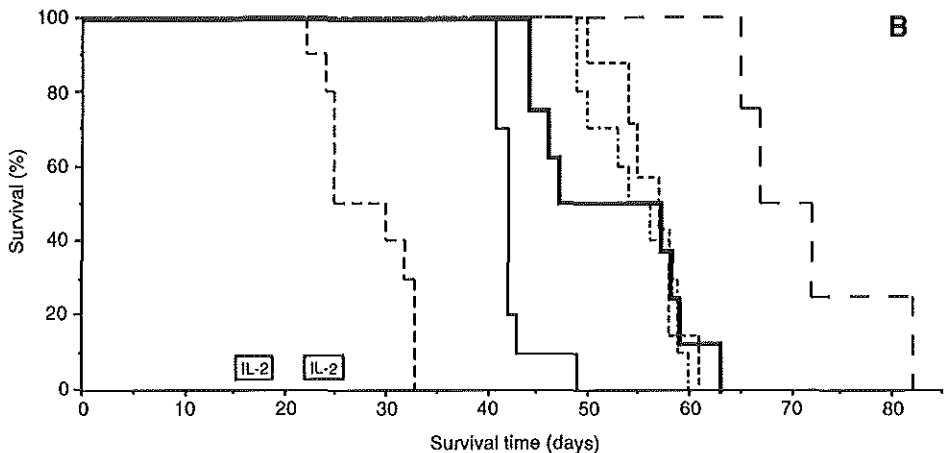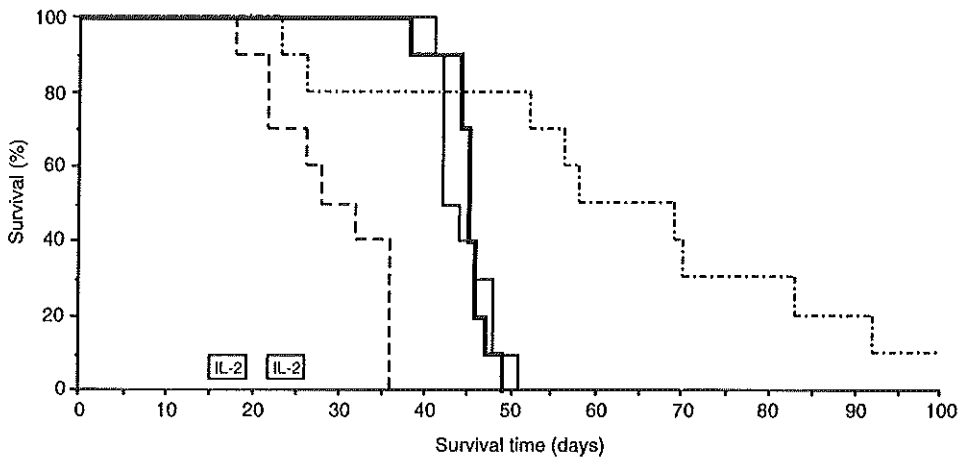**Figure 5.3** Optimal dose rhIL-2 treatment post allogeneic BMT. Overall survival curves of leukemic rats treated with allogeneic BMT ± rhIL-2 therapy (2x78,000 IU/day s.c. for 2x5 days) *versus* the leukemia control group and syngeneic transplanted animals (*n* =10 rats per group). The rats were inoculated with 10^7 BNML cells (i.v.) on day 0 and subjected to CY (i.p.; 80 mg/kg) and subsequently to TBI (7 Gy X-rays) and BMT (10^8 BM cells i.v.) on days 11 and 12, respectively. One animal survived longer than 150 days and was considered to be cured of leukemia; (_ _ _ _ _) 10^7 BNML i.v.; (_____) + syngeneic BMT; (▬▬▬▬) + allogeneic BMT; (_.._.._.._..) + allogeneic BMT + 2x78,000 IU IL-2 daily.

(correlating with 6.4 LCK) compared with allogeneic BMT alone (Figure 5.3). Thus, the intermediate dose of rhIL-2 after allogeneic BMT induced a highly significant antileukemic effect compared with the group treated with allogeneic BM alone (*p*=0.002), without inducing significant GVHD.

The results of syngeneic and allogeneic transplanted animals with or without rhIL-2 therapy post BMT are summarized in Table 5.1.

Table 5.1 RhIL-2 therapy after syngeneic or allogeneic BMT

Treatment (n = 10 rats)	Cause of death			Cure	MdST in days		Significance ILS
	BMT-related complications	GVHD	Leukemia		Overall survival	Leukemia-free survival	
10^7 BNML (i.v.)	-	-	10	-	27.5	27.5	-
+Syngeneic BMT	0	0	10	0	42.0	42.0	-
+2x130,000 IU IL-2/day	0	0	10	0	49.0	49.0	p = 0.03 [1]
+Allogeneic BMT I	2	0	8	0	46.5	52.0	p = 0.02 [1]
+Allogeneic BMT II	0	0	10	0	44.0	44.0	NS [1]
+2x 26,000 IU IL-2/day	0	0	10	0	55.0	55.0	NS [2]
+2x 52,000 IU IL-2/day	1	2	7	0	54.5	57.0	NS [2]
+2x 78,000 IU IL-2/day	2	0	7	1	63.5	69.5	p = 0.002 [3]
+2x104,000 IU IL-2/day 2	0	6	4	0	60.0	69.5	p = 0.0001

ILS = increase of life span; NS = not significant

[1] Compared with syngeneic transplanted animals
[2] Compared with allogeneic BMT I (= dose-finding experiment)
[3] Compared with allogeneic BMT II (= optimal dose experiment)

5.4.2.c Immunophenotyping of PBL post BMT

To study the engraftment of allogeneic BM with and without post BMT rhIL-2 treatment and a possible shift in lymphocyte (sub)populations in the PB during and after rhIL-2 administration, PB samples were obtained during the dose-finding experiment. PBL were isolated from blood samples taken weekly (from day 24 onwards) from two animals per experimental group receiving syngeneic or allogeneic marrow without post BMT treatment or treated with the highest dose of rhIL-2 studied, i.e. 2x130,000 IU rhIL-2/day or 2x104,000 IU rhIL-2/day, respectively.

1. Chimerism after allogeneic BMT. Immunophenotyping of PBL from rats not receiving rhIL-2 treatment after allogeneic BMT on days 24, 31 and 38 clearly indicated full donor chimerism ($\geq$95% U9F4$^+$ cells). Analysis of PBL from the same rats on days 45, 52 and 59 showed an increasing percentage of cells positively reacting with the MCA specific for the recipient rat strain and concomitantly, a decreasing percentage of U9F4$^+$ cells reflecting leukemia relapse (confirmed by Rm124 labeling). Labeling PBL of rats receiving rhIL-2 treatment (2x104,000 IU rhIL-2/day) after allogeneic BMT, showed full donor chimerism up to day 59. In these two rats no BNML cells could be detected till day 52; at day 59 one of these rats showed 5.0% Rm124$^+$ cells, indicating leukemia regrowth (data not shown).

2. Lymphocyte subsets. The PBL isolated were concomitantly immunophenotyped to define possible changes in percentages of lymphocyte (sub)type(s) induced by the rhIL-2 treatment, using MCA's specific for macrophages/ monocytes, NK cells and BNML cells, and

for the expression of class II, CD4, CD5, CD8 and CD25. During or after the rhIL-2 treatment post syngeneic BMT, no differences in MCA labeling of PBL could be detected comparing animals either or not treated with rhIL-2. During high dose rhIL-2 treatment after allogeneic BMT (day 24), an increase in the percentage macrophages/ monocytes and CD8+ cells was observed compared with allogeneic BMT alone (from 13.1% to 24.7% and from 23.1% to 44.6%, respectively), which disappeared immediately after rhIL-2 administration (day 31). No significant changes in expression of the other Ag studied were observed. All experimental groups showed a sharp increase in the percentage RM124+ cells upon leukemia relapse (from ≤1.0 to 20.0-45.0%; data not shown)

5.5 DISCUSSION

Controversial experimental data on IL-2 therapy post BMT have been reported. In syngeneic BMT in mice it has been postulated that the marrow graft should be activated with IL-2 (*ex vivo*) and IL-2 therapy should be initiated immediately after BMT to induce a syngeneic GVLR.[31] The mechanism of this approach is unknown and was observed to be transient. On the other hand, a maximal antileukemic effect of IL-2 therapy in minimal residual leukemia after murine syngeneic transplantation (unstimulated BM) was reported when IL-2 was given 2-3 weeks after BMT.[32]

In allogeneic BMT the addition of low doses of IL-2 during the first week of a murine GVHD could enhance the severity and mortality seen in a class I disparate, but not in a class II disparate GVHR presumably mediated by CD4+ cells.[33] Augmentation of GVHD induced by recombinant IL-2 was observed both in MHC disparate rats[34] and mice mismatched for minor histocompatibility Ag.[35] In contrast, a different group reported not to aggravate GVHD with IL-2 doses able to induce allospecific cytotoxic T lymphocytes (CTLs), using a different murine minor histoincompatibility model.[36] IL-2 is produced only within the first 2 days of a GVHR by the engrafted murine CD4+ T-cells,[37] coinciding with the induction phase of the GVHR.[38] It appears that IL-2 production plays a critical role in the early development of acute GVHD most likely by facilitating the development of donor-anti-host CTLs.[39]

The role of IL-2 in GVHD has recently become even more controversial. It was found in murine models that administration of high doses of IL-2 at the time of infusion of T-cell depleted syngeneic BM (to delay GVHD-related mortality) together with fully MHC mismatched allogeneic BM and allogeneic spleen cells, further reduces lethal GVHD, promotes alloengraftment and maintains the GVL.[40,41] However, initiation of IL-2 administration at later time points, e.g. day 7 post BMT, can exacerbate GVHD.[41] Further studies using mouse models for leukemia revealed that in fully MHC mismatched transplantation, early IL-2 treatment selectively inhibits the GVHD-producing CD4 activity, but does not inhibit the antileukemic effects of CD8+ T-cells.[42,43] Using an allogeneic murine

BMT model in which both GVL and GVHD were CD4-dependent, IL-2 treatment inhibited GVHD but not the antileukemic effects of CD4+ cells.[43] These findings suggest that the inhibitory effect of IL-2 is directed against a limited subset or function of CD4+ T-cells and that GVL and GVHD can be mediated through different CD4 mechanisms.[43]

The results obtained from the experiments described here indicate that the high dose of rhIL-2 applied post syngeneic BMT (2x130,000 IU/day for 2x5 days) induces a borderline significant or no antileukemic effect. The difference in the results obtained is most probably caused by slight biological variations between the experimental rats. Even though the dose of rhIL-2 applied was 1.25 times higher than the highest dose studied after allogeneic BMT, the treatment was well tolerated but no significant antileukemic effects could be observed. Higher doses of rhIL-2 after syngeneic BMT may induce a more pronounced antileukemic effect but were not studied in these experiments.

Dose-finding of rhIL-2 therapy post allogeneic BMT in the BNML model (resulting in full donor chimeras) revealed that doses up to 2x52,000 IU/day for 2x5 days did not induce a significant antileukemic effect. However, the dose of 2x52,000 IU Proleukin did induce deaths caused by GVHD (20%). After the rhIL-2 dose of 2x104,000 IU, 60% of the recipients died from GVHD. Nevertheless, the prolongation in the life-span of rats dying from leukemia indicated a highly significant antileukemic effect without signs of treatment induced toxicity. To study whether this significant antileukemic effect could be obtained without the induction of severe GVHD, the intermediate dose of 2x78,000 IU rhIL-2 was applied. The results clearly demonstrate that the intermediate Proleukin dose post allogeneic BMT induced the same median survival as was observed for animals dying from leukemia after 2x104,000 IU rhIL-2. In the dose-finding experiment, the allogeneic BM graft alone induced a GVL effect, resulting in an ILS of 10 days. As a result, the prolongation in survival induced by the rhIL-2 therapy compared with allogeneic BMT alone was less than in the experiment where the optimal dose of rhIL-2 was employed. In both experiments the ILS, compared with the *syngeneic* transplanted groups, was 27.5 days and the MdST was 69.5 days. Thus, a strong antileukemic effect could be induced in recipient rats with a relatively high leukemia cell load using 'the optimal dose' rhIL-2 (2x78,000 IU/day) post allogeneic BMT, without inducing evident GVHD. The GVLR induced by allogeneic marrow alone (dose-finding) is usually not observed and was, in this experiment, probably caused by biological variations in the rats. A higher allogeneic reactivity of the marrow lymphocytes may explain GVHD induction when 2x52,000 IU rhIL-2/day were administered in the dose-finding experiment compared with the absence of GVHD when 2x78,000 IU rhIL-2/day were applied in the 'optimal dose' experiment. These preclinical exercises demonstrate a dose-effect relationship: too low doses of IL-2 exert no effect, too high doses cause lethal GVHD and intermediate doses induce a significant GVLR without 'clinical' GVHD. Thus, when the recipient shows a high level of donor chimerism, the administration of IL-2 after allogeneic BMT may serve as a remission

induction therapy at leukemia relapse, in analogy with donor leukocyte transfusions.[44,45] Therefore, phase I studies are warranted.

The mechanism of the IL-2 induced antileukemic effect observed remains unclear. The 'optimal dose' rhIL-2 applied after allogeneic BMT is well tolerated and the MdST after the rhIL-2 treatment is 43.5 days. According to the standard growth curves of BNML this reflects less than 10^3 residual BNML cells at day 26 compared with the 2×10^5 residual leukemia cells at day 12 after the conditioning regimen (calculated using the MdST after syngeneic BMT; see Results section 'IL-2 therapy post syngeneic BMT'). This could mean that the induced ILS has been caused by a direct antileukemic effect which reduced the leukemia cell load another 2 logs in stead of the expected more than 4 logs increase in 14 days. In that case, a prolongation of the rhIL-2 treatment could have increased the cure rate. A different explanation could be a rhIL-2 induced leukemia growth suppression which is slowly fading after the treatment is stopped and finally results in leukemia relapse. Thus, the log leukemic cell *kill* (LCK) terminology should not be applied for the rhIL-2 induced antileukemic effects observed.

How does the 'optimal dose' of rhIL-2 applied in the experiments reported here relate to those administered to patients post BMT? Clinical trials report on a maximum tolerated rhIL-2 'induction' dose of 3.0×10^6 U/m^2/day (Roche) post autologous BMT.[10] Because 2.3 units of Roche rhIL-2 equals 6 IU, calculating the dosages from these trials in international units reveals 7.8×10^6 IU IL-2/m^2/day. Given the body surface area of an adult male of 70 kg to be 2 m^2, this correlates with 2.2×10^5 IU IL-2/kg/day. Thus the 'optimal dose' that was used after allogeneic BMT in the rat model was ($2 \times 78,000$ IU/250 g/day = 6.2×10^5 IU/kg/day) 2.8 times higher than used in the clinical studies. This comparison does not imply that, given our preclinical data on *allogeneic* BMT in the rat, the dose of rhIL-2 administered in patients after *autologous* BMT should be increased. It is simply meant to indicate that IL-2 regimens, both applied in rat and in humans, are of the same order of magnitude. Moreover, the 'tight' titration curve for antileukemic *versus* GVH effects in the rat model indicates that the dosing of IL-2 should be done with great caution and for each donor-recipient combination the optimal dose may be different.

As described, PBL were studied for the expression of rat strain specific class I Ag (chimerism). Concomitantly, these cells were immunophenotyped using MCA's specific for macrophages/monocytes, NK cells and BNML cells, and for the expression of class II, CD4, CD5, CD8 and CD25 to study possible changes in percentages of lymphocyte (sub)type(s) induced by the rhIL-2 treatment. During high dose rhIL-2 treatment after allogeneic BMT (day 24: $2 \times 104,000$ IU rhIL-2/day), an increase in macrophages/monocytes and CD8$^+$ cells was observed compared with allogeneic BMT alone. No significant changes in expression of the other Ag studied were observed. The increased percentage of CD8$^+$ cells is in agreement with our studies on the *in vitro* rhIL-2 incubation of rat splenocytes.[46] During or after the rhIL-2 treatment post syngeneic BMT no differences in MCA binding of PBL could be

detected compared with animals not treated with rhIL-2. These preliminary results indicate that macrophages/monocytes and CD8$^+$ lymphocytes may play a role in the antileukemic effects observed after high dose rhIL-2 treatment after allogeneic BMT.

Concerns have been raised whether IL-2 therapy could accelerate the growth of residual leukemia cells. Human *in vitro* and murine *in vivo* (immunosuppressed nu/nu mice as a model for human leukemia cell growth) data indicated that IL-2 does not promote the proliferation and growth of human acute leukemia cells of myeloid and lymphoid origin.[47] However, more recently, relapses were reported after IL-2 treatment of AML patients in CR, where the IL-2R α chain was detected on the surface of leukemia cells from such patients during relapse.[48] Similarly, *in vitro* proliferation of many human leukemia blasts in the monocytic lineage (FAB M4 and M5) was stimulated by IL-2, which was significantly correlated with the expression of the IL-2R β chain.[49]

Using the rat model for AML, resembling human promyelocytic leukemia (FAB-M3), no expression of the IL-2R β chain was detectable (CD25: MCA OX-39 [25]) and no growth stimulation of the BNML cells could be induced by rhIL-2 inoculations *in vivo* (data not shown). However, preliminary results in a rat model relevant for human acute lymphocytic leukemia (L4415; an immature T-cell leukemia growing in the WAG/Rij rat),[50] have shown that rhIL-2 therapy (2x130,000 IU/day, 2x5 days) significantly stimulates the growth of these IL-2 receptor bearing leukemia cells. Inoculation of 10^7 L4415 cells i.v. into untreated WAG/Rij rats on day 0 resulted in an MdST of 29.5 days, administration of 2x130,000 IU rhIL-2/day for 2x5 days s.c. (days 4-8 and days 11-15), reduced the life span to 23.5 days (p=0.02). These results lead to the conclusion that in human leukemias which express the IL-2R (α and/or β chain), IL-2 treatment might lead to acceleration of the regrowth of residual leukemia cells and should therefore be applied with great caution.

Using the BNML model, we have previously shown that the addition of critical numbers of allogeneic lymphocytes to the allogeneic graft, as a model for full marrow grafting in humans, induced a maximal GVLR of 2-3 LCK without symptoms of overt GVHD.[18] Further increased numbers of additional allogeneic splenocytes induced acute lethal GVHD. However, transplantation of allogeneic BM alone usually induces neither GVL nor GVHD, resembling human allogeneic, HLA-matched T-cell depleted BMT. Even though the mechanism(s) remain(s) unclear, in our rat model optimal IL-2 therapy after 'allogeneic T-cell depleted BMT' generates an antileukemic effect (the 25.5 days ILS correlates with 6.4 'LCK') which makes up for the loss of GVL after T-cell depletion (maximally 2-3 LCK).

Therefore, further IL-2 therapy studies in animal models to optimize the observed antileukemic effects or even inducing cures in minimal residual leukemia are needed. To elucidate the controversial data reported in the literature, future studies should include variation of the IL-2 treatment schedule (i.e. further increased doses of rhIL-2 post syngeneic BMT, different timing of treatment initiation, prolongation of optimal dose treatment and additional rhIL-2 maintenance treatment after allogeneic BMT), combination therapy with

other cytokines and the possible synergistic antileukemic effect of donor lymphocytes and IL-2 therapy.

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CHAPTER 6

GRAFT-VERSUS-LEUKEMIA IN RAT MHC-MISMATCHED BONE MARROW TRANSPLANTATION IS MERELY AN ALLOGENEIC EFFECT*

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6.1 SUMMARY

One of the major problems in the treatment of leukemia with bone marrow transplantation (BMT) remains leukemia relapse. It has been clearly shown that graft-versus-host disease (GVHD) is accompanied by a graft-versus-leukemia reaction (GVLR) which reduces the incidence of leukemia relapse. To date, discussion is still going on as to whether GVHD and GVLR are either two different reactions or are exerting their effects through the same mechanism(s). In two rat leukemia models, namely the Brown Norway acute myelocytic leukemia (BNML) and the WAG/Rij acute lymphocytic leukemia L4415, total body irradiation (TBI) was given to induce a state of so-called minimal residual disease (MRD). Subsequently, it was attempted to evoke a GVLR distinct from GVHD by using semi-allogeneic hybrid-to-parent or parent-to-hybrid BMT, with or without the addition of graded numbers of lymphocytes. In both leukemia models applying hybrid-to-parent BMT, the addition of high numbers of semi-allogeneic lymphocytes to the semi-allogeneic graft had no antileukemic effect. In parent-to-hybrid BMT, the grafts sharing their alloantigens with the leukemia cells did not induce an antileukemic effect, irrespective of the number of lymphocytes present in the graft or the induction of evident GVHD. When the parental graft was histoincompatible with the leukemia cells, transplantation of bone marrow alone induced a significant increase in life span (ILS) correlating with 2.8 log leukemia cell kill (LCK). Addition of graded numbers of histoincompatible splenocytes to the graft further increased the antileukemic effect (maximally correlating with 4.0 LCK) without signs of obvious GVHD or, with higher numbers of splenocytes, induced acute lethal GVHD. Thus, the results indicate that the GVLR observed post MHC mismatched BMT in these rat leukemia models for ALL and AML are mere allogeneic effects, inseparable from GVHD.

6.2 INTRODUCTION

In the treatment of hematological malignancies such as leukemia, allogeneic bone marrow transplantation (BMT) is being used with increasing frequency.[1,2] The success of BMT is limited by post-transplantation complications such as graft-versus-host disease (GVHD) and leukemia relapse. The GVHD incidence and intensity is dependent on the extend of genetic disparity (major and minor histocompatibility complex antigens) between the donor and host[3,4] as well as the quantity and quality of the lymphocytes present in the bone marrow (BM) graft.[5]

Studies applying animal models have shown that allogeneic T-cells in allogeneic BMT, which are the major contributors to GVHD,[6] are held responsible for a reduction in the incidence of leukemia relapse compared with isologous/autologous BMT, inducing a graft-versus-leukemia reaction (GVLR).[7-9]

Clinical data show that the magnitude of the GVLR correlates with GVHD severity.[10,11] T-cell depletion (TCD) of allogeneic donor BM decreases the incidence of both acute and chronic GVHD but at the same time significantly increases the rate of leukemia relapse.[12,13] Likewise, in a few cases the GVLR was shown to be abrogated by post BMT immunosuppression using cyclosporine A (CsA) to prevent GVHD.[14,15] The discontinuation of CsA at first signs of leukemia relapse induced complete hematologic and cytogenetic remission. Clearly, GVHD seems to be associated with the GVLR which eradicates residual leukemic cells. The question remains whether these two reactions are separable or whether it will be possible to optimize the beneficial GVL effects without increasing the risk of lethal GVHD.

In some animal models, the GVLR can be separated from apparent GVHD[16-18] leading to the speculation that GVL and GVH reactive cells are distinct. These preclinical data support the assumption that specific antigens (Ag) present on leukemic cells, are not shared by normal cells of the host. However, no leukemia associated specific surface markers have been identified on human leukemic cells. Alternatively, the same cells may mediate both GVL and GVH reactions, but quantitative differences in sensitivity between leukemic and normal cells could account for apparently distinct GVL/GVH reactions, including a 'threshold' effect. To date, no convincing clinical evidence has been reported on a GVLR separable from GVHD.

To study GVL in animal models relevant to human leukemia, experiments were performed applying the Brown Norway rat acute myelocytic leukemia (BNML)[19-21] and the L4415 acute lymphocytic leukemia growing in the inbred WAG/Rij rat strain.[22,23] Both leukemia models show a reproducible growth pattern upon intravenous (i.v.) transfer. The log-linear relationship between leukemia cell load and survival time allows an accurate calculation of the treatment induced antileukemic effect, in terms of log leukemic cell kill (LCK), by recording prolongation of survival.

Recent reports on our studies employing these rat models have shown that syngeneic BMT serves as a model for human autologous BMT (no GVL/GVH induction).[9] Transplantation of allogeneic MHC mismatched rat BM alone, containing just 5-8% lymphocytes, induced neither a significant GVLR nor GVHD and therefore resembles human allogeneic HLA-matched T-cell depleted BMT. The addition of critical numbers of (unseparated) allogeneic splenocytes to the allogeneic marrow graft evoked a significant GVLR in both models correlating with 2-3 LCK, without inducing overt GVHD. Acute lethal GVHD was induced when further increased numbers of lymphocytes were added.[9] Thus, transplantation of allogeneic rat marrow and splenocytes serves as a model for full marrow grafting in humans.

Subsequently, using the BNML model, studies on interleukin-2 (IL-2) administration post BMT have illustrated the capacity of optimal dose IL-2 therapy, after transplantation of allogeneic marrow only, to induce a significant antileukemic effect (correlating with 6.4 LCK) without the induction of 'clinically' evident GVHD.[24] The administration of higher doses of IL-2 induced acute (lethal) GVHD. Therefore, we concluded that the GVLR lost by T-cell depletion of the allogeneic graft, may be more than fully compensated by optimal IL-2 treatment after allogeneic T-cell depleted BMT.

To date, we have not been able to separate the GVLR from GVHD. To assess whether the *in vivo* observed GVLR is a specific antileukemic reaction or a secondary effect of GVHD, e.g. mediated by increased cytokine levels, semi-allogeneic BMT was studied. Semi-allogeneic hybrid-to-parent BMT does not lead to GVHD since the hybrid (F1(WAG/Rij x BN) or F1) lymphocytes will recognize the parental (P) Ag as 'self', but high numbers of hybrid (F1) lymphocytes may induce a specific antileukemic effect. Semi-allogeneic P-to-F1 BMT does induce GVHD, generating the opportunity to study the antileukemic effects of GVHD inducing lymphocytes, either or not sharing their major histocompatibility complex (MHC) Ag with the leukemia cells.

6.3 MATERIALS AND METHODS

Animals
Inbred male BN, WAG/Rij (Wistar Albino Glaxo / Rijswijk) and F1(WAG/Rij x BN) rats at 10-14 weeks of age (body weight: 200 - 250 g) were used in the experiments described. The rats were bred at and supplied by the (specified pathogen free) animal breeding facility of the Medical Biology Laboratory (MBL)-TNO (Rijswijk, The Netherlands) and housed in plastic cages with water and food pellets *ad libitum.*

BNML model
The BNML leukemia was induced in a female BN rat by 9,10-dimethyl-1,2-benzanthracene (DMBA). It shows a slow, reproducible growth pattern upon i.v. cellular transfer within the

BN rat strain and shares many characteristics with human acute (pro-) myelocytic leukemia: severe suppression of normal hemopoiesis, diffuse intravascular coagulation, response to chemotherapy, presence of clonogenic leukemic cells (*in vitro* and *in vivo*; ED_{50} = 25 cells) and no evidence for a virus as an etiological agent. The leukemia is passaged by i.v. cellular transplantation. The rat leukemia model for human AML has been described extensively.[19-21]

L4415 model

The L4415 lymphocytic leukemia was induced in a female WAG/Rij rat by 0.5 MeV total body neutron irradiation (dose; 5 cGy). Immunophenotyping has shown that the relatively slowly growing, non-immunogenic L4415 is an immature T-cell leukemia (CD4⁻, CD8⁻, CD5⁺). The L4415 leukemia is *in vivo* passaged by i.v. cellular transfer, showing a reproducible growth pattern. L4415 leukemia has been described in detail before.[22,23]

Preparation of cell suspensions

BNML or normal spleen cells. Monocellular suspensions were obtained by mincing the enlarged spleens of end stage leukemic animals or the spleens of untreated rats, and gently squeezing the suspension through nylon gauze. The cells were washed and suspended in Hanks' balanced salt solution (HBSS; Gibco, Paisley, UK). The number of nucleated cells was counted in Türks' staining solution (0.005% Crystal Violet, 1% acetic acid, in H_2O) using a Bürker-type hemocytometer and adjusted to the final cell concentration required.

Peripheral blood (PB) samples were obtained by orbital punctures. The blood was collected in small plastic tubes containing ethylene-diaminetetra-acetic acid (EDTA; Sigma, St. Louis, MO, USA) to prevent coagulation. When peripheral blood lymphocytes (PBL) were prepared for immunophenotypic analysis the erythrocytes were lysed first (lysing buffer: 0.15 M NH_4Cl, Merck, Darmstadt, Germany; 0.01 M $NaHCO_3$, Merck; 0.1 mM EDTA, Sigma). The suspension was then centrifuged, the pellet resuspended in HBSS and the PBL counted.

Bone marrow cells were isolated by repeated flushing of the cavity of tibiae and femora with HBSS. A single cell suspension was obtained by sieving the BM through nylon gauze. Subsequently the cells were washed, counted and the cell concentration adjusted.

Total body irradiation

To reduce the leukemia cell load and induce a state of so called 'minimal residual disease' (MRD; few leukemic cells survive the conditioning regimen[21]), lethal total body irradiation (TBI, 8.5 Gy) was applied. The rats were irradiated unilaterally in plastic cages, containing eight animals at a time, using a Philips X-ray generator (300 kV, 10 mA) at a dose rate of 48 $cGy.min^{-1}$. Subsequently, the rats were rescued by the inoculation of 10^8 BM cells +/- spleen cells (i.v.).

Chimerism: Immunophenotyping of PBL post BMT

Single cell suspensions of PBL, washed and resuspended in PBS/BSA (PBS, NPBI, Amsterdam, The Netherlands; 1% BSA, Sigma; 0.01% NaN3, Merck) were labeled with murine monoclonal antibodies (MCA's) directed to the BN (OX-27 [25]) or the WAG/Rij (U9F4 [26]) inbred rat strain specific cell surface Ag (RT-1.A^n and RT-1.A^u, respectively), or incubated with PBS/BSA serving as negative controls. Subsequently, the cells were incubated with FITC-conjugated rat anti-mouse Ig (Jackson Laboratories, Maine, U.S.A.) and analyzed on a FACScan flow cytometer operating with a single argon (488 nm) laser (Becton Dickinson, Mountain View, CA, USA). For data analysis, the FACScan research software program Consort 30 (Becton Dickinson) was used.

The OX-27 and U9F4 hybridoma cell lines were kindly donated by Dr. D. Mason (Oxford, U.K.). The antibodies were produced intraperitoneally (i.p.) in BALB/c mice and purified on a protein A column (Dr. P.H. van der Meide, Immunosciences, BPRC-TNO, Rijswijk, The Netherlands).

Survival time assay

This assay is based on the log-linear relation between the number of leukemia cells inoculated and the survival time of the rats.[21,27,20] The antileukemic effect of a particular treatment can be deduced from the increase of life span (ILS) of the treated group compared with the untreated leukemia control group (*n*=8 rats/group). A treatment inducing an ILS of 4.0 days corresponds with the extra survival time of animals with a 10-fold lower leukemia cell load and is referred to as a 1 LCK. In addition, gross pathology (e.g. spleen and liver weight, bone marrow smears) will confirm the cause of death (GVHD, take failures, leukemia).

Statistical Analysis

Survival time analysis of rats from the different experimental groups (*n*=8 rats), was performed according to Peto *et al*[28,29]

6.4 RESULTS

6.4.1 F1-to-parent BMT in the BNML model

BN rats were inoculated with 10^5 BNML cells on day 0, subjected to total body irradiation (TBI, 8.5 Gy X-rays) on day 7 and subsequently given a BMT on day 8.

The 17 days ILS observed after syngeneic BMT compared with the leukemia control group indicates the significant (*p*=0.001) antileukemic effect of the TBI, reflecting 4.3 log leukemic cell kill (LCK; Figure 6.1). To estimate the residual leukemic cell load at the time of BMT, the survival time after the irradiation was monitored. The median survival time (MdST) of
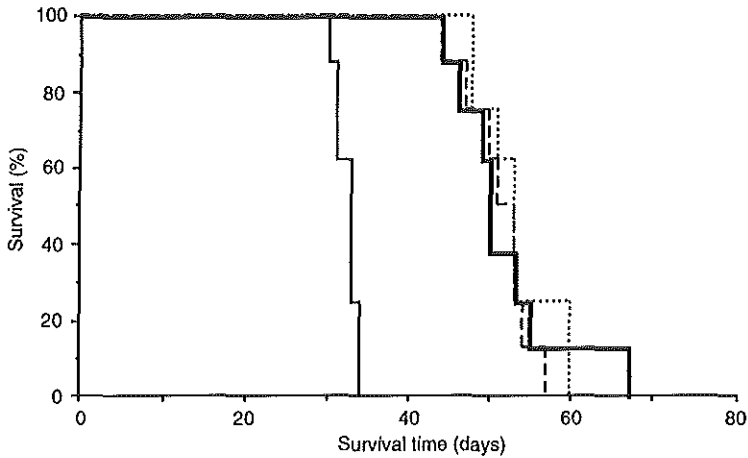
Figure 6.1 F1 to BN BMT. Survival curves of leukemic animals treated with semi-allogeneic BMT ± splenocytes *versus* syngeneic BMT and leukemia control groups (n =8 rats per group). The rats were inoculated with 10^5 BNML cells i.v. on day 0, subjected to TBI (8.5 Gy X-rays) on day 7 and to BMT (10^8 BM cells ± 10^8 splenocytes i.v.) on day 8. All transplanted rats died of leukemia relapse; (_____) 10^5 BNML i.v.; (▬▬▬▬▬) + syngeneic BMT; (.............) + F1 BMT; (─ ─ ─ ─ ─) + F1 BMT + 10^8 F1 splenocytes.

syngeneic transplanted animals was 43 days post-TBI, indicating that 10^2 leukemic cells survived the conditioning regimen (according to standard BNML growth curves), meeting the criteria for MRD.[20,21,27]

Transplantation with hybrid rat strain (F1) marrow induced a 3 days increase in the MdST compared with the rats transplanted with syngeneic marrow (p=not significant: NS). To evaluate the possible induction of an antileukemic effect of high numbers of semi-allogeneic lymphocytes, 10^8 F1 splenocytes were added to the F1 marrow graft because rat BM contains few T-lymphocytes (5-8% CD5$^+$ cells).[9] This high number of F1 splenocytes did not induce a significant difference in survival (MdST=52 days), compared with transplantation of syngeneic or F1 BM alone. All transplanted groups (n=8 rats) died of leukemia relapse (Figure 6.1).

6.4.2 F1-to-parent BMT in the L4415 model
WAG/Rij rats were inoculated with 10^6 L4415 cells at day 0, TBI and BMT were performed at days 7 and 8, respectively. TBI followed by syngeneic BMT yielded a significant ILS (p=0.001) indicating 3.6 LCK (13 days median ILS: data not shown). The MdST of the rats receiving a syngeneic graft was 24 days post-TBI with all rats dying of leukemia relapse, indicating a residual leukemic cell load of 5x10^3 cells.

Transplantation of F1 marrow did not induce a significant decrease in MdST (28.5 days: data not shown) compared with syngeneic BMT. Two (of eight) recipient rats died of aplasia whereas the other six animals died of leukemia relapse in the same range as the syngeneic transplanted group. Addition of 10^8 F1 splenocytes to the semi-allogeneic marrow inoculum

did not change the survival time compared with the recipient rats receiving semi-allogeneic BM only. All rats died from leukemia relapse. Thus, similar to the observation in the BNML model, no antileukemic effect could be observed in the rat ALL model after semi-allogeneic BMT ± high numbers of F1 lymphocytes.

6.4.3 Chimerism after F1-to-parent BMT
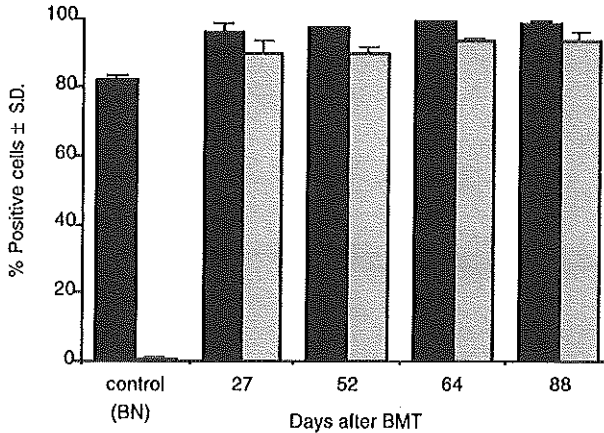
Figure 6.2 Immunophenotyping PBL of semi-allogeneic transplanted animals (F1 to BN BMT; BM only) at various timepoints post BMT *versus* untreated BN rats. Mean percentages positively reacting cells from 4 animals ± S.D. are represented. After BMT, all PBL show a high expression of both types parental class I Ag (as in F1 rats); (■) OX-27 (class I, BN); (☐) U9F4 (class I, WAG/Rij).

To establish the state of chimerism in recipients of F1 marrow, peripheral blood samples were drawn from four recipient rats at various time points after BMT. PBL were immuno-phenotyped using the rat strain specific class I MCA's OX-27 $(RT-1.A^n)$[25] and U9F4 $(RT-1.A^u)$.[26] The mean (+/- S.D.) percentage positive cells of the control (P) rats versus recipients of F1 BM cells alone, clearly illustrate full donor chimerism at each timepoint investigated after F1-to-P BMT both in the BNML model (Figure 6.2) and the L4415 model (data not shown).

6.4.4 Growth characteristics of the BNML and L4415 leukemia in F1 rats

F1 rats were inoculated i.v. with graded numbers of BNML and L4415 cells to define the correlation between leukemic cell dose and survival time.

The growth of BNML in F1 rats is accompanied by severe paralysis. Histological examination revealed growth of leukemia cells in all marrow compartments but limited in spleen and liver. Leukemia outgrowth from the vertebral bones led to compression of peripheral nerves, leading to the observed paralysis. First symptoms of paralysis of the front limbs were observed only 20 days after the inoculation of 10^7 leukemic cells, four days later

all rats were totally paralyzed and were therefore sacrificed. Since no reproducible dose-survival relation could be established for BNML growing in the hybrid rat strain, BNML was not further used in the parent-to-F1 transplantation studies.
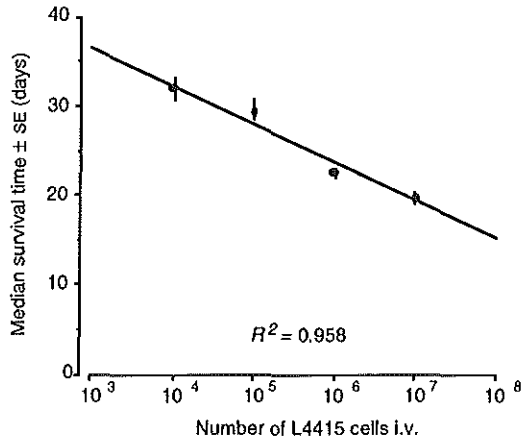
Figure 6.3 Correlation between L4415 leukemic cell dose inoculated (i.v.) in F1(WAG/Rij x BN) rats and survival time (n =8 rats per point). Logarithmic regression analysis results in a correlation coefficient of variation of 0.958 (R^2). Vertical bars indicate S.E.

Graded numbers of L4415 cells induced death of leukemia in all recipients. A significantly enlarged spleen ($\geq$5 g) frequently with spleen rupture, an increased liver weight ($\geq$20 g), gastrointestinal and pulmonary hemorrhage and enlargement of thymus and lymph nodes were prominent features at autopsy. The L4415 leukemia growth in F1 rats developed similar to that observed in the WAG/Rij rat strain[23] with a slightly slower growth pattern. As is shown in the correlation between the number of L4415 cells inoculated in the F1 and the survival time, 1 log leukemia cells correlates with 4.3 days in life span (Figure 6.3).

6.4.5 Parent-to-F1 BMT in the L4415 model

To evaluate the antileukemic efficacy of transplanted lymphocytes either sharing their Ag with the leukemia cells or expressing allogeneic MHC Ag, the L4415 acute lymphocytic leukemia growing in the F1(WAG/Rij x BN) has been used as a recipient of BN or WAG/Rij BMT.

6.4.5.a BN-to-F1 BMT

F1 rats were inoculated with 10^6 L4415 cells on day 0. Since the L4415 leukemia growth pattern in the hybrid rat strain is slightly slower, irradiation of the leukemic animals was postponed till day 14 and the BM graft was given on the same day. TBI followed by syngeneic (F1) BMT induced a significant ILS (15 days: p=0.002) indicating 3.5 LCK (Figure 6.4). The MdST of the rats receiving a syngeneic graft was 21 days post TBI with all

rats dying of leukemia relapse, indicating that 4×10^6 cells leukemic cell survived the conditioning regimen.

Transplantation of BN marrow induced a MdST of 47 days (Figure 6.4), hence an ILS of 12 days (correlating with 2.8 LCK) compared with the animals receiving a syngeneic graft ($p=0.002$). Addition of 10^5 (or 10^6: data not shown) parental splenocytes to the marrow graft increased the MdST 17 days (corresponding with 4.0 LCK) compared with syngeneic BMT,

Figure 6.4 BN to F1 BMT. Survival curves of leukemic animals treated with semi- allogeneic BMT ± splenocytes *versus* syngeneic BMT and leukemia control groups. The groups receiving syngeneic BM and semi-allogeneic BM+10^7 splenocytes existed of 6 recipient rats. All other groups consisted of 8 recipients. The rats were inoculated with 10^6 L4415 cells i.v. on day 0 and subjected to TBI (8.5 Gy X-rays) and BMT (10^8 BM cells ± splenocytes i.v.) on day 14. One rat from the group transplanted with BN marrow only was censored (dying of aplasia on day 29). The rats died from leukemia relapse but in the group receiving 10^7 splenocytes, all rats died from acute GVHD; (_____) 10^6 L4415 i.v.; (▬▬▬▬) + syngeneic BMT; (..........) + BN BMT; (_ _ _ _ _) + BN BMT + 10^5 splenocytes; (.—..—..—..) + BN BMT + 10^7 splenocytes.

with all rats dying from leukemia relapse. The highest dose of additional splenocytes studied i.e. 10^7 cells, reduced the MdST compared with the other groups receiving BN BMT (40 days), with all animals dying of acute GVHD (Figure 6.4). The observed signs of severe GVHD were red ears, paws and snout, hair loss, diarrhea, cachexia and a hunched appearance. At autopsy, no macroscopic evidence for leukemia regrowth was observed.

6.4.5.b. WAG/Rij-to-F1 BMT
As was shown in the BN to F1 transplantation, the residual leukemia cell load after the delayed radiotherapy and subsequent syngeneic BMT was relatively high. To further reduce the leukemia cell burden, the conditioning and BMT were performed 7 and 8 days after the inoculation of 10^6 L4415 cells, respectively. The significant ($p=0.001$) ILS induced by the irradiation and syngeneic BMT compared with the leukemia control group was 10 days (2.3 LCK; Figure 6.5). The MdST of the syngeneic (F1) transplanted group was 41.5 days (34.5

days post-TBI) reflecting a residual leukemic cell load of 2×10^3 with all rats dying from leukemia relapse.

Transplantation of WAG/Rij marrow resulted in a MdST of 42 days, the addition of 10^6 (or 10^7: data not shown) WAG/Rij splenocytes to the parental marrow graft reduced the MdST to 38 days (Figure 6.5). All rats died from recurrent leukemia. The transplantation of F1 rats with WAG/Rij marrow with the highest dose of additional spleen cells studied, i.e. 10^8 splenocytes, resulted in a MdST of 37.5 days with all rats dying with symptoms of severe GVHD. At autopsy, four of eight rats were found to have normal liver and spleen weights, whereas the other rats (4/8) showed significant enlargement of the spleen and liver, indicating leukemia regrowth concomitant with GVHD. Thus, no antileukemic effect could be observed after the addition of high numbers of semi-allogeneic splenocytes sharing their MHC Ag with the leukemic cells, even when lethal GVHD was induced.

An additional 'control' group of F1 rats was transplanted with BN marrow supplemented with 10^5 BN splenocytes to reproduce the significant antileukemic effect observed in the previous experiment, applying the postponed conditioning regimen. Once more a significant ($p=0.003$) ILS could be observed compared with the syngeneic transplanted group (12 days; correlating with 2.8 LCK), with all rats dying of leukemia relapse (Figure 6.5).

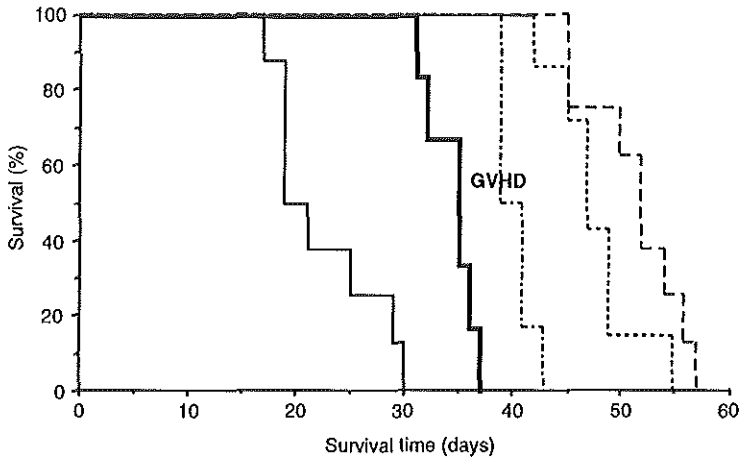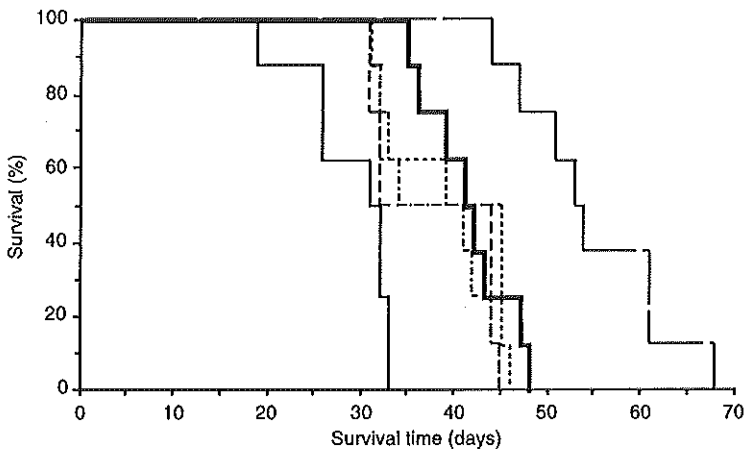**Figure 6.5** WAG/Rij to F1 BMT. Survival curves of leukemic animals treated with semi-allogeneic BMT ± splenocytes *versus* syngeneic BMT and leukemia control groups (*n* =8 rats per group). The rats were inoculated with 10^6 L4415 cells i.v. on day 0, subjected to TBI (8.5 Gy X-rays) on day 7 and to BMT (10^8 BM cells ± splenocytes i.v.) on day 8. The rats died from leukemia relapse but in the group receiving 10^8 splenocytes, all rats died from acute GVHD; (——————) 10^6 L4415 i.v.; (▬▬▬▬▬▬) + syngeneic BMT; (············) + WAG/Rij BMT; (— — — — —) + WAG/Rij BMT + 10^6 splenocytes; (—··—··—··) + WAG/Rij BMT + 10^8 splenocytes; (——— ——) + BN BMT + 10^5 splenocytes.

6.4.6 Chimerism after parent-to-F1 BMT

Peripheral blood samples were drawn from four recipient rats at various time points after parental BMT to establish the state of chimerism. PBL were immunophenotyped using the rat strain specific MCA's (OX-27 [25] and U9F4 [26]). The mean (+/- S.D.) percentage positive cells of untreated control (F1) rats versus recipients of parental strain BM cells alone, clearly indicate full donor chimerism at each timepoint investigated after semi-allogeneic BMT, both after transplantation with BN marrow (Figure 6.6) and WAG/Rij marrow (data not shown).
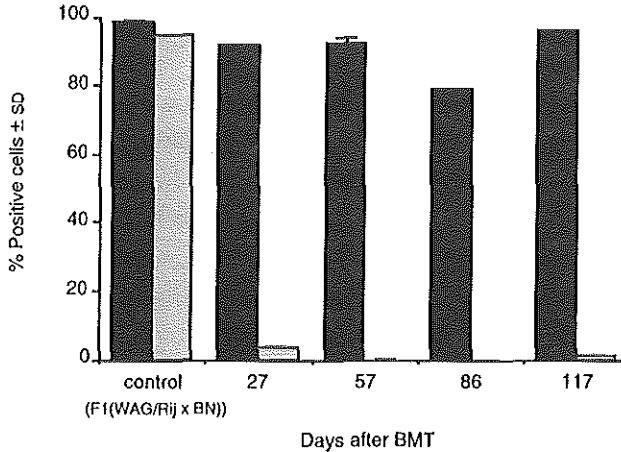
Figure 6.6 Immunophenotyping PBL of semi-allogeneic transplanted animals (BN to F1 BMT; BM only) at various timepoints post BMT *versus* untreated F1 rats. Mean percentages positively reacting cells from 4 animals ± S.D. are represented. After BMT, all PBL show a high expression of BN class I Ag and low ('background level') of WAG/Rij class I Ag; (▇) OX-27 (class I, BN); (▢) U9F4 (class I, WAG/Rij).

6.5 DISCUSSION

To date the pathophysiological mechanism of the GVLR has not been well defined. The involvement of immunocompetent donor T-cells in the GVLR is well established, both in animal models[7,8] and in man.[12,13] It is not clear whether the mechanisms of the GVLR are separable from those operative in GVHD. Evidence has been reported about at least two elements of the GVLR; one that is dependent on the presence and the intensity of GVHD[30] and the other one operating without clinically overt GVHD.[18,31] The latter would allow enhancing the GVLR while minimizing GVHD. In this way a more safe and complete eradication of leukemic cells still present after the conditioning regimen may be achieved. However, to determine whether this can be accomplished, a better understanding of the mechanisms of the GVLR and its mediators is warranted.

Using the BNML and the L4415 rat leukemia models, we have previously shown that the addition of critical numbers of allogeneic (MHC mismatched) unseparated lymphocytes to the

allogeneic graft, as a model for human allogeneic HLA matched full marrow grafting, induced a maximal GVLR correlating with 2-3 LCK without symptoms of obvious GVHD.[9] Further increased numbers of additional allogeneic splenocytes induced acute lethal GVHD. Using the BNML model, studies on administration of IL-2 post BMT have shown that optimal IL-2 therapy after 'allogeneic T-cell depleted BMT' generates an antileukemic effect correlating with 6.4 LCK, which makes up for the loss of GVL after T-cell depletion.[24] Higher doses of IL-2 after allogeneic BMT induced acute lethal GVHD. The present paper describes our studies on GVL and GVHD in the BNML and L4415 rat leukemia models using semi-allogeneic hybrid-to-parent and parent-to-hybrid BMT with and without the addition of graded numbers of splenocytes.

In both models, the survival time of leukemic BN and WAG/Rij rats treated with syngeneic BMT indicated a state of MRD after the conditioning regimen (10^2 and 3×10^5 residual leukemia cells, respectively). Neither the transplantation of hybrid strain marrow only nor the addition of high numbers of semi-allogeneic splenocytes to the F1 graft significantly changed the life span of the recipient rats compared with the rats given a syngeneic marrow transplant. Thus, these data indicate that hybrid-to-parent BMT, even when high numbers of F1 lymphocytes are present in the graft, did not induce GVL.

Since the BNML caused severe paralysis before antileukemia effects could be evaluated in the hybrid rat strain, parent-to-F1 BMT was not further studied using the BNML model. L4415 leukemia was shown to have a similar growth pattern in the hybrid rat strain as in the parental rat strain. Thus, the hybrid rat that shared half of the alloantigens with the parent-type tumor cells was immunologically incapable of rejecting the L4415 leukemia cells. This model permitted the generation of GVHD in a hybrid recipient by using either parent as a donor whereby the GVHD producing effector cells were either histocompatible or incompatible with the leukemia cells.

Syngeneic BMT after delayed TBI in F1 rats carrying L4415 leukemia, indicated a relatively high residual leukemia cell load (4×10^6 cells). Nevertheless, transplantation of BN marrow only resulted in a significant ILS compared with syngeneic BMT. Addition of critical numbers (10^5 or 10^6) of BN splenocytes further increased the survival time (correlating with 4.0 LCK) without inducing GVHD, whereas higher numbers of parental lymphocytes induced acute lethal GVHD.

To induce MRD, the hybrid strain recipients of L4415 leukemia were subjected to TBI on day 7. The MdST (post TBI) of rats given a syngeneic marrow transplant indicated a residual leukemia cell load of 2×10^3 cells. Transplantation of WAG/Rij BM ± the addition of high numbers of parental splenocytes (up to 10^7) did not induce an antileukemic reaction compared with syngeneic BMT, and all rats died from leukemia relapse. The addition of 10^8 WAG/Rij splenocytes induced acute GVHD. At autopsy, 50% of these recipients showed leukemia specific characteristics such as hepatosplenomegaly, indicating that the leukemia growth was not hampered by GVHD. In the same experiment, a group of recipient F1 rats

was transplanted with BN marrow and 10^5 additional splenocytes. Again, these rats were shown to have a significantly increased life span compared with the syngeneic transplanted rats, without 'clinical' signs of GVHD. These data indicate that semi-allogeneic lymphocytes sharing their alloantigens with the leukemia cells are not capable to induce an antileukemic effect, even when they do induce acute GVHD. Thus, the GVL effects observed after MHC mismatched allogeneic BMT in these rat models for leukemia are mere allogeneic effects, confirming results of Tutschka *et al.*[32]

These results seem to be in contrast with several reports mentioning the *in vitro* induction of cytotoxic T-lymphocytes (CTLs) specific for allogeneic leukemia cells or nonleukemic cells, or lysing both leukemic and nonleukemic target cells,[33,34] and their correlation to the GVLR *in vivo* in a murine model.[35,36] These observations were made in studies on MHC matched, minor histocompatibility Ag mismatched transplantations, whereas we have studied MHC mismatched BMT. Though the reports on allogeneic MHC matched leukemia specific CTLs are promising, their role in the GVL effects observed *in vivo* still has to be proven. As the higher reactivity of MHC mismatched lymphocytes seems to be logical (greater disparity), it still needs to be confirmed that the *in vitro* 'leukemia specific' CTLs are capable to exert their effect *in vivo* through that same leukemia specific pathway. For *in vivo* application, the cell numbers need to be largely expanded and the specificity could be lost. Even when these cells are able to induce an evaluable *in vivo* antileukemic effect, this may turn out to be an alloreaction of minor histocompatibility Ag mismatched lymphocytes, comparable to the 'threshold' effect exerted by MHC mismatched lymphocytes. Nevertheless, the 'threshold' effect induced by minor histocompatibility mismatched lymphocytes probably reveals a wider therapeutical window compared with MHC mismatched lymphocytes.

In fact, donor buffy coat transfusions in patients with chronic myelocytic leukemia showing leukemia relapse after HLA matched allogeneic BMT have recently been reported to induce a GVLR. The majority of patients had complete hematologic and cytogenetic remission persisting 28-91 weeks[37,38] but most of these patients also developed acute or chronic GVHD which responded well to immunosuppression without losing its antileukemic efficacy.[39,40] These data confirm that minor histocompatibility mismatched lymphocytes are capable of inducing a significant GVLR, often accompanied by controllable GVHD.

Further studies on the nature of lymphocyte (sub-) populations and cytokine involvement in GVLR and GVHD in response to major- and/or minor histocompatibility complex Ag in allogeneic BMT are essential to further develop and refine therapeutic approaches. Currently, several possible treatment strategies to optimize the GVLR without the induction of severe GVHD, e.g. IL-2 therapy after T-cell depleted allogeneic BMT[24], are encouraging.

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CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

7.1 INTRODUCTION

Allogeneic bone marrow transplantation (BMT) transfers both hemopoietic stem cells and alloreactive donor lymphocytes. The latter are capable of responding to host antigens, leading to a form of adoptive immunotherapy (**Chapter 1**). The antileukemic effect mediated by immunocompetent donor cells is known as the graft-versus-leukemia (GVL) effect and has been well characterized in animals.[1-3] In humans, the presence of the GVL reaction has been supported by indirect evidence that relapse rates are significantly higher after transplantation of T-cell depleted allogeneic donor bone marrow (BM)[4-6] and autologous/isologous grafts[5] compared with allogeneic, 'full' marrow transplantation. The occurrence of graft-versus-host disease (GVHD) after BMT is associated with lower relapse rates,[4-8] and patients with a relapse of leukemia may enter remission when GVHD follows the withdrawal of immunosuppression (**Chapter 1**).[9-11]

Leading investigators in the field of experimental and clinical BMT for leukemia have expressed the opinion that elucidation of the nature of the GVL effect and its therapeutic exploitation should be one of the highest priorities among attempts to improve cure rates.[7,12,13] An issue which has not yet been finally resolved is whether GVL independent of GVHD can be documented and whether clinical approaches to amplify GVL without GVHD can be developed. Although several experimental data as well as a recent analysis from the International Bone Marrow Transplant Registry (IBMTR) suggest that some of the GVL effects observed may be independent from GVHD, identification of leukemia-specific effector cells and leukemia-specific target molecules that may serve as antigens for donor-type T lymphocytes have not yet been clearly documented.[14]

7.2 GVL IN THE RAT LEUKEMIA MODELS

Transplantable leukemias growing in animals, preferentially inbred rodent strains, offer the opportunity to reproducibly manipulate initial leukemia cell dose and intervene in leukemia growth. Animal leukemias are relevant when they share many characteristics with human leukemias, e.g. specific homing, low antigenicity and sensitivity to drugs and radiation. The studies reported here were performed with the acute myelocytic leukemia (AML)

transplantable in the inbred Brown Norway rat strain (BNML)[15-17] and the L4415 acute lymphocytic leukemia (ALL) transplantable in the inbred WAG/Rij rat strain.[18,19] Both leukemia models show a reproducible growth pattern upon intravenous (i.v.) transfer. Survival time following i.v. inoculation is inversely related to the logarithm of the number of leukemic cells in the inoculum. This log-linear relationship between leukemic cell load and survival time allows an accurate estimation of the treatment-induced antileukemic effect, by recording prolongation of survival. As opposed to many other experimental rodent leukemias, one of the many characteristics which these rat leukemias have in common with human leukemia is that they seem to be non-immunogenic (**Chapter 2**).
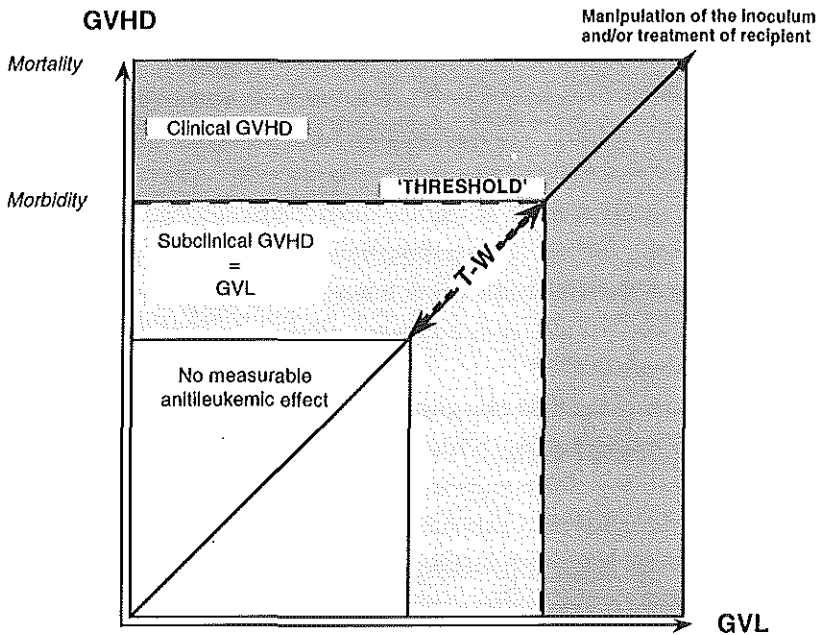
Figure 7.1 Schematic representation of the hypothesis that GVL (within the therapeutical window) = subclinical GVHD. Different susceptibility to the donor lymphocytes between leukemic and normal cells causes the 'threshold'-effect which may lead to the confusion that GVL and GVHD are two different reactions; T-W, therapeutic window.

We showed that it was not possible in these leukemias to induce an antileukemic effect with syngeneic (**Chapter 3**) or semi-allogeneic (F1 to parent; **Chapter 6**) BMT. Since the transfer of fully MHC-mismatched allogeneic marrow does (normally) not induce GVL or GVHD, the transplantation of allogeneic BM in the rats resembles human allogeneic HLA-matched T-cell depleted BMT (**Chapter 3**). The addition of increasing numbers of allogeneic spleen cells, as a source of lymphocytes, to the BM graft induces GVL and, at higher numbers, lethal acute GVHD. Thus, the transplantation of allogeneic rat marrow with additional splenocytes serves as a model for 'full' marrow grafting in man. In these models the

same cells seem to induce/mediate both GVL and GVH reactions, but quantitative differences in sensitivity of leukemic and normal cells appear to account for a 'threshold' effect, leading to a measurable GVL effect before GVHD becomes evident. The GVL effect further increases beyond the 'threshold' with increasing spleen cell numbers, but concomitantly GVH-morbidity emerges. The therapeutic window was found to be narrow, the resulting increase in life span correlates with 2-3 log leukemic cell kill. Therefore we hypothesize that GVL, within the therapeutical window, is merely a subclinical GVH reaction (Figure 7.1). Nevertheless, a significant GVL effect without the induction of overt GVHD could be observed when critical numbers of allogeneic splenocytes were added to the marrow graft (**Chapter 3**). Unfortunately, the separation of the GVL from GVHD could no longer be achieved after a microbiological change of the rat intestinal microflora. However, the results are in concert with data reported by other investigators who used partially depleted BM. Results of their studies suggest that there may be an 'optimal' number of T-cells residing in the allogeneic graft, reducing the incidence and severity of GVHD to tolerable levels while preserving a GVL effect.[20-22]

7.3 DEPLETION OF LYMPHOCYTE SUBSETS

It is generally accepted that T-cells are the major contributors to the GVL effect and to GVHD. Assuming that there is not only a quantitative effect of allogeneic T-cells on GVL and/or GVHD but also a qualitative difference between the T-cells, an approach to prevent GVHD different from reducing the T-cell numbers may be depletion of a specific lymphocyte subset. In an attempt to separate the observed GVL and GVHD inducing capacity, lymphocyte subsets were depleted from the allogeneic spleen cells prior to transplantation into leukemic rats using the BNML model (**Chapter 3**). Lymphocyte depletion was performed with monoclonal antibodies (MCA's) specific for CD4 and CD8, and magnetic beads coated with a MCA binding the Fc of the CD4 or CD8 specific murine MCA's. The one step, two log depletion of CD4 or CD8 positive cells, was cumbersome but feasible. Subsequently, the CD4 or CD8 negative fractions of splenocytes were added to the marrow graft, correcting for the percentages of CD4 and CD8 negative cells present in the unseparated spleen cell suspension.

It was clearly shown that the depletion of CD4+ cells completely prevented GVHD. When CD8+ lymphocytes were depleted from the spleen cell suspension, the incidence of lethal GVHD was still 100%, but the progression of the disease was reduced in all recipient rats. Unfortunately, in the experiment adding CD4 depleted or CD8 depleted splenocytes to the allogeneic marrow graft, no evident GVL effect could be observed in the rats receiving the critical dose of unseparated allogeneic spleen cells normally inducing the maximal GVL. Therefore we can not deduce any conclusions on the depletion of CD4 or CD8 positive cells

as regards the GVL effect. Attempts to obtain prove of a pivotal role of CD4^{+} T-cells in the GVL effect failed because of the inability to reproduce the separation of GVL and GVHD (see above). In view of the close linkage of GVHD and GVL, it seems nevertheless very likely that CD4^{+} T-cells are also responsible for the antileukemic effect.

Our conclusions are in agreement with experimental data reported by Truitt et al. and Sykes et al. They have shown that CD4^{+} T-cells play a critical role in causing GVHD in <u>fully mismatched</u> murine strain combinations, and that CD8^{+} T-cells given alone do not cause acute GVHD.[23-25] Moreover, it was proven in these murine models that CD4^{+} cells can contribute to GVL effects.[23] It was reported by Korngold et al. that both CD4^{+} and CD8^{+} T lymphocytes play a crucial role in GVHD in mice. It appears that the T-cell subset involved was dependent on the MHC or mH antigen differences between donor and recipient.[26] In some mH antigen disparate strain combinations both subsets appear to be equally involved.[27] A promising clinical trial of selective donor CD8^{+} cell depletion provided information on T-cell subsets mediating GVHD and GVL in man (<u>HLA-identical</u> sibling BMT for CML in chronic phase). This study demonstrated a reduction in, but not absence of, GVHD, whereas leukemia relapse rates were low.[28,29] When relapse rates remain low with adequate follow-up, CD8 depletion of the allogeneic marrow graft or addition of CD4^{+} cells to the T-cell depleted graft seems to be favorable in the clinical setting (HLA-matched BMT) compared with depletion of all T-cells or CD4 depletion of the marrow graft.

7.4 GVL AND LAK ACTIVITY

Some authors have described an association between NK activity and GVHD,[30-32] and donor NK cells or LAK cells have been implicated as effectors of GVHD.

Other studies indicated that NK cells and LAK cells may play a role in the GVL reaction.[5,33,34] These cells are able to lyse, most efficiently in the presence of IL-2, a variety of tumor cells with limited effect on normal cells. This lytic activity is not restricted to solid tumors, but also acute lymphocytic leukemia (ALL) and acute myelocytic leukemia (AML) blasts are reported to be susceptible to the lytic activity of autologous and allogeneic LAK cells *in vitro* .[35-39] Clinical studies confirm these preclinical results for acute leukemias.[40,41] Other studies suggest that in AML, but not in ALL, the LAK cell compartment may play a role in the clinical course and overall outcome of the disease.[42]

We investigated whether lymphokine activated killer (LAK) cells play a role in the GVL effect observed in our rat models after allogeneic BMT (**Chapter 4**). Splenocytes from WAG/Rij and BN rats were activated *in vitro* by recombinant human interleukin-2 (rhIL-2). The WAG/Rij and BN derived LAK cells were not capable of lysing the rat leukemia cells in contrast to significant cytolytic activity on the rat solid tumor cell lines. To reveal whether LAK cytokine production could mediate leukemic cell lysis, graded numbers of LAK cells

and leukemic cells were co-cultivated. This resulted in moderate suppression of leukemic colony formation. Thus, in the rat model, the GVL effect observed in allogeneic BMT is most probably not due to a direct antileukemic effect of LAK cells. These results are in concert with our T-cell depletion data indicating that GVHD (and GVL) effects were abolished by the depletion of CD4+ cells.

It was shown recently that acute GVHD may serve to increase the lytic activity of NK cells but does not result in increased numbers of LAK precursors. LAK precursor frequencies are below normal during the first two months after BMT, a finding not previously recognized from bulk culture LAK studies. The role of LAK effectors in GVL may rather involve the degree of cellular activation than the number of cells activated.[43]

7.5 GVL EFFECTS INDUCED BY IL-2

Numerous reports both on preclinical[24,44-51] and clinical studies[52-55] have stressed the importance of IL-2 in GVL effects and GVHD.

To explore a possible increase in the GVL effect without aggravating lethal GVHD by post-BMT immunotherapy, IL-2 administration has been studied in the BNML model (**Chapter 5**). The results indicated that animals with minimal residual leukemia treated with high doses of IL-2 post syngeneic BMT (equivalent to human autologous BMT), showed a small, borderline significant antileukemic effect. Future studies on the addition of syngeneic lymphocytes, either or not activated with IL-2 in the presence of irradiated BNML cells, to the BM graft combined with high dose IL-2 therapy, will reveal whether IL-2 administration after autologous BMT may lead to antileukemic effects. A recent report mentioned an alternative beneficial procedure for IL-2 therapy and autologous BMT. Transplantation of BM autografts activated by culture in IL-2 followed by administration of IL-2 represents a novel approach in an attempt to combine *ex vivo* purging and post-transplant *in vivo* immunotherapy. Initial clinical results have suggested its feasibility.[56]

Table 7.1 The rat as a model for human allogeneic BMT

Rat model	Human equivalent	GVL
Full marrow	T-cell depleted BMT	-
Full marrow + splenocytes (A)	Full marrow	+
Full marrow + IL-2 (B)	T-cell depleted BMT + IL-2	+++

Log leukemic cell kill (LCK) in the BNML model:
(A) 2-3
(B) 6.4

Repeated IL-2 treatment initiated 3 days after allogeneic BMT in the BNML model, resulted either in no significant antileukemic effect or in lethal GVHD when 'low' or 'high' doses were administered, respectively. An intermediate dose (2x78,000 IU/day) of Proleukin® however, induced a significant antileukemic effect amounting to an increase in life span correlating with 6.4 log leukemic cell kill (LCK) without the induction of GVHD (**Chapter 5**). We have shown that the maximal GVLR (without overt GVHD), induced by the addition of critical numbers of lymphocytes to the allogeneic graft, corresponds with a 2-3 LCK. Therefore, it appears that the GVLR lost by T-cell depletion of the allogeneic graft, may be more than fully compensated by IL-2 treatment post allogeneic T-cell depleted BMT (Table 7.1). Thus, our data show that a major contribution of IL-2 treatment to the GVL effects after allogeneic T-cell depleted BMT may be feasible, significantly widening the therapeutic window of clinical allogeneic BMT.

In studies with transplantable leukemia in mice, Sykes et al. observed that after transplantation of fully MHC mismatched BM and additional allogeneic lymphocytes, early IL-2 treatment (initiated on the day of BMT) selectively inhibits GVHD induced by CD4$^+$ cells, but does not affect the antileukemic effects of CD8$^+$ T-cells.[24,51] In BMT, using different mouse combinations, in which both GVL and GVHD were CD4 dependent, IL-2 treatment inhibited GVHD but not the antileukemic effects of CD4$^+$ cells.[51] These findings suggest that the inhibitory effect of IL-2 is directed against a limited subset or function of CD4$^+$ T-cells and that GVL and GVHD can be mediated through different CD4 mechanisms. Finally, these authors have shown that the type of CD4 activities mediating GVHD is determined by the particular histoincompatibilities between donor and host,[25] and that IL-2 preserves CD4 as well as CD8 mediated GVL effects.[57] However, initiation of IL-2 administration at later time points, e.g. day 7 post BMT, can exacerbate GVHD.[48,49] Confirmation of these complicated interactions have to be awaited before the implications for clinical practice can be evaluated.

In conclusion, after syngeneic BMT of leukemic rats it remains dubious whether IL-2 may lead to a GVL effect. Further studies on the addition of syngeneic lymphocytes combined with high dose IL-2 treatment will solve this question. The data of Sykes et al. point to the GVHD suppressive effects without losing the GVL efficacy of immediate IL-2 administration in allogeneic 'full' marrow grafting. They have shown that this response to IL-2 is highly dependent on the timing of administration and the degree of histoincompatibility between the donor and recipient. Nevertheless, this approach seems to bear a high risk of GVHD. Our data indicate that IL-2 therapy post allogeneic T-cell depleted BMT provides a significantly wider therapeutical window compared with allogeneic 'full' marrow grafting. Moreover, promising results of a phase II study, using low dose IL-2 after allogeneic T-cell depleted BMT for hematologic malignancies, were recently published.[55] Thus, a randomized controlled trial of IL-2 immunotherapy after allogeneic (HLA-identical), T-cell depleted BMT for leukemia is warranted.

7.6 GVL EFFECT; LEUKEMIA-SPECIFIC OR MERELY AN ALLOGENEIC REACTION?

To date, discussion is still going on whether the GVL effect and GVHD are two different reactions or that both effects are caused via the same mechanism(s). Both the T-cell subset depletion studies and the IL-2 therapy studies have so far failed to prove a distinct GVL reaction apart from GVHD. Both reactions are highly dependent on the histocompatibility of the donor and the recipient. Even when they are MHC-matched, the minor histocompatibility complex antigens may lead to both GVL and GVH reactions.

Using the BNML and the L4415 rat leukemia, it was attempted to evoke a GVLR distinct from GVHD by using semi-allogeneic hybrid-to-parent or parent-to-hybrid BMT (**Chapter 6**). Firstly, in hybrid-to-parent transplantation, no GVHD can be induced. Similarly, the addition of large numbers of lymphocytes to the hybrid marrow graft did not induce a GVL effect.

In parent-to-hybrid BMT, the grafts sharing their alloantigens with the leukemia cells did not induce an antileukemic effect, irrespective of the number of lymphocytes present in the graft or the induction of (lethal) GVHD. When the parental graft was histoincompatible with the leukemic cells, transplantation of bone marrow alone induced a significant increase in life span. Addition of critical numbers of histoincompatible splenocytes to the graft further increased the antileukemic effect without obvious signs of GVHD. The antileukemic effect of BM only might be explained by the fact that the leukemic cells are the exclusive cells being completely mismatched to the donor lymphocytes compared with the haploidentical recipient cells, increasing the GVL/GVHD 'threshold'. Higher numbers of splenocytes induced acute lethal GVHD. The results provide the most compelling evidence that the GVL effect, observed post MHC-mismatched BMT in these rat leukemia models for ALL and AML, are mere allogeneic effects, unseparable from GVHD. These data again point at a quantitative difference in sensitivity of leukemic and normal cells accounting for apparently distinct GVL/GVH reactions, confirming our earlier (**Chapter 3**) hypothesized 'threshold' effect.

Another approach to separating the beneficial from the harmful effects of allogeneic T-cells comprises the *in vitro* expansion and cloning of donor T-cells that specifically react to host leukemia cells, but that do not recognize non-malignant recipient cells. It has been demonstrated that leukemia 'specific' CTL lines and clones could be generated from the peripheral blood of MHC identical siblings of patients with leukemia, which do not recognize non-malignant cells from the same patient *in vitro*.[58,59] The specific recognition of some leukemias/lymphomas by effector cells might be explained by the expression of so called fusion proteins, encoded for by gene rearrangements as a result of chromosomal translocations. Well known examples are BCR-ABL, as a result of the translocation t(9;22) in Philadelphia chromosome (Ph1) positive CML and ALL, the E2A-PBX protein associated with t(1;19) in pre-B cell ALL, PML-RAR after t(15;17) in acute promyelocytic leukemia (PML) and DEK-CAN in t(6;9) AML.[60-62] The AML cells used to stimulate the 'specific'

CTL clone exhibited the chromosomal translocation t(8;16). Nevertheless, the specificity was not associated with this translocation since the other AML samples recognized by the CTL clone did not display t(8;16).[59] To explore the possibility of clinical application of donor-derived CTL lines directed against the leukemic cells from patients who relapsed after allogeneic BMT, Falkenburg et al. performed a pilot study using eight donor-recipient combinations.[63] In five of these cases, the CTL lines showed reactivity with the leukemic cells, but not with the IL-2-stimulated lymphocytes from the same individual, illustrating a 'relative specificity' for leukemic cells. However, this does not exclude possible reactivity with antigens expressed on the target organs of GVHD.[63] It can be imagined that such leukemia 'specific' T-cells are quite rare in normal peripheral blood. Their separation from T-cells that do induce GVHD, subsequent *in vitro* cloning and expansion may be impractical in the setting of BMT.[64]

A less sophisticated but, so far, more applicable method is the administration of donor buffy coat cells to induce remission in patients whose CML and AML had relapsed after allogeneic BMT.[65-73] Although details of the protocols varied, it has been shown that unseparated allogeneic buffy coat mononuclear cells have a striking capacity to destroy CML *in vivo* . Even though acute GVHD occurred in a high percentage of the patients (grade II-IV in 51%), mortality was directly attributed to GVHD in only one patient (= 2.2%). A retrospective comparison of the disease free survival reported in these studies, with the results of a second BMT shows that both the relapse rate and therapy-induced mortality are substantially lower with buffy coat infusions.[74,75] It appears that the GVL reaction is more effective in chronic-phase CML than in other hematologic malignancies or advanced CML. However, retrospective analyses do support the presence of a GVL effect in non-Hodgkin's lymphoma[76] and the acute leukemias.[5,69] Thus, further studies in these hematologic disorders are needed to reveal the feasibility of reliably inducing leukemia remission with donor buffy coat infusions, without the induction of severe (lethal) GVHD.

7.7 FUTURE PROSPECTS

Ongoing studies will disclose whether it will be possible to optimize the currently used culture, stimulation and cloning techniques to obtain leukemia specific CTL lines, preferentially for each single patient (in remission), on such short notice that residual leukemia can be eliminated before the number of proliferating target cells exceeds the limits of 'immunotherapy'. Newly discovered cytokines and cytokine-therapy studies will continuously improve our knowledge on and insight in the necessities of *ex vivo* lymphocyte culturing, stimulation and cloning, and the prevention of anergy and/or apoptosis once these cells are retransplanted.

In the meantime, appropriate follow-up of the patients already treated, together with further studies on the administration of donor buffy coat lymphocytes to CML patients and patients with acute leukemia in relapse after allogeneic BMT will strive to improve this approach. Until now, only Drobyski et al.[67] have attempted to administer a 'standard' number of donor T-cells. Complete molecular remissions were reported with as few as 0.34×10^8 cells/kg whereas some patients receiving far larger doses showed no response. It will be important to establish a 'threshold' dose of effectors, in combination with the degree of histoincompatibility, to administer a sufficient number of cells to induce a remission but as few as possible to limit the risk of severe GVHD.[77]

Currently, a new exciting approach to explore a wider therapeutic window of GVL is being studied. Transducing cells with the Herpes Simplex thymidine kinase (HSV-tk) gene makes these cells susceptible to destruction by ganciclovir, not affecting non-transduced cells. The drug will be phosphorylated by HSV-tk to its monophosphate derivative, which finally will be phosphorylated to ganciclovir-triphosphate, a competitive inhibitor of DNA synthesis,[78] resulting in selective cell death of HSV-tk expressing cells. The approach has been proven highly efficient in the treatment of transfected (experimental) tumor cells.[79] To apply the antileukemic effect of GVHD without adverse effects of GVHD, HSV-tk expressing donor T-cells may be added to the allogeneic (T-cell depleted) marrow graft to induce GVL and/or GVHD. GVHD will be held within tolerable limits by the application of ganciclovir, selectively killing the multiplying lymphocytes causing GVL and GVHD. This approach may allow the induction of significant GVL without inducing lethal GVHD. Crucial in the preclinical development (BNML and L4415 leukemia models) of this 'suicide gene' treatment will be the efficacy of donor lymphocyte HSV-tk gene transfection, efficient lymphocyte depletion of the allogeneic graft to prevent uncontrollable GVHD and the optimal timing of the immediate abrogation of the GVL/GVH reaction to maximally benefit from its anti-leukemic effect.

In the ongoing development of new drugs for the treatment of human diseases, compounds may emerge which further improve the treatment of leukemia. As a striking example, dinaline (4-amino-N-(2'-aminophenyl)benzamide) was initially described as a potent anticonvulsive agent.[80] The drug and its acetylated form acetyldinaline (ACD) have been shown to be immunosuppressive[81] and highly effective against slowly growing experimental tumors[82] and the BNML.[83] In this rat model, ACD shows an exceptional sparing effect on normal hemopoiesis and unlike most others drugs when given post-BMT, ACD does not jeopardize a (syngeneic) marrow graft. Optimal anti-leukemic efficacy is obtained after repeated daily oral administration for a prolonged period of time, inducing an 8 log leukemic cell kill with less than a 1 log normal stem cell kill.[84,85] Preliminary results indicated that a brief ACD treatment after allogeneic BMT plus high numbers of splenocytes, reduced the incidence of acute lethal GVHD from 100% to 37.5% while its antileukemic efficacy (5 log leukemic cell kill) is preserved. Further studies in the BNML model are therefore warranted on the efficacy

of ACD as an anti-leukemic, immunosuppressive and marrow ablative conditioning agent, which may allow reduced doses of total body irradiation, and on its efficacy in eradicating minimal residual leukemia and GVHD in the post-BMT period. Currently, ACD phase-I clinical studies are being conducted.

7.8 FINAL REMARKS

Returning to the title of this thesis, as concerned to the clinical applicability of GVL, the following can be concluded. We hypothesize that the GVL effects without evident symptoms of GVHD, observed in our rat models as well as in the reported experimental and clinical studies, are mere subclinical graft-versus-host effects. These GVL effects are occasionally interpreted as a distinct reaction, misled by the quantitative differences in sensitivity of leukemic and normal cells for the allogeneic lymphocytes, accounting for a 'threshold' effect (Figure 7.1).

The intensity of the GVL effect induced in MHC-compatible allogeneic BMT varies among different leukemias and donor-host strain combinations in mice.[86] Similarly, it was shown that human leukemic cells show an extreme variability in their allostimulatory capacity and that HLA-DR expression plays a central role in determining leukemic allo-recognition.[87] Therefore one can imagine that it is impossible to standardize allogeneic BMT in man. Since it seems that acute GVHD is easier to prevent than to treat once it is established,[88] it is strongly discouraged to deliberately induce GVHD for its GVL effect until reliable strategies for the abrogation of GVHD are developed. However, an exception should be made for BMT in the treatment of CML, in which a limited T-cell depletion (one-log instead of two log) of the marrow graft seems to be optimal. Optimization of the treatment pre and post allogeneic T-cell depleted, HLA identical BMT should be the objective for future studies to further reduce relapse rates in the treatment of leukemia. The studies in the BNML model indicate that ACD treatment in conjunction with the current conditioning regimen, or even in combination with reduced doses of TBI, seems to be the pre-BMT treatment of choice. Apart from the optimal conditioning regimen, T-cell depleted HLA-identical BMT and post-BMT IL-2 therapy may be the best approach to reduce the incidence of acute leukemia and CML relapse to date. At molecular recurrence of leukemia (PCR), donor buffy coat transfusions ($\pm$ 1-2 x 10^8 mononuclear cells/kg) should be administered, followed by ACD treatment when symptoms of severe GVHD are observed. Studies on the application of lymphocytes carrying suicide genes,[89] will allow the deliberate induction of GVL without risking the detrimental effects of GVHD in the near future.

HOOFDSTUK 7

## SAMENVATTING EN DISCUSSIE

7.1 INTRODUCTIE

Met een donor (allogene) beenmerg transplantatie (BMT) worden zowel bloedvormende stamcellen als alloreaktieve lymfocyten getransplanteerd. Deze laatste soort cellen zijn in staat om te reageren tegen ontvanger-antigenen (o.a. aanwezig op leukemie cellen), hetgeen leidt tot een vorm van immunotherapie (**Hoofdstuk 1**). Het antileukemische effect gemedieerd door deze immunocompetente cellen staat bekend als de transplantaat-tegen-leukemie reaktie (graft-versus-leukemia; GVL) en is goed gekarakteriseerd in proefdieren.[1-3] In de mens is het bestaan van een GVL reaktie indirect aangetoond door een hogere incidentie van leukemie recidief na transplantatie van T-cel gedepleteerd allogeen[4-6] of autoloog (patiënt-eigen) beenmerg (BM)[5] in vergelijking met een ongedepleteerd allogeen transplantaat. Het optreden van een transplantaat-tegen-gastheer/ontvanger ziekte (omgekeerde afstotingsreaktie: graft-versus-host disease; GVHD) na BMT is geassocieerd met een lagere incidentie van leukemie recidief,[4-8] patiënten met een dergelijk leukemie recidief kunnen in remissie komen wanneer een immunosuppressieve behandeling gestopt wordt (**Hoofdstuk 1**).[9-11]

Vooraanstaande onderzoekers in het veld van experimentele en klinische BMT in de behandeling van leukemie menen dat de opheldering van het mechanisme van de GVL reaktie en haar therapeutische bruikbaarheid een zeer hoge prioriteit heeft om de genezingskansen te verhogen.[7,12,13] Onduidelijk is nog of GVL onafhankelijk van GVHD verloopt en of het klinisch mogelijk is de GVL reaktie te optimaliseren zonder GVHD te induceren. Hoewel experimentele gegevens alsook een recente analyse van de Internationale Beenmerg Transplantatie Registratie (IBMTR) suggereren dat sommige van de GVL reakties los zouden staan van GVHD, zijn leukemie-specifieke effector cellen of leukemie-specifieke target moleculen die zouden kunnen dienen als antigenen voor donor T-lymfocyten nog niet duidelijk beschreven.[14]

7.2 GVL IN DE RATTELEUKEMIE MODELLEN

Transplanteerbare proefdier-leukemieën, bij voorkeur groeiend in inteelt knaagdierstammen, bieden de mogelijkheid reproduceerbaar de initiële hoeveelheid leukemie cellen te bepalen en

de leukemie-groei te kunnen manipuleren. Proefdier-leukemieën zijn relevant wanneer zij veel gemeen hebben met humane leukemieën, zoals specifieke groeilokaties, een lage antigeniciteit en gevoeligheid voor cytostatica en/of bestraling. De hier beschreven studies zijn uitgevoerd met de acute myeloide leukemie (AML) transplanteerbaar in de inteelt Brown Norway rattestam (BNML),[15-17] en de L4415 acute lymfatische leukemie (ALL) transplanteerbaar in de inteelt WAG/Rij rattestam.[18,19] Beide leukemiemodellen hebben een reproduceerbaar groeipatroon na intraveneuze (i.v.) toediening van leukemiecellen. De overlevingstijd na i.v. inoculatie is omgekeerd evenredig gerelateerd aan het aantal leukemiecellen dat is toegediend. De lineaire correlatie tussen de hoeveelheid leukemiecellen en de overleving maakt een nauwkeurige berekening van het antileukemische effect van een toegepaste behandeling mogelijk door de toename in de overlevingstijd te bepalen. In tegenstelling tot vele andere experimentele knaagdier leukemieën, zijn deze ratteleukemieën net als humane leukemieën niet immunogeen (**Hoofdstuk 2**).
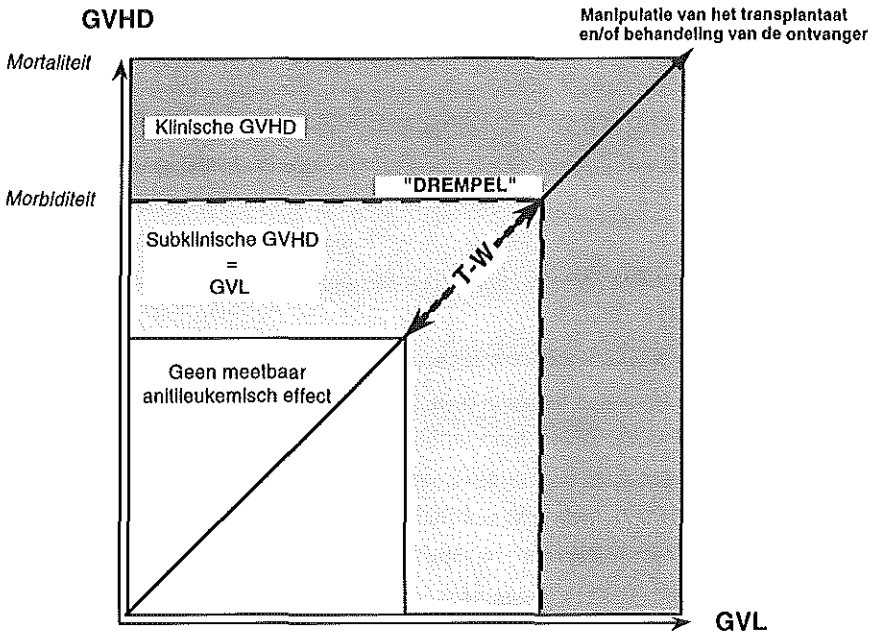
Figuur 7.1 Schematische weergave van de hypothese dat GVL (binnen de therapeutische marge) gelijk is aan subklinische GVHD. Verschil in de gevoeligheid voor donor lymfocyten tussen leukemische en normale cellen resulteert in een "drempel" effect, hetgeen kan leiden tot de verwarring dat GVL en GVHD twee verschillende reakties zouden zijn; T-W, therapeutisch "window" of marge.

We hebben aangetoond dat het niet mogelijk was in deze ratteleukemieën een antileukemisch effect te induceren met syngene (identieke rattestam; **Hoofdstuk 3**) of semi-allogene (kruising/hybride naar ouderstam; **Hoofdstuk 6**) BMT. Daar de transplantatie van volledig MHC-verschillend allogeen beenmerg in de rat geen GVL of GVHD induceert,

vergelijken we de transplantatie van allogeen BM in de rat met allogene HLA-identieke T-cel gedepleteerde BMT in de mens (**Hoofdstuk 3**). De toevoeging van toenemende hoeveelheden donor miltcellen, als een bron voor lymfocyten, aan het beenmerg transplantaat induceert GVL en, bij grote aantallen, letale acute GVHD. Daarom wordt de transplantatie van allogeen ratte-beenmerg met toevoeging van miltcellen beschouwd als een model voor ongedepleteerde BMT in de mens. In deze modellen lijken GVL en GVHD geïnduceerd/gemedieerd door dezelfde celpopulatie(s). Echter, kwantitatieve verschillen in (donor lymfocyten) gevoeligheid tussen leukemische en normale cellen lijkt te resulteren in een "drempel" effect, hetgeen leidt tot een meetbaar GVL effect voordat GVHD waarneembaar wordt. De antileukemische reaktiviteit neemt verder toe (over de "drempel") bij een toename van het aantal donor miltcellen, maar tegelijkertijd worden dan de symptomen van GVHD evident. De therapeutische marge blijkt klein, de resulterende toename in overlevingstijd correleert met een afname van 2-3 log aan leukemiecellen. Om die reden veronderstellen we dat GVL, binnen de therapeutisch marge, niets anders is dan subklinische GVHD (Figuur 7.1). Desalniettemin kon een significant antileukemisch effect worden waargenomen wanneer een specifiek aantal allogene miltcellen werd toegevoegd aan het beenmerg transplantaat (**Hoofdstuk 3**). Deze "scheiding" van GVL en GVHD kon echter niet langer bewerkstelligd worden nadat de microflora in de darmen van de ratten was gewijzigd. Toch zijn de data in overeenstemming met die welke gerapporteerd werden door andere onderzoekers die partieel gedepleteerde BM transplantaten gebruikten. Die resultaten suggereren dat er een "optimale" hoeveelheid donor lymfocyten in het transplantaat zou kunnen zijn, die de incidentie en ernst van GVHD zou verminderen tot een toelaatbaar niveau zonder het antileukemische effect te verliezen.[20-22]

7.3 DEPLETIE VAN LYMFOCYTEN SUBPOPULATIES

Het wordt algemeen aangenomen dat donor T-cellen de belangrijkste mediatoren zijn van de antileukemische reaktie en GVHD. Verondersteld dat er niet alleen een kwantitatief effect van allogene T-cellen op de GVL en/of GVH reaktie is maar bovendien een kwalitatief verschil tussen de T-cellen, dan zou een alternatieve benadering voor de vermindering van het aantal T-cellen ter voorkoming van GVHD, het depleteren van specifieke subpopulaties van lymfocyten kunnen zijn. In een poging de waargenomen GVL en GVHD inducerende capaciteit te scheiden zijn lymfocyten subpopulaties gedepleteerd van de allogene donor miltcellen voor de transplantatie in het BNML model (**Hoofdstuk 3**). De depletie werd uitgevoerd m.b.v. monoklonale antilichamen (MCA's) specifiek voor CD4 en CD8 , en magnetische bollen bedekt met een MCA die de Fc van de CD4 en CD8 MCA's bindt. De een-staps, twee log depletie van CD4 of CD8 positieve lymfocyten was bewerkelijk maar uitvoerbaar. Vervolgens werd de CD4 of CD8 negatieve fraktie van de donor miltcellen

toegevoegd aan het BM transplantaat, gecorrigeerd voor het percentage CD4 of CD8 negatieve cellen aanwezig in de oorspronkelijke miltcelsuspensie.

Duidelijk werd dat de depletie van CD4 positieve cellen de GVHD-inductie volledig voorkwam. De verwijdering van CD8 positieve cellen kon de GVHD niet voorkomen, wel werd de progressie van de ziekte in alle ratten vertraagd. In dit experiment werd geen duidelijk antileukemisch effect waargenomen met de dosis donor miltcellen die normaal gesproken de maximale GVL induceert. Om die reden kunnen we geen conclusie trekken uit de depletie van CD4 of CD8 positieve cellen voor wat betreft het GVL effect. Doordat GVL en GVH effecten niet langer onafhankelijk waarneembaar bleken (zie boven), is een cruciale rol van CD4 positieve cellen in de antileukemische reaktie niet bewezen. Maar, met het oog op de duidelijke link tussen GVL en GVH, lijkt het toch aannemelijk dat in deze rattemodellen de CD4 positieve cellen het antileukemische effect bewerkstelligen.

Onze conclusies zijn in overeenstemming met experimentele gegevens gerapporteerd door Truitt et al. en Sykes et al. Zij hebben aangetoond dat CD4 positieve cellen een belangrijke rol spelen in GVHD in volledig <u>MHC-verschillende</u> muizen (donor-ontvanger) combinaties, en dat toediening van allogene CD8 positieve T-cellen alleen geen acute GVHD induceert.[23-25] Bovendien is het in deze muizemodellen bewezen dat CD4 positieve cellen een bijdrage kunnen leveren aan het GVL effect.[23] Korngold et al. hebben beschreven dat zowel CD4 als CD8 positieve cellen een belangrijke rol spelen bij GVHD in muizen. Het blijkt dat de verantwoordelijke T-cel subpopulatie wordt bepaald door de MHC of minor histocompatibility (mH) antigenen-verschillen tussen de donor en de ontvanger.[26] In sommige mH verschillende muizestammen zijn beide subpopulaties in gelijke mate betrokken.[27]

Een veelbelovend klinisch onderzoek van selectieve CD8 depletie gaf meer duidelijkheid betreffende T subpopulaties die GVL en GVHD mediëeren (<u>HLA-identieke</u> broer/zus BMT voor CML in chronische fase). Deze studie demonstreert een vermindering in, maar geen afwezigheid van, GVHD, terwijl de incidentie van leukemie recidief laag was.[28,29] Wanneer de incidentie van leukemie recidief laag blijft gedurende een adequate vervolgtijd, lijkt CD8 depletie van het allogene transplantaat of de toevoeging van CD4 positieve cellen aan T-cel gedepleteerd beenmerg de juiste keuze bij HLA-identieke BMT, in vergelijking met depletie van alle T-cellen of CD4 positieve cellen.

7.4 GVL EN LAK AKTIVITEIT

Enkele auteurs hebben een associatie tussen NK aktiviteit en GVHD beschreven,[30-32] en donor NK cellen of LAK cellen als effectorcellen in GVHD aangeduid.

Anderen menen dat NK cellen en LAK cellen mogelijk een rol spelen in de GVL reaktie.[5,33,34] Deze cellen zijn, het meest efficiënt in de aanwezigheid van IL-2, in staat om

sommige tumorcellen te lyseren met een gering effect op normale cellen. Deze cel-lysis is niet beperkt tot solide tumoren, maar ook blasten van ALL en AML zijn gevoelig gebleken voor de lytische aktiviteit van autologe en allogene LAK cellen *in vitro*.[35-39] Klinische studies bevestigen deze preklinische resultaten betreffende acute leukemieën.[40,41] Andere studies suggereren dat in AML, maar niet in ALL, de LAK cellen een belangrijke rol spelen in het verloop van de ziekte.[42]

Wij hebben bestudeerd of lymfokine-geaktiveerde killer (LAK) cellen een rol spelen in de GVL reaktie waargenomen in de rattemodellen na allogene BMT (**Hoofdstuk 4**). Miltcellen van WAG/Rij en BN ratten werden geaktiveerd *in vitro* met recombinant humaan interleukine-2 (rhIL-2). De ratte LAK cellen bleken niet in staat de ratteleukemieën te lyseren, terwijl dat wel het geval was in ratte solide tumorcellijnen. Of cytokinen geproduceerd door LAK cellen eventueel leukemiecel lysis zou mediëren werd bestudeerd door verschillende hoeveelheden ratteleukemiecellen en LAK cellen te co-cultiveren. Dit resulteerde in een lichte suppressie van de koloniegroei. Dus lijkt de GVL reaktie waargenomen in de rattemodellen na allogene BMT niet te berusten op een direct antileukemisch effect van LAK cellen. Deze resultaten zijn in overeenstemming met onze T-cel depletie data die impliceren dat GVHD (en GVL) werden voorkomen door de depletie van CD4 positieve cellen.

Recent is beschreven dat acute GVHD mogelijk de lytische aktiviteit van NK cellen doet toenemen maar niet de aantallen LAK precursorcellen verhoogt. LAK precursor aantallen zijn lager dan normaal gedurende de eerste twee maanden na BMT. De rol van LAK effector cellen in GVL hangt waarschijnlijk meer samen met de mate van cellulaire aktiviteit dan met de aantallen geaktiveerde cellen.[43]

7.5 GVL EFFECTEN GEÏNDUCEERD DOOR IL-2

In vele preklinische[24,44-51] en klinische studies[52-55] is de rol van IL-2 in GVL effecten en GVHD onderstreept.

Om mogelijke versterking van het GVL effect zonder de inductie van letale GVHD m.b.v. immunotherapie te bestuderen, werd IL-2 behandeling post-BMT toegepast in het BNML model (**Hoofdstuk 5**). De resultaten duiden op de inductie van een klein antileukemisch effect in ratten met minimale residuele leukemie behandeld met hoge doses IL-2 na syngene BMT (equivalent van humane autologe BMT). Studies naar de toediening van syngene lymfocyten, al dan niet geaktiveerd met IL-2 in de aanwezigheid van bestraalde BNML cellen, aan het BM transplantaat gecombineerd met hoge dosis IL-2 therapie zouden kunnen uitsluiten of IL-2 behandeling na autologe BMT kan leiden tot antileukemische effecten. Onlangs is er een publikatie omtrent IL-2 therapie en autologe BMT verschenen. Transplantatie van autoloog, met IL-2 geaktiveerd BM, gevolgd door IL-2 toediening is een

nieuwe benadering welke *ex vivo* purgeren en *in vivo* immunotherapie combineert. De eerst klinische resultaten bevestigen de toepasbaarheid.[56]

Herhaalde IL-2 toediening vanaf 3 dagen na allogene BMT in het BNML model, heeft geen significant antileukemisch effect of letale acute GVHD als gevolg wanneer er, respectievelijk, "lage" of "hoge" doses IL-2 werden gebruikt. Een tussenliggend dosis (2x78,000 IU/day) Proleukin® resulteert wel in een significant antileukemisch effect, met een toename in overlevingstijd die correleert met een afname in leukemiecellen van 6.4 log, zonder de inductie van GVHD (**Hoofdstuk 5**). We hebben aangetoond dat de maximale GVL reactie (zonder duidelijke GVHD), geïnduceerd door toediening van cruciale aantallen donor lymfocyten aan het allogene transplantaat, overeenkomt met 2-3 log leukemiecel afname. Het blijkt dat de GVL reactie, die verdwijnt na T-cel depletie van het allogene transplantaat, waarschijnlijk meer dan volledig gecompenseerd kan worden door IL-2 toediening na allogene T-cel gedepleteerde BMT (Tabel 7.1). Onze data duiden dus op een mogelijk belangrijke bijdrage van IL-2 aan de GVL reactie na allogene T-cel gedepleteerde BMT, hetgeen de therapeutische marge van klinische allogene BMT duidelijk vergroot.

Tabel 7.1 De rat als een model voor humane allogene BMT

Rattemodel	Humane equivalent	GVL
Ongedepleteerd BM	T-cel gedepleteerde BMT	-
Ongedepleteerd BM + miltcellen (A)	Ongedepleteerd BM	+
Ongedepleteerd BM + IL-2 (B)	T-cel gedepleteerde BMT + IL-2	+++

Log leukemiecel afname in het BNML model:
(A) 2-3
(B) 6.4

In studies met transplanteerbare leukemie in muizen, hebben Sykes et al. geobserveerd dat na transplantatie van compleet MHC-verschillend BM en toegevoegde allogene lymfocyten, vroege behandeling met IL-2 (vanaf de dag van BMT) selectief de door CD4 positieve cellen geïnduceerde GVHD remt, maar niet de antileukemische effecten van CD8 positieve cellen beïnvloedt.[24,51] Bij BMT, gebruik makend van verschillende muizestam combinaties in welke zowel GVL als GVHD CD4 afhankelijk waren, remde IL-2 behandeling GVHD maar niet de antileukemische effecten van CD4 positieve cellen.[51] Deze bevindingen suggereren dat het inhiberende effect van IL-2 is gericht tegen een gelimiteerde subset of functie van CD4 positieve cellen, en dat GVL en GVHD gemedieërd kunnen worden door verschillende CD4 mechanismen. Tenslotte hebben deze auteurs aangetoond dat de CD4 aktiviteit welke de GVHD medieërt wordt bepaald door de histo-incompatibiliteit tussen donor en ontvanger,[25] en dat IL-2 zowel CD4 als CD8 gemedieërde GVL aktiviteiten in stand houdt.[57] Wanneer de toediening van IL-2 langer na BMT wordt gestart, bijvoorbeeld na 7 dagen, kan IL-2 de ernst

van GVHD juist doen toenemen.[48,49] Bevestiging van deze gecompliceerde interakties moet worden afgewacht alvorens implicaties voor de kliniek kunnen worden geëvalueerd.

Concluderend, blijft het dubieus of IL-2 behandeling een GVL effect kan induceren na syngene BMT in leukemische ratten. Nieuwe studies naar de toediening van syngene lymfocyten gecombineerd met hoge dosis IL-2 behandeling zal deze vraag beantwoorden. De resultaten van Sykes et al. duiden op GVHD-suppressieve effecten van onmiddellijke initiatie van IL-2 behandeling na allogene, niet T-cel gedepleteerde BMT zonder vermindering van het GVL effect. Zij hebben aangetoond dat deze reaktie op IL-2 zeer afhankelijk is van het tijdstip van toediening en de mate van histo-incompatibiliteit tussen de donor en de ontvanger. Desalniettemin lijkt deze benadering een grote kans op ernstige GVHD te veroorzaken. Onze data duiden er op dat IL-2 behandeling na allogene, T-cel gedepleteerde BMT een significant grotere therapeutische marge geeft dan allogene niet T-cel gedepleteerde BMT. Bovendien zijn er onlangs veelbelovende gegevens van een fase II studie gepubliceerd betreffende lage dosis IL-2 behandeling na allogene T-cel gedepleteerde BMT voor hematologische maligniteiten.[55] Dus, een gerandomiseerde studie naar IL-2 immunotherapie na allogene (HLA-identieke) T-cel gedepleteerde BMT voor leukemie is gewenst.

7.6 GVL EFFECT; LEUKEMIE-SPECIFIEK OF EEN ALLOGENE REAKTIE?

Tot op heden is de discussie nog altijd gaande of het GVL effect en GVHD twee verschillende reakties zijn of dat beide reakties volgens dezelfde mechanismen verlopen. De T-cel depletie studies en de IL-2 experimenten hebben tot nu toe geen van beide een GVL effect los van GVHD kunnen aantonen. Beide reakties zijn zeer afhankelijk van de mate van histo-incompatibiliteit tussen donor en ontvanger. Zelfs wanneer deze MHC-identiek zijn kunnen verschillen in minor histocompatibiliteit antigenen aanleiding geven tot GVL en GVH reakties.

Met de BNML en de L4415 ratteleukemie werd getracht een GVL reaktie te induceren los van GVHD door semi-allogene (hybride-naar-ouder of ouder-naar-hybride) BMT toe te passen (**Hoofdstuk 6**). Het is niet mogelijk om in een hybride-naar-ouder transplantatie GVHD te induceren. Ook de toediening van grote aantallen lymfocyten aan het hybride BM transplantaat bleek niet te resulteren in een antileukemisch (GVL) effect.

In de ouder-naar-hybride transplantatie waarbij het transplantaat dezelfde alloantigenen draagt als de leukemie cellen werd geen GVL effect waargenomen, onafhankelijk van de aantallen toegevoegde lymfocyten of de inductie van (letale) GVHD. Wanneer het ouder-transplantaat histo-incompatibel was met de leukemiecellen was het BM transplantaat zonder toevoeging van lymfocyten in staat een antileukemisch effect te induceren. Toevoeging van kritische aantallen semi-allogene lymfocyten kon de GVL verder doen toenemen zonder verschijnselen van ernstige GVHD. Het antileukemische effect van BM zonder toevoeging

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van donor lymfocyten zou verklaard kunnen worden door het feit dat de leukemiecellen in deze setting de enige cellen zijn die volledig MHC-afwijkend zijn ten opzichte van de haplo-identieke ontvanger, hetgeen de GVL/GVHD "drempel" verhoogt. Hogere aantallen lymfocyten in het transplantaat induceerde acute letale GVHD. Deze resultaten geven het overtuigende bewijs dat het GVL effect, waargenomen na MHC-afwijkende BMT in deze rattemodellen voor ALL en AML, berust op allogene reakties, en dus niet te scheiden is van GVHD. De data duiden opnieuw op een kwantitatief verschil in gevoeligheid van leukemische en normale cellen, wat leidt tot schijnbaar verschillende GVL en GVH reakties, hetgeen onze eerder geponeerde (**Hoofdstuk 3**) "drempel" hypothese bevestigd.

Een andere benadering om de gunstige van de kwalijke effecten van allogene T-cellen te scheiden is de *in vitro* expansie en klonering van donor T-cellen die specifiek reageren met leukemiecellen, maar niet met de niet-maligne ontvanger cellen. Men heeft aangetoond dat leukemie "specifieke" cytotoxisch T-cel (CTL) lijnen en klonen gegenereerd kunnen worden uit perifeer bloed van MHC-identieke familieleden van patiënten met leukemie, welke niet reageren met niet-maligne cellen van diezelfde patiënt *in vitro*. [58,59] De specifieke herkenning van sommige leukemieën/lymfomen door effectorcellen zou verklaard kunnen worden door de expressie van zogenaamde fusie proteïnen, gecodeerd door genrearrangeringen die een gevolg zijn van chromosomale translokaties. Bekende voorbeelden zijn BCR-ABL, een gevolg van de translokatie t(9;22) in Philadelphia chromosoom (Ph1) positieve CML en ALL, het E2A-PBX eiwit geassocieerd met t(1;19) in pre-B cel ALL, PML-RAR bij t(15;17) in acute promyelocyten leukemie (PML) en DEK-CAN in t(6;9) AML. [60-62] De AML cellen gebruikt om de "specifieke" CTL klonen te stimuleren zijn gekenmerkt door t(8;16). Desondanks was de specificiteit niet geassocieerd met deze translokatie daar de andere leukemieën die herkend werden door deze CTL niet de t(8;16) vertoonden. [59] Om inzicht te krijgen in de mogelijke klinische toepassing van donor CTL lijnen welke reageren met de leukemieën van patiënten die een leukemie recidief vertonen na allogene BMT, hebben Falkenburg et al. een pilot studie uitgevoerd waarbij acht verschillende donor-ontvanger combinaties gebruikt werden. [63] In vijf van deze gevallen bleken de CTL lijnen reaktief met de leukemiecellen en niet met de IL-2 geaktiveerde lymfocyten van hetzelfde individu, hetgeen de "relatieve specificiteit" voor leukemiecellen demonstreert. Dit sluit niet uit dat deze cellen mogelijk toch reageren met antigenen van de target organen van GVHD. [63] Het lijkt aannemelijk dat deze "leukemie-specifieke" T-cellen slechts in zeer lage aantallen voorkomen in normaal perifeer bloed. Het scheiden van deze T-cellen van cellen die wel GVHD induceren, en vervolgens de *in vitro* klonering en vermeerdering zouden onpraktisch kunnen blijken voor de toepassing in BMT. [64]

Een minder verfijnde, maar tot nu toe beter toepasbare methode om een remissie te induceren in patiënten met een CML of AML recidief na allogene BMT is de toediening van donor buffy coat cellen. [65-73] Hoewel details in de protocollen variëren, is bewezen dat allogene buffy coat mononucleaire cellen CML *in vivo* kunnen uitroeien. Zelfs wanneer

ernstige acute GVHD optrad (graad II-IV in 51% van de patiënten), was GVHD slechts in één patiënt de directe doodsoorzaak (=2.2%). Een retrospectief onderzoek naar de ziektevrije overleving in deze studies in vergelijking met die na een tweede BMT, toont dat zowel de recidief-percentages als de therapie-gerelateerde mortaliteit substantieel lager liggen na buffy coat transfusies.[74,75] Het blijkt dat de GVL reactie effectiever is in chronische fase CML dan in andere hematologische maligniteiten of progressieve CML. Toch duiden retrospectieve analyses ook op een GVL effect in non-Hodgkin lymfomen[76] en acute leukemieën.[5,69] Verdere studies naar een betrouwbare remissie-inductie met donor buffy coat transfusies, zonder ernstige (letale) GVHD te induceren, zijn thans van groot belang.

7.7 TOEKOMSTVERWACHTING

Lopende studies zullen ophelderen of het mogelijk is de gebruikte kweek-, stimulatie- en klonerings-technieken te optimaliseren om leukemie specifieke CTL lijnen te genereren, bij voorkeur voor elke afzonderlijke patiënt (in remissie), en wel in een dermate korte tijd dat de residuele leukemie geëlimineerd kan worden alvorens deze tot grote celaantallen uitgroeit en niet langer met immunotherapie te behandelen is. Nieuw ontdekte cytokines en cytokine-therapie studies zullen voortdurend de kennis verbeteren betreffende de _ex vivo_ kweek, stimulatie en klonering van lymfocyten, en de preventie van anergie en/of apoptosis van deze cellen na toediening.

Intussen zal een adequate vervolgtijd van patiënten die reeds behandeld zijn en nieuwe studies naar de toediening van donor buffy coat lymfocyten aan patiënten met een leukemie recidief na allogene BMT leiden tot een mogelijke verbetering van deze behandeling. Tot nu toe hebben alleen Drobyski et al.[67] getracht een standaard aantal donor T-cellen toe te dienen. Complete moleculaire remissie werd geïnduceerd met "slechts" 0.34×10^8 cellen/kg, terwijl sommige patiënten die een veel hogere dosis ontvingen geen respons vertoonden. Het is belangrijk om een mogelijk "optimale" dosis te bepalen in combinatie met de mate van histo-incompatibiliteit, en om een hoeveelheid cellen toe te dienen voldoende om een remissie te induceren, maar tevens zo klein mogelijk om de kans op ernstige GVHD te beperken.[77]

Momenteel wordt er een nieuwe therapie bestudeerd ter vergroting van de therapeutische marge van GVL. Door cellen te transduceren met het Herpes Simplex thymidine kinase (HSV-tk) gen worden zij gevoelig voor destructie door ganciclovir, hetgeen de niet-getransduceerde cellen niet schaadt. Ganciclovir wordt gefosforyleerd door HSV-tk tot een monofosfaat derivaat, wat uiteindelijk wordt gefosforyleerd tot ganciclovir-trifosfaat, een competitieve remmer van de DNA-synthese,[78] met als gevolg een selectieve celdood van de HSV-tk expresserende cellen. Bewezen is dat deze benadering zeer efficiënt is in de behandeling van getransfecteerde (experimentele) tumorcellen.[79] Om het antileukemische effect van GVHD te benutten zonder de nadelige effecten van GVHD zouden HSV-tk

expresserende donor T-cellen kunnen worden toegevoegd aan het allogene (T-cel gedepleteerde) BM transplantaat om GVL en/of GVHD te induceren. GVHD zal dan binnen toelaatbare grenzen gehouden worden door behandeling met ganciclovir, waarbij selectief de delende lymfocyten die GVL en GVHD veroorzaken worden geëlimineerd. Deze behandeling zou het mogelijk maken een significant antileukemisch effect te induceren zonder letale GVHD. Cruciaal in de preklinische ontwikkeling (BNML en L4415 leukemie modèllen) van deze "suïcide gen" behandeling zal de transfectie-efficiëntie zijn van de donor lymfocyten met het HSV-tk gen, het efficiënt depleteren van de lymfocyten uit het allogene transplantaat om oncontroleerbare GVHD te voorkomen en de optimale timing van de onmiddellijke stopzetting van de GVL/GVH reactie om het antileukemische effect optimaal te kunnen benutten.

In de voortdurende ontwikkeling van nieuwe medicijnen voor de behandeling van humane ziektes kunnen ook nieuwe middelen worden gegenereerd die de behandeling van leukemie verder verbeteren. Een opvallend voorbeeld hiervan is dinaline (4-amino-N-(2'-aminophenyl) benzamide), een middel dat oorspronkelijk werd gepresenteerd als een uiterst probaat middel tegen convulsies.[80] Het is bewezen dat dit geneesmiddel en zijn geacetyleerde vorm acetyldinaline (ACD) sterk immunosuppressief zijn[81] en zeer effectief tegen langzaam groeiende experimentele tumoren[82] en de BNML.[83] In dit rattemodel toont ACD een exceptioneel sparend effect op normale hemopoiese en in tegenstelling tot de meeste andere middelen vormt ACD geen gevaar voor het (syngene) BM transplantaat wanneer het post-BMT worden gegeven. Een optimaal antileukemisch effect wordt bereikt door herhaalde dagelijkse orale toediening gedurende een langere periode, hetgeen het leukemiecel aantal met 8 log doet afnemen met tegelijkertijd slechts minder dan 1 log vermindering van de normale hemopoietische stamcellen.[84,85] Preliminaire resultaten laten zien dat een korte behandeling met ACD na allogene BMT met hoge aantallen allogene lymfocyten, de incidentie van acute letale GVHD reduceert van 100% tot 37.5% terwijl het antileukemische effect (5 log afname in leukemiecellen) bewaard blijft. Verdere studies met het BNML model zijn dus nodig naar de efficiëntie van ACD als een antileukemisch, immunosuppressief en BM ablatief middel (hetgeen een lagere dosis totale lichaamsbestraling zou toestaan) en op de eradicatie van minimale residuele leukemie en GVHD in de post-BMT periode. Momenteel wordt er een fase I studie met ACD uitgevoerd.

7.8 TENSLOTTE

Om terug te komen op de titel van dit proefschrift, voor wat betreft de klinische toepasbaarheid van GVL, kan het volgende worden geconcludeerd. Het GVL effect zonder duidelijke symptomen van GVHD, waargenomen in ons rattemodel evenals in gerapporteerde experimentele en klinische studies, is niets meer of minder dan een subklinisch GVH-effect.

Sommige onderzoekers beschouwen het GVL effect (zonder symptomen van GVHD) als een losstaande reaktie, misleid door de kwantitatieve verschillen in gevoeligheid van leukemische en normale cellen voor de allogene lymfocyten, resulterend in een "drempel-effect" (Figuur 7.1).

De intensiteit van het GVL effect geïnduceerd in MHC-identieke allogene BMT varieert tussen de verschillende leukemieën en donor-ontvanger combinaties in muizen.[86] Vergelijkbaar vertonen humane leukemieën een extreme variabiliteit in allo-stimulatie en speelt HLA-DR expressie een centrale rol in de bepaling van de allo-herkenning van leukemiecellen.[87] Om die redenen is het onmogelijk om allogene BMT in patiënten te standaardiseren. Omdat het eenvoudiger blijkt om GVHD te voorkomen dan om een aktieve GVH reaktie te stoppen,[88] wordt het sterk afgeraden om moedwillig een GVHD te induceren om zijn antileukemische effect, totdat betrouwbare methoden zijn ontwikkeld om GVHD te stoppen. Een uitzondering zou gemaakt kunnen worden voor BMT bij de behandeling van CML, waarbij een beperkte T-cel depletie (één log in plaats van twee log) van het beenmerg transplantaat optimaal lijkt te zijn. Optimalisatie van de behandeling voor en na allogene T-cel gedepleteerde, HLA-identieke BMT zou de spil van verdere studies ter vermindering van leukemie recidieven in de behandeling van leukemie moeten zijn. De studies in het BNML model duiden erop dat ACD behandeling in combinatie met de huidige conditionerings regimes, of zelfs in combinatie met gereduceerde doses totale lichaamsbestraling, de optimale behandeling zou zijn pre-BMT. Behalve optimale conditionering, zou momenteel T-cel gedepleteerde HLA-identieke BMT en post-BMT IL-2 behandeling de beste benadering kunnen zijn in de behandeling van CML en acute leukemieën. Bij moleculaire detectie van een leukemie recidief (PCR) zouden buffy coat transfusies (± 1-2 x 10^8 mononucleaire cellen/kg) toegediend moeten worden, gevolgd door ACD behandeling wanneer symptomen van acute GVHD waargenomen worden. Studies naar de toepassing van lymfocyten die het "suïcide gen" tot expressie brengen,[89] zouden in de nabije toekomst kunnen leiden tot de bewuste inductie van GVL zonder de catastrofale gevolgen van GVHD te riskeren.

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ABBREVIATIONS

ACD : acetyldinaline
ADCC : antibody-dependent cellular cytotoxicity
Ag : antigen
ALL : acute lymphocytic leukmemia
AML : acute myelocytic leukemia
APC : antigen presenting cell
ATG : antithymocyte globulin
BM : bone marrow
BMT : bone marrow transplantation
BN : Brown Norway rat
BNML : Brown Norway acute myelocytic leukemia
Bu : busulfan
CD : cluster of differentiation
CFU-S : colony forming unit-spleen
CLL : chronic lymphocytic leukemia
CML : chronic myelocytic leukemia
CMV : cytomegalovirus
CNS : central nervous system
CR : complete remisson
CsA : cyclosporine A
CTL : cytotoxic T lymphocyte
Cy : cyclophosphamide
D_o : dose inducing a surviving fraction of 0.37
DFS : disease free survival
DIC : diffuse intravascular coagulation
DMBA : 9,10-dimethyl-1,2-benzanthracene

EBV : Epstein-Barr virus
ED_{50} : 50% effective dose (affecting 50% of the animals)
ELAM : endothelial leukocyte adhesion molecule=E-selectin
F1 : hybrid strain
FACS : fluorescence activated cell sorter
f-factor : fraction of cells entering the spleen
GM-CSF : granulocyte-macrophage colony stimulating factor
GVH : graft-versus-host
GVHD : graft-versus-host disease
GVL : graft-versus-leukemia
GVLR : graft-versus-leukemia reaction
Gy : Gray
HLA : human leukocyte antigen
HSV : herpes simplex virus
HSV-tk : herpes simplex virus-thymidine kinase
ICAM : intercellular adhesion molecule
IFN : interferon
Ig : immunoglobulin
IL : interleukin
IL-1ra : interleukin-1 receptor antagonist
IL-2R : interleukin-2 receptor
ILS : increase of life span
i.p. : intraperitoneal

i.t.	: intrathecal	PB	: peripheral blood
IU	: international unit	PBL	: peripheral blood lymphocytes
i.v.	: intravenous	PCR	: polymerase chain reaction
LAK	: lymphokine activated killer	PLA_2	: phospholipase A_2
LCFU-S	: leukemic-colony forming unit-spleen	PNH	: paroxysmale nachtelijke hemoglobinurie
LCK	: log leukemic cell kill	R^2	: correlation coefficient of variation
LFA	: leukocyte function antigen		
LGL	: large granular lymphocyte	s.c.	: subcutaneous
LN	: lymph node	SCA	: spleen-colony-assay
MCA	: monoclonal antibody	SCID	: severe combined immunodeficiency disease
MDS	: myelodysplastic syndrome		
MdST	: median survival time	S.D.	: standard deviation
mH	: minor histocompatibility complex	S.E.	: standard error
		SPF	: specified pathogen free
MHC	: major histocompatibility complex	TBI	: total body irradiation
		TCD	: T-cell depletion
MRD	: minimal residual disease	TCR	: T-cell receptor
mRNA	: messenger ribonucleic acid	TGF	: transforming growth factor
n	: number in group	Th	: T helper cell
N	: extrapolation number	Thp	: T helper precursor cell
NK cell	: natural killer cell	TNF	: tumor necrosis factor
NKSF	: natural killer cell stimulatory factor	VOD	: veno-occlusive disease
		WAG/Rij	: Wistar Albino Glaxo rat (bred in Rijswijk)
NS	: not significant		
p	: probability	WBC	: white blood cells
P	: parent		

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 27 juni 1962 te Schiedam. Nadat de diploma's HAVO en VWO behaald waren aan de scholengemeenschap Etten-Leur, werd in 1982 met de studie Biologie aangevangen. Gedurende deze studie werden de drie hoofdvakken Moleculaire Celbiologie o.l.v. Dr. Verhoef (Rijksuniversiteit Utrecht, 14 maanden), Immunologie o.l.v. Dr. Sterenberg en Prof. Dr. den Otter (RIVM, Bilthoven, 9 maanden) en Genetica o.l.v. Dr. Ostrer en Prof. Dr. Voorma (Universiteit van Florida, Gainesville, USA, 10 maanden) doorlopen. Het doctoraal examen Medische Biologie (B5*) werd in februari 1989 behaald.

Van april 1989 tot september 1994 werd het hier beschreven promotieonderzoek verricht binnen het Instituut voor Toegepaste Radiobiologie en Immunologie (ITRI) - TNO te Rijswijk en vanaf januari 1993, het Instituut voor Hematologie van de Erasmus Universiteit Rotterdam, o.l.v. Prof. Dr. A. Hagenbeek en Prof. Dr. D.W. van Bekkum.

Sedert september 1994 is de auteur aangesteld als wetenschappelijk medewerker (post-doc) binnen de afdeling Hematologie van het Academisch Ziekenhuis Leiden.

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