T LYMPHOCYTES AND CYTOKINES IN GRAFT-VERSUS-HOST DISEASE

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A study in mice with emphasis on prevention and treatment

T LYMFOCYTEN EN CYTOKINEN BIJ
GRAFT-VERSUS-HOST ZIEKTE

PROEFSCHRIFT

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aan de Erasmus Universiteit Rotterdam
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Wat hebt gij, dat gij niet hebt ontvangen ?

1 Korinthe 4: 7b

Aan Arianne
Aan Kees
Aan mijn ouders

T LYMPHOCYTES AND CYTOKINES IN GRAFT-VERSUS-HOST DISEASE

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Graft-versus-host disease

1.1 General introduction

Bone marrow transplantation (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 (1). Following transplantation, donor stem cells engraft and reconstitute hematopoiesis and immunity in the recipient. For transplantation, hemopoietic cells from various sources can be used: from the host (autologous), from an identical twin (syngeneic) or from related or unrelated donors (allogeneic). For the treatment of malignancies, allogeneic, syngeneic as well as autologous BMT can be applied. For treatment of severe immunodeficiencies and metabolic diseases, allogeneic BMT is indicated. Worldwide over 4,000 patients per year receive an allogeneic BMT, 73% for leukemia, 11% for other malignant diseases, 9% for severe aplastic anemia, 3% for immunodeficiencies, 2% for thalassemia major, and less than 2% for inborn errors of metabolism. A similar number of patients receive an autologous BMT (1,2). In general, autologous BMT can only be performed when the bone marrow is not involved in the disease. In the case of leukemia, however, autologous bone marrow can be used when taken during remission. Alternatively, the bone marrow can be manipulated *in vitro* to remove (residual) leukemic cells (2). The period after BMT can be complicated by serious problems (Table 1).

Table 1. Potential complications of bone marrow transplantation*.

Toxicity of the preparative regimen
Graft rejection or failure
Hemorrhagic diathesis
Acute and chronic graft-versus-host disease
Interstitial pneumonitis
Opportunistic infections
Secondary malignancy

* adapted from reference 2.

In allogeneic BMT, donor and recipient should be compatible for the major histocompatibility complex (MHC) because of the high incidence of severe acute graft-versus-host disease (GVHD) and graft rejection after MHC incompatible BMT (3,4). For only about one third of the patients that would benefit from allogeneic BMT an MHC-identical sibling donor is available. Therefore, it is necessary to improve prevention and treatment of GVHD, which is still the major complication of allogeneic BMT. This would allow successful BMT for a greater number of patients.

The aim of the work described in this thesis was to improve insight into the complex syndrome of GVHD and especially in the role of T lymphocytes and cytokines. Furthermore, we investigated whether blood transfusion of the prospective donors of hemopoietic cells could reduce GVHD. This possibility was considered because of the well-known beneficial effect of blood transfusion in kidney and heart transplantation (5,6).

1.2 Graft-versus-host disease after bone marrow transplantation

At present, GVHD remains the major cause of morbidity and mortality after allogeneic BMT. GVHD can occur not only after BMT, but also in a number of other situations (Table 2). About 50% of the patients receiving an allogeneic bone marrow graft from an HLA identical sibling donor will develop some degree of GVHD. Approximately 25% of them will not survive the BMT procedure, due to GVHD or complications of the immunosuppressive treatment of GVHD (7).

The syndrome that is presently well-known as GVHD, was firstly described by Barnes and Loutit (8). Cohen, Vos and Van Bekkum were the first to introduce the name GVHD, postulating the idea that the disease was a consequence of the reaction of

Table 2. Procedures associated with a high risk of GVHD*.

Procedure	groups at risk
Solid-organ transplantation	recipients of small-bowel transplants
Transfusion of unirradiated blood products	neonates and fetuses patients with congenital immunodeficiency syndromes patients receiving immunosuppressive or chemoradiotherapy patients receiving blood donations from partially HLA identical, HLA homozygous donors

* adapted from reference 14.

transplanted donor cells to host tissues (9). In 1966 Billingham formulated three requirements for the development of GVHD (10):

- the graft must contain immunologically competent cells;
- the recipient must express foreign tissue antigens;
- the recipient should be unable to reject the transplanted cells.

During the 1970s it became more and more clear that GVHD was primarily caused by mature T lymphocytes residing in the transplanted graft (11). GVHD can develop when donor and host are mismatched for MHC antigens, but also when they are fully compatible. It has been suggested that minor histocompatibility antigens may elicit the GVH reaction in this case (12). In mice, in certain donor-recipient combinations minor histocompatibility antigens appeared to be able to induce severe GVHD (13).

More recently, it has been reported that GVHD can even occur after syngeneic BMT. GVHD might be the result of inappropriate recognition of self-antigens (14,15). It can be questioned, whether in the latter case the term GVHD is correctly used. For this purpose Bos et al. proposed to use the term BMT-associated immune disease (15).

Two distinct syndromes can be distinguished: acute and chronic GVHD. In humans, acute GVHD may develop at three weeks after allogeneic BMT, whereas chronic GVHD typically develops 6-12 months after transplantation (2). In rodents, acute GVHD usually starts 1-2 weeks after allogeneic reconstitution, whereas chronic GVHD starts at 1 month (16). Acute GVHD is closely correlated to the number of mature T cells within the graft. It has been suggested that chronic GVHD in humans might be caused by newly developed donor-derived T lymphocytes in the host or by long-lived lymphocytes of donor origin that have been sensitized to unknown antigens of the host (15). In rodents, chronic GVHD seems to be more closely related to the number of mature T lymphocytes within the graft than in humans.

The occurrence of GVHD can be associated with an anti-leukemic effect. In patients with GVHD, a decreased incidence of relapse of malignant disease was found. It has been suggested that this effect is caused by donor (T) lymphocytes that are able to recognize residual tumor cells, which bear host histocompatibility antigens, and destroy the tumor cells. This phenomenon has been termed graft-versus-leukemia (GVL) effect. Current investigations of several groups are aimed to explore whether it is possible to prevent GVHD, while sparing the GVL effect (17,18).

1.3 T lymphocytes in graft-versus-host disease

Many investigators have shown that T lymphocytes play an essential role both in transplant rejection and GVHD (14,19-22). In general, T cells can recognize antigens

only when presented by antigen-presenting cells (APC). Various cell types have the capacity to present antigen. Dendritic cells and macrophages are APC that probably play an important role in transplant rejection and GVHD. The recognition of antigens presented by APC occurs in the context of class I or class II MHC determinants. This phenomenon is called MHC restriction. In humans, the MHC is known as the Human Leucocyte Antigen (HLA) system. The murine MHC is named Histocompatibility (H)-2 complex. It is likely that foreign antigens are processed to peptides, which are bound in the groove of MHC molecules. These MHC-peptide complexes are recognized by T cells (23).

Class I and class II molecules have similarities in their structure and function, but differ in their fine structure and also in their expression on various cell types. Class I molecules are constitutively expressed on almost all nucleated cells (24), whereas class II molecules are expressed only on certain cell types, such as B lymphocytes, macrophages, monocytes, Langerhans cells and dendritic cells. They can be induced on T lymphocytes, endothelial cells, renal tubular cells, pancreatic and thyroid cells as well as several other cell types by certain cytokines, especially interferon-gamma (IFN-γ) (21,25).

T lymphocytes can be divided into two subclasses, based on their cell surface antigen expression: a $CD4^+$ T cell subset and a $CD8^+$ T cell subset. $CD4^+$ T cells are class II restricted, whereas $CD8^+$ T cells are class I restricted. Although previously it has been thought that the phenotype of a T cell correlated with its function, it has now become clear that phenotype and function are not correlated (26). Helper functions are usually performed by $CD4^+$ T cells, but can also be performed by $CD8^+$ T cells, whereas cytotoxic/suppressor functions are usually mediated by $CD8^+$ T cells, but sometimes also by $CD4^+$ T cells.

T lymphocytes can also be subdivided based on their cytokine profile (Table 3). In this context two $CD4^+$ T helper (Th) cell subsets can be recognized: a Th1 and Th2 subset. Th1 cells produce a.o. interleukin (IL)-2 and IFN-γ, whereas Th2 cells produce a.o. 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 in a Th0 cell, which secretes IL-2 and IFN-γ as well as IL-4 and IL-5 (27). The cytokine profile of $CD8^+$ T cells resembles that of the Th1 subset, but their capacity to produce IL-2 is limited (28). Although these data are obtained from *in vitro* studies, evidence is increasing that at least certain strong immune responses in mice and humans are preferentially mediated by Th1 or Th2 cells (29). Th1 and Th2 cells crossregulate each other. Th2 cells inhibit the cytokine synthesis of Th1 cells by IL-10 (originally named Cytokine Synthesis Inhibitory Factor). Moreover, Th2 cells inhibit the cytokine synthesis of cytotoxic T lymphocytes (CTL) which have a Th1 like cytokine profile.

Table 3. Cytokine secretion patterns of mouse T lymphocytes*.

	Thp	Th0	Th1	Th2	CTL
IL-2	++	++	++	-	+/-
IFN-γ	-	++	++	-	++
LT			++	-	+
TNF-α			++	+	+
IL-4	-	++	-	++	-
IL-5	-	++	-	++	-
IL-6	-		-	++	-
IL-10	-	++	-	++	-

* according to reference 31.

On the other hand, Th1 cells inhibit the proliferation of Th2 cells by the production of IFN-γ (30,31).

T lymphocytes use a specific cell surface receptor, which is built up from two subunits: the α and β chain to recognize antigens that are presented by APC. Other cell surface molecules contribute to the interaction between T cells and APC: co-receptors such as CD4 and CD8, accessory molecules such as CD44 and CD45, lymphocyte function-associated antigens such as CD11a/CD18 (LFA-1), CD2 (LFA-2) (21,32-34), and adhesion molecules such as ICAM-1, ICAM-2 and ELAM-1. Probably they intensify the contact between the T cell and APC. Some of them might also play a role in signal transduction.

Upon the contact of T cells with (allo)antigens, T cell receptor signal transduction occurs via the CD3 complex, which consists of transmembrane polypeptide chains that are associated with the T cell receptor. This leads to intracellular activation and a cascade of events that results in the transcription of genes for cytokines 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 IFN-γ, IL-3, IL-4, IL-5 and IL-6 production starts. As a consequence, T lymphocytes differentiate, thereby gaining specialized effector functions and/or cytokine production profiles (21).

Both $CD4^+$ and $CD8^+$ T lymphocytes have been shown to be involved in GVHD in mice. It appeared that the T cell subset involved was dependent on the MHC or non-MHC 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 were able to cause lethal GVHD in a class I disparate strain

combination (35). In minor histocompatibility disparate strain combinations, either the $CD4^+$ T subset or the $CD8^+$ T cell subset can play a dominant role and in some minor disparate strain combinations both subsets appear to be equally involved (20).

1.4 Cytokines in graft-versus-host disease

Cytokines are important mediators in the immune system and evidence is increasing that they are also involved in organ transplant rejection and GVHD. The number of cytokines reported to play a role in transplant rejection and GVHD steadily increases. It is likely that different cytokines play a role in different phases of the GVH reaction. Ferrara and Deeg distinguish an afferent phase, in which IL-1 and IL-2 are involved and an efferent or effector phase in which other cytokines, such as IFN-γ and tumor necrosis factor (TNF)-α are implicated (14).

Animal studies indicate an important role for IL-2 which is necessary for T cell proliferation and differentiation (21). It was found that anti-IL-2R monoclonal antibody (mAb) treatment was able to prevent not only a local GVH response (36), but also a systemic GVH reaction (37). Preliminary data in human BMT also indicate that anti-IL-2R mAb may be useful in the prevention or even treatment of GVHD. Experimental data further showed that exogenous IL-2 administration can enhance the mortality of lethal GVHD (38-41). The effect of exogenous IL-2 seems to be dependent on the time of administration. Sykes et al. reported even an inhibitory effect of IL-2 when administered before the induction of GVHD (41).

Mowat et al. (42) were able to demonstrate that anti-IFN-γ mAb were able to decrease the severity of gut lesions due to GVHD, although this treatment did not improve the survival. It is well known that IFN-γ can enhance the expression of cell surface molecules, such as class II, thereby potentiating the immune response (43). IFN-γ can also activate other cell types that might play a role, such as natural killer cells and macrophages. IFN-γ could be demonstrated in a human skin explant model, which showed lesions characteristic for GVHD and can be used to test the *in vitro* reactivity of sensitized lymphocytes on a skin explant. Anti-IFN-γ mAb inhibited the development of the skin lesions in this model (44).

In the same model, TNF-α production was demonstrated and anti-TNF-α mAb were able to inhibit the appearance of skin lesions. A role for TNF-α in cutaneous lesions of GVHD was also suggested by others (45). TNF-α might be the cause of intestinal lesions in acute GVHD as well (45). Elevated serum levels of TNF-α were found in human BMT prior to the development of GVHD (46,47). The GVH reaction in newborn mice could be prevented by administration of antibodies to TNF-α (48).

IFN-γ and TNF-α might act synergistically in the up-regulation of class I and class II antigen expression (49). TNF-α might act also indirectly by activating CTL and macrophages (50). Cohen suggested that endotoxin from the gut microflora, upon entering the circulation, might be a strong stimulus for the induction of TNF-α, which in turn might result in lesions that are characteristic for GVHD. Passive immunization against endotoxin was able to protect mice from GVHD (51). This might also be the explanation for the fact that germ-free mice are less susceptible for developing GVHD (16).

When GVHD was induced by injection of parental lymphoid cells into F1 hybrid recipients, high IgE levels were induced, which could be prevented by treatment of the recipients with anti-IL-4 (52). However, in irradiated recipients suffering from GVHD no elevated IgE levels were found. It is likely that other (presently unknown) cytokines play a role as well. Further studies are necessary to determine their role and the phase of GVHD in which they are involved, and the possibilities to intervene in the cascade.

1.5 Pathology of graft-versus-host disease

The principal target organs of acute GVHD both in humans and animal species are the skin, liver, gastrointestinal tract and lymphoid tissues (53-55). At present, it is unknown, why especially these organ systems are involved. It has been suggested that primitive cell surface antigens on undifferentiated epithelial cells are the targets (15,56). The incidence and severity of GVHD is influenced by the degree of prior skin, liver or gastrointestinal injury, caused by drugs, radiation or infection. The first symptoms in humans most commonly involve the skin. A pruritic maculopapular rash on the palms, soles and ears can be found, which can progress to total-body erythema. Gastrointestinal symptoms include anorexia, nausea, (bloody) diarrhoea, abdominal pain and even paralytic ileus. When the liver is involved, hyperbilirubinemia and elevated serum levels of liver enzymes may be found and coagulation can be abnormal.

The grading of GVHD is based on clinical changes in skin, intestinal tract and liver (Table 4). Sometimes the diagnosis is difficult to make, since several complications of BMT have clinical features that are easily confused with GVHD, such as drug and radiation toxicity and bacterial and viral infections (55).

Chronic GVHD primarily involves the immune system, skin and liver. The number of $CD4^+$ T cells decreases and the antibody production of B cells is impaired. Skin lesions resemble widespread lichen planus with papulosquamous dermatitis. Severe chronic GVHD often leads to a syndrome which has similarities with some autoimmune diseases (54,57). The hepatic lesions are similar to those found in acute GVHD.

Table 4. Clinical stages of GVHD*.

Stage	skin	liver (bilirubin)	gut (ml diarrhoea)
1	rash < 25%	2 - 3.5	500 - 1000
2	rash 25-50%	3.5 - 8	1000 - 1500
3	erythroderma	8 - 15	1500 - 2500
4	bullae, desquamation		>1500

Overall clinical stage

Stage 1	cutaneous GVHD only - skin: stage 2 or greater, positive skin biopsy
Stage 2	skin: stage 1-3; liver and/or gut: stage 1; positive skin biopsy
Stage 2o	orange GVHD only - liver or gut disease with negative skin biopsy
Stage 2s	severe skin GVHD: stage 4, positive skin biopsy
Stage 3	skin: stage 2-4; liver and/or gut: stage 2-4; positive skin biopsy (only one organ system stage 3 or greater)
Stage 4	skin: stage 3 or 4; liver and gut: stage 2-4; positive skin biopsy (two or more systems stage 3 or greater)

* adapted from reference 7.

1.6 Prevention and treatment of graft-versus-host disease

To prevent the development of GVHD, immunosuppressive drugs such as cyclophosphamide and methotrexate have been used, however, with limited results (58,59). In addition, polyclonal anti-T cell reagents such as anti-thymocyte globulin (ATG) were introduced (60-62). In the late 1970s cyclosporin A (CsA) was developed and beneficially employed (63). Subsequently, combination treatments have led to the best results (5). However, the overall results remained disappointing since the immunosuppressive agents used were non-specific and proved to have serious side-effects. This led to the introduction of the use of T cell depleted grafts. This procedure strongly decreased the incidence of GVHD, but had also major drawbacks. Patients that underwent T cell depleted BMT were more prone to infection and more frequently suffered from delayed 'take' of the graft and graft rejection. Moreover, in patients transplanted for leukemia, the incidence of relapses increased, possibly due to abrogation of the GVL effect.

New strategies have to be developed for the prevention and treatment of GVHD, based on an improved insight into the underlying mechanisms. More and more mAb are employed in experimental and clinical organ transplantation. It is likely that mAb will be included in future therapeutic approaches in human BMT as well. Animal studies, especially in mice, have shown that anti-T cell mAb can be used effectively

to prevent marrow rejection and GVHD when given to the recipients prior to their reconstitution, even in completely allogeneic strain combinations (64,65). It appeared that anti-T cell mAb can vary considerably in their efficacy *in vivo* (66). It has been suggested that the effectiveness of mAb therapy is related to the epitope recognized by the mAb, the antigen density of the epitope on the target cell and the isotype and affinity of the mAb (66-68). Presumably this is also the case for mAb against human determinants (69). A number of variables require further study: the timing and dose, the effect of anti-T cell subset mAb, the influence of the isotype, the long-term effects, the interference with currently used immunosuppressive treatment and the mechanism of action. Encouraging preliminary data indicate that mAb treatment of the recipient might also be useful in human BMT (70,71).

Not only anti-T cell mAb, but also anti-cytokine mAb, anti-cytokine receptor mAb and mAb against accessory molecules are promising, since preliminary data indicate that they are able to improve the survival of GVHD (37,72,73).

Although mAb can be useful to prevent and treat GVHD, it has been suggested that mAb treatment might hamper the engraftment of transplanted hemopoietic cells. Both anti-T cell and anti-LFA-1 mAb have been implicated in this respect. Anti-T cell mAb might directly attack the hemopoietic cells, since it has been demonstrated that hemopoietic precursor cells may share T cell epitopes such as the Thy-1 antigen (74). It has also been suggested that the CD4 antigen might be present on (a subpopulation of) hemopoietic precursors. Theoretically, anti-Thy-1 and anti-CD4 treatment might therefore eliminate these cells *in vivo*. However, in practice this seems not to be a major problem because of the low expression of these antigens on hemopoietic precursor cells. This is supported by the observation that anti-Thy-1 treatment of recipient mice prior to their reconstitution led to complete and stable chimerism (66,69).

1.7 Introduction to the experimental work

The aim of the work presented in this thesis was to improve the insight into the complex syndrome indicated with the term GVHD and secondly to study some putative prophylactic and therapeutic modalities. In the experiments mice were used of which the microbiological status was carefully monitored and controlled. We employed a delayed-type hypersensitivity (DTH) assay (75), clinical symptoms and mortality to monitor GVH reactivity and GVHD. Since, according to Van Bekkum mouse bone marrow is a relatively poor source of T lymphocytes as compared to human bone marrow (54), spleen cells were used to induce GVHD. The DTH assay was based on the observation that spleen cells from allogeneically reconstituted mice displayed

specific DTH reactivity to recipient alloantigens. GVHD was evaluated by careful examination of the mice for the development of signs of GVHD, such as diarrhoea, weight loss, hunched posture, decreased physical activity, skin lesions and mortality. A control group, which was only irradiated, was always included.

The experiments described in Chapter 2 were based on previous work from our laboratory. Previously we have shown that donor pretreatment with a large dose of irradiated recipient-specific spleen cells suppressed the anti-recipient DTH response. Since in the clinical situation blood transfusion is commonly used, we first studied whether donor pretreatment with a recipient-specific blood transfusion was also able to suppress the anti-recipient DTH response. We investigated the role of various blood cell fractions, the dose-response relationship and the role of major and minor histocompatibility antigens. We also determined the phenotype of the cells that mediated suppression (Chapter 2.1).

We further assessed the effect of donor blood transfusion on the development of clinical symptoms and mortality of GVHD, which is more relevant to the clinical situation. We investigated the role of MHC differences, the dose-response relationship, the time-dependence and the specificity. We found that donor blood transfusion indeed could reduce GVHD (Chapter 2.2).

We also addressed the mechanism of the observed effect. We investigated whether the effect was to be ascribed to a veto cell mechanism or a suppressor cell mechanism. We studied the role of $CD4^+$ and $CD8^+$ T cells. The possible role of the Th1 and Th2 subsets is discussed (Chapter 2.3).

T cells are known to be the major cause of GVHD. In the literature, a strong beneficial effect of anti-T cell mAb on GVHD in mice was reported, both when used to treat donor marrow *in vitro* and when given to the recipients *before* bone marrow infusion. Rat mAb of the IgG2b subclass and mouse mAb of the IgG2a subclass appeared to be the most effective (66). We hypothesized, that anti-T cell subset mAb treatment could also be useful when given *after* bone marrow infusion. Chapter 3.1 deals with the effect of such *in vivo* treatment of the recipients *after* reconstitution with allogeneic lympho-hemopoietic cells, with mAb against all T cells and T cell subsets. The advantage of *in vivo* treatment of the recipients is that presumably both residual T lymphocytes of the host and transplanted T lymphocytes of the donor are eliminated. Elimination of donor T cells only would increase the risk of graft rejection, whereas elimination of recipient T cells only would increase the risk of GVHD. We determined the dose-response relationship, the timing and the effect of delayed treatment and employed mAb against all T cells as well as to T cell subsets.

In Chapter 3.2 we studied the effect of anti-T cell subset mAb in more detail and determined their capacity to deplete their target cell population *in vivo*. We also

evaluated long-term stable chimeras for the degree of chimerism and the state of tolerance.

In several studies reported in the literature, the *in vivo* effect of mAb depended on the isotype and specificity of the mAb employed. Therefore, we compared the effectiveness of several rat-anti-mouse mAb. We determined their capacity to eliminate their target cells *in vivo* and their effect on morbidity and mortality of GVHD (Chapter 3.3).

In recent years it has become more and more clear, that cytokines are important mediators in graft rejection and GVHD. Therefore, we investigated in the same model which cytokines were involved. First we studied the levels of various cytokines during GVHD in serum samples and culture supernatant of spleen cells. Thereafter we investigated the effect of *in vivo* treatment with recombinant cytokines, with anti-cytokine mAb, and with anti-cytokine receptor mAb (Chapter 4).

In Chapter 5 the results of the experimental work presented in this thesis are discussed in relation to the relevant international literature.

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Chapter 2

Effect of donor pretreatment with a recipient-specific blood transfusion on graft-versus-host reactivity and graft-versus-host disease

Chapter 2.1

Suppression of graft-versus-host reactivity by a single host-specific blood transfusion to prospective donors of hemopoietic cells*

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Summary

Delayed-type hypersensitivity (DTH) responses against recipient's histocompatibility antigens can occur early in the course of a graft-versus-host (GVH) reaction in lethally irradiated allogeneically reconstituted mice. This reactivity could be suppressed by a single host-specific blood transfusion to the prospective donors of allogeneic spleen cells. Maximum suppression was found when the blood transfusion was given 4 or 5 days before the mice were used to reconstitute lethally irradiated hosts. Whole blood and purified white blood cells were capable of inducing suppression, whereas purified red blood cells, plasma and serum were not. Suppression was already detectable after administration of 1 µl of whole blood and virtually complete at a dose of 1 ml. Irradiation of the blood reduced but did not abrogate its capacity to induce suppression. Purified B and purified T lymphocytes appeared equally effective in inducing suppression. Two helper T (Th) cell clones, a Th1 and a Th2 clone were able to induce suppression as well. A high dose of recombinant IL-2, injected daily for 5 days after reconstitution, did not abrogate or reduce the suppression. Suppression could be induced by H-2 as well as non-H-2 alloantigens, separately or together. A pure H-2 incompatible transfusion was more effective in inducing suppression than a pure non-H-2 incompatible one. Suppression appeared to be a dominant phenomenon and was mediated by a Thy-1$^+$, CD4$^+$, CD8$^-$ spleen cell population. This T cell population had its origin in the transfused donor, which excludes the possible involvement of blood-derived veto cells.

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Introduction

Blood transfusions are widely used in clinical practice. In organ transplantation, transfusion of prospective recipients in general improves the graft survival (1,2). In bone marrow transplantation (BMT) blood transfusion of prospective recipients is disadvantageous. Patients who have received blood transfusions before undergoing BMT, e.g. in the case of aplastic anemia, are at high risk for the occurrence of marrow graft rejection, which increases with the number of transfusions (3).

This discrepancy led us to investigate the effect of blood transfusion in a preclinical BMT model in mice. Previously we have studied suppression of host-versus-graft (HVG) reactivity by a donor-specific blood transfusion (4). In the present study we investigated the blood transfusion effect under GVH conditions. We studied pretreatment of the donors of hemopoietic cells with a host-specific blood transfusion since our principal aim was to prevent graft-versus-host disease (GVHD) and not graft rejection. A number of experimental studies have reported improved survival of GVHD in a minor alloantigen disparate strain combination, after transplantation of hemopoietic cells from donors that were immunized against host alloantigens (5-7). Thus far a beneficial effect of allogeneic blood transfusion in BMT, has not been documented.

The various constituents of whole blood that are possibly involved in the blood transfusion effect in organ transplantation are leukocytes, red blood cells, dendritic cells, platelets and the plasma component (8-10). The amount of blood used for transfusion plays an important role (11). Both major and minor alloantigens have the capacity to induce suppression. In a rat kidney transplantation model allogeneic help was necessary for the activation of suppression by minor alloantigens only (12).

In this study we deal with the effect of host-specific blood transfusion on the anti-host DTH reaction early after transplantation of spleen cells to lethally irradiated recipients. Some of our preliminary data were reported elsewhere (13). In this study, we investigated in more detail the effect of the various fractions of whole blood, the dose-response relationship and the role of major and minor alloantigens. We also determined the phenotypic characteristics of the suppressive cell population involved. Moreover, we investigated whether the suppression could be reversed by administration of IL-2. The mechanism of the induced state of suppression is discussed.

Materials and methods

Mice

(C57BL/Ka x CBA/Rij)F1 (H-$2^{b/q}$), CBA/Rij (H-2^{q}) and C57BL/Ka (H-2^{b}) female mice were

purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. BALB/c (H-2^d) and DBA/2 (H-2^d) female mice were purchased from Bomholtgard, Ry, Denmark. BALB.B (H-2^b), B10.D2 (H-2^d) and C57BL/Ka-BL-1 (H-2^b) female and male mice were bred at our facility. Mice were 8-20 weeks old when they entered the experiments.

Induction of GVH-reactivity

GVH reactions were induced in lethally irradiated mice by intravenous (i.v.) injection of 3×10^7 nucleated allogeneic spleen cells from suppressed or non-suppressed donors (see below), prepared as described earlier (14), within 4 h after irradiation (Figure 1). Irradiation was performed in a Philips-Müller MG 300 X-ray apparatus.

Assay for anti-host DTH

Spleen cells from mice in which a GVH reaction was induced (see above) were tested in a transfer system for anti-host DTH reactivity (Figure 1).

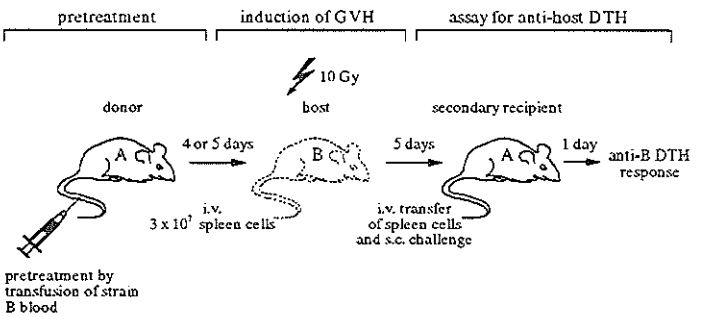

Figure 1. Experimental set-up of the experiments, involving three separate phases: (a) donor pretreatment; (b) induction of GVH; and (c) assay for anti-host DTH by transferring spleen cells from mice subjected to GVH to naive secondary recipients and subsequent challenge with host-type spleen cells.

Thus, five days after the i.v. injection of allogeneic spleen cells into lethally irradiated hosts, their spleens were removed, pooled and prepared to single cell suspensions. The obtained cells were i.v. transferred into secondary recipients that were syngeneic to the donors of the spleen cells that had induced the GVH reaction. To prevent embolism the secondary recipients were intraperitoneally (i.p.) injected with 15 U heparin (Thromboliquine, Organon Teknika, Boxtel, The Netherlands), 30 min before transfer. The secondary recipients were challenged in the dorsum of the right hind foot with 2×10^7 host-type spleen cells, within 4 h after transfer. The subsequent anti-host DTH response was measured 24 h later with a foot thickness meter with 0.05 mm accuracy. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the secondary recipients

minus that of challenge control mice that only received the challenge injection. The 'background' increase of foot thickness of these challenge control mice ranged between 15 and 25%.

Donor pretreatment by an allogeneic blood transfusion

Blood was collected by cardiac puncture immediately after the mice had been sacrificed using carbon dioxide, and put in heparinized tubes. Pretreatment was given by i.v. injection of the amount of blood indicated in the legend to the figures, 4 or 5 days before the mice were used as donors of spleen cells (Figure 1).

Fractionation of whole blood

To purify the white cell fraction, whole heparinized blood was centrifuged at 3000 rpm for 10 min. The plasma and the buffy coat were removed separately. The buffy coat was centrifuged again and red blood cells were subsequently eliminated by treatment with 0.155 M NH_4Cl. Erythrocytes were purified by filtration of 5 times diluted whole blood through a 5 µm filter (Millipore, Etten-Leur, The Netherlands). To obtain serum, blood was allowed to clot for 45 min at 37°C. After centrifugation for 20 min at 3000 rpm the serum was collected.

Purification of B and T lymphocytes and T cell depletion

T cells were purified using the indirect panning technique. Briefly, plastic 10 cm petri dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) were coated overnight with rabbit-anti-rat immunoglobulin (Dakopatts, Glostrup, Denmark) in PBS at a concentration of 20 µg per ml. The dishes were washed once with PBS and incubated for 30 min with PBS containing 1% FCS. Spleen cells (3×10^7/dish), previously incubated with the anti-B220 mAb (clone RA3 6B2) of rat origin (kindly provided by the group of Dr. W. van Ewijk from our department), were incubated on the coated dishes for 1.5 h. The non-adherent cells were collected. After washing, the cells were analyzed using a flow-cytofluorometer (FACScan, Becton Dickinson, Mountain View, CA). Contaminating B cells were <1%. B cells were purified by treatment of the spleen cell suspension with anti-Thy-1.2 mAb (clone F7D5), purchased from Serotec, Oxford, U.K., and complement, as earlier described (14). Contaminating T cells were <3%. The latter procedure was also used for selective elimination of T cells.

T cell clones

Helper T (Th) cell clones of BALB/c origin and specific for rabbit gammaglobulin (15) representing a Th1 (D1.1) and a Th2 (CDC-35) subpopulation were kindly provided by Dr. R.L. Coffman (DNAX, Palo Alto, CA). Resting clones were stimulated for 24 h with antigen (5 µg/ml) and a source of antigen presenting cells (20 Gy irradiated BALB/c spleen cells). Cells were cultured for 7 days and collected by separation on a Histopaque 1.119 gradient (Sigma, St. Louis, MO), washed 3 times and used for i.v. injection.

IL-2 treatment

Recombinant IL-2 was kindly provided by Dr. G. Malainer (Sandoz Forschungsinstitut, Wien, Austria). IL-2 was injected subcutaneously (s.c.) in the inguinal region. The IL-2 activity was checked in an *in vitro* proliferation assay and expressed in U/ml. One U is the amount of IL-2 that results in half maximum ^{3}H-thymidine incorporation of a CTLL line.

Data analysis

Differences between groups were analyzed using the 2-tailed Student's t-test. Values of $p<0.05$ were considered significant.

Results

Suppression by various fractions of whole blood and the influence of irradiation

In the DBA/2 - (C57BL x CBA)F1 H-2 and non-H-2 disparate strain combination, transfusion of 0.5 ml of whole blood, or a number of white and red blood cells together or purified white blood cells only, equivalent to 0.5 ml of blood, consistently induced significant suppression of the anti-host DTH response ($p<0.05$), whereas transfusion of red blood cells did not (Figure 2). This was confirmed in an experiment using the BALB/c - C57BL/Ka strain combination in which various doses were compared (Figure 3, *right*). Serum and plasma (data not shown) were ineffective. Irradiation of whole blood reduced but did not completely abrogate the capacity to induce suppression (Figure 2).

Dose response relationship

BALB/c mice were transfused with varying amounts of H-2 and non-H-2 disparate C57BL/Ka whole blood ranging from 0.01 µl to 1.0 ml before their use as donors of spleen cells for transplantation to irradiated C57BL/Ka mice. Suppression was significant at a dose of 1 µl ($p<0.05$), and virtually complete at a dose of 1.0 ml (Figure 3, *left*). When purified blood cells were used for transfusion, the number of cells was adjusted to the number determined in the same volume of whole blood. It appeared that white blood cells alone were as effective as whole blood, whereas red blood cells alone did not induce suppression (Figure 3, *right*).

donor	transfusion of donor	irradiated host	% specific increase of foot thickness
DBA/2	-	(C57BL x CBA)F_1	
DBA/2	irradiated whole blood	(C57BL x CBA)F_1	
DBA/2	whole blood	(C57BL x CBA)F_1	
DBA/2	white and red blood cells	(C57BL x CBA)F_1	
DBA/2	white blood cells	(C57BL x CBA)F_1	
DBA/2	red blood cells	(C57BL x CBA)F_1	
DBA/2	serum	(C57BL x CBA)F_1	

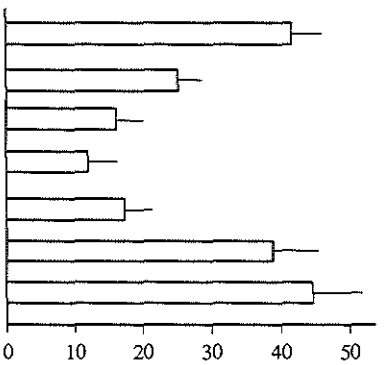

Figure 2. Suppression of anti-host DTH by various fractions of whole blood and the effect of irradiation. DBA/2 donor mice were transfused with 0.5 ml of whole blood derived from (C57BL x CBA)F1 mice or with white and red blood cells, purified white blood cells, purified red blood cells, or serum, in an amount equivalent to 0.5 ml whole blood, or received 0.5 ml irradiated whole blood. Each column represents the arithmetic mean of the anti-host DTH response ± 1 SEM (n=6).

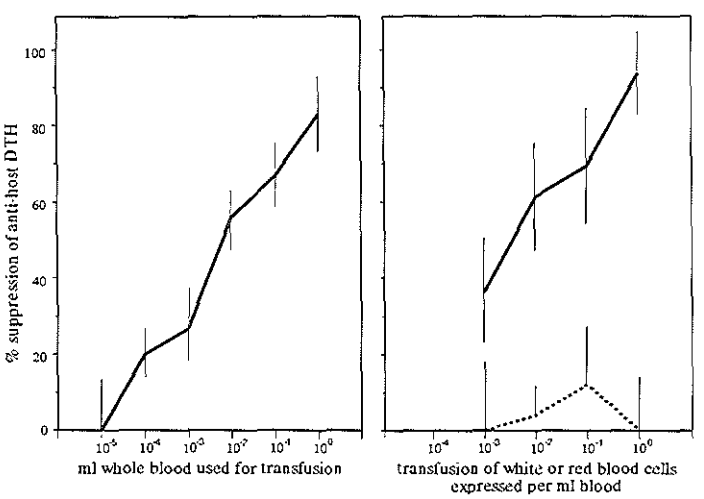

Figure 3. Dose response relationship. *Left.* BALB/c donor mice received varying doses of blood of C57BL/Ka origin. *Right.* BALB/c donor mice received various doses of purified white (solid line) or red (broken line) blood cells of C57BL/Ka origin. Each point represents the arithmetic mean of the suppressive effect ± 1 SEM (n = 6), calculated as a percentage of the control group in which no suppression was induced. The anti-host DTH response of this control group, given as the specific increase of foot thickness, was 30.7 ± 5.1.

Suppression by purified B and T lymphocytes and a Th1 and Th2 clone

BALB/c donor mice received an amount of B or T cells (purified by the panning technique) of (C57BL x CBA)F1 origin as indicated. These BALB/c mice were used five days later to reconstitute lethally irradiated (C57BL x CBA)F1 hosts. From Figure 4 (*upper part*) it can be concluded that purified B cells as well as purified T cells were able to induce suppression at a dose of 10^6 cells ($p<0.05$), whereas a dose of 10^5 cells was ineffective. Both lymphocyte subsets appeared equally effective. As a source of pure T cells we further used a Th1 clone and a Th2 clone (15) to induce suppression. Figure 4 (*lower part*) shows that both the Th1 and the Th2 clone were able to induce suppression.

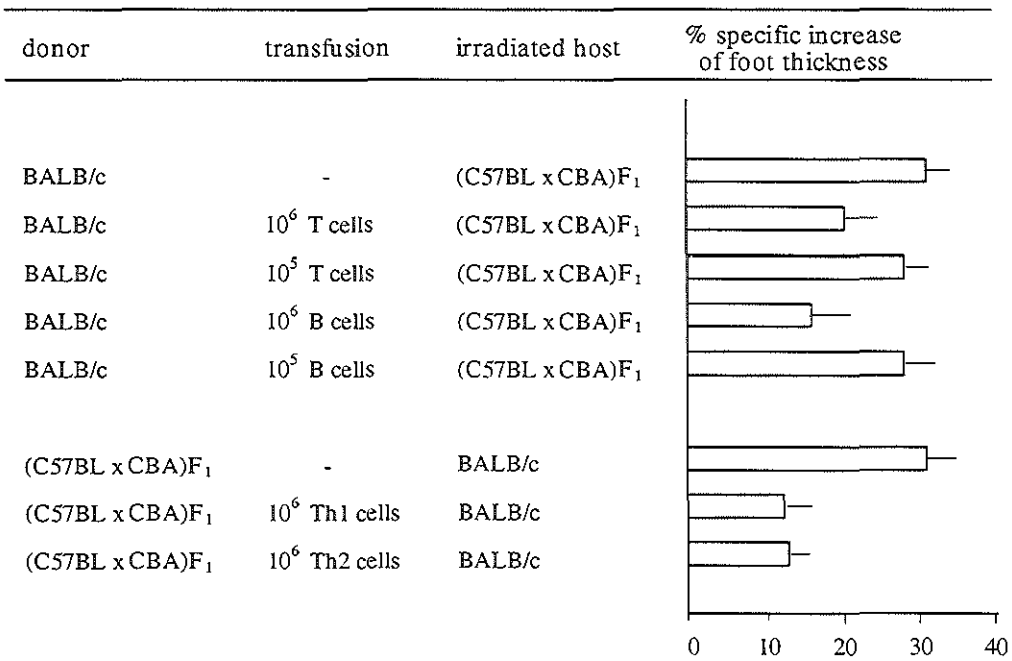

Figure 4. Suppression of the anti-host DTH response by purified B or T lymphocytes. *Upper part.* BALB/c donor mice were either untreated or received the indicated number of purified B or T lymphocytes of (C57BL x CBA)F1 origin. *Lower part.* (C57BL x CBA)F1 donor mice were either untreated or received 10^6 cells either from a Th1 clone (D1.1) or from a Th2 clone (CDC-35), both of BALB/c origin. Each column represents the arithmetic mean of the anti-host DTH response ± 1 SEM (n = 6).

The role of major and minor transplantation antigens

BALB/c donors were transfused with 0.1 ml of C57BL/Ka (non-H-2 plus H-2 incompatible), BALB.B (H-2 incompatible) or B10.D2 (non-H-2 incompatible) whole blood. Their spleen cells were used to reconstitute lethally irradiated C57BL/Ka (non-H-2 plus H-2 incompatible) hosts. It appeared that a non-H-2 as well as an H-2 mismatched transfusion induced suppression (Figure 5). An H-2 incompatible blood transfusion was more potent in inducing suppression than a non-H-2 incompatible one, while the combination of an H-2 and non-H-2 disparate blood transfusion was as effective as an H-2 incompatible transfusion alone.

donor	transfusion	disparity	irradiated host	% specific increase of foot thickness
BALB/c	-	-	C57BL/Ka	
BALB/c	C57BL/Ka	H-2 + non-H-2	C57BL/Ka	
BALB/c	BALB.B	H-2	C57BL/Ka	
BALB/c	B10.D2	non-H-2	C57BL/Ka	

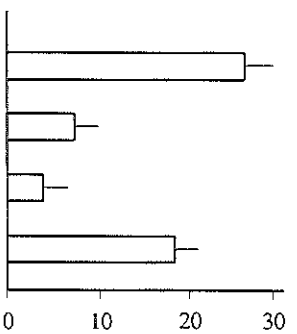

Figure 5. The role of major and minor transplantation antigens in the induction of suppression of the anti-host DTH response. BALB/c donors were untransfused or transfused with 0.1 ml of whole blood from (non-H-2 plus H-2 disparate) C57BL/Ka, (H-2 disparate) BALB.B or (non-H-2 disparate) B10.D2 mice. Their spleen cells were used to reconstitute lethally irradiated C57BL/Ka hosts. Each column represents the arithmetic mean of the anti-host DTH response ± 1 SEM (n = 6).

Phenotype of the spleen cell population involved in suppression

DBA/2 donor mice were transfused with 0.5 ml of (C57BL x CBA)F1 blood. Spleen cells from these suppressed donors were either treated with complement (C) only, as a control, or with either anti-Thy-1.2, anti-CD4 or anti-CD8 and C. A mixture of these treated spleen cells and normal spleen cells (capable to induce GVH) was used to reconstitute lethally irradiated hosts. Injection of the mixture of naive spleen cells and

suppressive spleen cells treated with C only, resulted in suppression (Figure 6, *upper part*, line 3). Treatment of the suppressive spleen cells with anti-Thy-1.2 (line 2) or anti-CD4 (line 4) and C abrogated the suppression, whereas anti-CD8 and C did not (line 5). After transfer of a suppressive spleen cell population treated with either anti-CD4 or anti-CD8 (containing $CD4^-$, $CD8^+$ and $CD4^+$, $CD8^-$ cells, respectively) suppression was present (line 6). Together these data indicate that a $CD4^+$, $CD8^-$ T cell subpopulation mediated the suppression and that the suppressive effect is dominant over cells capable of eliciting a GVH-related DTH response.

donor	blood transfusion of donor	treatment of suppressive cells	irradiated host	% specific increase of foot thickness
DBA/2	-	-	(C57BL x CBA)F_1	
DBA/2	(C57BL x CBA)F_1	anti-Thy-1.2 + C	(C57BL x CBA)F_1	
DBA/2	(C57BL x CBA)F_1	C	(C57BL x CBA)F_1	
DBA/2	(C57BL x CBA)F_1	anti-CD4 + C	(C57BL x CBA)F_1	
DBA/2	(C57BL x CBA)F_1	anti-CD8 + C	(C57BL x CBA)F_1	
DBA/2	(C57BL x CBA)F_1	anti-CD4 + C anti-CD8 + C	(C57BL x CBA)F_1	
BALB/c	-	-	C57BL/Ka-BL-1	
BALB/c (Thy-1.2)	C57BL/Ka-BL-1 (Thy-1.1)	C	C57BL/Ka-BL-1	
BALB/c	C57BL/Ka-BL-1	anti-Thy-1.2 + C	C57BL/Ka-BL-1	

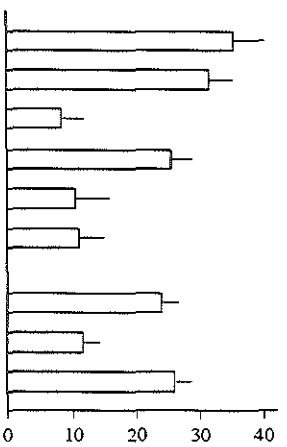

Figure 6. Phenotype of the suppressor cell population. *Upper part.* Spleen cells from suppressed donors were treated with the indicated mAb, subsequently mixed (1:1) with spleen cells from naive donors and used to reconstitute lethally irradiated hosts. Each column represents the arithmetic mean of the anti-host DTH response ± 1 SEM (n = 6). *Lower part.* BALB/c (Thy-1.2) donors received a transfusion of 1 ml of blood from C57BL/Ka-BL-1 (Thy-1.1) mice. Their spleen cells were treated with anti-Thy-1.2 and C, mixed (1:1) with naive BALB/c spleen cells and used to reconstitute lethally irradiated C57BL/Ka-BL-1 hosts. Each column represents the arithmetic mean of the anti-host DTH response ± 1 SEM (n = 6).

Mediation of suppression by T cells from the transfused donor

We further wanted to discriminate between a mechanism of suppression mediated by cells from the transfused mice and a veto cell mechanism mediated by cells derived

from the transfused blood. Therefore, BALB/c (Thy-1.2) spleen cell donors were either untransfused or transfused with blood from C57BL/Ka-BL-1 (Thy-1.1) mice. Spleen cells from the transfused BALB/c mice were treated with anti-Thy-1.2 and C or C only. The residual cells were used together with naive BALB/c spleen cells (1:1) to reconstitute lethally irradiated C57BL/Ka-BL-1 hosts. As can be seen in Figure 6 (*lower part*), suppression was abrogated by the anti-Thy-1.2 treatment. Thus suppressive cells of BALB/c origin, the transfused donor, were responsible for the observed suppression.

The effect of IL-2 on suppression

We further studied the effect of exogenous IL-2 administration on suppression. BALB/c donor mice were transfused with 0.5 ml of (C57BL x CBA)F1 whole blood. After five days their spleen cells were used to reconstitute lethally irradiated (C57BL x CBA)F1 hosts. These hosts subsequently received s.c. injections of 5,000, 50,000 (data not shown) or 100,000 U of recombinant IL-2, for five consecutive days. At 24 h after the last IL-2 injection, spleen cells from these hosts were prepared and transferred to naive secondary recipients which were challenged within 4 h after transfer. A control group of hosts that had received spleen cells from untransfused donors was similarly treated. Figure 7 shows that even high dose IL-2 treatment did not reverse the blood transfusion-induced suppression (line 4) and did not influence the anti-host DTH response induced by cells from non-transfused donors (line 2).

Discussion

From this study it can be concluded that the anti-host immune response after transplantation of allogeneic spleen cells to lethally irradiated hosts is suppressed in a specific way by a single host-specific blood transfusion given to the prospective donors of hemopoietic cells. Thus the beneficial effect of blood transfusion, already well-known in organ transplantation, can also be demonstrated under GVH conditions.

It was reported by Halle-Pannenko et al. that specific immunization to host alloantigens in the non-H-2 incompatible B10.D2 - (DBA/2 x B10.D2)F1 strain combination improved the survival from lethal GVHD in mice (5). The suppressive effect could merely be ascribed to alloimmunization against Mls^a encoded antigens (6). They found slight but significant improvement after immunization with non-specific H-2 alloantigens. In line with these data we demonstrated that suppression specific for

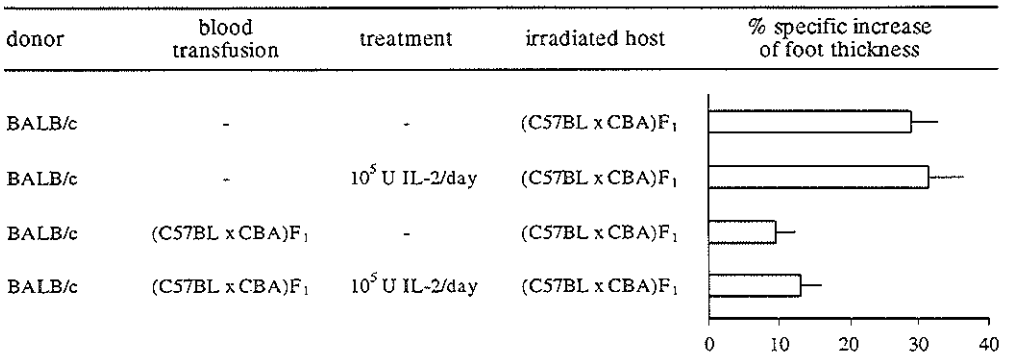

Figure 7. The effect of recombinant IL-2 on the (suppressed) anti-host DTH response. BALB/c donors were untransfused or transfused with 0.5 ml of whole blood. Their spleen cells were used to reconstitute lethally irradiated (C57BL x CBA)F1 hosts that were s.c. injected with 100,000 U of recombinant IL-2 for 5 consecutive days. Each column represents the arithmetic mean of the anti-host DTH response ± 1 SEM (n=6).

the histocompatibility antigens used for the induction of suppression could be induced also in H-2, H-2 plus non-H-2 and H-2I-A (class II) disparate strain combinations (13). We did not find evidence of a non-specific effect, since third-party blood transfusion was ineffective.

In the non-H-2 and H-2 incompatible but Mls identical BALB/c - C57BL/Ka strain combination, we found evidence that both H-2 and non-H-2 alloantigens are able to induce suppression. H-2 alloantigens appeared to be more potent in the induction of suppression than non-H-2 alloantigens. Mls disparity was not necessary for the induction of suppression. In our model, minor antigens induced suppression without the need for allogeneic help (possibly lymphokine production), as described in a rat kidney transplant model (12).

We further determined the capability of various fractions of whole blood to induce suppression. Only white blood cells were able to induce suppression, whereas red blood cells and serum were not. This may imply that in order to induce suppression, together with the allogeneic (class I and minor) histocompatibility antigens that are also present on red blood cells, a second signal generated by the white blood cells is necessary. In the literature there is no concordance concerning the role of red blood cells in immunosuppression. We could determine suppression of DTH after red blood cell transfusion under HVG conditions, but not under GVH conditions (4,13). This indicates that the requirements for suppression are different under HVG and GVH conditions. Subsequently we investigated whether purified B and T lymphocytes were able to induce suppression. Both lymphocyte subsets appeared to be equally effective.

As a source of pure T cells we also used two *in vitro* maintained helper T cell clones, a Th1 and a Th2 clone and investigated their capacity to induce suppression. These clones were used 1 week after restimulation, during the resting phase in which the lymphokine secretion had stopped. Both clones induced significant suppression. This argues against a role for lymphokines in the induction of suppression. Further studies are needed to elucidate the requirements to induce suppression by allogeneic blood cells.

An important question deals with the mechanism of suppression. Is this a dominant phenomenon? Is there a role for (functional) clonal deletion? Or is there a role for veto cells? We have clear evidence supporting the first hypothesis, since T cells from suppressed mice consistently inhibited the GVH inducing capacity of normal spleen cells. Furthermore, we demonstrated the phenomenon of bystander suppression (16), which is incompatible with the clonal deletion theory. The data are also at variance with suppression by veto cells. Veto cells were described to be largely $CD8^+$ (17). However, the obtained results were consistent with $CD4^+$, $CD8^-$ T cells mediating the suppression. Furthermore, using Thy-1 congeneic mice we were able to demonstrate that the cells mediating the suppression in our system were not blood-derived but originated in the transfused mice. Together these data indicate that our blood transfusion protocol indeed induces dominant suppression that is not caused by veto cells.

Recently, Dallman et al. described the effect of IL-2 on blood transfusion-induced suppression in a rat kidney transplantation model. A single daily dose of 50,000 U of IL-2 appeared to be sufficient to reverse the suppression (18). Blood transfusion may lead to a state of IL-2 deficiency or relative unresponsiveness, clinically becoming clear in a decreased transplant rejection response. Therefore, we investigated the effect of administration of recombinant IL-2, first using a comparable dose (based on animal weight) of 5,000 U of IL-2. Since this dose of IL-2 did not reverse the blood transfusion effect, we used higher doses up to 100,000 U of IL-2 as well. Even this high-dose treatment did not abrogate suppression, which indicates that the blood transfusion effect in this model is not (only) due to a deficient IL-2 production or relative unresponsiveness to IL-2. Further studies are needed to clarify the role of the various lymphokines in the blood transfusion effect.

Acknowledgments

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Chapter 2.2

Improved survival from potentially lethal graft-versus-host disease by donor pretreatment with a recipient-specific blood transfusion

I. Requirements for induction and specificity of the effect*

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Summary

Pretreatment of prospective donors of hemopoietic cells with a single recipient-specific blood transfusion can significantly decrease the morbidity and mortality of graft-versus-host disease (GVHD) in lethally irradiated, allogeneically reconstituted mice. This beneficial effect of donor pretreatment could be demonstrated in donor-recipient strain combinations that were H-2 plus non-H-2, H-2, or only class II disparate, but not in the class I disparate C57BL - B6.C-H-2^{bm1} strain combination. The effect was proportional to the amount of recipient-strain blood used for transfusion. Donor transfusion with a single dose of 1 ml recipient-specific whole blood resulted in minimum GVHD, lower doses being less or not effective. The interval between donor pretreatment and the use of their hemopoietic cells for reconstitution appeared to be important. The best survival was found at an interval of 4 days. Multiple transfusion was not more effective than a single one. We compared the effectiveness of whole blood and irradiated spleen cells for donor pretreatment. Both protocols have been shown previously to suppress anti-recipient delayed-type hypersensitivity (DTH) reactivity. It appeared that the blood transfusion protocol was superior to the spleen cell protocol. The beneficial effect appeared to be recipient-specific, since a third-party blood transfusion did not improve GVHD. We found that the beneficial effect of donor blood transfusion was due to suppression of the anti-host immune response. The donor blood

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transfusion was able to induce bystander suppression to alloantigens that were not used for the induction of suppression, provided they were co-expressed with the specific alloantigens by the recipients. This also indicates that, although the induction of suppression is specific, the ultimate suppressive effect is non-specific.

Introduction

Blood transfusions have a profound effect on the immune system. For instance, pretransplant blood transfusions can strongly improve organ transplant survival (1), whereas perioperative blood transfusions decrease the recurrence of Crohn's disease (2). On the other hand, blood transfusion can negatively influence the outcome of malignant and infectious diseases (3). Many studies have been dedicated to the blood transfusion effect. In recent times, the beneficial effect of pretransplant blood transfusion has become a matter of debate. Due to new and improved immunosuppressive agents such as cyclosporine A, the transplant survival has increased considerably and the transfusion effect has become difficult to demonstrate (4,5). However, in a recent study clear evidence was found for a blood transfusion effect in patients under cyclosporine A treatment, provided the blood transfusions were HLA-DR matched (6). This stresses the importance of studies aimed to elucidate the requirements for the beneficial blood transfusion effect.

In human bone marrow transplantation (BMT), a disadvantageous effect of recipient transfusion has been reported. Especially in multiple transfused patients the incidence of bone marrow graft rejection increased (7). On the other hand, it was recently reported that recipient transfusion shortly before BMT decreased the incidence of chronic GVHD (8).

In previous studies from our laboratory, allogeneic blood and irradiated spleen cell transfusion were shown to suppress DTH responses to alloantigens, both under host-versus-graft (HVG) and GVH conditions (9-12). The present study was undertaken to evaluate the effect of donor pretreatment with a recipient-specific blood transfusion on clinical symptoms and the survival of lethal GVHD. A beneficial effect of donor pretreatment with allogeneic spleen cells has been reported by others in a non-H-2 incompatible strain combination (13). With the increasing use of HLA - mismatched donors, it is relevant to investigate whether donor pretreatment can enhance the survival in strain combinations mismatched for H-2 (subregion) antigens.

In this article we report data concerning the impact of histocompatibility differences between donor and recipient, the dose-response relationship, time-dependence and specificity of the blood transfusion effect.

Materials and methods

Mice

(C57BL/Ka x CBA/Rij)F1 (H-$2^{b/q}$), CBA/Rij (H-2^{q}) and C57BL/Ka (H-2^{b}) mice were purchased from the Institute for Applied Radiobiology and Immunology (ITRI) TNO, Rijswijk, The Netherlands. BALB.B (H-2^{b}), BALB.K (H-2^{k}) and B10.D2 (H-2^{d}) mice were purchased from Harlan-CPB, Zeist, The Netherlands. BALB/c (H-2^{d}), (BALB/c x BALB.K)F1 (H-$2^{d/k}$), B6.C-H-2^{bm1} (H-2^{bm1}) and B6.C-H-2^{bm12} (H-2^{bm12}) mice were bred at our own department. Recipient mice were age-matched and 12-24 weeks old at the start of the experiments. Mice were kept 2 per cage in light-cycled rooms and had access to acidified water and pelleted food *ad libitum*.

Preparation of cell suspensions

Mice were killed using carbon dioxide. Spleen and bone marrow cell suspensions were prepared in phosphate-buffered BSS. Nucleated cell concentrations were determined with a Coulter Counter model ZB1. Viability of the cell suspensions as determined by the trypan blue exclusion method was >90%.

Irradiation, reconstitution and monitoring for GVHD

Lethally irradiated (C57BL x CBA)F1 (10 Gy) and BALB (7.5 Gy) mice were reconstituted by i.v. injection of both 10^7 allogeneic spleen cells and 10^7 allogeneic bone marrow cells obtained from (at least two) pretreated (see below) or naive donors within 24 h after irradiation (Figure 1).

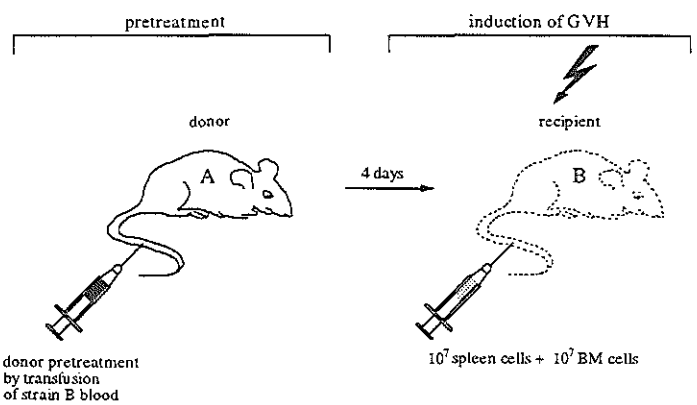

Figure 1. Set-up of the experiments. Donor mice (A) were pretreated with recipient-specific (B) whole blood. Four days later donor spleen and bone marrow cells were used to reconstitute lethally irradiated recipients (B), that were subsequently monitored for signs of GVHD and mortality.

Irradiation was performed in a Cesium-137 source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) with a dose rate of 1.15 Gy/min. Mice were examined daily for the development of signs of GVHD, such as decreased physical activity, hunched posture, skin lesions, wasting and diarrhoea, and for mortality. Mice judged moribund were killed. The body weight was determined at least twice weekly for the first 3-4 weeks. Thereafter, the frequency was decreased, dependent on the changes in body weight per group. Control mice reconstituted with the same number of syngeneic cells survived >250 days. Radiation controls died between days 10 and 23.

Induction of suppression by allogeneic blood transfusion

Blood was collected by cardiac puncture and stored in heparinized tubes. Suppression was induced by i.v. injection of 1 ml of allogeneic blood, 4 days before the mice were used as donors of spleen and bone marrow cells, unless otherwise indicated. Since syngeneic transfusion did not influence the survival (data not shown), in later experiments control donor mice were not transfused.

Data analysis

Differences between groups were analyzed using the Wilcoxon-Mann-Whitney statistic. Values of $p<0.05$ were considered as significant.

Results

The effect of donor pretreatment with a recipient-specific blood transfusion

BALB/c donors were pretreated with 1 ml of (C57BL x CBA)F1 whole blood. After 4 days, 10^7 spleen and 10^7 bone marrow cells of these pretreated mice were used to

Table 1. Evaluation of clinical symptoms of GVHD in recipients after donor treatment with a recipient-specific blood transfusion.

Donor pretreatment	days after reconstitution							
	0	3	6	9	13	16	21	28
Blood transfusion	-	-	-	+	+	+	-	-
None	-	-	++	+++	†			

(C57BL x CBA)F1 recipients were lethally irradiated and reconstituted with 10^7 BALB/c spleen and 10^7 bone marrow cells from naive or pretreated donors. The mice were daily monitored for symptoms of GVHD. Each experimental group consisted of 10 mice.

- absence of disease + first symptoms of disease ++ moderate disease +++ severe disease † death

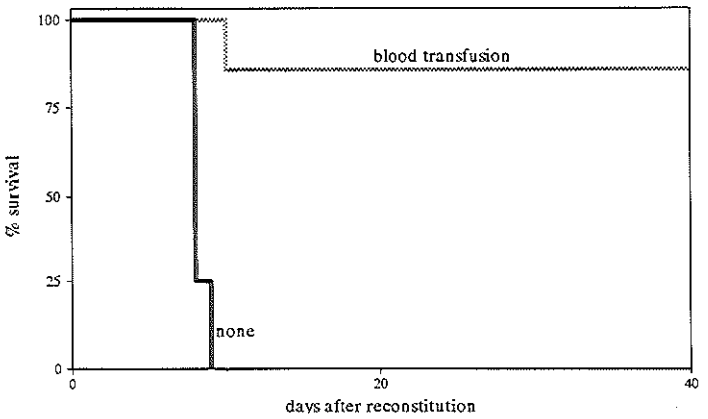

Figure 2. The effect of donor blood transfusion on the survival. BALB/c donor mice were either or not pretreated with a recipient-specific (C57BL x CBA)F1 blood transfusion. After 4 days the donors were sacrificed and used for reconstitution of lethally irradiated (C57BL x CBA)F1 recipients. Each experimental group consisted of 10 mice.

Table 2. Effect of donor blood transfusion with recipient-specific blood on the body weight of mice during GVHD.

Donor pretreatment	days after reconstitution							
	0	3	6	9	13	16	21	28
Blood transfusion (% body weight loss)	0	7	9	18	16	9	3	12
None (% body weight loss)	0	14	19	24	†			

(C57BL x CBA)F1 recipients were lethally irradiated and reconstituted with 10^7 BALB/c spleen and 10^7 bone marrow cells from naive or pretreated donors. The percentage body weight loss was determined at various time points after reconstitution. Each experimental group consisted of 10 mice.

† death

reconstitute lethally irradiated (C57BL x CBA)F1 recipients. As a control, naive BALB/c mice were used as donors. Figure 2 shows that the donor pretreatment significantly improved the survival. Table 1 shows the effect on the clinical symptoms of GVHD. Table 2 shows the effect of donor pretreatment on the body weight. Together the data indicate that donor pretreatment with a recipient-specific blood transfusion decreases the severity of clinical symptoms of GVHD and improves the survival.

The effect of donor pretreatment in H-2, non-H-2, class I and class II disparate strain combinations

We further studied whether the blood transfusion effect could be demonstrated in donor-recipient strain combinations mismatched for H-2, non-H-2, class I or class II alloantigens. We used the H-2 disparate BALB.K - BALB/c strain combination (Figure 3A) and the reversed strain combination (Figure 6), the H-2 compatible but non-H-2 disparate B10.D2 - BALB/c strain combination (Figure 3B), the class I disparate C57BL - bm1 strain combination (Figure 3C) and the class II disparate C57BL - bm12

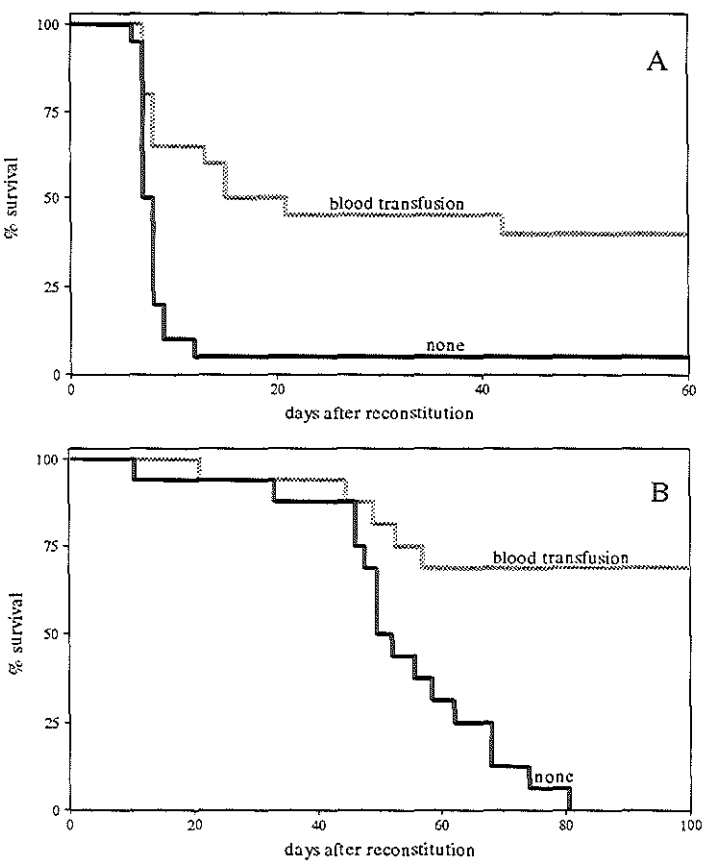

Figure 3. The effect of donor blood transfusion in H-2, non-H-2, class I and class II disparate strain combinations. The effect of donorpretreatment with a recipient-specific blood transfusion was investigated in the H-2 disparate BALB.K - BALB/c strain combination (A) and in the H-2 compatible but non-H-2 incompatible B10.D2 - BALB/c strain combination (B), *continued next page.*

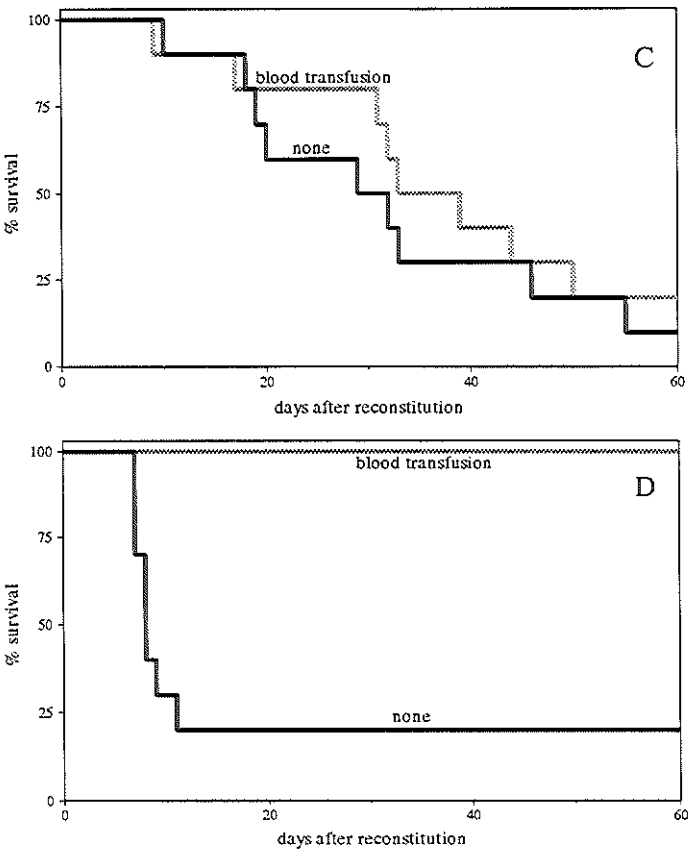

Figure 3. *(continued).* The effect of donor pretreatment with a recipient-specific blood transfusion in the class I disparate C57BL - bm1 strain combination (C), and in the class II disparate C57BL - bm12 strain combination (D). The experimental groups consisted of 10-20 mice. In A, B and D: $p<0.005$; in C: $p>0.05$.

strain combination (Figure 3D). It appeared that donor pretreatment had a beneficial effect in all donor-recipient strain combinations tested, except the class I mismatched C57BL - bm1 strain combination.

Dose-response relationship

BALB/c donors were untreated or transfused with 0.1 or 1 ml of H-2 and non-H-2 disparate, recipient-specific whole blood. Significant improvement of the survival was found after donor transfusion with the higher dose of 1 ml, whereas transfusion with 0.1 ml did not ($p=0.27$) lead to enhanced survival (Figure 4). A similar dose-response relationship was found in the BALB.K - BALB/c strain combination (data not shown).

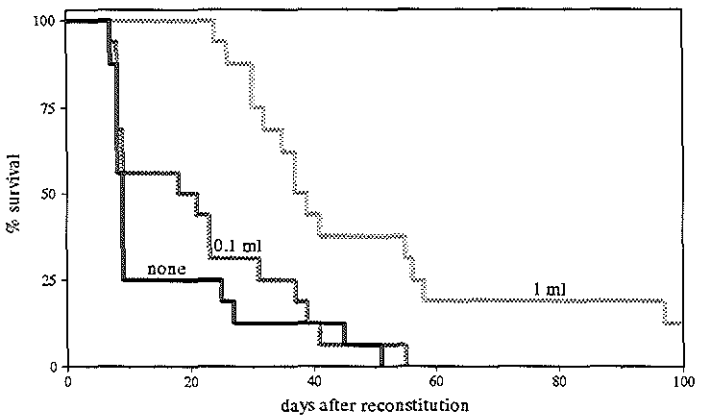

Figure 4. Dose-response relationship of recipient-specific blood transfusion of the donors. BALB/c donors were not transfused (solid line) or transfused with 0.1 or 1 ml (C57BL x CBA)F1 whole blood,4 days before use of their spleen cells to reconstitute lethally irradiated BALB/c recipients (n=16).

Interval between donor pretreatment and donor use for reconstitution

BALB/c donor mice were transfused with 1 ml of (C57BL x CBA)F1 whole blood. Their spleen and bone marrow cells were used to reconstitute lethally irradiated (C57BL x CBA)F1 recipients, either 4 hours or 1, 4, 7 or 28 days after donor blood transfusion. Figure 5 shows the results for intervals of 4, 7 and 28 days. The strongest

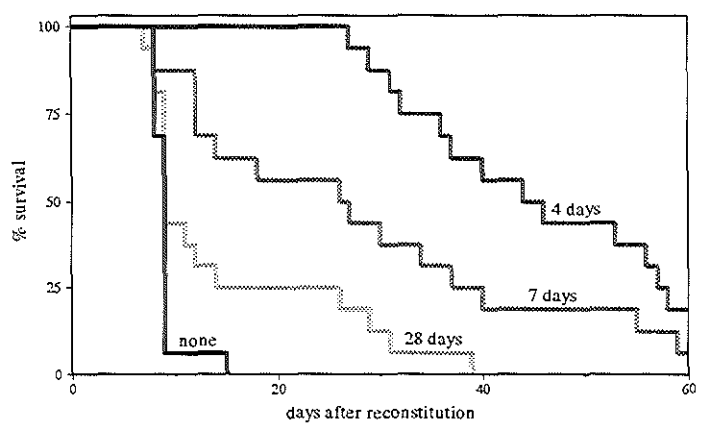

Figure 5. Interval between donor transfusion and their use for reconstitution. BALB/c donor mice were untreated (solid line) or transfused with 1 ml of (C57BL x CBA)F1 whole blood. After 4, 7 or 28 days they were used for reconstitution of lethally irradiated (C57BL x CBA)F1 hosts (n=16).

beneficial effect of donor blood transfusion was found after an interval of 4 days ($p<0.005$). After an interval of 1 or 7 days the effect was also significant ($p<0.005$). No significant effect could be demonstrated at 4 hours ($p=0.48$) or 28 days ($p=0.08$) after transfusion.

Comparison of the effect of donor transfusion with either whole blood or irradiated spleen cells

BALB/c donor mice were either not transfused, transfused with 1 ml of recipient-specific (C57BL x CBA)F1 whole blood or transfused with 5 x 10^7 irradiated (20 Gy) recipient-specific spleen cells. After four days, 10^7 spleen and 10^7 bone marrow cells from these BALB/c donor mice were used to reconstitute lethally irradiated H-2 and non-H-2 disparate (C57BL x CBA)F1 recipients. Figure 6 shows that donor blood transfusion improved the survival significantly ($p<0.005$). Transfusion of the donors with 5 x 10^7 irradiated spleen cells also significantly improved the recipients' survival, but the effect was less pronounced ($p=0.01$) than that of blood transfusion.

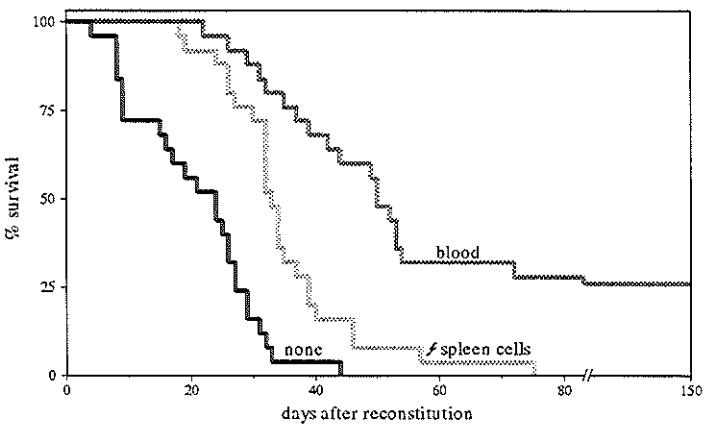

Figure 6. Improved survival after donor transfusion with recipient-specific blood or spleen cells. BALB/c donor mice were either untreated,transfused with 1 ml of recipient-specific (C57BL x CBA)F1 whole blood or transfused with 5 x 10^7 irradiated recipient-specific (C57BL x CBA)F1 spleen cells, 4 days before their use for reconstitution of lethally irradiated (C57BL x CBA)F1 recipients (n=25).

Multiple blood transfusions

The influence of donor pretreatment with multiple blood transfusions as compared to a single transfusion, was investigated. Therefore, BALB/c donors were treated 3 times at weekly intervals with 1 ml of recipient-specific (C57BL x CBA)F1 blood or with a single blood transfusion. Seven days after the last transfusion, they were used to reconstitute lethally irradiated (C57BL x CBA)F1 recipients. Figure 7 shows that multiple donor transfusions were not more effective than a single transfusion. A single donor transfusion seemed to be even slightly better, but the difference was not significant (p=0.08).

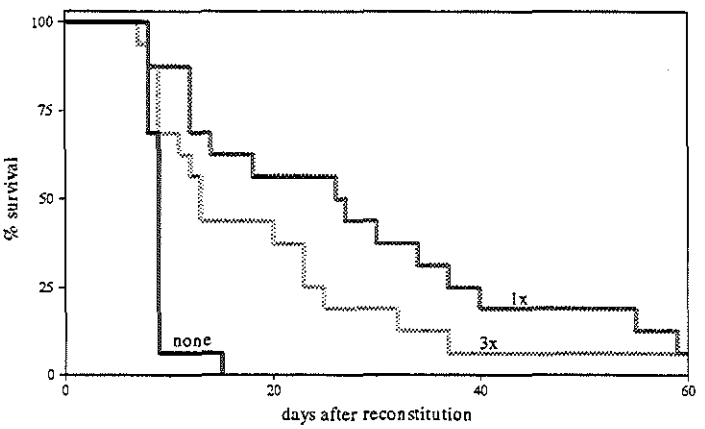

Figure 7. Survival after multiple blood transfusion of the donors. BALB/c donor mice were untransfused or received 1 or 3 transfusions with 1 ml of recipient-specific (C57BL x CBA)F1 whole blood at weekly intervals. Seven days after the last transfusion they were used for reconstitution (n=16).

Specificity of the blood transfusion effect

To study whether the effect of donor pretreatment with a blood transfusion was specific, BALB/c donors were transfused with either recipient-specific (BALB/c x BALB.K)F1 blood or third-party BALB.B blood and four days later used for reconstitution of lethally irradiated (BALB/c x BALB.K)F1 hosts. The recipient-specific (BALB/c x BALB.K)F1 blood transfusion strongly improved the survival, whereas the third-party BALB.B transfusion did not (Figure 8). No sensitizing effect was found either.

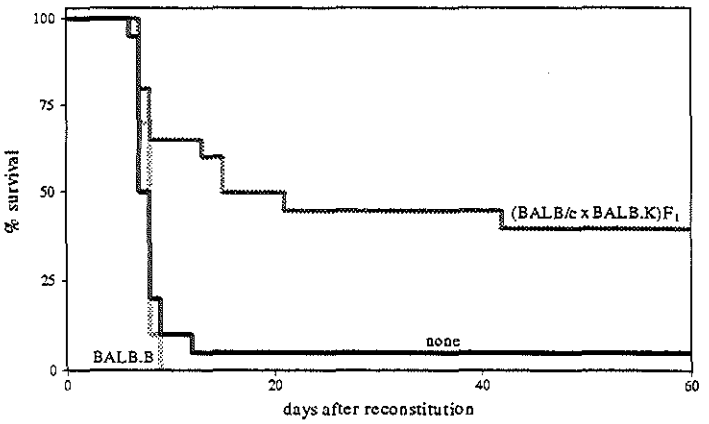

Figure 8. Specificity of the blood transfusion effect. BALB/c donor mice were either untransfused, transfused with recipient-specific (BALB/c x BALB.K)F1 blood or with third-party BALB.B blood. After 4 days their spleen and bone marrow cells were used for reconstitution of lethally irradiated (BALB/c x BALB.K)F1 recipients (n=16).

Bystander suppression

We further investigated whether bystander suppression could be demonstrated in this model of GVHD. Therefore, BALB/c donor mice were transfused with 1 ml of blood derived from either (C57BL x CBA)F1, C57BL or CBA mice. Four days later the

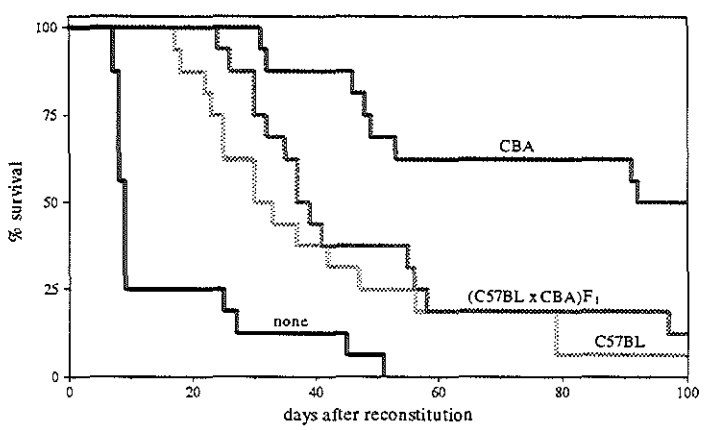

Figure 9. Bystander suppression of GVHD. BALB/c donor mice were untreated, transfused with 1 ml of (C57BL x CBA)F1 whole blood, or with 1 ml of C57BL or CBA whole blood before their use to reconstitute lethally irradiated (C57BL x CBA)F1 recipients (n=16).

transfused BALB/c donors were used for reconstitution of lethally irradiated (C57BL x CBA)F1 recipients. The results are presented in Figure 9. Improved survival not only occurred after donor transfusion with a recipient-specific (C57BL x CBA)F1 blood transfusion ($p<0.005$), but also after donor transfusion with CBA blood, lacking the C57BL antigens ($p<0.005$) or with C57BL blood, lacking the CBA antigens ($p<0.005$). Transfusion with CBA blood appeared to be more effective than transfusion with C57BL or (C57BL x CBA)F1 blood ($p<0.05$). This indicates, that the GVH reaction to third-party alloantigens that were co-expressed with the alloantigens used for donor blood transfusion, was decreased.

Discussion

We have earlier shown that donor pretreatment with a recipient-specific spleen cell or blood transfusion suppressed the anti-recipient DTH response (11,12). The data presented in this report show that donor pretreatment with a recipient-specific blood transfusion also decreases the morbidity and mortality of lethal GVHD. This beneficial effect of donor blood transfusion on GVHD could be demonstrated in various donor-recipient strain combinations, mismatched for H-2 plus non-H-2, H-2, non-H-2, or class II antigens. Donor blood transfusion appeared to lead to improvement of acute as well as of chronic GVHD.

In a similar model, Halle-Pannenko et al. studied the effect of donor pretreatment with 2 x 10^7 allogeneic spleen cells on the survival of minor histoincompatible recipients, which resulted in enhanced survival (13,14). This could merely be ascribed to alloimmunization against Mls encoded antigens. Immunization with third-party H-2 antigens was able to enhance this effect. We were able to demonstrate a beneficial effect of donor blood transfusion in Mls identical strain combinations, differing for non-H-2, H-2, or class II antigens. This indicates that other antigens than Mls antigens can play a role as well.

Interestingly, in the class I disparate, Mls identical C57BL - B6.C-H-2^{bm1} strain combination no improvement of GVHD was found. This might be due to the alloantigens and, as a consequence, the donor T cell subset involved. It has been shown that $CD8^+$ T cells mediate GVHD in a class I incompatible strain combination, whereas $CD4^+$ T cells mediate GVHD in a class II incompatible strain combination (15). We have earlier shown that $CD4^+$ T cells induced lethal GVHD in the BALB/c - (C57BL x CBA)F1 strain combination, whereas $CD8^+$ T cells did not (16). This was also found in the BALB/c - BALB.K strain combination (unpublished observations). It might be that the $CD4^+$ T cell subset is more susceptible to the effect of allogeneic blood

transfusion than the $CD8^+$ T cell subset.

The blood transfusion effect appeared to be dose-related, as was the effect on anti-recipient DTH reactivity (12). However, for the beneficial effect on the survival at least a 100 times higher dose of blood cells was required, corresponding with 0.8×10^7 - 2×10^7 white blood cells. From these data it can be concluded, that it is more easy to influence the anti-recipient DTH reactivity, which is only one of the effector mechanisms of GVHD, than the whole complex syndrome of GVHD. White blood cells probably are required to induce the blood transfusion effect, which is in line with our observations in the DTH model (12) and most reports in the literature.

The interval between donor blood transfusion and reconstitution was found to be critical. As determined in a DTH model, under HVG conditions more than one year after blood transfusion still a reduced DTH response to the alloantigens of the transfused blood cells is found (10). Under GVH conditions, donor blood transfusion reduces anti-host DTH for about 28 days (12). However, the beneficial effect on the survival of mice subjected to GVHD was found at an interval of 1, 4 or 7 days only. This is in line with data from others (13). This might indicate that allogeneic blood transfusion has a differential effect on various T lymphocyte subpopulations.

There has been a lot of controversy in the literature about the number of transfusions required for an optimum effect (17). In our model multiple transfusions were not better than a single one.

We compared two protocols that have been developed in our laboratory and have been shown to suppress DTH reactivity to alloantigens both under HVG and GVH conditions. It appeared that the blood transfusion protocol was superior to the other protocol which consists of the transfusion of 5×10^7 irradiated spleen cells. The difference in effectiveness might be due to the irradiation applied to the spleen cells to be used for transfusion. This procedure inhibits their proliferation and presumably shortens their half-life after transfusion. On the other hand, it is equally well possible that the different constitution of the white cell population in blood and spleen accounts for the differential effect.

An important issue is the specificity of the blood transfusion effect. Although there are reports claiming a non-specific effect, in our models we only found evidence for a specific effect. A third-party transfusion proved to be completely ineffective. A beneficial effect of 'non-specific' transfusion might in fact be due to sharing of (unknown) minor or major histocompatibility antigens. This suggestion is in line with evidence from earlier studies on the phenomenon of bystander suppression (18,19). This implies suppression of the response to third-party alloantigens that are not used for pretreatment, but are co-expressed with the alloantigens used for pretreatment, at the time of induction of GVHD. We were able to demonstrate this phenomenon also

in the present study. This implies that the induction and activation of the suppressive effect is specific and requires sharing of alloantigens between the blood (donor) and the prospective recipient, but that the ultimate suppressive effect is non-specific (6). The data presented in Figure 9 also show that alloantigens vary in their capacity to improve the survival since a CBA blood transfusion was considerably better than a C57BL blood transfusion. Madsen et al. have shown similar data on the suppression induced by L cells transfected with K^b and D^b genes (20). Furthermore, they found a relation with the degree of expression of the alloantigens involved.

The data presented here show that donor pretreatment with a recipient-specific blood transfusion can decrease the morbidity and enhance the survival of mice suffering from potentially lethal GVHD. We did not find evidence for significant sensitization in our studies. Since blood transfusion is inevitable in clinical practice and because of its potential beneficial but also harmful effects in transplantation, the conditions that determine the ultimate outcome should be further elucidated. Studies are in progress, to clarify the mechanism underlying the beneficial effect of blood transfusion in GVHD.

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Chapter 2.3

Improved survival from potentially lethal graft-versus-host disease by donor pretreatment with a recipient-specific blood transfusion

II. Evidence for a principal role of the CD4$^+$ T cell subset*

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Summary

Pretreatment of prospective donors of hemopoietic cells with a single recipient-specific blood transfusion can significantly decrease the morbidity and mortality of potentially lethal graft-versus-host disease (GVHD) in lethally irradiated, allogeneically reconstituted mice. In a previous report we described the requirements for induction of this blood transfusion effect. In the present study we addressed in particular the mechanism underlying this effect. The beneficial effect of blood transfusion appeared to be due to the white blood cell population in the transfused blood. X-irradiation (20 Gy) of the blood prior to transfusion did not abrogate the effect, which makes a veto cell mechanism unlikely. The blood transfusion effect in this model appeared to be mediated by the CD4$^+$ T cell subset, since purified CD4$^+$ spleen cells from transfused donors caused considerably less morbidity and mortality than naive CD4$^+$ spleen cells. Apparently CD8$^+$ cells were not involved, because their absence did not affect the beneficial effect. This observation was further confirmed by the finding that treatment of recipient mice that were reconstituted with spleen cells from transfused donors with anti-CD8 mAb did not abrogate the blood transfusion effect. Interestingly, the blood transfusion effect was enhanced by administration of anti-CD4 mAb to the recipients. The anti-CD4 mAb might impair the interaction between T cells and antigen-presenting cells, resulting in functional inactivation.

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Introduction

In previous studies from our laboratory, we found that allogeneic blood and spleen cell transfusion can suppress delayed-type hypersensitivity (DTH) responses to the specific alloantigens, both under host-versus-graft and under GVH conditions (1,2). In both situations the suppression was caused by T cells with a dominant suppressive effect. Recently, we have shown that donor pretreatment with a recipient blood transfusion strongly decreased the morbidity and enhanced the survival from potentially lethal GVHD (3). The present study was addressed to the mechanism of this beneficial effect of donor blood transfusion on GVHD and the role of T cell subsets. We report on the role of red and white blood cells and the effect of irradiation of the transfused blood. We further investigated the role of the $CD4^+$ and $CD8^+$ T cell subsets.

Materials and methods

Mice and monoclonal antibodies

(C57BL/Ka x CBA/Rij)F1 ($H\text{-}2^{b/q}$) and BALB/c ($H\text{-}2^d$) mice were bred at our department. Recipient mice were 10-18 weeks of age at the start of the experiments. For *in vivo* treatment, rat IgG2a anti-CD4 (H129.19) mAb (4) or rat IgG2b anti-CD8 (YTS 169.4) mAb (5) were purified from ascitic fluid. For *in vitro* incubation and subsequent cell panning or immunofluorescence, culture supernatant of the IgG2a rat anti-mouse hybridomas anti-B220 (RA3 6B2), anti-CD4 (H129.19) or anti-CD8 (53-6.72) was kindly provided by Prof. Dr. W. van Ewijk from our department. For staining, we used optimally titrated FITC-labelled rabbit-anti-rat $F(ab')_2$ fragments (Organon-Teknika Cappel, Turnhout, Belgium) as a second step antibody.

IL-2 treatment

Recombinant IL-2 was kindly provided by Dr. G. Malainer (Sandoz Forschungsinstitut, Wien, Austria). IL-2 was injected s.c. in the inguinal region. The IL-2 activity was checked in an *in vitro* proliferation assay and expressed in U/ml. One U is the amount of IL-2 that results in half maximum ^{3}H-thymidine incorporation of a CTLL line.

Other procedures and statistical analysis

Preparation of cell suspensions, blood transfusion, induction of GVHD and purification of $CD4^+$ T cells were performed as earlier described (3,6). Differences between groups were analyzed using the Wilcoxon-Mann-Whitney statistic. Values of $p<0.05$ were considered as significant.

Results

The role of white and red blood cells and the effect of irradiation

We determined which blood cell fraction was responsible for the blood transfusion effect after reconstitution of lethally irradiated mice with allogeneic spleen cells. Therefore, BALB/c donor mice were transfused with a number of purified white blood cells, purified red blood cells, or platelets equivalent to 1 ml whole blood. Control donor mice were transfused with 1 ml of whole blood for comparison. After 4 days 10^7 spleen cells from the donor mice were injected into lethally irradiated (C57BL x CBA)F1 recipients. Table 1A shows that transfusion of purified white blood cells was at least as effective as transfusion of whole blood ($p<0.005$). Red blood cell transfusion did not significantly improve the survival ($p>0.05$) nor did platelet transfusion (data not shown). Irradiation of whole blood did not decrease its ability to enhance the survival.

The effect of coadministration of cells from transfused and naive donors

We investigated whether the blood transfusion effect was a dominant phenomenon, by studying whether cells from transfused donors were able to inhibit the development of lethal GVHD upon coadministration with naive cells. Therefore, lethally irradiated (C57BL x CBA)F1 mice were reconstituted with 10^7 spleen cells from naive BALB/c donors, with 10^7 spleen cells from transfused BALB/c donors, or with the combination of both. It appeared that spleen cells from transfused donors were unable to inhibit or decrease the induction of lethal GVHD by spleen cells from naive donors (Table 1B).

The involvement of CD4⁺ and CD8⁺ T cells in the blood transfusion effect

We further determined which T cell subpopulation was involved in the blood transfusion effect. Therefore, we used purified CD4⁺ T cells from transfused BALB/c donors and from naive donors, as a control, to reconstitute lethally irradiated (C57BL x CBA)F1 recipients. Upon reconstitution with purified CD4⁺ T cells the blood transfusion effect could still be demonstrated (Table 1C). This indicates that CD8⁺ T cells were not required. This was further investigated, using a procedure which eliminates CD8⁺

Table 1. Induction and transfer of the blood transfusion effect.

	Donor pretreatment	reconstitution	% survival		
			day 20	day 40	day 60
A.	Whole blood	10^7 SC	88	56	19
	White blood cells	10^7 SC	94	63	56
	Red blood cells	10^7 SC	56	19	19
	Irradiated whole blood	10^7 SC	94	50	25
	None	10^7 SC	19	0	0
B.	Whole blood	10^7 SC	69	69	n.d.
	Whole blood	10^7 SC + 10^7 naive SC	6	0	n.d.
	None	10^7 SC	6	0	n.d.
C.	Whole blood	2×10^6 $CD4^+$ SC	100	80	60
	None	2×10^6 $CD4^+$ SC	60	50	0

BALB/c donor mice were pretreated and their spleen cells (SC) were used four days later for reconstitution of lethally irradiated (C57BL x CBA)F1 recipients. (A). Donor pretreatment consisted of various blood cell fractions equivalent to 1 ml blood. (B). Recipients were reconstituted with SC from transfused and SC from naive donors. (C). Recipients were reconstituted with purified $CD4^+$ SC.

T cells *in vivo*. Therefore, recipient mice were injected with 400 µg of a depleting anti-CD8 mAb, the day before reconstitution with spleen cells from naive or transfused donors. The treatment eliminated >99% of the $CD8^+$ T cells (data not shown). Figure 1 shows that also this procedure did not abrogate the beneficial effect of donor blood transfusion. Anti-CD8 treatment did not significantly influence the survival of both groups ($p>0.05$). Since in the strain combination used only $CD4^+$ T cells, but not $CD8^+$ T cells can induce lethal GVHD (4), we did not use purified $CD8^+$ T cells for reconstitution, or eliminate $CD4^+$ T cells *in vivo*. This would eliminate the T cells necessary for induction of GVHD and make it impossible to evaluate the effect on donor pretreatment.

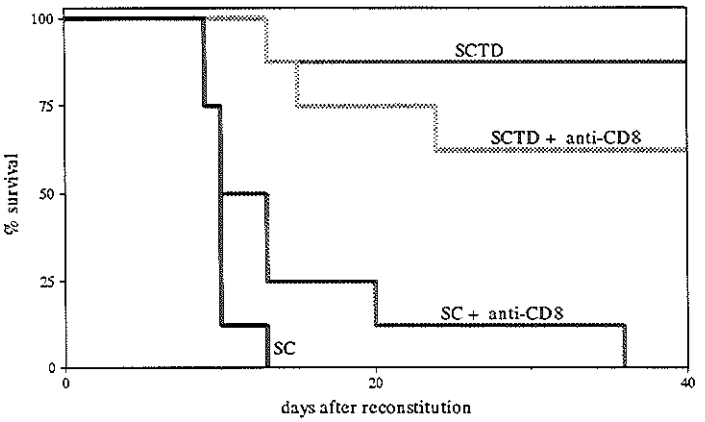

Figure 1. Effect of anti-CD8 treatment in vivo. Lethally irradiated (C57BL x CBA)F1 recipients were reconstituted with spleen cells obtained from naive (SC) or transfused (SCTD) BALB/c donors. A part of each group of recipients was treated with 400 μg of anti-CD8 mAb i.p. the day before reconstitution. Each experimental group consisted of 8 mice.

Enhancement of the blood transfusion effect by anti-CD4 mAb

Since donor blood transfusion led to long-term survival of usually only a minority of the recipients, we investigated whether it was possible to enhance the blood transfusion effect. Based on the finding that the CD4⁺ T cell subset appeared to be primarily involved in the blood transfusion effect in this model and on data from the literature (7), we administered anti-CD4 mAb *in vivo*. Therefore, (C57BL x CBA)F1 mice were lethally irradiated and reconstituted with spleen cells from naive or transfused BALB/c donors. These recipients were treated with a single dose of 200 μg of non-depleting (data not shown) anti-CD4 mAb, the day before they were reconstituted. It appeared that the combination of donor transfusion and recipient treatment with anti-CD4 mAb was significantly better than each treatment given separately and led to strongly reduced body weight loss (data not shown) and enhanced survival (Figure 2).

Discussion

We have recently demonstrated that donor pretreatment with a single recipient-specific blood transfusion decreased the severity and mortality of GVHD after allogeneic reconstitution (3). The present report deals with the mechanism of the observed

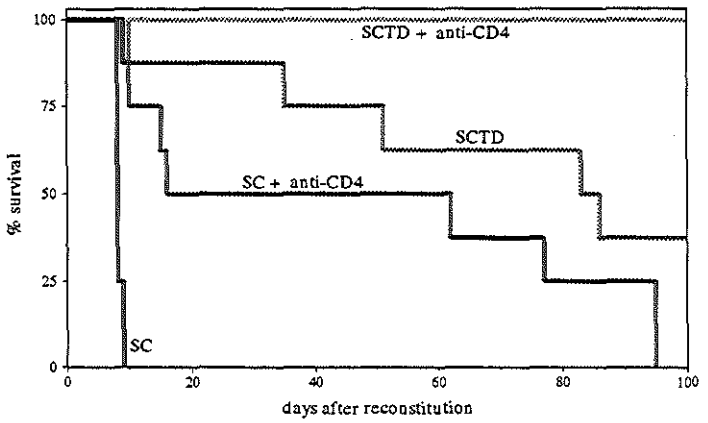

Figure 2. Enhancement of the blood transfusion effect by anti-CD4 mAb. (C57BL x CBA)F1 mice were lethally irradiated and reconstituted with spleen cells from naive (SC) or transfused (SCTD) BALB/c donors. The day before reconstitution a part of each group of recipients was injected i.p. with 200 μg of anti-CD4 mAb. Each experimental group consisted of 8 mice.

beneficial effect of donor blood transfusion and the involvement of T cell subsets.

White blood cells appeared to be necessary for the induction of the beneficial effect, which is in line with our previous data in a DTH model and most reports from the literature (1,2,8). This might be related to the types of histocompatibility antigens present on these cells, but also to the degree of expression of these antigens (9). Furthermore, white cells can produce cytokines and other factors that might play a role. However, in our studies on the suppression of anti-host DTH during GVHD we did not find evidence for the latter suggestion (2). Irradiation of the blood did not abrogate its ability to improve the survival, which is in line with data reported by others (10). This makes it unlikely that so-called veto cells play a role, since these were reported to be radiosensitive (11).

We were not able to show a dominant suppressive effect by spleen cells from transfused donors, as could earlier be demonstrated in the DTH model (2). This might be due to the large histocompatibility differences in the strain combinations used. In a similar model, Halle-Pannenko et al. found enhanced survival in a minor histoincompatible strain combination after donor pretreatment with allogeneic spleen cells (12). The beneficial effect in their study was primarily due to alloimmunization against Mls^a antigens. Although they were able to show a dominant suppressive effect using spleen cells from alloimmunized and naive donors at a ratio of 1:1, the effect was more pronounced using nylon-wool purified T cells from alloimmunized donors. It might be that in our model an even larger number of cells from transfused donors is required.

Our data clearly indicate that transfer of the blood transfusion effect is not dependent on $CD8^+$ T cells. The blood transfusion could be demonstrated after depletion of $CD8^+$ T cells *in vitro* as well as after elimination of $CD8^+$ T cells *in vivo*. These results reinforce the conclusion that a veto cell mechanism is unlikely the cause of the observed suppression of GVHD, as veto cells were found to be $CD8^+$ (11). These data also exclude the involvement of $CD8^+$ suppressor T cells.

We have earlier found that $CD4^+$ T cells play a central role in the induction of lethal GVHD in this strain combination (6). The fact that $CD4^+$ T cells from transfused donors caused reduced morbidity and mortality suggests that the pretreatment results in functional impairment of $CD4^+$ T cells. The absolute number of $CD4^+$ T cells in the spleen of transfused mice appeared to be unchanged (data not shown). Since $CD8^+$ T cells probably are not responsible for this effect, $CD4^+$ T cells seem on the one hand to mediate suppression and on the other hand to be the target.

Recently, it has become clear that $CD4^+$ T cells can be subdivided in a Th1 and Th2 subset, based upon their cytokine production profile. The Th1 and Th2 subset crossregulate each other (13). Th2 cells are able to inhibit the cytokine production of Th1 cells by IL-10 that probably acts via the antigen-presenting cell (13). Donor pretreatment might lead to a disturbed balance between Th1 and Th2 cells as was recently suggested by Wood et al. (14). It might also be that donor pretreatment induces alterations in the responsiveness to cytokines either or not as a consequence of a disturbed balance between the Th1 and Th2 subset. This could be demonstrated for IL-2 in a rat heart transplantation model and appeared to be associated with down-regulation of the IL-2R (15). Despite this, we were unable to reverse the blood transfusion effect by repeated administration of high doses of IL-2 (data not shown), as was possible in the above model. Similar IL-2 treatment appeared to be able to enhance the morbidity and mortality of GVHD (manuscript in preparation). However, other cytokines might be involved as well, since recently Soulillou reported the involvement of IFN-γ in the same rat heart transplantation model (16).

Although donor blood transfusion strongly improved the survival from potentially lethal GVHD, the long-term results were disappointing, since in most experiments only a minority of the recipients became long-term survivors. Therefore, we investigated the possibility to enhance the survival. Pearson et al. recently reported in a murine heart transplantation model, that the combination of donor-specific blood transfusion and anti-CD4 mAb treatment was superior as compared to each treatment alone (7). In our model it appeared also possible to enhance the blood transfusion effect by recipient treatment with anti-CD4 mAb. It can be speculated that blood transfusion induces partial unresponsiveness of $CD4^+$ T cells, which can be enhanced by blocking $CD4^+$ T cells with anti-CD4 mAb. It has to be noticed that the anti-CD4 mAb we used are non-

depleting, in contrast to the mAb used by Pearson et al. Since they used a very low dose of 25 μg, it is likely that the enhanced survival in their model was also due to blockade of $CD4^+$ T cells, rather than to depletion. The mice that became long-term survivors after donor blood transfusion were fully repopulated with donor-type cells, which was also found by others (10).

Taken together the data indicate that donor blood transfusion induces functional impairment of $CD4^+$ T cells, which results in enhanced survival from potentially lethal GVHD. Apparently the effect is not due to altered IL-2 production or sensitivity. We did not find evidence for a role of $CD8^+$ (suppressor) T cells and excluded the possible involvement of veto cells. Therefore, it is possible that the beneficial blood transfusion effect is the result of inhibition of Th1 cells by Th2 cells. Further studies are required to confirm this hypothesis.

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Chapter 3

Effect of anti-T cell (subset) treatment on lethal graft-versus-host disease

Chapter 3.1

Prevention of lethal graft-versus-host disease by a single low dose of anti-T cell monoclonal antibody to the allograft recipients*

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Summary

We investigated the capacity of monoclonal antibody (mAb) treatment to prevent graft-versus-host disease (GVHD) in lethally irradiated , allogeneically reconstituted mice, employing anti-T cell (subset) mAb and a fully allogeneic strain combination. In this strain combination purified $CD4^+$ cells were able to induce a lethal GVH reaction, whereas purified $CD8^+$ cells were not. In the same strain combination, a single intraperitoneal injection of IgG2b anti-Thy-1 mAb, one day after reconstitution, caused a dose-dependent improvement of the survival. A single injection of a dose as low as 12.5 µg per mouse was already effective. Intravenous and intraperitoneal administration of the mAb appeared equally effective. For effective prevention of GVHD the treatment could be postponed until the 4^{th} day after transplantation, but treatment delayed until day 6 was no longer effective. Treatment with IgG2b mAb specific for either helper or cytotoxic T cells also led to improvement of GVHD and survival, but was less effective than treatment with anti-Thy-1 mAb. Clinically, there was a difference in the effectiveness of anti-CD4 and anti-CD8 treatment, since symptoms of GVHD started earlier in the anti-CD8-treated group and the survival was better in the anti-CD4-treated group. These results press for prospective clinical studies employing anti-T cell mAb treatment early after allogeneic bone marrow transplantation (BMT), especially in HLA mismatched cases.

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Introduction

MAb are becoming increasingly important in the prevention and treatment of transplant rejection (1). MAb directed to T cells are particularly important, since these cells play a major role both in organ transplant rejection and GVHD after allogeneic BMT. MAb directed to activation markers like IL-2R are also employed (2). In clinical BMT, mAb are used in many centers to deplete the bone marrow graft to be transplanted of mature T cells that would otherwise cause GVHD, especially in (partly) mismatched donor-recipient pairs. This procedure considerably decreases the risk of GVHD. Nevertheless, T cell depletion of the graft cannot completely prevent the development of GVHD. Moreover, T cell depletion of the graft has two major disadvantages. First, it causes a significantly increased risk of unsuccessful take of the bone marrow graft. Second, in leukemia patients it increases the chance of recurrence of the leukemia. The overall survival after allogeneic BMT for leukemia did not increase after the introduction of T cell depletion of the graft (3). Additional strategies have to be developed that successfully decrease or treat GVHD, but do not lead to increased graft rejection or leukemia recurrence.

Experimental studies show that mAb can be very effective *in vivo*. When mAb of the appropriate specificity and immunoglobulin subclass were used, *in vivo* pretreatment of thymectomized donor mice or of recipient mice could decrease or abolish GVHD. Particularly rat mAb of the IgG2b and murine mAb of the IgG2a subclass appeared effective which may be due to their capacity to bind to the C1 protein of the complement system (4-6).

Thus far, there are very few reports on the use of mAb to treat or prevent GVHD by administration to the recipients *after* transplantation of allogeneic hematopoietic cells.

We investigated whether unmodified IgG2b anti-T cell (subset) mAb could effectively reduce GVHD when given *after* reconstitution. We used allogeneic spleen cells in our mouse model since mouse bone marrow is relatively poor in T cells unlike human bone marrow. We focussed on the route of administration, the dose-response relationship and the timing. Moreover, we studied the effect of mAb specific for either helper T (Th) or cytotoxic T (Tc) cells. The results proved to be extremely encouraging for low-dose mAb treatment administered during the first days after transplantation.

Materials and methods

Mice

(C57BL/Ka x CBA/Rij)F1 (H-$2^{b/q}$) and C3H/Law (H-2^{k}) mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. BALB/c (H-2^{d}) mice were bred at our own department. Recipient mice were age-matched and at least 12-18 weeks old when entered in the experiments. Mice were kept 2 per cage in light-cycled rooms and had access to acidified water and pelleted food *ad libitum*.

Preparation of cell suspensions

Mice were killed using carbon dioxide. Spleen cell suspensions were prepared in BSS. Nucleated cell concentrations were determined with a Coulter Counter model ZB1. Viability of the cell suspensions as determined by the trypan blue exclusion method was >95%.

Induction of GVH

GVH reactions were induced in lethally irradiated (9.5 Gy) mice by i.v. injection of 10^7 nucleated allogeneic spleen cells within 24 h after irradiation. Irradiation was performed in a Cesium-137 source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) with a dose rate of 1.15 Gy/min. Mice were examined daily for the development of signs of GVHD, such as hung posture, wasting, skin lesions, and diarrhoea, and for mortality. The body weight was determined twice weekly. Control mice reconstituted with syngeneic cells survived >250 days. Radiation controls died between days 11 and 22.

Purification of T cell subsets

T cells were purified using a two-step indirect panning technique, originally described by Mage et al. (7). Briefly, plastic petri dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) were coated overnight at 4°C with rabbit-anti-rat immunoglobulin (Dakopatts, Glostrup, Denmark) in PBS at a concentration of 20 μg/ml. The dishes were washed once with PBS and subsequently incubated for 30 min with PBS containing 1% FCS. Spleen cells were incubated with the relevant mAb for 30 min at 4°C, washed with PBS - 1% FCS and incubated on the petri dishes (3 x 10^7 cells/dish) for 1.5 h. The non adherent cells were collected and incubated with the second mAb for 30 min. After washing with PBS - 1% FCS, they were incubated on the petri dishes for 1.5 h. Thereafter the obtained non-adherent cells were analyzed using a flowcytofluorometer (FACScan, Becton Dickinson, Mountain View, CA).

Chimerism

To test whether the long-term chimeras had become tolerant for the donor antigens, they were grafted with a specific BALB/c skin graft and a third-party C3H/Law skin graft. The C3H/Law skin grafts were rejected normally whereas the BALB/c grafts were not rejected. To determine the degree of chimerism on the B cell level, spleen cells of the long term chimeras were stained with the FITC-labelled Y3P anti-class II mAb, kindly provided by Dr. A. Kruisbeek. The percentage of Y3P positive cells was compared with the percentage of immunoglobulin positive cells as determined with a FITC-labelled goat-anti-mouse-immunoglobulin antiserum. Using this method the degree of B cell chimerism was determined to be >99%.

Monoclonal antibodies

For *in vivo* treatment, purified rat IgG2b anti-Thy-1 (YTS 154.7), IgG2b anti-CD4 (YTS 191.5), IgG2b anti-CD8 (YTS 169.4) mAb were purchased from Sera-Lab., Ltd., Sussex, U.K. The time and route of administration is indicated in the Results section. For *in vitro* incubation and subsequent cell panning, culture supernatant of the hybridomas RA3 6B2 (IgG2a anti-B220), H129.19 (IgG2a anti-CD4) or 53-6.72 (IgG2a anti-CD8) was kindly provided by the group of Dr. W. van Ewijk from our department.

Data analysis

Differences between groups were analyzed using the Wilcoxon-Mann-Whitney statistic. Values of $p<0.05$ were considered as significant.

Results

Induction of lethal GVHD by purified T cell subsets

Lethally irradiated (C57BL x CBA)F1 recipient mice were reconstituted with either 2 x 10^6 purified $CD4^+$ T cells (containing <1% $CD8^+$ cells) or 1 x 10^6 purified $CD8^+$ T cells (containing about 1.5% $CD4^+$ cells) or with 2 x 10^6 $CD4^+$ T cells and 1 x 10^6 $CD8^+$ T cells of H-2 plus non-H-2 incompatible BALB/c donors. The number of cells of the respective T cell subsets corresponds with their number as present in 10^7 unseparated spleen cells. Figure 1 shows that reconstitution with $CD4^+$ T cells induces a GVH reaction that is lethal in 100% of the recipients within 23 days, whereas after reconstitution with $CD8^+$ T cells 80% of the mice became long-term survivors. In fact, in the latter group no signs of GVHD were found at all. Two mice of this group died on days 26 and 43, respectively, but without signs of GVHD. If both subsets together

were used for reconstitution a lethal GVH reaction was induced resulting in 100% mortality within 34 days. For comparison, a control group of (C57BL x CBA)F1 mice was reconstituted with 10^7 unseparated allogeneic BALB/c spleen cells. The survival of this group was comparable to the group reconstituted with both T cell subsets (data not shown).

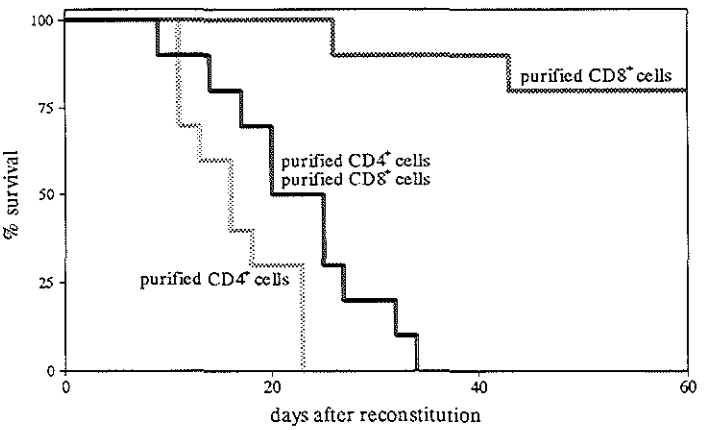

Figure 1. Induction of GVH by purified T cell subsets. Lethally irradiated (C57BL x CBA)F1 recipients were reconstituted with either 2 x 10^6 purified $CD4^+$ T cells or 1 x 10^6 purified $CD8^+$ BALB/c T cells, or with the combination of both subsets. Each group consisted of 10 mice. Survival of the $CD8^+$ T cell reconstituted group was significantly better than that of the $CD4^+$ T cell or $CD4^+$ T cell and $CD8^+$ T cell reconstituted group ($p<0.05$).

Abrogation of lethal GVHD by injection of 100 μg of anti-Thy-1

Lethally irradiated (C57BL x CBA)F1 recipient mice were reconstituted with 10^7 allogeneic BALB/c spleen cells. After 24 h they received either an i.p. or an i.v. injection of 100 μg anti-Thy-1 mAb. This treatment totally prevented GVH-related weight loss, morbidity and mortality. All mAb-treated mice became long-term survivors without signs of disease (Table 1). No difference in effectivity was found after i.p. or i.v. administration with regard to clinical symptoms and body weight.

Dose-response relationship

The dose-response relationship was investigated in irradiated (C57BL x CBA)F1 mice

Table 1. Effect of i.v. versus i.p. treatment of mice with anti-Thy-1 mAb on the mean survival time (MST) of allogeneically reconstituted irradiated mice.

Recipient strain	donor	anti-Thy-1 treatment	MST
(C57BL x CBA)F1	BALB/c	100 µg i.p.	>250 days (n=5)
(C57BL x CBA)F1	BALB/c	100 µg i.v.	>250 days (n=5)
(C57BL x CBA)F1	BALB/c	none	44.9 ± 5.0 days (n=10)

reconstituted with 10^7 BALB/c spleen cells and treated 24 h later with a single i.p. dose of anti-Thy-1 ranging from 12.5 to 100 µg. As can been seen in Figure 2, a dose of 12.5 µg of anti-Thy-1 already significantly prolonged survival. Higher doses caused a dose-related better survival. The increased survival was inversely correlated with the decrease in weight. In this particular experiment a dose of 100 µg did not completely prevent mortality.

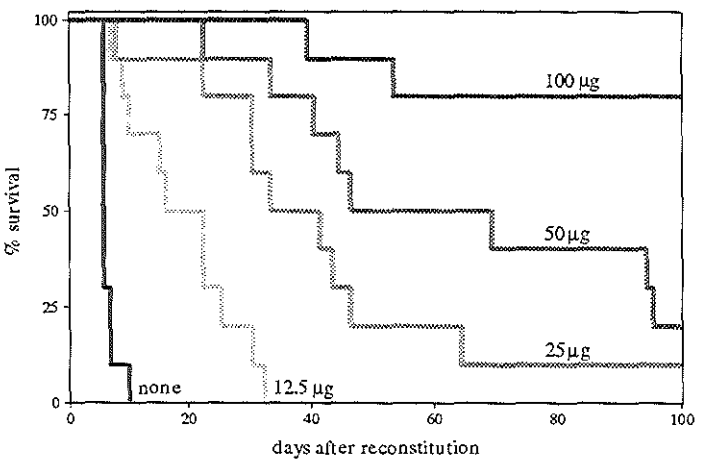

Figure 2. Dose-response relationship. Lethally irradiated (C57BL x CBA)F1 recipients were reconstituted with 10^7 allogeneic BALB/c spleen cells and received a single dose (indicated in the figure) of anti-Thy-1 i.p. 24 h after reconstitution. Each group consisted of 10 mice.

Timing of monoclonal antibody treatment

Next we studied the effect of the interval between reconstitution and mAb treatment on the recipients' survival. Lethally irradiated (C57BL x CBA)F1 recipients were

Table 2. Effect of the interval between allogeneic reconstitution and anti-Thy-1 mAb treatment on the MST of irradiated mice.

Recipient strain	donor	anti-Thy-1 (100 μg i.p.)	MST
(C57BL x CBA)F1	BALB/c	day 1	>250 days (n=5)
(C57BL x CBA)F1	BALB/c	day 4	>250 days (n=5)
(C57BL x CBA)F1	BALB/c	day 6	34.0 ± 15.0 days (n=5)
(C57BL x CBA)F1	BALB/c	none	44.9 ± 5.0 days (n=10)

reconstituted with 10^7 allogeneic BALB/c spleen cells. Recipients were treated with anti-Thy-1 at 1, 4 or 6 days after reconstitution. Table 2 shows that delayed mAb treatment up to 4 days after allogeneic spleen cell reconstitution could prevent mortality. However, treatment postponed until day 6 led to a mean survival time not exceeding that of untreated controls.

Effect of anti-T cell subset monoclonal antibodies

Anti-Thy-1 treatment affects both the $CD4^+,CD8^-$ Th cell subset and the $CD4^-CD8^+$ Tc cell subset. Our data indicate a dominant role in GVHD for the $CD4^+,CD8^-$ subset in the BALB/c - (C57BL x CBA)F1 strain combination. To confirm this, we tried to eliminate each of these T cell subsets separately by i.p. administration of anti-CD4 and anti-CD8 mAb to irradiated (C57BL x CBA)F1 recipients reconstituted the day before with 10^7 BALB/c spleen cells, using the same amount (100 μg) of purified IgG2b protein. Figures 3A and 3B show that anti-CD4 treatment as well as anti-CD8 treatment improved the survival considerably as compared to the untreated control group. When comparing Figure 3A with Figure 3B, it can be seen that there is variation between the experiments with respect to the onset of mortality. However, both in acute and somewhat more chronic GVHD mAb treatment was effective. Compared to the anti-Thy-1 treatment both the anti-CD4 and the anti-CD8 treatment were less effective in improving GVHD and survival. Onset of clinical symptoms differed between the anti-CD4- and the anti-CD8-treated group. The long- term survival in the anti-CD4-treated group shown in Figure 3A was 30% (3/10), while in the anti-CD8-treated group no mice survived (0/10).

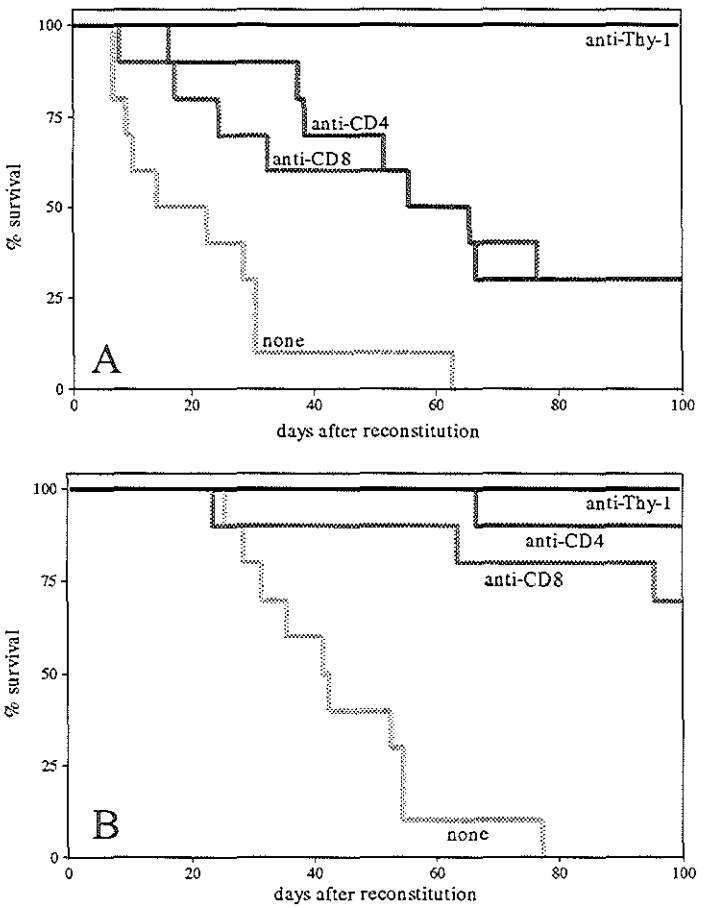

Figure 3. Treatment with anti-T cell subset mAb. Lethally irradiated (C57BL x CBA)F1 recipients were reconstituted with 10^7 BALB/c spleen cells. After 24 h they received an i.p. injection of either 100 μg of anti-Thy-1, 100 μg of anti-CD4 or 100 μg of anti-CD8. Survival curves of two independent experiments are given (A and B). Each group consisted of 10 mice.

Discussion

One of the most important complications of allogeneic BMT is the development of GVHD. T cells in the transplanted graft play a central role in this complex disease. Therefore, a lot of attention has been directed to methods to deplete the graft of T cells *before* reconstitution. Although the employment of T cell-depleted grafts indeed led

to a considerably decreased risk of GVHD, the procedure appeared to have important drawbacks. In patients receiving T cell-depleted grafts for the treatment of leukemia, the risk of graft rejection on one hand and of recurrence of the leukemia on the other hand was strongly enhanced. For this reason the overall survival did not increase after the introduction of transplantation of T cell-depleted bone marrow for the treatment of leukemia (3). A new strategy might include the use of anti-T cell mAb shortly after transplantation. Such application of anti-T cell mAb would affect donor as well as host T lymphocytes, thereby preventing both GVHD and graft rejection.

Our results indicate that i.v. or i.p. administration of anti-T cell mAb can indeed effectively prevent GVHD and GVH-related mortality, using a single low dose of 100 μg of anti-Thy-1 up to 4 days after allogeneic spleen cell reconstitution. In some experiments this dose did not prevent mortality completely. This might be due to a higher T cell content of the inoculum used to reconstitute the recipients which in our experience varies slightly between various donor mice. Despite this, an extremely powerful effect on survival was found in all experiments. It should be mentioned that we used a fully allogeneic (H-2 plus non-H-2 disparate) strain combination for our studies. Lower doses were also effective and even a dose as low as 12.5 μg led to significant prolongation of survival. Such a beneficial effect of mAb therapy shortly *after* reconstitution has not been described before.

Anti-Thy-1 mAb have been used successfully by administration *before* reconstitution to deplete recipients of their T cells, thereby preventing marrow rejection and GVHD (8). In the latter study improved survival times were reported after administration of mAb 20 h before allogeneic reconstitution. However, a dose of 100 μg was not effective in these experiments. Moreover, injection of the mAb 24 h after reconstitution did not result in prolonged survival at all. This is in sharp contrast with our results. Most likely the effectiveness of the relatively low doses of mAb in our experiments can be explained by the fact that shortly after allogeneic reconstitution the number of viable T cells is still low. It can be speculated that this is partly due to competition between cells in the graft and remaining T cells in the host occurring during this early phase. These T cells might have an increased susceptibility to the anti-Thy-1 mAb treatment because of their activation. This might explain the effectiveness of low dose mAb during the first few days after transplantation. The difference between the above-mentioned literature data and our results might be due to the difference in the number of spleen cells used for reconstitution, which was five times less in our experiments. On the other hand, the dose of mAb we used was at least five times lower, so that this cannot fully explain the discrepancy.

Our study indicates that treatment can be postponed for some days after transplantation to be effective, although this period is limited. A single low-dose injection of anti-

Thy-1 cannot, however, prevent an ongoing GVH reaction. This might be due to the higher number of T cells at later stages. One can speculate that the delay time can be increased by increasing the dose of mAb. It also possible that at later stages cell types other than T cells become more and more involved in the GVH reaction, so that other treatments should also be used to reduce GVHD.

From a number of studies it has become clear that both the $CD4^+$,$CD8^-$ Th cell subset and the $CD4^-$, $CD8^+$ Tc cell subset play a role in transplant rejection. The degree of involvement of each subset is dependent on the antigenic differences between donor and recipient (9-11). In the BALB/c - (C57BL x CBA)F1 fully allogeneic strain combination, we found that purified $CD4^+$,$CD8^-$ T cells were able to induce a lethal GVH reaction, whereas $CD4^-$, $CD8^+$ T cells were not. This argues for a dominant role of the $CD4^+$ T cell subset. Therefore, we expected an effect of treatment with anti-CD4 mAb. This indeed was found to be the case, although the treatment did not result in 100% survival (Figure 2). Surprisingly, anti-CD8 treatment also substantially decreased the mortality rate, although the clinical symptoms of GVHD started at the same time or even earlier than in the non-treated group. This suggests a role for $CD4^-$, $CD8^+$ T cells as well. Since $CD8^+$ T cells in this strain combination are not able to induce a lethal GVH reaction themselves, they might be dependent on the help of $CD4^+$ T cells or their lymphokines.

The difference in survival between the anti-Thy-1-treated group and the anti-CD4-treated group was striking. The dose of anti-CD4 mAb used, although an equal amount of purified protein, was not as efficient as the same dose of anti-Thy-1. In this respect it should be mentioned that the antigen density on the cell surface is probably important (12) and this may differ for the respective antigens. Subclass differences cannot be involved , since all mAb used were of the IgG2b subclass.

This study has considerable implications for clinical allogeneic BMT. Potent murine anti-human T cell mAb have already been developed and used with success in organ transplantation. This study provides evidence that these mAb can also be favorably employed in allogeneic BMT. Possibly, there will be a role for anti-T cell subset mAb as well. It can be speculated that the anti-T cell mAb treatment can be used successfully in early stages of GVHD as well as in the prevention strategy after allogeneic BMT. With the increasing number of HLA mismatched allogeneic BMT, the incidence and severity of GVHD have increased comparably and it is likely that anti-T cell mAb treatment can be useful also in this field.

Acknowledgments

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Chapter 3.2

Effective monoclonal antibody treatment of lethal graft-versus-host disease in mice

Role of T cell subsets and evidence for the induction of a state of tolerance based on suppression*

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Summary

We earlier demonstrated that lethal graft-versus-host disease (GVHD) in the fully allogeneic BALB/c (donor) - (C57BL x CBA)F1 (recipient) mouse strain combination can be prevented by a single dose of IgG2b monoclonal antibodies (mAb) directed to T cells. This observation was further addressed in the present study. We investigated the influence of the timing of mAb administration, the effect of anti-T cell subset mAb and the state of tolerance in the mice that had become long-term chimeras.

Anti-Thy-1 treatment of the recipients, either one day before reconstitution, two hours before reconstitution or one day after reconstitution, almost completely prevented lethal GVHD. A single dose of 100 μg of anti-Thy-1 was equally effective as four daily doses of 25 μg each. However, treatment with intervals of 4 days between the doses of 25 μg was less effective.

To clarify the role of T cell subsets, we injected the recipients with mAb either directed to all T cells or to the $CD4^+$ or $CD8^+$ T cell subset only. Using a dose of 100 μg mAb, anti-CD4 treatment appeared to be less effective than anti-Thy-1 treatment, whereas anti-CD8 treatment was not effective at all. A dose of 200 μg anti-CD4 was equally effective as a dose of 100 μg anti-Thy-1. The difference in effectiveness between anti-CD4 and anti-CD8 was not due to less efficient depletion of the $CD8^+$ T

* *Submitted for publication.*

cell subset, since anti-CD8 mAb were even more effective in that respect.

All mice that became long-term survivors remained free of signs of GVHD and were >99% repopulated with donor-type cells. We used spleen cells from these BALB/c into (C57BL x CBA)F1 chimeric mice either after successful anti-Thy-1 or anti-CD4 treatment to reconstitute lethally irradiated BALB/c, BALB.K or (C57BL x CBA)F1 recipients. Lethal GVHD developed in the BALB.K and (C57BL x CBA)F1 recipients, but not in the BALB/c recipients. This suggests that a state of tolerance was maintained in the chimeric mice, which was not based on clonal deletion of alloreactive T cells, but possibly on active suppression. This was further evaluated in stable chimeras that were sublethally (6 Gy) irradiated or treated with a high dose (5×10^7 or 2×10^8) naive BALB/c spleen cells, or injected with anti-CD8 mAb. This treatment did not induce GVHD. The absence of reactivity of these BALB/c spleen cells in the chimeric situation was not due to altered alloantigen expression, since when stable chimeras were subjected to a second lethal irradiation and subsequent reconstitution with naive BALB/c spleen cells, even a low dose of 10^7 spleen cells induced a graft-versus-host (GVH) reaction. This further suggests that the cells responsible for the tolerant state in stable chimeras are sensitive to high-dose irradiation.

Together these results indicate that *in vivo* anti-T cell (subset) mAb treatment of recipients can effectively prevent lethal GVHD and leads to a state of complete chimerism and tolerance, which is not due to clonal deletion of alloreactive T cells, but to active suppression.

Introduction

The occurrence of GVHD is still a major complication of allogeneic bone marrow transplantation (BMT). This complex disease is caused by mature T cells in the graft that recognize foreign antigens of the host (1). In mice it has been shown that $CD4^+$ T cells as well as $CD8^+$ T cells can play a predominant role in the GVH reaction depending on the histocompatibility differences between donor and recipient (2-4). Purified $CD4^+$ T cells are able to induce a lethal GVH reaction in class II disparate hosts, whereas purified $CD8^+$ cells are able to cause lethal GVHD in class I disparate recipients (4). Although T cell depletion of the bone marrow graft reduces the incidence and severity of GVHD, this procedure increases the risk of graft rejection and, in the case of leukemic patients, also the risk of recurrence of malignant disease (5). Therefore, new prophylactic and therapeutic approaches have to be developed, based upon the increasing knowledge of the cells involved in GVHD, the factors they can produce (cytokines), and the phase(s) of the disease they are involved.

In solid organ transplantation in humans, anti-T cell mAb are increasingly used to prevent and treat rejection episodes (6,7). In human BMT, mAb treatment is still infrequently used, but it is likely that mAb treatment will play a role in future treatment protocols. Animal studies indicate that anti-T cell mAb treatment can be very effective in the treatment of GVHD, either by pretreatment of donor cells, or when given to the recipients as prophylactic treatment (8-10) or early in the course of a GVH reaction (11). Rat mAb of the IgG2b subclass appeared to be very effective in the prevention of graft rejection and GVHD in mice. This is presumably due to their capacity to bind to the C1 protein of the complement system and to eliminate the respective cell population *in vivo* (12). Although administration of anti-T cell mAb of this subclass leads to depletion of T cells *in vivo*, the immunosuppressive properties are not only based on elimination of the cell population, since the depletion is not always complete (12). Anti-CD4, but also anti-LFA-1 mAb appeared to be able to facilitate the induction of tolerance to foreign antigens (13-15).

We reported earlier that low-dose anti-Thy-1 mAb treatment prevented lethal GVHD (11). In the present study we extended these experiments and analyzed in detail the role of the $CD4^+$ and $CD8^+$ T cell subset by evaluation of T cell subsets in the spleen early in the course of GVHD as well as by studying the effect of *in vivo* administration of mAb against T cell subsets. These findings were related to clinical symptoms of GVHD and the survival. We also investigated the *in vivo* T cell depleting capacity of anti-Thy-1, anti-CD4 and anti-CD8 mAb. Furthermore, we evaluated the state of chimerism and nature of tolerance in mice that had become long-term stable chimeras, either after successful anti-Thy-1 or anti-CD4 treatment.

Materials and methods

Mice

(C57BL/Ka x CBA/Rij)F1 ($H\text{-}2^{b/q}$), BALB/c ($H\text{-}2^{d}$) and BALB.K ($H\text{-}2^{k}$) mice were bred at the Department of Immunology of the Erasmus University. Recipient mice were age-matched and 12-20 weeks old at the start of the experiments. Mice were kept 2 per cage in light-cycled rooms and had access to acidified water and pelleted food *ad libitum*.

Preparation of cell suspensions

Mice were sacrificed using carbon dioxide. Spleen cell suspensions were prepared in balanced salt solution as described previously (16). Nucleated cell concentrations were determined with a Coulter Counter model ZB1. Viability of the cell suspensions as determined by trypan blue exclusion was >95%.

Induction of GVH

GVH reactions were induced in lethally irradiated mice ((C57BL x CBA)F1 mice: 10 Gy, BALB mice: 7.5 Gy) by i.v. injection of 10^7 allogeneic spleen cells within 24 h after irradiation. Irradiation was performed in a Cesium-137 source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) with a dose rate of 1.15 Gy/min. Mice were examined daily for mortality and the development of signs of GVHD, such as hunched posture, decreased physical activity, wasting, skin lesions, and diarrhoea. The body weight was determined two to three times per week in the first 4 weeks. Thereafter the frequency was decreased depending on the health status of the mice. Mice judged moribund were killed. Irradiated control mice of each of the strain combinations involved, that were reconstituted with syngeneic cells survived >250 days without signs of disease. Non-reconstituted (C57BL x CBA)F1 and BALB radiation controls died between days 8 and 22.

Antibodies and conjugates

For *in vivo* treatment, purified rat-anti-mouse anti-Thy-1 (YTS 154.7), anti-CD4 (YTS 191.5), and anti-CD8 (YTS 169.4) mAb of the IgG2b subclass were purchased from Sera-lab, Ltd., Sussex, U.K. The mAb were administered by i.p. injection. For staining, culture supernatants of the rat-anti-mouse hybridomas 59-AD2.2 (anti-Thy-1), H129.19 (anti-CD4) and 53-6.72 (anti-CD8), and of the hamster-anti-mouse hybridoma 500-A2 (anti-CD3) were kindly provided by Prof. Dr. W. van Ewijk from our department. As second step antibody we used optimally titrated FITC-labelled rabbit-anti-rat $F(ab')_2$ fragments and an FITC-labelled goat-anti-hamster-IgG antiserum (Organon-Teknika Cappel, Turnhout, Belgium). To the final dilution of the conjugate, 2% of normal mouse serum was added to reduce background fluorescence levels.

Collection of blood

Blood samples obtained by tail bleeding were collected in heparinized tubes and stored at room temperature, until the start of the immunofluorescent staining.

Immunofluorescence of spleen and blood cell samples

From spleen cell suspensions (10^7 nucleated cells/ml), 25 µl was aliquotted in 96 well round bottom microtiter plates (Nunc, Roskilde, Denmark). Subsequently, 50 µl of hybridoma tissue culture supernatant was added to each well. The cells were incubated for 30 min at 4°C. After this incubation the cells were washed 3 times with PBS containing 5% FCS and 20 mM azide (PBS-FCS-azide). The pellets were resuspended and 50 µl of the appropriate conjugate was added per well. After incubation for 30 min at 4°C, the cells were washed 3 times with PBS-FCS-azide, resuspended in 100 µl of isotonic fluid, put in centrifuge tubes (Falcon, Becton Dickinson, Lincoln Park, NJ) and analyzed using a flowcytofluorometer (FACScan, Becton Dickinson, Mountain View, CA).

In the case of peripheral blood samples the procedure was adapted. Briefly, an amount of 25 µl peripheral blood was put in centrifuge tubes and 50 µl of hybridoma tissue culture supernatant was added. After incubation for 10 min at room temperature, the samples were washed twice with PBS-

FCS-azide. The pellets were resuspended and 50 µl of the appropriate conjugate was added. After incubation for 10 min at room temperature, the samples were washed twice with PBS-FCS-azide. The erythrocytes were lysed using 1 ml of FACS lysing solution (Becton Dickinson, Lincoln Park, NJ). After washing with PBS-FCS-azide, the pellets were resuspended in 100 µl isotonic fluid and the samples were analyzed.

Data analysis

The experiments were performed 2 to 3 times, except the experiments presented in Table 4, which could only be performed once, due to the restricted number of chimeras available. Data from representative experiments are given. Differences between groups were analyzed using the Wilcoxon-Mann-Whitney statistic. Values of $p<0.05$ were considered as significant.

Results

Single versus multiple dose treatment

(C57BL x CBA)F1 recipients were lethally irradiated and subsequently reconstituted with 10^7 BALB/c spleen cells. Anti-Thy-1 mAb treatment was started one day after reconstitution and given as a single dose of 100 µg, as four daily doses of 25 µg each, as a single dose of 25 µg or as 4 doses of 25 µg, given with an interval of four days. Figure 1 shows the results which indicate that each treatment resulted in strongly improved survival. Treatment with a single dose of 100 µg was as effective as four daily doses of 25 µg each. A single dose of 25 µg however, was significantly less effective. Treatment with 4 doses of 25 µg of anti-Thy-1 at intervals of four days, starting on day 1, was not more effective than a single dose of 25 µg.

Timing of monoclonal antibody treatment

We investigated the effect of the timing of anti-Thy-1 mAb treatment on lethal GVHD. Therefore, (C57BL x CBA)F1 recipients were lethally irradiated and reconstituted with 10^7 BALB/c spleen cells. Treatment with a dose of 100 µg of anti-Thy-1 was given either the day before reconstitution (day -1), two hours before reconstitution (day 0), or the day after reconstitution (day 1). Table 1 shows that treatment at day -1, 0 or 1 resulted in strongly enhanced survival. Treatment given at day -1 was slightly, but not significantly, better than treatment given at day 0 or 1.

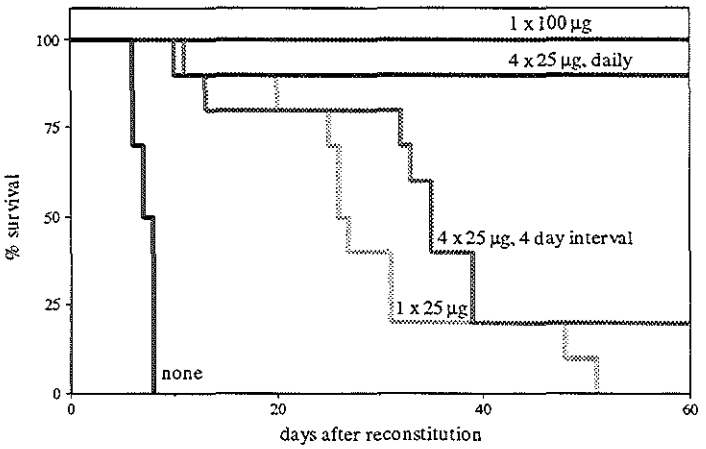

Figure 1. Effect of a single versus multiple dose treatment with anti-Thy-1 mAb on GVHD. One day after reconstitution of lethally irradiated (C57BL x CBA)F1 mice with 10^7 BALB/c spleen cells the recipients received either 100 µg or 25 µg of anti-Thy-1 i.p. Another group received four daily doses of 25 µg, starting on day 1, and again another group of mice received four doses of 25 µg of anti-Thy-1 with an interval of four days between each injection (n=10).

Table 1. Effect of the moment of anti-Thy-1 mAb treatment on GVH-related mortality.

Recipient strain	donor	anti-Thy-1 treatment	% survival		
			day 20	day 60	day 100
(C57BL x CBA)F1	BALB/c	day -1	100	90	90
(C57BL x CBA)F1	BALB/c	day 0	100	70	60
(C57BL x CBA)F1	BALB/c	day 1	100	60	60

(C57BL x CBA)F1 recipient mice were lethally irradiated and subsequently reconstituted with 10^7 BALB/c spleen cells. Treatment of recipients with 100 µg of anti-Thy-1 was given the day before reconstitution (day -1), the day of reconstitution (day 0), or one day after reconstitution (day 1). Each experimental group consisted of 10 mice.

T cell subsets in the spleens of mice after allogeneic and syngeneic reconstitution

To study the involvement of the $CD4^+$ and $CD8^+$ T cell subset in this model, (C57BL x CBA)F1 mice were lethally irradiated and reconstituted either with 10^7 allogeneic or with 10^7 syngeneic spleen cells, as a control. At day 4, 5, 6, 7 and 8 after reconstitution,

spleen cells from 2 mice of each group were analyzed for the number of Thy-1, CD4, CD8 and CD3 positive cells. The results are presented in Figure 2A. In the allogeneically

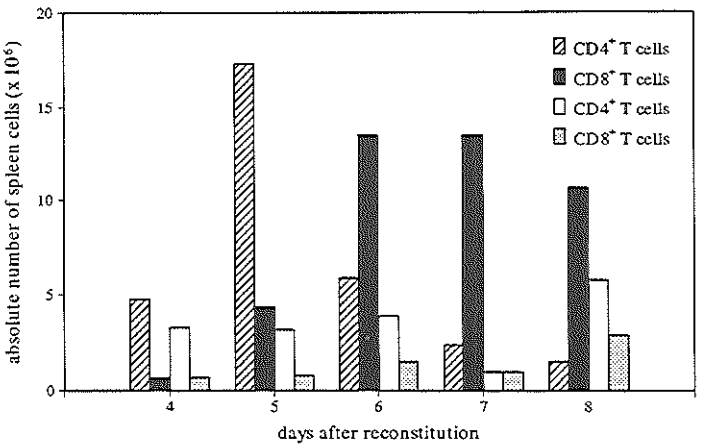

Figure 2A. Evaluation of T cell subsets after allogeneic and syngeneic reconstitution. (C57BL x CBA)F1 mice were lethally irradiated and reconstituted with 10^7 syngeneic or allogeneic spleen cells. At days 4, 5, 6, 7 and 8, the spleens of two mice of each group were analyzed for the presence of CD4 (allogeneic ▨, syngeneic □) and CD8 (allogeneic ■, syngeneic ▩) positive T cells.

reconstituted mice, the number of $CD4^+$ T cells increased strongly with a peak at day 5, followed by a peak in the number $CD8^+$ T cells at day 6. Similar changes, although somewhat less pronounced, were found in the lymph nodes (data not shown). Signs of disease in the allogeneically reconstituted mice were found from day 7 onward. The median survival time of the allogeneically reconstituted group was 11 days (range 8 - 47 days). In the syngeneically reconstituted mice the number of $CD4^+$ and $CD8^+$ T cells remained stable. No morbidity or mortality occurred. The initial increase in the number of $CD4^+$ T cells and the subsequent increase in the number of $CD8^+$ T cells after allogeneic reconstitution might reflect the sequential activation and proliferation of $CD4^+$ and $CD8^+$ T cells during GVHD. This was further investigated in an experiment in which we reconstituted lethally irradiated (C57BL x CBA)F1 mice with increasing numbers of allogeneic BALB/c cells. The results are presented in Figure 2B. After injection with only 3×10^6 allogeneic spleen cells, the numbers of $CD4^+$ and $CD8^+$ T cells did not increase at all. Only very mild and temporary symptoms of disease occurred in this group. However, after injection of 10^7 allogeneic cells, a strong increase in the numbers of $CD4^+$ and $CD8^+$ cells was found. The peak in the number of $CD8^+$ T cells occurred already at day 7. These changes were followed by symptoms

of severe GVHD from day 8. In the group reconstituted with 3 x 10^7 allogeneic cells there was even a more rapid increase in the numbers of $CD4^+$ and $CD8^+$ T cells, with a peak in the $CD8^+$ T cell number already at day 5. These changes were accompanied by symptoms of severe GVHD from day 6.

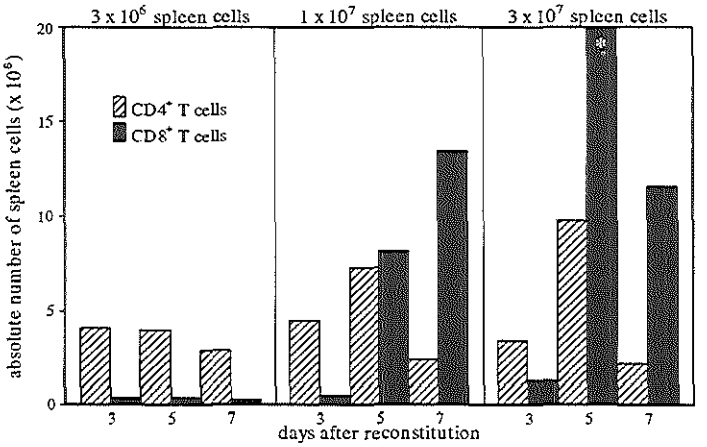

Figure 2B. Evaluation of T cell subsets after allogeneic reconstitution. (C57BL x CBA)F1 mice were lethally irradiated and reconstituted either with 3 x 10^6, 1 x 10^7 or 3 x 10^7 allogeneic spleen cells. At days 3, 5 and 7 two mice of each group were analyzed for the presence of CD4 and CD8 positive cells. (*Absolute value 27.1).

Comparison of the effects of anti-Thy-1, anti-CD4 and anti-CD8

The role of T cell subsets was further evaluated by *in vivo* administration of mAb directed to individual T cell subsets. (C57BL x CBA)F1 recipients were lethally irradiated, reconstituted with 10^7 BALB/c spleen cells and treated with 100 µg of either anti-Thy-1, anti-CD4, anti-CD8 or were untreated. As shown in Figure 3, an acute GVH reaction developed in the untreated group, which was 100% lethal within 8 days. Treatment with anti-Thy-1 resulted in 100% survival at 60 days. Anti-CD4 treatment also significantly improved the survival, although less than anti-Thy-1 treatment. The survival of the anti-CD8 treated group did not exceed that of the untreated group. To exclude that the difference in effectiveness of anti-CD4 and anti-CD8 was due to the dose of mAb used, these experiments were repeated with a double dose of 200 µg. Anti-Thy-1 treatment again resulted in 100% survival (Figure 4, *upper part*). The higher dose of anti-CD4 appeared to be as effective as the anti-Thy-1 treatment. The higher dose of anti-CD8, however, was still ineffective. A similar effect was seen on the body weight (Figure 4, *lower part*) and the clinical symptoms (Table 2).

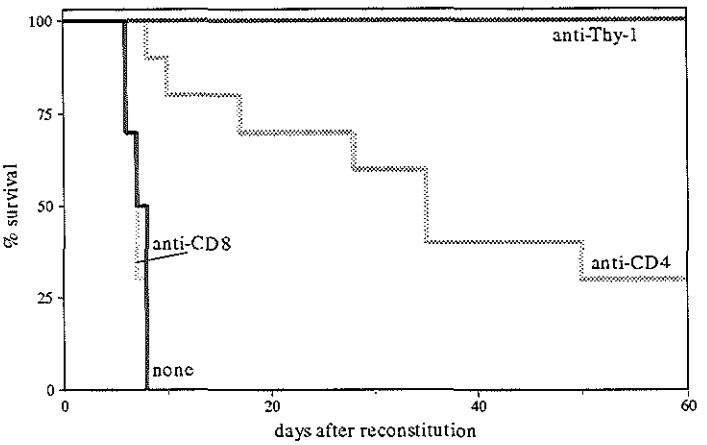

Figure 3. Comparison of the effect of anti-Thy-1, anti-CD4 and anti-CD8 mAb treatment on the survival. Lethally irradiated (C57BL x CBA)F1 recipients were reconstituted with 10^7 BALB/c spleen cells. After 24h the mice received a single i.p. injection of either 100 µg anti-Thy-1, 100 µg anti-CD4 or 100 µg anti-CD8. Each experimental group consisted of 10 mice.

Table 2. Evaluation of clinical symptoms of GVHD in recipients either treated with anti-Thy-1, anti-CD4, anti-CD8 or untreated.

Treatment	days after reconstitution							
	0	6	7	8	9	10	14	21
Anti-Thy-1 (100 µg/recipient)	-	-	-	-	-	-	-	-
Anti-CD4 (200 µg/recipient)	-	-	-	-	-	-	-	-
Anti-CD8 (200 µg/recipient)	-	-	+	++	+++	†		
None	-	-	+	++	+++	†		

(C57BL x CBA)F1 recipients were lethally irradiated, reconstituted with 10^7 BALB/c spleen cells and treated with the indicated amount of mAb. The mice were daily monitored for symptoms of GVHD. Each experimental group consisted of 10 mice.

\- absence of disease + first symptoms of disease ++ moderate disease +++ severe disease
† death

We further investigated whether the difference in anti-CD4 and anti-CD8 treatment was due to a different ability of the mAb to deplete their target cell population *in vivo*. Therefore, we analyzed spleen cells of 2 mice from each group for the presence of Thy-1, CD4 and CD8 positive cells 7 days after allogeneic reconstitution.

Table 3 shows that anti-Thy-1 treatment eliminated almost all T cells. Anti-CD4 treatment resulted in less effective elimination of CD4 positive T cells. Despite this, all mice treated with anti-CD4 survived. Interestingly, in this group there was also a strong decrease in the percentage of CD8 positive T cells. Anti-CD8 treatment eliminated the CD8 positive cells almost completely. Apparently the anti-Thy-1 and anti-CD8 mAb used were more effective in eliminating their target cell population *in vivo* than anti-CD4 mAb. Thus the differences observed between the anti-CD4 and anti-CD8 treated groups are not related to their depleting effect *in vivo*.

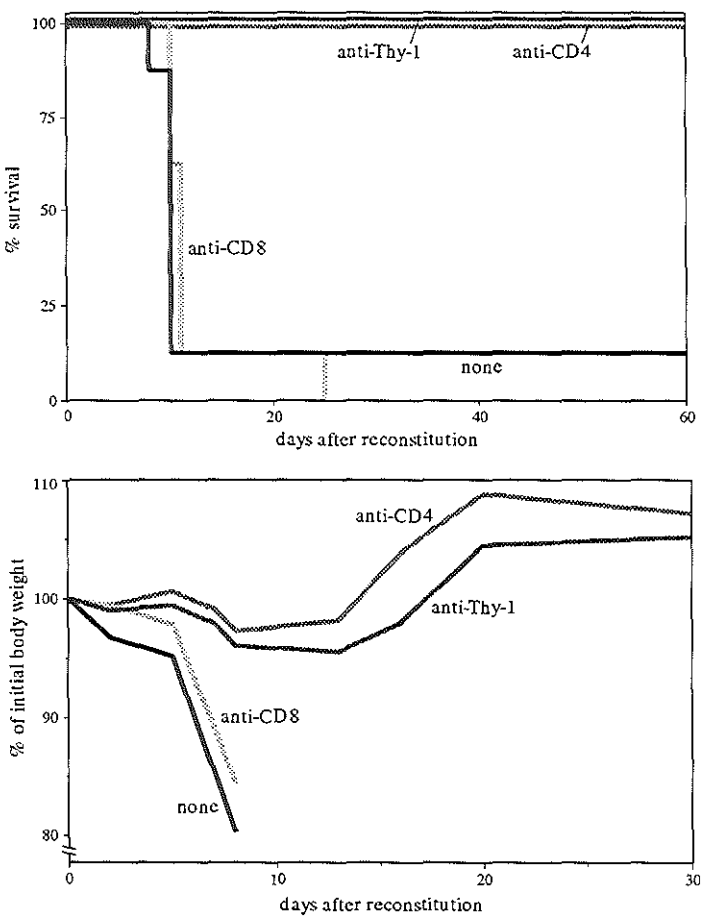

Figure 4. Dose response relationship of anti-Thy-1, anti-CD4 and anti-CD8 treatment. (C57BL x CBA)F1 recipient mice were lethally irradiated, reconstituted with 10^7 BALB/c spleen cells and treated with either 100 µg anti-Thy-1, 200 µg anti-CD4 or 200 µg anti-CD8. Each experimental group consisted of 8 mice. *Upper part.* The effect on survival. *Lower part.* The effect on body weight.

Table 3. FACS analysis of spleen cells after *in vivo* treatment with anti-CD4, anti-CD8 or anti-Thy-1

Treatment	% positive spleen cells at day 7		
	Thy-1	CD4	CD8
Anti-Thy-1 (100 μg/recipient)	3	3	<1
Anti-CD4 (200 μg/recipient)	24	8	10
Anti-CD8 (200 μg/recipient)	56	58	<1
None	80	15	65

(C57BL x CBA)F1 mice were lethally irradiated, treated with anti-Thy-1, anti-CD4 or anti-CD8 and reconstituted with 10^7 BALB/c spleen cells. The pooled spleen cells of two mice of each of the four groups were analyzed for the presence of Thy-1, CD4 or CD8 positive cells, 7 days after reconstitution.

State of chimerism and tolerance in long-term stable chimeras

All irradiated (C57BL x CBA)F1 mice reconstituted with BALB/c spleen cells that had become long-term survivors following anti-Thy-1 treatment were >99% repopulated with donor-type (BALB/c derived) cells, as determined at least 100 days after reconstitution. The percentages of T and B lymphocytes of the long-term chimeric mice were comparable to those of naive BALB/c mice (data not shown).

We evaluated the state of tolerance in long-term stable chimeras. Therefore we used 10^7 spleen cells from such long-term chimeric mice to reconstitute lethally irradiated BALB/c, BALB.K and (C57BL x CBA)F1 recipients, and evaluated their capacity to induce lethal GVHD in these secondary recipients. It appeared that the spleen cells from the chimeric mice induced an acute and lethal GVH reaction in lethally irradiated BALB.K and (C57BL x CBA)F1 recipients, but not in lethally irradiated BALB/c recipients (Figure 5). This confirms that the lymphoid cells of the chimeric mice were indeed of BALB/c origin. Furthermore, the data indicate that the chimeric cells have retained their capacity to recognize the recipient-type alloantigens and to mount a lethal GVH response, which excludes the possibility of clonal deletion.

To prove that the (C57BL x CBA)F1 chimeras do express their original H-2 antigens, we subjected them to a second lethal irradiation and reconstituted them with 10^7 BALB/c spleen cells. As a control, naive (C57BL x CBA)F1 mice were lethally irradiated and reconstituted. The results of this experiment are shown in Figure 6. Both groups of mice developed acute GVHD. There was no significant difference between the two groups. The mortality of the control group (naive recipients) in this experiment was less rapid than in the other experiments. This was probably due to the fact that the

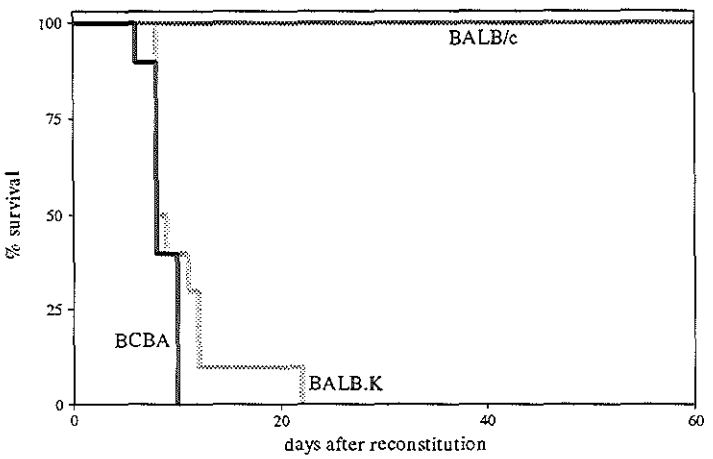

Figure 5. Evaluation of the state of tolerance in long-term chimeric mice. Spleen cells from long-term stable chimeras, obtained after reconstitution of lethally irradiated (C57BL x CBA)F1 recipients with 10^7 BALB/c spleen cells and treatment with 100 µg of anti-Thy-1, were evaluated for specific immune reactivity. Therefore, 126 days after irradiation and reconstitution 10^7 spleen cells of these chimeras were transferred to lethally irradiated BALB/c, BALB.K or (C57BL x CBA)F1 mice (n=10).

number of T cells in the spleens of BALB/c donor mice can vary to some extent (unpublished observations). Mortality in both groups of reconstituted mice was due to GVHD and not to the (re)irradiation procedure alone, since chimeric and naive radiation control mice died between days 9-11 and 8-14, respectively (data not shown).

We further investigated whether the tolerant state in established chimeras could be broken down. Therefore, we injected them either with large numbers i.e. 5×10^7 or 2×10^8 of naive BALB/c spleen cells, subjected them to sublethal (6 Gy) irradiation or treated them with 1 mg of anti-CD8 mAb, which eliminated all $CD8^+$ T cells *in vivo*. None of these mice developed symptoms of GVHD and we could not detect any significant decrease in body weight (Table 4).

Discussion

Until now mAb are not regularly included in prophylactic or therapeutic approaches after allogeneic BMT in humans to prevent or treat GVHD. This in spite of the promising results in clinical organ transplantation and also in experimental models for GVHD. We earlier reported the strong beneficial effect of even low doses of anti-T cell

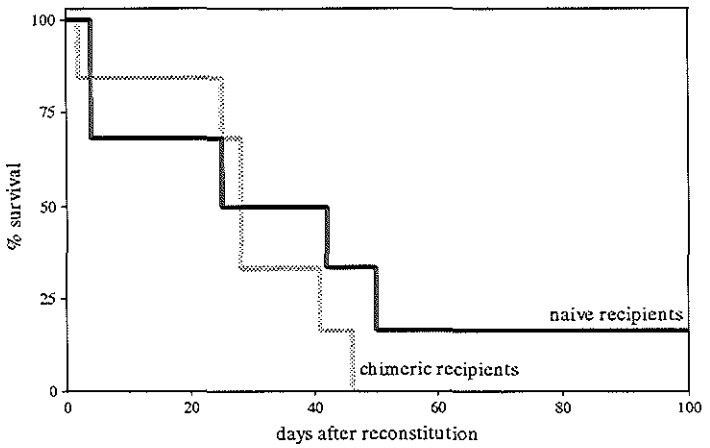

Figure 6. Effect of lethal irradiation and reconstitution on the survival of long-term stable chimeras. Chimeric BALB/c into (C57BL x CBA)F1 mice or naive (C57BL x CBA)F1 mice were lethally irradiated and 24 h later injected with 10^7 naive BALB/c spleen cells (n=6).

Table 4. Effect of injection of donor cells, sublethal irradiation and anti-CD8 treatment on the state of tolerance in long-term chimeras.

Treatment of chimeras	signs of GVHD		% of initial body weight		% survival	
	day 10	day 30	day 10	day 30	day 10	day 30
5×10^7 spleen cells	-	-	100	102	100	100
2×10^8 spleen cells	-	-	99	104	100	100
Sublethal irradiation (6 Gy)	-	-	100	110	100	100
1 mg of anti-CD8 mAb	-	-	103	110	100	100

Long-term stable chimeras were injected with either 5×10^7 (n=4) or 2×10^8 (n=4) BALB/c spleen cells, or were sublethally irradiated (n=5), or received 1 mg of anti-CD8 mAb (n=5).

mAb on acute GVHD in a murine model (11). It appeared that low-dose treatment could be postponed for some days with similar effectiveness, but that treatment postponed until day 6 did not lead to improved survival. In this paper we demonstrate that treatment given either one day before (day -1), two hours before (day 0) or the day after reconstitution (day 1) is equally effective in improving the survival. We also show that a higher dose of mAb can be given in four smaller doses with equal effectiveness, provided the interval between the doses is short. Animal studies (8-10) and preliminary

reports in human BMT demonstrate a beneficial effect of mAb treatment particularly when given as prophylaxis or as treatment of low-grade GVHD (17,18), but not in established GVHD (19). This might be due to the fact that T cells play a central role in the induction phase of GVHD, but that later in the course of GVHD other cell types become more and more involved (1). A high incidence of Epstein-Barr virus-associated lymphoproliferative disorders was reported as a severe side-effect in humans after delayed mAb treatment. This was not found when mAb were given as prophylactic treatment (18,20). In our mice no lymphoproliferative disorders were found even at 1 year after allogeneic reconstitution. Taken together, these data indicate that mAb treatment preferably should be given as prophylaxis, or early in the course of GVHD.

We further investigated the role of the CD4 and CD8 positive T cell subset in our model. Analysis of T cell subsets in the spleen early after reconstitution revealed striking results. After allogeneic reconstitution, there is a strong increase in the number of $CD4^+$ T cells which peaks at day 5. The number of $CD8^+$ T cells also increases strongly with a peak one day later. $CD8^+$ T cells than become the predominant T cell phenotype in the spleen. This in contrast to the situation after syngeneic reconstitution (Figure 2A). The increase in the number of T cells and the time of inversion of the CD4/CD8 ratio and of development of overt GVHD was dependent on the number of allogeneic cells used for reconstitution (Figure 2B). Apparently, when the number of T cells transplanted is sufficient, firstly a peak of $CD4^+$ T cells can be found, which is followed by a peak in the number of $CD8^+$ T cells, which precedes the development of overt GVHD. It is likely that in the latter two groups (reconstituted with 1×10^7 or 3×10^7) the CD4 peak occurred before day 5. These data suggest that in this strain combination, firstly $CD4^+$ T cells become activated, proliferate, and initiate the GVH reaction. They probably in turn activate $CD8^+$ T cells which become involved later in the course of GVHD. It is unlikely that these cells are NK cells, since mouse NK cells, in contrast to human NK cells, do not express CD8 (21). To our knowledge, there are no reports thus far describing the events in spleen and lymph nodes *early* after allogeneic reconstitution, which to our opinion might reflect the sequential activation of cell types involved in the induction of GVHD (2).

The available data in humans are scarce and concern the period later after reconstitution, since the peripheral blood white cell count is evaluable only at some weeks after BMT. Moreover the data are influenced by various therapeutic interventions. Another drawback is the variability between patients which might be caused a.o. by different antigenic disparities between donors and recipients (3). Atkinson et al. demonstrated in 3 patients, which were followed individually, an initial high CD4/CD8 ratio at day 14 after BMT, which reversed between days 42 and 70, after which it remained low. However, they were not able to detect significant differences between

patients after allogeneic or syngeneic BMT (22). Friedrich et al. also reported abnormalities in the T cell subsets after allogeneic BMT, a.o. a low CD4/CD8 ratio which was already present between days 10 - 30 after BMT, both in patients suffering from GVHD and patients free of symptoms of GVHD (23). This stresses the importance of short intervals between the samples, especially in the period early after BMT, and follow-up of individual patients.

Further evidence for a sequential involvement of $CD4^+$ and $CD8^+$ T cells was provided by the finding that anti-CD4 mAb treatment not only decreased the number of $CD4^+$ T cells, but also strongly reduced the number of $CD8^+$ T cells. Probably, in the absence of (a suitable number of) $CD4^+$ T cells, $CD8^+$ T cells are not activated and GVHD does not develop. Despite this, even in the absence of detectable $CD8^+$ T cells, as in the case of anti-CD8 treatment, acute GVHD can develop. This suggests that $CD8^+$ T cells are not an absolute requirement for the induction of lethal GVHD. This is in harmony with our earlier observation that purified $CD4^+$ T cells are able to induce a lethal GVH reaction in this strain combination, in contrast to purified $CD8^+$ T cells (10).

Although after anti-CD4 treatment still a substantial number of $CD4^+$ T cells were detectable, no symptoms of GVHD were found. Apparently, to prevent GVHD it is not necessary to deplete all ($CD4^+$) T cells, which is also suggested by our finding that after allogeneic reconstitution with low numbers of allogeneic spleen cells, no increase in the number of $CD4^+$ and $CD8^+$ T cells occurred and no GVHD developed (Figure 2B). That anti-CD8 mAb are even better than anti-CD4 mAb in depleting their targets *in vivo*, is in line with data reported by Cobbold et al., who demonstrated that anti-CD8 treatment resulted in 96% specific depletion, whereas anti-CD4 treatment resulted in 86% depletion (24). This might be related to the antigen density on the target cells (25). Using FACScan analysis we found that the number of binding sites on $CD8^+$ T cells was approximately 2 times higher than that on $CD4^+$ T cells (data not shown). Furthermore, the effect of anti-CD4 *in vivo* might not be based on depletion of target cells only. Anti-CD4 mAb are known to have tolerance facilitating properties (13,14), which can be demonstrated even in the absence of depletion (15). Recently Wood et al. reported that injection of non-depleting anti-CD4 mAb improved cardiac allograft survival in mice (26). The mechanism of induction of tolerance by anti-CD4 mAb is unknown. It was suggested that tolerance might be induced by confronting T cells with antigen and blocking at the same time their accessory molecules, thus preventing costimulatory signals (15). On the other hand, anti-CD4 mAb might deliver negative signals, resulting in the inactivation of $CD4^+$ T cells (27). Recently Wood et al. suggested that anti-CD4 treatment might cause a disturbance of the Th1/Th2 balance (26). The amount of anti-CD4 mAb, although not high enough to eliminate all $CD4^+$ T cells, might be sufficient to inhibit their GVHD inducing potential. Studies are in progress to determine whether

non-depleting mAb are also effective in this model.

We also investigated the state of tolerance in long-term stable chimeras to determine whether the observed tolerance was due to clonal deletion of alloreactive T cells or to another mechanism. We therefore used spleen cells from chimeric mice to reconstitute lethally irradiated recipients, syngeneic to the original donor or the host, as well as a third-party group. The chimeric spleen cells were able to induce a strong GVH reaction, comparable to that of naive spleen cells, directed to the original host and the third-party control, but not to the original donor. This excludes clonal deletion as the mechanism of tolerance in these chimeric mice. Tutschka et al. (28,29), reported the occurrence of non-specific suppressor cells in a rat model, as determined *in vitro*, which appeared 40 days after grafting and were replaced by specific suppressor cells by day 250. Upon transfer, only cells from chimeras at 250 days after grafting were able to inhibit the induction of GVHD by naive cells. Auchincloss et al. (30,31) were also able to demonstrate suppressor cells of CML and MLR reactivity in mouse bone marrow chimeras, but only up to 2 months after reconstitution, with maximum suppressive activity at 2 weeks. The fact that we used cells from chimeras at later time points after reconstitution, might explain the absence of a transferable suppressive effect in our model, as the suppressor cell population meanwhile might have been reduced and should first be expanded to be detectable (28). The observation of differences between *in vitro* and *in vivo* assays for suppression suggests the importance of an intact microenvironment for the maintenance of suppression in certain models.

We did not find differences in mice that became long-term stable chimeras either after anti-Thy-1 or anti-CD4 treatment. Cells from chimeras after anti-CD4 treatment were equally effective in inducing acute GVHD in secondary recipients syngeneic to the original hosts as cells from chimeras after anti-Thy-1 treatment. This suggests that the observed effect of anti-CD4 treatment is mostly based on its depleting effect and not on its tolerizing properties.

The absence of GVH reactivity in stable chimeras, despite the presence of potentially GVH reactive cells might be due to a decreased H-2 antigen expression in these long-term chimeras. This was further evaluated in an experiment whereby stable chimeras were subjected to a second lethal irradiation and subsequently injected with naive spleen cells, able to induce a lethal GVH reaction in the original (C57BL x CBA)F1 recipients. It appeared that acute GVHD developed in the chimeric mice, similarly as in the control group. This indicates that the absence of symptoms of GVHD in the chimeric mice was not likely due to altered expression of the original H-2 antigens. This was also suggested by Tutschka et al. (28). Tolerance was not broken by transfer of even a high dose of naive allogeneic spleen cells into the chimeras, which is in harmony with data reported by Tutschka et al. (28). However, they did break down

tolerance using sensitized spleen cells. Furthermore, elimination of $CD8^+$ T cells did not abrogate tolerance, which makes a veto cell mechanism unlikely (32). Our data suggest that in the chimeras a suppressive mechanism is operating that inhibits the development of lethal GVHD by potentially alloreactive T cells.

Acknowledgments

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Chapter 3.3

Prevention of graft-versus-host disease in mice by rat anti-CD3 and anti-CD4 monoclonal antibodies of the IgG2a and IgG2b isotypes*

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Summary

We and others have earlier demonstrated that lethal graft-versus-host disease (GVHD) in mice can be prevented by rat-anti-mouse T cell (subset) monoclonal antibodies (mAb) of the IgG2b isotype. Rat IgG2b mAb were reported to have a strong capacity to eliminate their target cells *in vivo*, in contrast to rat mAb of other isotypes.

The present study assesses the role of the specificity and the isotype of rat-anti-mouse T cell (subset) mAb in GVHD in more detail. We compared the effect of *in vivo* administration of anti-CD4 and anti-CD3 mAb of the IgG2a and IgG2b subclasses. It appeared that all mAb tested caused depletion, although the degree of depletion of the IgG2a anti-CD4 mAb was less than that of the other mAb employed. At a dose of 200 μg, both anti-CD4 mAb decreased body weight loss and improved the survival, the IgG2b mAb being more effective. For a similar effect a 5 times higher dose (1 mg) of the IgG2a anti-CD4 mAb was required. At this dose, complete and long-term survival was achieved. At a dose of 200 μg IgG2a and IgG2b anti-CD3 mAb were equally effective in decreasing GVH-related body weight loss and mortality. All mice that became long-term survivors were fully repopulated with donor-type cells. These data indicate that both IgG2a and IgG2b rat anti-CD4 and anti-CD3 mAb can give rise to long-term survival of irradiated and allogeneically reconstituted mice.

* *Submitted for publication*

Introduction

Until now GVHD is a major complication of allogeneic bone marrow transplantation (BMT). It has become clear that mature donor T lymphocytes transplanted together with the hemopoietic stem cells play a dominant role (1,2). Animal studies have shown that anti-T cell mAb treatment can be used for the prevention and treatment of GVHD. Both *in vitro* treatment of the graft before transplantation (3) and *in vivo* treatment of recipients before or after reconstitution (4-6) have been shown to be effective.

The effectiveness of a particular mAb *in vivo* appeared to be related to the isotype. Rat IgG2b mAb have been shown to be very effective in the prevention of GVHD in murine models, in contrast to rat IgG2a, IgG2c and IgM mAb. This was claimed to be due to their differential capacity to deplete their target cell population *in vivo* (3,4,7). In a recent study, however, Darby et al. showed that a rat IgG2a anti-CD4 mAb caused little or no depletion, but improved heart allograft survival in mice (8). This could be important in the clinical situation where it might be preferable to achieve immunosuppression without profound depletion as the latter might increase the susceptibility for infectious disease. Therefore, we investigated the effect of anti-CD4 and anti-CD3 mAb of the rat IgG2a and IgG2b isotypes on the development of GVHD. Furthermore, we evaluated the effect of *in vivo* administration of these mAb on the T cell subsets in the recipients' spleen.

Materials and methods

Mice

(C57BL/Ka x CBA/Rij)F1 (H-$2^{b/q}$) and BALB/c (H-2^{d}) mice were bred at the Department of Immunology of the Erasmus University. Recipient mice were age-matched and 12-20 weeks old at the start of the experiments. Donor mice were 10-16 weeks of age. During the experiments, mice were kept 2 per cage in light-cycled rooms and had access to acidified water and pelleted food *ad libitum*.

Preparation of cell suspensions

Mice were killed using carbon dioxide. Spleen cell suspensions were prepared in BSS. Nucleated cell concentrations were determined with a Coulter Counter model ZB1. Viability of the cell suspensions as determined by trypan blue exclusion was >90%.

Induction of GVH

GVH reactions were induced in lethally irradiated (10 Gy) mice by i.v. injection of 10^7 allogeneic spleen cells within 24 h after irradiation. Irradiation was performed in a Cesium-137 source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) with a dose rate of 1.15 Gy/min. Mice were examined daily for the development of signs of GVHD, such as ruffled fur, hunched posture, decreased physical activity, wasting, skin lesions and diarrhoea, and for mortality. The body weight was determined two or three times per week during the first 4 weeks. Thereafter the frequency was

decreased depending on the health status of the mice. Mice judged moribund were killed. Irradiated control mice reconstituted with syngeneic cells survived >250 days without signs of disease. Radiation controls died between days 10 and 22.

Antibodies and conjugates

For *in vivo* treatment and *in vitro* binding studies, rat-anti-mouse mAb were purified from ascitic fluid by protein G (Pierce Europe, Oud-Beijerland, The Netherlands) affinity chromatography. We used the IgG2b anti-CD4 (YTS 191.5) and anti-CD3 (17A2) mAb, and the IgG2a anti-CD4 (H129.19) and anti-CD3 (KT3) mAb (5.9-11). The amount of protein was determined by OD_{280} spectrofotometry and the purity was determined by SDS phast gel electrophoresis (PhastSystem, Pharmacia, Uppsala, Sweden). The mAb were administered by intraperitoneal (i.p.) injection 4 h after irradiation on the day before reconstitution. For double staining of cell suspensions, a phycoerythrin-labelled anti-Thy-1 mAb (30H12) and a FITC-labelled anti-CD4 mAb (RM-4-5) were purchased from Pharmingen, San Diego, CA. We further used FITC-labelled anti-CD8 (53-6.72) and anti-CD3 (KT3) mAb kindly provided by Prof. Dr. W. van Ewijk from our department.

Double immunofluorescence of spleen cell samples

From spleen cell suspensions (10^7 nucleated cells/ml), 25 µl was aliquotted in 96 well round bottom microtiter plates (Nunc, Roskilde, Denmark). Subsequently, 25 µl of the relevant FITC and phycoerythrin-labelled mAb was added to each well. The cells were incubated for 30 min at 4°C. After this incubation the cells were washed 3 times with PBS containing 5% FCS and 20 mM azide (PBS-FCS-azide), resuspended in 100 µl of isotonic fluid, and analyzed using a flowcytofluorometer (FACScan, Becton Dickinson, Mountain View, CA).

Comparison of binding by different mAb

For comparison of the binding of the respective mAb, cells were labelled with mAb for 30 min. After washing with PBS-FCS-azide the samples were incubated for 30 min with a polyclonal FITC-labelled rabbit-anti-rat-IgG $F(ab')_2$ antiserum (Organon-Teknika Cappel, Turnhout, Belgium). Fluorescence intensity was determined by FACScan analysis. Antibody binding was quantified by interpolation on a calibration scale, using quantitative micro beads standards (Becton Dickinson). Antibody binding is expressed as soluble FITC molecular equivalents (mol. eq. FITC).

Chimerism

To determine the degree of chimerism, peripheral blood cells were stained with FITC-conjugated mouse-anti-mouse H-$2K^d$ mAb (clone SF1-1.1) or mouse-anti-mouse H-$2K^b$ mAb (clone AF6-88.5), purchased from Pharmingen.

Data analysis

Differences between groups were analyzed using the Wilcoxon-Mann-Whitney statistic. Values of $p<0.05$ were considered significant.

Results

Effect of 200 µg of IgG2a and IgG2b anti-CD4 mAb on body weight and survival

We compared the capacity of the IgG2a and IgG2b anti-CD4 mAb to enhance the survival from potentially lethal GVHD. Therefore, (C57BL x CBA)F1 mice were

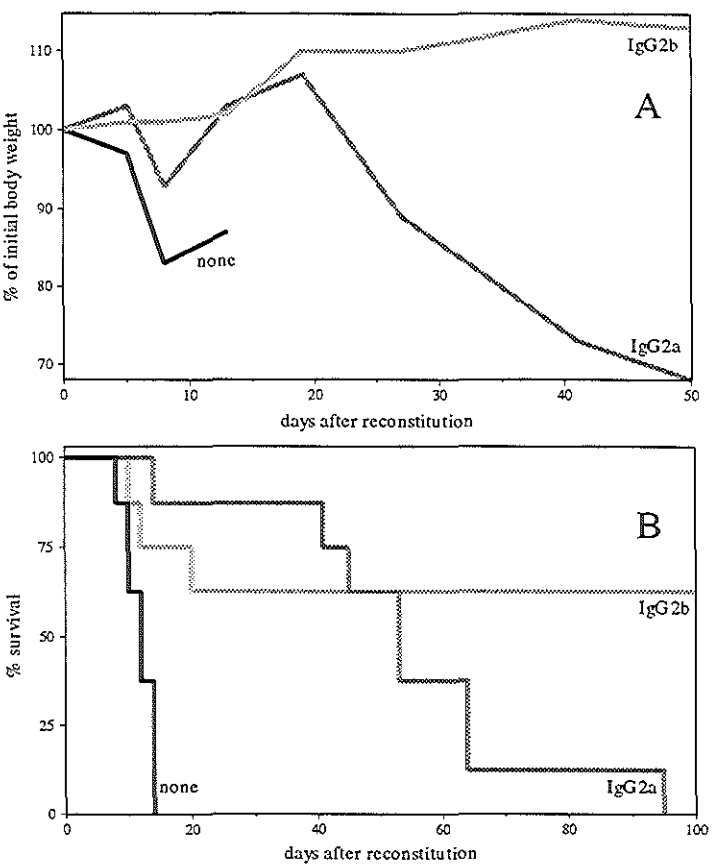

Figure 1. Effect of IgG2a and IgG2b anti-CD4 mAb on body weight (A) and survival (B). Groups of lethally irradiated (C57BL x CBA)F1 mice were reconstituted with 10^7 BALB/c spleen cells. The day before, a group was treated with 200 µg of IgG2a anti-CD4 mAb and another group with IgG2b anti-CD4 mAb (n=8).

lethally irradiated and reconstituted with 10^7 BALB/c spleen cells. The day before reconstitution all mice received 200 μg of mAb i.p., either of the IgG2a or the IgG2b isotype. It appeared that both the IgG2a and IgG2b anti-CD4 mAb strongly improved the survival, but the IgG2a mAb was less effective than the IgG2b mAb. The IgG2a anti-CD4 treated mice lost body weight at about day 20 (Figure 1A) and developed other clinical symptoms of GVHD (hunched posture, diarrhoea, wasting) at day 32, while after the initial loss of one animal on day 15, cumulative mortality started on day 40 (Figure 1B). Of the IgG2b treated group only 3 mice developed signs of GVHD, lost body weight (data not included in Figure 1A) and died (Figure 1B). The other mice of this group neither lost body weight nor developed other symptoms of GVHD.

Dose-response relationship of IgG2a anti-CD4 mAb treatment

Since IgG2a anti-CD4 mAb were less effective than IgG2b mAb, we studied whether this was dose-dependent. Therefore, groups of (C57BL x CBA)F1 mice were lethally irradiated and reconstituted with 10^7 BALB/c spleen cells. One day before reconstitution the recipient mice were injected with either 0.2, 0.5, 1 or 2 mg of IgG2a anti-CD4 mAb. From Figure 2 it can be concluded that at doses of 1 and 2 mg the mortality of GVHD was completely prevented. The effect lasted for >100 days.

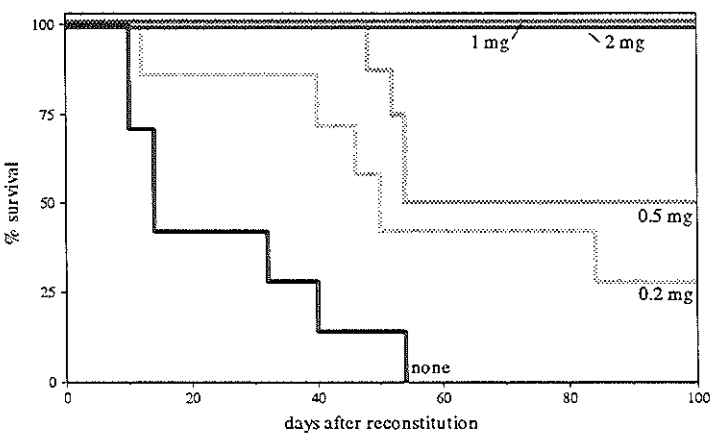

Figure 2. Dose-response relationship of IgG2a anti-CD4 mAb. (C57BL x CBA)F1 mice were lethally irradiated and reconstituted with 10^7 BALB/c spleen cells. Groups of mice were either untreated (n=7) or treated with a dose of either 0.2 (n=7), 0.5 (n=8), 1 (n=5) or 2 mg (n=5) of IgG2a anti-CD4 mAb.

Effect of 200 µg of IgG2a and IgG2b anti-CD3 mAb on body weight and survival

The effect of 200 µg of IgG2a and of IgG2b anti-CD3 mAb was studied in the same experimental set-up. It appeared that at the dose tested both anti-CD3 mAb strongly reduced the occurrence of signs of GVHD like body weight loss (Figure 3A) and mortality (Figure 3B). Differences between the two groups were not significant.

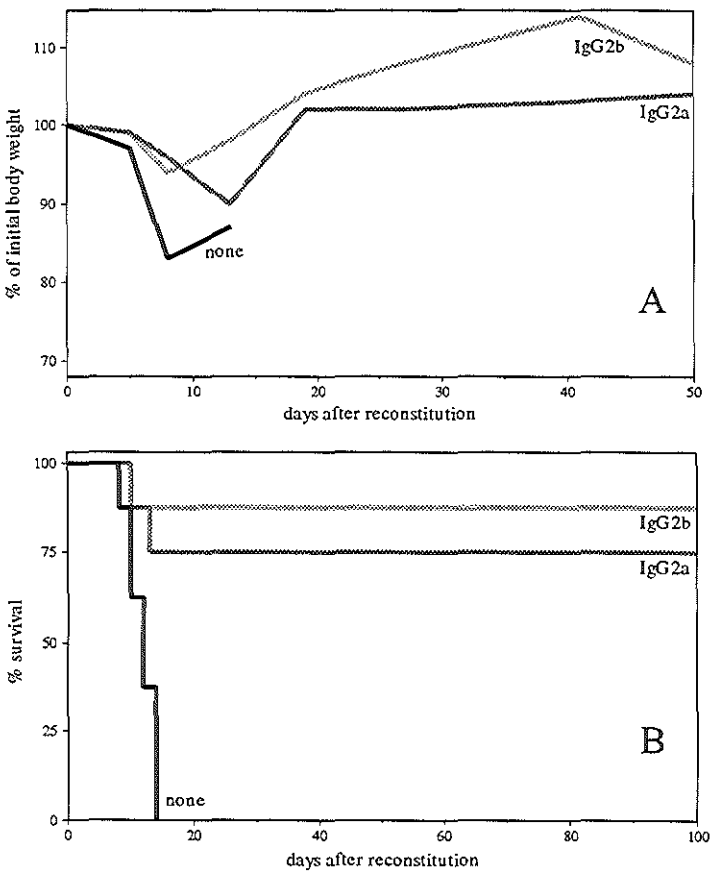

Figure 3. Effect of IgG2a and IgG2b anti-CD3 mAb on body weight (A) and survival (B). Lethally irradiated (C57BL x CBA)F1 mice were reconstituted with 10^7 BALB/c spleen cells. Groups of 8 mice were treated with 200 µg of IgG2a anti-CD3 mAb or 200 µg of IgG2b anti-CD3 mAb.

Effect of in vivo administration of IgG2a and IgG2b anti-CD4 and anti-CD3 mAb on T cell subsets in the spleen

We analyzed the effect of rat IgG2a and IgG2b anti-CD4 mAb on the various T cell subsets in the same experimental set-up as used for analyzing the effect on GVHD related morbidity and mortality. The day before reconstitution the recipients received an i.p. injection of either 200 μg IgG2a or 200 μg IgG2b anti-CD4 mAb. At day 4, 5 and 7 after reconstitution, three mice of each group were sacrificed and their spleen cells were analyzed using a double staining technique for Thy-1 and either CD4, CD8 or CD3. The results presented in Figure 4 show the absolute number of Thy-1$^+$, Thy-1$^+$CD4$^+$, Thy-1$^+$CD8$^+$ and Thy-1$^+$CD3$^+$ cells. The number of Thy-1$^+$CD4$^-$CD8$^-$ cells can be calculated by substracting the number of Thy-1$^+$CD4$^+$ and Thy-1$^+$CD8$^+$ from the total number of Thy-1$^+$ cells. It appeared that a dose of 200 μg of IgG2b anti-CD4 mAb strongly depleted the CD4$^+$ T cell subset, whereas a similar dose of IgG2a anti-CD4 mAb caused significantly less depletion, as indicated by the number of splenic Thy-1$^+$ cells. In both groups the reduction of the number of T cells not only concerned the CD4$^+$ T cell subset, but also the CD8$^+$ T cell subset.

In addition to the depletion of CD4$^+$T cells, the mAb caused modulation of the CD4 determinant. This is apparent from the fact that after anti-CD4 treatment a significant number of Thy-1$^+$ CD4$^-$CD8$^-$ cells was present (Figure 4A and 4B), which suggests the disappearance of CD4 from the membrane of a substantial number of T cells. Moreover, the expression of CD4 on the remaining CD4$^+$ T cells appeared to be weaker according to FACScan analysis (data not shown). By comparison of the IgG2a and IgG2b mAb it appeared that the IgG2b mAb caused considerably less modulation than the IgG2a mAb. At day 7 the CD4 molecule reappeared in the IgG2b anti-CD4 treated group (data not shown). In contrast to the IgG2a anti-CD4 mAb, the IgG2b anti-CD4 mAb caused also modulation of the CD3 antigen. Figure 4 also shows the depletion and modulation by anti-CD3 mAb. Both anti-CD3 mAb strongly depleted the CD3$^+$ T cell population. Moreover, both mAb caused considerable modulation. At day 7 the CD3 molecule started to reappear in both groups.

Degree of binding of anti-CD3 and anti-CD4 mAb to donor-type T cells

We determined the degree of binding of the various mAb used to donor-type cells. Therefore, different aliquots of BALB/c spleen cells were incubated with 10 μg of one of the four purified mAb employed in this study. Subsequently all samples were incubated with the same FITC-labelled rabbit-anti-rat IgG antiserum. The results

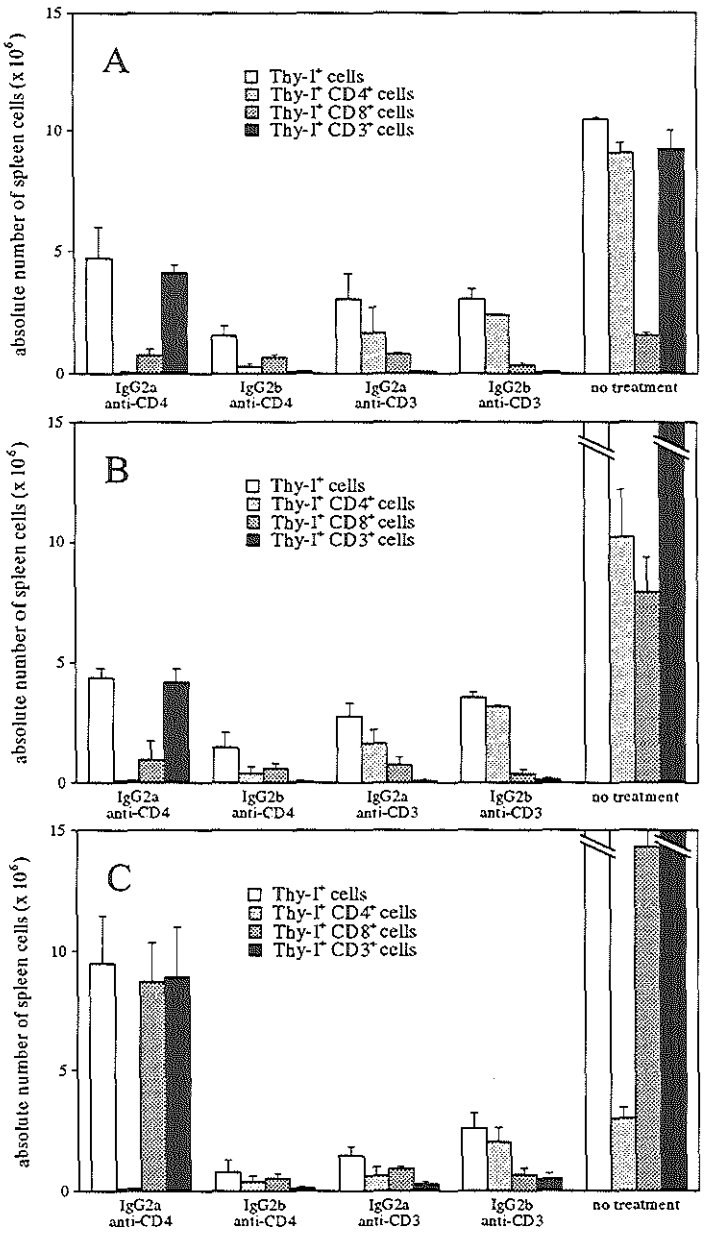

Figure 4. Effect of *in vivo* administration of anti-CD4 and anti-CD3 mAb on T cell subsets in the spleen. (C57BL x CBA)F1 mice were lethally irradiated and reconstituted with 10^7 BALB/c spleen cells. The day before reconstitution they were i.p. injected with either 200 µg of IgG2a or 200 µg of IgG2b anti-CD4 or anti-CD3 mAb. At days 4 (A), 5 (B) and 7 (C) after reconstitution the spleens of three mice per group were analyzed for the absolute number of single positive Thy-1$^+$ cells and of double positive Thy-1$^+$CD4$^+$, Thy-1$^+$CD8$^+$ and Thy-1$^+$CD3$^+$ cells.

presented in Table 1 show that the degree of binding of IgG2a anti-CD4 mAb was two times higher than that of the IgG2b anti-CD4 mAb. By comparison of the binding of the IgG2a and IgG2b anti-CD3 mAb it was found that the binding of IgG2a anti-CD3 mAb was four times higher than that of the IgG2b mAb (Table 1).

Table 1. Fluorescence intensity of binding of IgG2a and IgG2b anti-CD4 and anti-CD3 mAb.

	CD4		CD3	
mAb (isotype)	H129.19 (IgG2a)	YTS191 (IgG2b)	KT3 (IgG2a)	17A2 (IgG2b)
Mol equivalent FITC	1.6×10^5	0.9×10^5	4.5×10^4	1.1×10^4

Pooled BALB/c donor spleen cells from two mice were incubated with 10 μg/ml of the indicated mAb for 30 min and subsequently with a rabbit-anti-rat-FITC conjugate. The figures represent the mol equivalent FITC bound after incubation with the various mAb.

Chimerism

Long-term surviving mice after successful anti-CD4 and anti-CD3 mAb treatment were evaluated for the degree of repopulation with donor- and/or recipient-type cells. Table 2 shows that long-term survivors of either treatment group were completely repopulated with donor-type cells.

Table 2. Evaluation of the degree of chimerism of long-term stable chimeras after IgG2a or IgG2b anti-CD4 or anti-CD3 mAb treatment.

marker	Treatment			
	H129.19	YTS 191	KT3	17A2
H-2K^b	<1*	<1	1 ± 0.5	<1
H-2K^d	99 ± 0.5	99 ± 0.1	98 ± 0.6	99 ± 0.4

Long-term stable chimeras, at least 200 days after reconstitution, were evaluated for the percentage of H-2K^b (recipient-type) and H-2K^d (donor-type) positive cells (n=3).

* % positive cells

Discussion

MAb are becoming increasingly important as therapeutic agents. The murine-anti-human CD3 mAb OKT3 has been most widely used in clinical organ transplantation. OKT3 treatment appeared to be able to improve cardiac and renal allograft survival (12,13). Preliminary data suggest that OKT3 might be of value in human BMT as well (14,15). However, the use of OKT3 especially at first dose is accompanied by serious side-effects, due to T cell activation. This results in the release of cytokines such as IL-2, IL-6, IFN-γ and TNF-α, which can cause considerable morbidity (16,17). OKT3 administration after BMT appeared to be associated with an increased incidence of Epstein-Barr virus (EBV)-induced lymphoproliferative disorders (15,18), which might be a.o. a consequence of the T cell activation and cytokine release. Another drawback is the fact that OKT3 elicits a strong humoral response, which occasionally hampers further use (19). In the case of BMT this might be less a problem, because of the profound immunodeficiency after BMT. The induction of T cell activation and of a humoral response might be related to the isotype of the mAb, since Hirsch et al. found in a murine model that both of these complications could be prevented using $F(ab')_2$ fragments (20).

We investigated the effect of a.o. two rat-anti-mouse CD3 mAb, one of the IgG2a and the other of the IgG2b subclass, in a murine model for GVHD. At a dose of 200 µg, both of these mAb were similarly effective and reduced the morbidity and mortality of GVHD greatly. Moreover, both mAb caused strong depletion of T cells. This indicates that, in contrast to reports from Thierfelder et al., also rat IgG2a mAb can eliminate their target cells *in vivo*. The data presented in Figure 4 suggest that both mAb cause modulation of the CD3 molecule. This has already been described for other anti-CD3 mAb (21,22). Anti-CD3 treatment in our model was not associated with side-effects. This might be due to the fact that we used rat mAb in contrast to Hirsch et al., who used a hamster mAb (22). Preliminary data indicate that this hamster mAb 145-2C11 causes substantial cytokine release and, probably as a consequence, morbidity in our model. Our data stimulate studies to search for anti-CD3 mAb for human use that do not lead to T cell activation. This might also decrease the risk of EBV-induced lymphoproliferative disorders.

Previous studies from our laboratory suggest that mAb to other T cell antigens, such as CD4 and Thy-1, might be used for treatment of GVHD as well (6,23). In the present study we compared a rat IgG2a and IgG2b anti-CD4 mAb. Both anti-CD4 mAb were able to decrease the morbidity and mortality of GVHD. IgG2a mAb were approximately 5 times less effective, which is in harmony with data from others (8). Both mAb caused depletion, although the IgG2a mAb was less effective in this respect.

On the other hand, the IgG2a mAb appeared to induce stronger modulation of the CD4 antigen than the IgG2b mAb (Figure 4 and data not shown).

These data indicate that not only anti-CD3 but also anti-CD4 mAb should be considered for use in humans. Animal studies have already shown the effectiveness of anti-CD4 mAb *in vivo* (24). Preliminary data suggest that anti-CD4 mAb treatment increases renal allograft survival also in humans (25). Furthermore, anti-CD4 treatment thus far was not associated with morbidity (24,25).

Taken together these data indicate that anti-CD3 mAb treatment is not necessarily associated with severe morbidity. Moreover, our data show that anti-CD4 treatment might be a good alternative for anti-CD3 treatment for prevention and treatment of GVHD. It can be speculated that anti-CD4 treatment may be less effective in donor-recipient combinations in which $CD4^+$ T cells play a less dominant role. This requires further studies.

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Chapter 4

Role of cytokines in graft-versus-host disease

Chapter 4.1

Cytokines in lethal graft-versus-host disease

Analysis of serum levels after allogeneic and syngeneic reconstitution*

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Introduction

Graft-versus-host disease (GVHD) is caused by donor T lymphocytes that recognize foreign antigens on host tissues. This leads to T cell activation, which involves a cascade of events including the transcription of genes for cytokines and their receptors and the production of cytokines (1,2). One of the first cytokines to appear is IL-2. IL-2 production enhances the IL-2 receptor expression and leads to T cell proliferation. As a further step, differentiation of T cells occurs, which results in the production of a certain pattern of cytokines. These cytokines influence the expression of cell surface antigens and adhesion molecules, and are able to activate other cell types such as cytotoxic T cells, macrophages and natural killer cells, which might act as effector cells in tissue destruction (2). Insight into the sequential expression of the various cytokines involved might enable more effective treatment of GVHD. Therefore, we investigated the occurrence of cytokines in a murine model for acute GVHD. We addressed in particular the period early after allogeneic reconstitution.

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Materials and methods

Mice

(C57BL/Ka x CBA/Rij)F1 (H-$2^{b/q}$) and BALB/c (H-2^{d}) mice were bred at the Department of Immunology of the Erasmus University. The mice were 12-18 weeks old at the start of the experiments. Mice were kept 2 per cage in light-cycled rooms with access to acidified water and pelleted food *ad libitum*.

Determination of cytokines

Serum samples and supernatants from spleen cells cultured for 24 h with Con A (1 μg/ml) (3) were assayed for cytokine activity. IL-2 was determined by a proliferative assay using an IL-2 dependent CTLL cell line, *in vitro* maintained in medium supplemented with rhIL-2. We used rhIL-2 as a standard (gift from Sandoz Forschungsinstitut, Wien, Austria). For the detection of IL-6, the B9 cell line was used (4). We used rmIL-6 as a standard (British Biotechnology, Abingdon, UK). TNF-α levels were determined by a cytotoxicity assay on WEHI164 cells (5). We used rh-TNF-α as a standard (gift from BASF/Knoll, Ludwigshafen, Germany). The proliferative or cytotoxic activity was measured with the MTT assay (6). IFN-γ was determined in a sandwich ELISA, using a rat-anti-mouse IFN-γ monoclonal antibody (XMG1.2) as a catching antibody (Ab) and a polyclonal rabbit-anti-mouse IFN-γ Ab as a second step. We used rmIFN-γ purified from CHO cells transfected with the mIFN-γ gene as a standard (kind gift from Dr. R.L. Coffman, DNAX Research Institute, Palo Alto, CA).

Other procedures

Preparation of cell suspensions, induction of GVHD, collection of serum samples and data analysis were performed as described by us in earlier studies (7,8).

Results and discussion

Serum samples obtained from lethally irradiated (C57BL x CBA)F1 mice in which a lethal graft-versus-host reaction was induced by injection of 10^7 allogeneic BALB/c spleen cells, were analyzed for cytokine activity. As a control, serum samples from similarly treated mice injected with 10^7 syngeneic spleen cells were used. The results are summarized in Table 1. Since mortality occurred at day 8, from that day on the number of mice in each group became too small for statistical analysis. Therefore, no data are presented after day 8. It appeared that IL-6 levels increased at day 4 in the allogeneically reconstituted mice, in contrast to syngeneically reconstituted mice in

which no rise was found. Serum IFN-γ levels increased strongly in the allogeneically reconstituted mice between day 4 and 5, reaching a peak level on day 6. No rise was seen in the syngeneically reconstituted mice. We further determined the TNF-α activity in sera of both groups of mice. Significantly increased TNF-α levels were found at day 7 after allogeneic reconstitution. Serum IL-2 levels were below the detection limit (0.1 U/ml).

Since the detection of cytokines in the serum might be hampered by inhibitory or binding factors, e.g. soluble receptors, we further analyzed tissue culture supernatants of spleen cells 24 h after culture in the presence of Con A. A significantly higher activity of IL-6, IFN-γ and TNF-α was found in spleen cell supernatants from allogeneically reconstituted mice as compared to syngeneically reconstituted mice, showing a similar

Table 1. Symptoms of GVHD in relation to serum cytokine levels after allogeneic and syngeneic reconstitution of lethally irradiated mice.

	days after reconstitution					
	3	4	5	6	7	8
Symptoms after allogeneic reconstitution	-	-	-	+	++	+++
IL-2	-	-	-	-	-	-
IL-6	-	+	+	++	++	+++
IFN-γ	-	-	++	+++	++	++
TNF-α	-	n.d.	-	n.d.	+++	n.d.
Symptoms after syngeneic reconstitution	-	-	-	-	-	-
IL-2	-	-	-	-	-	-
IL-6	-	-	-	-	-	-
IFN-γ	-	-	-	-	-	-
TNF-α	-	n.d.	-	n.d.	+	n.d.

(C57BL x CBA)F1 mice were lethally irradiated and reconstituted either with 10^7 allogeneic BALB/c spleen cells or with 10^7 syngeneic spleen cells. At various days after reconstitution, serum samples were obtained from mice of each group and analyzed for cytokine activity. Furthermore clinical symptoms were evaluated in both groups of mice.

- not detectable + low/light ++ moderate +++ high/severe n.d. not determined

time-dependence as the serum levels. IL-2 levels were below the detection limit.

Evaluation of clinical symptoms in both groups revealed that in the allogeneically reconstituted mice symptoms of acute GVHD were present at about day 6. Mortality in these mice occurred between 8 and 24 days after reconstitution, with a mean survival time of 12.5 ± 4.9 days. In the syngeneically reconstituted mice no signs of disease were found (Table 1).

Taken together, the above data indicate that a rise in IFN-γ and IL-6 levels, detectable both in serum and culture supernatants preceded the clinical symptoms and mortality in acute GVHD. The clinical symptoms of GVHD seemed to be most closely associated with a rise in serum TNF-α activity. It is likely that the expression of these cytokines was preceded by cytokines that appeared earlier, especially IL-2. Although we could not detect IL-2 activity, possibly due to the fact that IL-2 is rapidly consumed, an important role for IL-2 was suggested by preliminary data showing decreased morbidity and mortality in acute GVHD after the *in vivo* administration of anti-IL-2 or anti-IL-2 receptor mAb. This stresses the importance of the use of complementary assay systems to determine the role of cytokines. Studies are underway to determine whether other cytokines also play a role in this complex disease.

Acknowledgements

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Chapter 4.2

Cytokines in acute graft-versus-host disease in mice

Analysis of cytokine levels and the effect of anti-cytokine and anti-cytokine-receptor monoclonal antibody treatment*

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Summary

Evidence is increasing that cytokines play a central role in graft-versus-host (GVH) reactions. We investigated in a murine model for acute lethal graft-versus-host disease (GVHD) the role of a number of cytokines that have been suggested to play a role in transplant rejection or GVHD. In this study we focussed on the role of IL-1, IL-2, IL-4, IL-6, IFN-γ and TNF-α.

Lethally irradiated (C57BL x CBA)F1 mice were reconstituted with either 10^7 allogeneic BALB/c spleen cells or a similar number of syngeneic spleen cells, as a control. Clinical symptoms of GVHD were found in the allogeneically reconstituted group at day 6, whereas mortality occurred at day 8. The mean survival time of allogeneically reconstituted mice was 12.5 days. Syngeneically reconstituted mice survived at least 250 days without signs of disease. At various days after reconstitution serum samples were analyzed for the presence of cytokines. We found a rise of serum IL-6 levels in allogeneically reconstituted mice at day 4, with a peak on day 7, in contrast to the syngeneic control group in which no rise was seen at all. IFN-γ levels increased strongly at day 5. Serum TNF-α levels were significantly increased in the allogeneically reconstituted mice on day 7. Serum IL-2 and IL-4 levels were below the detection limit. Elevated IL-6, IFN-γ and TNF-α levels were also found in the supernatant of Con A stimulated spleen cells from allogeneically reconstituted mice. The expression of mRNA for cytokines was also studied in spleen cells. Differences

* *Submitted for publication.*

between the two groups were found for the levels of IL-1α, IL-2, IL-6, IFN-γ and TNF-α as detected by reverse transcription polymerase chain reaction.

Administration of recombinant cytokines to allogeneically reconstituted mice could affect the development of GVHD. It appeared that administration of IL-2 strongly increased the morbidity and mortality. IL-1α and TNF-α administration, on the other hand, hardly influenced the survival. Administration of antibodies against IL-2 or the IL-2 receptor decreased the morbidity and mortality. Anti-IL-6, anti-IFN-γ and anti-TNF-α mAb, on the other hand, did not affect GVHD.

Introduction

Graft-versus-host disease (GVHD) is the result of activation of transplanted donor T cells by alloantigens on host tissues (1). The recognition of these antigens by T lymphocytes initiates a cascade of events which leads to the production of cytokines and the expression of their receptors. One of the first cytokines produced by activated T cells is IL-2. This cytokine enhances the expression of the IL-2 receptor and induces proliferation and activation (2). Activated T lymphocytes produce a variety of cytokines (3). This production of cytokines leads to enhancement or decrease of the expression of particular cell surface molecules, including homing receptors and adhesion molecules and the recruitment and activation of other cell types such as B lymphocytes, macrophages and natural killer cells (1,2). These cell types might also be involved in the process that leads to clinically overt GVHD and tissue destruction.

The number of cytokines that are reported to play a role in experimental as well as clinical GVHD steadily increases (4-8). The data from the literature, however, are not unequivocal. Moreover, many studies deal with only one particular cytokine. To improve current diagnostic and therapeutic approaches more insight is necessary in the sequential involvement of the cells that play a role in GVHD, the cytokines they produce, their interactions and their regulation. Therefore, we extended our investigations on the cellular aspects of murine GVHD (9-13) with studies on the role of cytokines using a model in which $CD4^+$ T cells play a predominant role (13,14). We analyzed serum samples and supernatants of spleen cell cultures for the presence of various cytokines and spleen cell suspensions for the expression of mRNA for cytokines. The role of these cytokines in morbidity and mortality of GVHD was further assessed by treating allogeneically reconstituted mice with recombinant cytokines and monoclonal antibodies to cytokines and cytokine receptors.

Materials and methods

Mice

(C57BL/Ka x CBA/Rij)F1 (H-$2^{b/q}$) and BALB/c (H-2^{d}) mice were bred at the Department of Immunology of the Erasmus University. Recipient mice were age-matched and 12-18 weeks old when entered in the experiments. During the experiments, mice were kept 2 per cage in light-cycled rooms and had access to acidified water and pelleted food *ad libitum*.

Preparation of cell suspensions and collection of serum samples

Mice were sacrificed using carbon dioxide. Spleen cell suspensions were prepared in BSS. Nucleated cell concentrations were determined with a Coulter Counter model ZB1. Viability of the cell suspensions as determined by the trypan blue exclusion method was >90%. Blood collected by cardiac puncture was allowed to clot overnight at 4°C and centrifuged for 5 min in an Eppendorf centrifuge (International Equipment Company, Needham, MA). Serum samples were aliquotted and stored at -70°C. For each cytokine all samples were assayed simultaneously.

Tissue culture

For the determination of cytokines in spleen cell supernatants, spleen cells were cultured in RPMI 1640 tissue culture medium, supplemented with 10% FCS, glutamin and antibiotics according to Cleveland *et al.* (15). A number of 5 x 10^5 cells/well was seeded in five-fold in 96 well flat-bottom microtiter plates (Falcon, Becton Dickinson, Lincoln Park, NJ) and cultured for 24 h at 37°C and 5% CO_2 in the presence or absence of Con A (1 μg/ml). The samples were centrifuged and the supernatants were collected and stored in aliquots at -70°C.

Recombinant cytokines

Recombinant cytokines were administered *in vivo* by s.c. injection in the inguinal region, in a volume of 0.25 - 0.5 ml BSS. Recombinant human (rh) IL-2 was kindly provided by the Sandoz Forschungsinstitut, Wien, Austria. The rhIL-1α used was a gift from Hoffmann-La Roche, Mijdrecht, The Netherlands. Recombinant murine (rm) IFN-γ and rmIL-4 were a kind gift from Dr. R.L. Coffman (DNAX Research Institute, Palo Alto, CA). The rmIL-6 was purchased from British Biotechnology, Abingdon, UK. Human rTNF-α was a gift from BASF/Knoll, Ludwigshafen, Germany.

Determination of cytokines

IL-2, IL-6 and TNF-α were determined in a bioassay using CTLL-2, B9 and WEHI163 (clone13) indicator cells, respectively, as described previously (16-18). The MTT assay was used to quantitate

the cytokine activity. IL-4 and IFN-γ were determined in ELISA as described (19,20).

MTT assay

We used the procedure as earlier described (21) with minor modifications. Briefly, 30 µl MTT-solution (5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, St. Louis, MO) in PBS) was added to each well. Plates were incubated for 3.5 h at 37°C with 5% CO_2. The supernatant was discarded and 100 µl DMSO (Merck, Darmstadt, Germany) was added to each well. The plates were monitored in a Titertek Multiskan MCC 96-well ELISA plate reader (Flow Laboratories, Ayrshire, Scotland) at a wavelength of 510 nm. The experimental values were corrected for background values.

Detection of cytokine mRNA

RNA was isolated by gradient centrifugation on 5.7 M cesiumchloride for 18 h in an ultracentrifuge (Sorvall, Du Pont, Newtown, CT) as described (22). After ethanol precipitation 1 µg RNA was treated with DNase 1 and used in a reverse transcriptase reaction as described (23). For amplification 35 cycles (1 min at 94°C for denaturation, 2 min at 55°C for annealing and 3 min at 72°C for primer extension) were performed with 5% of cDNA mixture, using a DNA thermal cycler (Perkin-Elmer, Gouda, The Netherlands). For all samples sense and anti-sense primersets were used as described (24).

Antibodies and LPS

For *in vivo* treatment, anti-IL-2 (S4B6.1), anti-IL-6 (MP 20F3), anti-IFN-γ (XMG 1.2) and anti-TNF-α (XT22) producing hybridomas were used. These were a kind gift from Dr. R.L. Coffman. An anti-IL-2R (PC61) producing hybridoma was obtained from the American Type Culture Collection. The mAb were purified from ascites by protein G (Pierce Europe, Oud-Beijerland, The Netherlands) affinity chromatography and subsequently administered by i.p. injection.

The Ab used in the ELISA, anti-IL-4 (11B11 and BVD-24 G2.3), anti-IFN-γ (XMG 1.2) and polyclonal anti-IFN-γ Ab were kindly provided by Dr. R. L. Coffman and purified from culture supernatant by protein G affinity chromatography.

LPS-B (*Salmonella typhosa* 0901) was purchased from Difco Laboratories, Detroit, MI. Mice received a dose of 100 µg i.p. dissolved in BSS.

Other procedures and statistical analysis

GVHD was induced as earlier described by us (13). Differences between groups were analyzed using the Student's *t* test or the Wilcoxon-Mann-Whitney statistic. Values of $p<0.05$ were considered as significant.

Results

Serum cytokine levels after allogeneic and syngeneic reconstitution

A group of (C57BL x CBA)F1 mice was lethally irradiated and reconstituted with 10^7 allogeneic BALB/c spleen cells. As a control, a group of lethally irradiated (C57BL x CBA)F1 mice was reconstituted with 10^7 syngeneic spleen cells. Both groups were examined for the development of signs of GVHD and mortality. Signs of disease were detectable in the allogeneically reconstituted mice from day 6 onwards, whereas mortality occurred from day 8 onwards. The mean survival time was 12.5 ± 4.9 days. The syngeneically reconstituted mice survived >250 days without any symptom of disease. From both groups 5 mice were sacrificed at 1, 2, 3, 4, 5, 6, 7, 8 or 10 days after reconstitution. Serum samples from these mice were analyzed for the presence of IL-2, IL-4, IL-6, IFN-γ and TNF-α. Figure 1A shows the IL-6 levels in serum, which increased at day 4 after allogeneic reconstitution. In syngeneically reconstituted mice no rise in IL-6 levels was seen. Serum IFN-γ levels in the allogeneically reconstituted mice increased at day 4 with a peak on day 5 (Figure 1B). In the syngeneically reconstituted mice no rise of IFN-γ was seen. TNF-α levels were significantly different between the two groups on day 7 (Figure 1C). IL-2 and IL-4 levels were below the detection limit: <0.1 U/ml (data not shown).

Cytokines in spleen cell supernatants

(C57BL x CBA)F1 mice were lethally irradiated and reconstituted with either 10^7 BALB/c or syngeneic spleen cells. On days 3, 5 and 7 after reconstitution spleen cells from 3 mice of each group were cultured in the presence of Con A. After 24h culture, levels of IL-2, IL-4, IL-6, IFN-γ and TNF-α were determined in the supernatants. Figure 2 (A-C) shows that IL-6, IFN-γ and TNF-α levels in the allogeneically reconstituted mice were increased as compared to syngeneically reconstituted mice. Similar but less pronounced results were obtained after *in vitro* culture of spleen cells in the presence of IL-2 or anti-CD3 mAb (data not shown).

Detection of cytokine mRNA

From both groups of mice on days 1, 2, 4, 5 and 7 after reconstitution spleen cell suspensions were analyzed for the presence of cytokine mRNA. As shown in Table 1, in the allogeneically reconstituted mice mRNA levels of IL-1α, IL-6 and TNF-α were

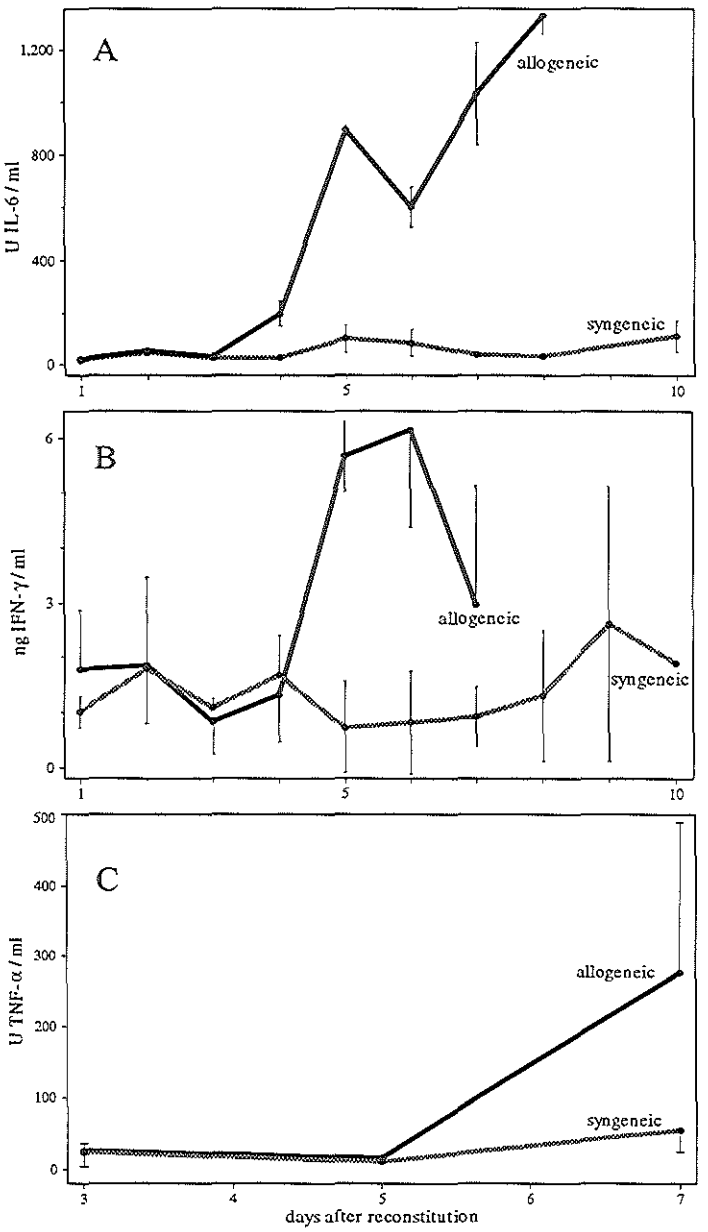

Figure 1. Serum IL-6, IFN-γ and TNF-α levels in acute GVHD. Lethally irradiated (C57BL x CBA)F1 mice were reconstituted with either 10^7 allogeneic BALB/c spleen cells or 10^7 syngeneic spleen cells. From each of these two groups the serum level of IL-6 (A) and IFN-γ (B) was examined from day 1 until day 10 after reconstitution. TNF-α serum levels (C) were determined on days 3, 5 and 7. Each point represents the arithmetic mean ± 1 SEM (n=5).

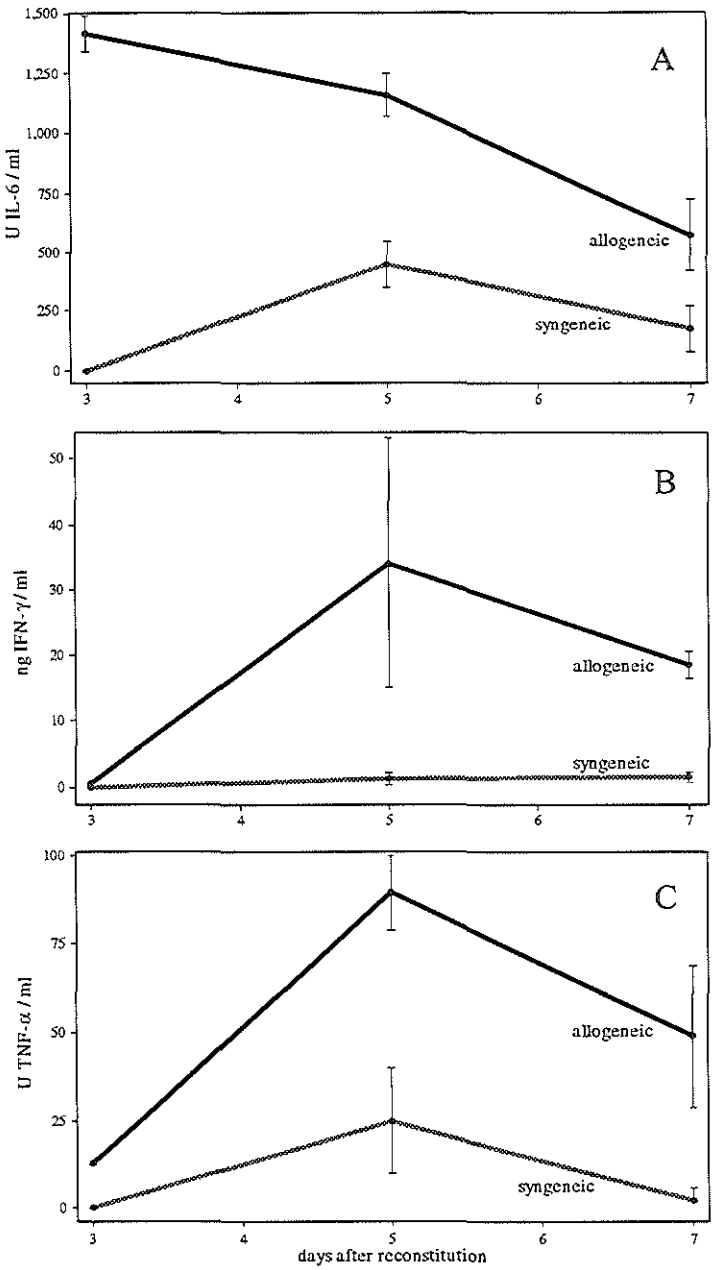

Figure 2. IL-6 (A), IFN-γ (B) and TNF-α (C) levels in the supernatant of 24 h spleen cell cultures. From allogeneically and syngeneically reconstituted mice spleen cells were cultured for 24 h in the presence of Con A. Each point represents the arithmetic mean of the cytokine level ± 1 SEM (n=3).

enhanced at day 1, while mRNA levels of IL-2 and IFN-γ were enhanced at days 4 and 5, respectively, as compared to the syngeneically reconstituted group. On day 7 the mRNA levels were more or less similar in both groups.

Table 1. mRNA expression for IL-1α, IL-2, IL-6, IFN-γ and TNF-α during GVHD.

Cytokine	days after reconstitution									
	1		2		4		5		7	
	allo	syn	allo	syn	allo	syn	allo	syn	allo	syn
IL-1α	+	-	+	-	+	-	+	-	±	+
IL-2	-	-	-	-	+	-	+	-	+	+
IL-6	±	-	±	-	+	-	+	-	-	±
IFN-γ	-	-	-	-	-	-	+	-	±	±
TNF-α	±	-	+	-	+	-	+	-	±	+

(C57BL x CBA)F1 mice were lethally irradiated and reconstituted with 10^7 allogeneic (allo) BALB/c or syngeneic (syn) spleen cells. At days 1, 2, 4, 5 and 7 after reconstitution, pooled spleen cells of both groups were analyzed for the expression of mRNA for IL-1α, IL-2, IL-6, IFN-γ and TNF-α (n=5).

- negative ± weakly positive + positive

Effect of administration of recombinant cytokines

We investigated the effect of administration of rhIL-2 to (C57BL x CBA)F1 mice that were lethally irradiated and reconstituted with 10^7 BALB/c spleen cells. IL-2 was injected twice daily at a dose of 50,000 IU, either from day 0 to 3 or from day 6 to 9 after reconstitution. The results are shown in Figure 3. IL-2 administration from day 6 to 9 resulted in strongly increased mortality as compared to the non-treated control group. IL-2 injection from day 0 to 3 also led to increased mortality, but significantly less than in the group treated from day 6 to 9. Treatment with a lower dose of 5,000 IU twice daily did not influence the morbidity or mortality in this model of GVHD (data not shown).

The effect of rhIL-1α was studied after a single dose of 400 ng IL-1α on day -1, 3 or 6 . It appeared that IL-1α treatment did not influence the survival (Figure 3).

The effect of a single gift of 2 μg of rhTNF-α was studied after injection on either day -1, 3 or 6. TNF-α administration neither increased nor decreased the survival (Figure 3).

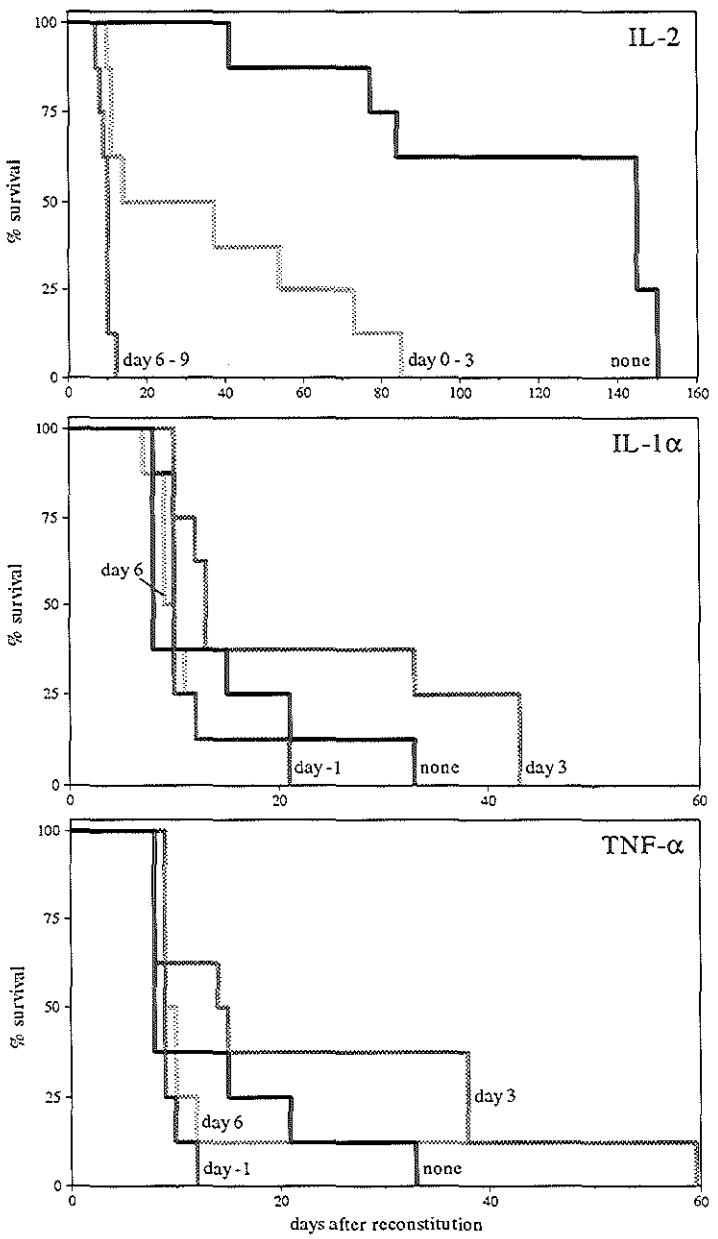

Figure 3. (A). Effect of administration of rhIL-2 *in vivo* on GVHD. Lethally irradiated (C57BL x CBA)F1 mice were reconstituted with 10^7 BALB/c spleen cells and subsequently treated twice daily with 50.000 IU rhIL-2 s.c. at days 0 to 3 or 6 to 9 after reconstitution (n=8). (B). Effect of rhIL-1α. GVHD was induced as indicated. A dose of 400 ng of rhIL-1α was given either on day -1, 3 or 6 after reconstitution (n=8). (C). Effect of rhTNF-α. GVHD was induced as indicated. A dose of 2 μg of rhTNF-α was given on either day -1, 3 or 6 after reconstitution (n=8).

Effect of injection of LPS

In vivo injection of LPS is known to induce IL-1, IL-6 and TNF-α production. LPS might be involved in the pathogenesis of GVHD (6). We studied whether injection of LPS at various days after reconstitution influenced the mortality of GVHD in the BALB/c - (C57BL x CBA)F1 strain combination. Therefore, groups of mice received 100 μg LPS either on day -1, 0, 1, 3 or 6 after allogeneic reconstitution. It appeared that the effect of LPS administration was dependent on the time of injection. LPS enhanced GVHD and mortality when injected on day 1, 3 or 6 after reconstitution. The effect was stronger when the LPS was administered at a later time point after reconstitution. In contrast, injection of LPS on day -1 appeared to inhibit GVHD (Figure 4).

Effect of anti-cytokine and anti-cytokine receptor mAb

(C57BL x CBA)F1 mice were lethally irradiated, reconstituted with 10^7 BALB/c spleen cells and injected with 1 mg of anti-IFN-γ mAb either as a single dose or as a daily dose for 7 days. Both treatment modalities did not decrease the morbidity and mortality of GVHD (Table 2A). The effect of anti-IL-6 mAb was studied after either a single dose of 1 mg or 3 daily doses of 1 mg. Neither treatment decreased the morbidity or mortality (Table 2B). The effect of anti-TNF-α mAb was studied after either a single dose of 2 mg on day -1 or injection of 2 mg on days -1, 1, 3 and 5. Table 2C shows that also anti-TNF-α mAb were not able to enhance the survival.

Since serum analysis indicated the possible involvement of IL-6, IFN-γ and TNF-α, but treatment with mAb directed to a single cytokine did not enhance the survival, we decided to investigate the effect of combined treatment with anti-IL-6, anti-IFN-γ and anti-TNF-α mAb. This was done by administering 2 mg of each of these mAb to recipient mice the day before reconstitution. From Table 2D it can be concluded that also this combined treatment did not result in a significant increase of the survival.

The effect of anti-IL-2 and anti-IL-2R mAb on the development of GVHD was investigated by injecting the relevant mAb the day before reconstitution. As shown in Figure 5, both mAb were able to enhance the survival significantly ($p<0.05$). However, mortality did occur starting day 38. This indicates that the inhibitory effect of anti-IL-2 and anti-IL-2R mAb was only temporary at the doses tested.

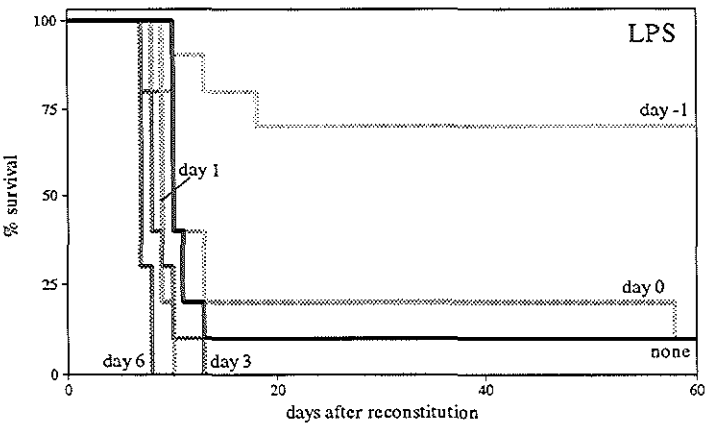

Figure 4. Effect of LPS on the development of acute GVHD. Lethally irradiated (C57BL x CBA)F1 mice reconstituted with 10^7 BALB/c spleen cells were i.p. injected with 100 µg of LPS on either day-1, 0, 1, 3 or 6 after reconstitution (n=10).

Table 2. Summary of the effects of treatment with various anti-cytokine mAb on the survival of lethally irradiated, allogeneically reconstituted mice.

Antibody		treatment	day	days after reconstitution			
				5	10	20	30
A.	anti-IFN-γ	1 mg (n=10)	-1	100 *	40	20	0
	anti-IFN-γ	7 × 1 mg (n=10)	-1 to 6	100	20	0	0
	-	- (n=10)		100	40	0	0
B.	anti-IL-6	1 mg (n=5)	-1	80	20	0	0
	anti-IL-6	3 × 1 mg (n=5)	-1,1,3	100	20	0	0
	-	- (n=5)		100	60	0	0
C.	anti-TNF-α	2 mg (n=5)	-1	100	100	100	0
	anti-TNF-α	4 × 2 mg (n=5)	-1,1,3,5	100	100	80	0
	-	- (n=5)		100	80	60	0
D.	anti-IFN-γ anti-TNF-α anti-IL-6	2+2+2 mg (n=5)	-1	80	0	0	0
	-	- (n=5)		60	40	0	0

(C57BL x CBA)F1 mice were lethally irradiated and reconstituted with 10^7 BALB/c spleen cells. Recipient mice were treated as indicated.

* Numbers represent the percentage survival at 5, 10, 20 and 30 days after reconstitution.

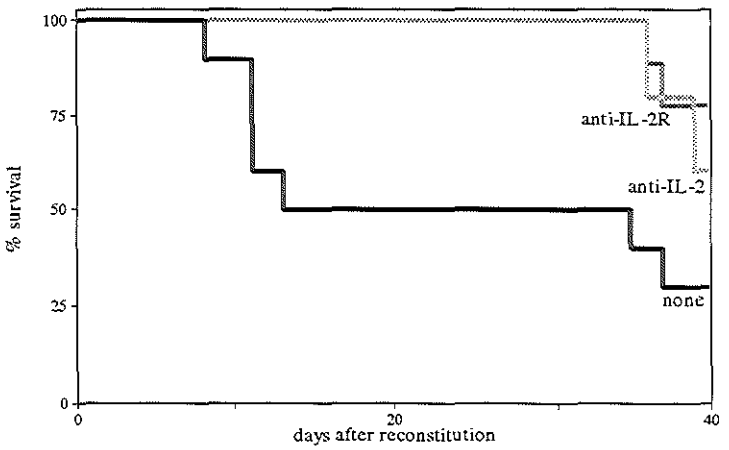

Figure 5. Effect of anti-IL-2 and anti-IL-2R mAb treatment. Lethally irradiated (C57BL x CBA)F1 mice reconstituted with 10^7 BALB/c spleen cells were injected with 0.1 ml anti-IL-2 ascitic fluid or 1 mg anti-IL-2R mAb one day before reconstitution (n=10).

Discussion

Evidence is increasing that cytokines are involved both in transplant rejection and GVHD. In GVHD, a role for IL-1, IL-2, IFN-γ and TNF-α has been postulated (4-8). Blocking of IL-1 activity led to enhanced survival from GVHD in mice (8). Blocking of IL-1 might interfere with early T cell activation (2). A number of studies suggests that also TNF-α plays a role in the pathogenesis of GVHD (25,26). Elevated serum levels of TNF-α preceded clinical symptoms of GVHD (9,27). Most studies, however, deal only with one particular cytokine. We studied several cytokines that are potentially involved in GVHD, by analysis of serum and culture supernatant and by *in vivo* administration of recombinant cytokines or anti-cytokine and anti-cytokine receptor mAb. In the murine model used, we have earlier demonstrated that $CD4^+$ T cells play a dominant role in the induction of GVHD (13).

Analysis of serum and supernatant of Con A stimulated spleen cell cultures revealed that after allogeneic, but not syngeneic reconstitution, IL-6, IFN-γ and TNF-α levels increased. The peak of IFN-γ activity coincided with a strong increase in the number of $CD4^+$ T cells in the spleen (14) and also with anti-host DTH reactivity (12). This might reflect Th1 activity, as an early event in the development of GVHD. The peak of IL-6 activity coincided with a strong increase in the number of $CD8^+$ T cells (data not shown). These observations might reflect the sequential involvement of $CD4^+$

T cells and $CD8^+$ T cells and their cytokines in our model. Ford et al. have shown in a sponge matrix allograft model that the occurrence of IL-6 coincided with cytotoxic T cell development (28).

The impact of the balance between Th1 and Th2 cells for the induction and pathogenesis of GVHD is still unclear. Umland et al. found evidence for predominance of a Th2 response in a model for chronic GVHD (29). In our model IL-2 and IL-4 were not detectable in the serum, possibly as the result of rapid consumption. However, the involvement of IL-2 is implicated by: a) the finding of enhanced IL-2 mRNA expression after allogeneic reconstitution; b) the observation that exogenous IL-2 enhanced the mortality; and c) the observation that anti-IL-2 and anti-IL-2R mAb were able to decrease the morbidity and mortality of GVHD. Enhanced mortality of GVHD by exogenous IL-2 administration was also reported by others (30-32). The fact that IL-2 administration at a later stage (days 6 to 9) enhanced the mortality more strongly then IL-2 administered during the first few days after reconstitution, suggests that the strongest effect of exogenous IL-2 is exerted on $CD8^+$ T cells. In the early phase of GVHD, $CD4^+$ T cells are activated and give rise to IL-2. They might be less dependent on exogenous IL-2, since they can produce IL-2 themselves. Sykes et al. found that IL-2 treatment given early after allogeneic reconstitution even delayed the development of GVHD (30). This effect was most clearly found in chronic GVHD and was dependent on the dose of IL-2. Recently, they were able to demonstrate that early IL-2 administration had an inhibitory effect on donor T cells (33). We did not find a beneficial effect of early IL-2 administration.

The administration of LPS, which is known to induce the sequential production of a number of cytokines, a.o. IL-1 and TNF-α (34), also influenced the morbidity and mortality of GVHD. The earlier after reconstitution the LPS was administered, the less the mortality was enhanced. Administration of LPS one day before allogeneic reconstitution even delayed the development of GVHD. We also studied the effect of exogenous IL-1 and TNF-α administration. However, it appeared that at the dose tested, neither IL-1α nor TNF-α influenced the survival.

Our data demonstrate that the production of cytokines was dependent on T cells, since anti-Thy-1 treatment reduced the serum IFN-γ and IL-6 levels to a level comparable to that in syngeneically reconstituted mice (data not shown).

More insight into the involvement of cytokines in GVHD can also be obtained by studying the effects of neutralizing mAb directed to cytokines or their receptors. We studied the effect of anti-IL-6, anti-IFN-γ and anti-TNF-α mAb. Even repeated or combined administration of these mAb in doses that are known to exert distinct *in vivo* effects (35,36) did not influence the morbidity or mortality of GVHD in our model. Thus far, most reports dealing with administration of a single mAb to a particular

cytokine show that a beneficial effect depends on coadministration of other immunosuppressive agents, such as cyclosporin A (37). It has also been described that specific lesions, e.g. gut lesions, due to GVHD can improve upon anti-IFN-γ treatment only, but decreased mortality has not been found (4).

It can be speculated that once a GVH reaction has been initiated, a variety of cytokines is released which have overlapping activities. Blocking of the activity of only one particular cytokine will be ineffective in that case. Another possibility is that the local amount of cytokines produced in GVHD is so high that even higher systemic doses are required. The development of anti-cytokine receptor mAb offers new perspectives, since these might be more potent in blocking cytokine activity. Finally, it cannot be excluded that in GVHD also other cytokines than we studied here are involved. Recently, IL-7 was reported to be involved in tumor rejection (38) whereas IL-8 levels were found to be elevated after liver transplantation (39).

The observation that an anti-IL-2 mAb so far is the only anti-cytokine mAb that is able to enhance the survival, might be due to the critical role of IL-2 early in T cell activation (2). Similarly, anti-IL-2R mAb were able to reduce the morbidity and mortality of GVHD. It is likely that blockade of the cytokine cascade is most easy during the induction phase. Preliminary data in human also show a beneficial effect of anti-IL-2R treatment in GVHD (40). The results of serum and culture supernatant analysis suggest that, besides IL-2, IL-6, IFN-γ and TNF-α are involved as well. The observation that serum IL-6, IFN-γ and TNF-α levels are elevated before symptoms of GVHD become apparent, suggests that careful evaluation of cytokines in serum might be useful for the early detection of GVHD. It has to be stressed, however, that elevation of cytokine levels is not restricted to alloreactivity but occurs also during bacterial and viral infections and that determination of cytokines at present can be used only to support current diagnostic procedures.

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Chapter 5

General discussion

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The nature of graft-versus-host disease (GVHD) is still not fully understood. Despite the fact that HLA typing and mixed lymphocyte reaction (MLR) are routinely performed to evaluate donor-recipient histoincompatibility and donor-recipient combinations are maximally matched, severe GVHD occurs even in HLA identical, MLR negative donor-recipient pairs (1). Therefore, it is important to improve our insight into this complex disease to be able to develop better prophylactic, diagnostic and therapeutic approaches.

The first part of the experimental work of this thesis was dedicated to the possibility to induce specific immunosuppression of graft-versus-host (GVH) reactivity by allogeneic blood transfusion. Therefore, we used the fully allogeneic strain combination BALB/c - (C57BL x CBA)F1. The idea was based on the observation of Marquet et al. in 1971 in a rat model, that renal and cardiac allograft recipients that had received pretransplant blood transfusions showed a better graft survival than untransfused recipients (2). A similar phenomenon was reported two years later in humans (3). In previous studies from our laboratory we found in line with these observations that delayed-type hypersensitivity (DTH) to alloantigens was suppressed after pretreatment of the recipients with donor-specific spleen cell or blood transfusion (4,5). We could demonstrate specific immunosuppression not only under host-versus-graft (HVG), but also under GVH conditions, since GVH-related DTH reactivity was suppressed after donor pretreatment with a recipient-specific spleen cell (6-8) or blood transfusion (Chapter 2.1). In addition, we found that after donor pretreatment with a recipient-specific blood transfusion, clinical symptoms of GVHD were less severe and the mortality was decreased (Chapter 2.2). These findings are in harmony with those of Halle-Pannenko et al. who studied the effect of donor pretreatment with a spleen cell or blood transfusion on the development of GVHD in a particular minor histoincompatible strain combination (9). In their model the effect of donor pretreatment could be ascribed primarily to alloimmunization against Mls determinants. Our data show that the blood transfusion effect can also be induced in (Mls identical) H-2, H-2 plus non-H-2 and class II disparate strain combinations.

In our experiments the blood transfusion effect appeared to be specific (5, Chapter 2.1), which is in line with data from murine, rat and some human studies under HVG conditions (2,10-12). Although in clinical organ transplantation mostly random transfusions are given, the actual influence might still be specific, since the blood to be transfused is often obtained from a pool of donors, which increases the chance of sharing (unknown) histocompatibility antigens between organ transplant and transfused blood. Sharing of only some major or minor alloantigens between the transfused blood and the graft appeared sufficient to improve kidney allograft survival in rats (13). We found a similar phenomenon in GVHD (Chapter 2.1 and 2.2).

The mechanism underlying the blood transfusion effect is, in spite of many efforts, still unknown. Clonal deletion, clonal anergy, a veto cell mechanism and the involvement of suppressor cells have been postulated (14-17). Most studies indicate that T cells are primarily involved as targets. Some groups reported that after blood transfusion the MLR and cell mediated lympholysis were reduced (10,18). These two types of *in vitro* assays can be seen as *in vitro* tests for helper and cytotoxic T cell activity, respectively. Others reported a decrease in the frequency of helper and cytotoxic T lymphocyte precursor cells (16,19). In a rat heart allograft model it was found that the sensivity for the cytokines IL-2 and IFN-γ was altered after allogeneic blood transfusions (20,21). We found that donor blood transfusion induced suppression of the GVH-related DTH response to alloantigens expressed by the blood cells. This suppression was mediated by $CD4^+$ T cells (Chapter 2.1). In addition, donor blood transfusion reduced the morbidity and mortality of GVHD. As GVHD in our model was mediated primarily by $CD4^+$ T cells (Chapter 3.2), the above observations suggest that these $CD4^+$ T cells are the primary targets of the blood transfusion effect. The observation that $CD4^+$ T cells mediate suppression as well as are target of the suppresion is compatible with the recent finding that the $CD4^+$ T cell subsets Th1 and Th2 can crossregulate each other (22). Blood transfusion might alter the balance between the Th1 and Th2 subset in favor of the latter, resulting in a suppressed Th1 activity.

The second part of the experimental work of this thesis deals with the role of T cell subsets in GVHD and the effect of anti-T cell (subset) monoclonal antibody (mAb) treatment. Once it became obvious that T cells play a primary role in GVHD, protocols were developed to eliminate these cells *in vitro* from the donor bone marrow prior to BMT. Polyclonal antilymphocyte serum (ALS) was partially effective, but the results were not very reproducible. Moreover ALS caused damage to hemopoietic stem cells (23). T cell depletion of the graft using physical methods or mAb appeared to be a reliable and effective way to prevent GVHD (24), although the procedure had two major disadvantages: the increased rate of bone marrow graft rejection and, in leukemia

patients, leukemia relapse (25). Both of these problems may be circumvented by more intensive conditioning (24). Our murine studies indicate that it is also possible to prevent GVHD by treatment *in vivo* of the recipients with anti-T cell (subset) mAb (Chapter 3.1). The timing of mAb treatment was found to be crucial for the therapeutic effect. In the case of use of anti-T cell mAb (anti-Thy-1), we found that treatment of the recipient mice could be given also *after* hemopoietic reconstitution, although the effective period was limited to the first few days. The observation that treatment could be postponed for 4 days, without a decrease in effectiveness, in a model in which mortality occurred already at day 8 in non-treated mice, indicates that the effective period is long in terms of progression of GVHD. Since GVHD in humans usually develops several weeks to months after BMT, it would be worthwhile to investigate whether mAb treatment might be effective even when started at appearance of the first clinical symptoms. This was suggested by early studies on the effects of ALS in rhesus monkeys (23). Nevertheless, the above data and preliminary data in humans indicate that mAb treatment is most effective when given as prophylaxis or early in the course of GVHD (26-28).

Data from the literature show that next to $CD4^+$ T cells also $CD8^+$ T cells can be involved in GVHD. The relative contribution of each subset might be dependent on the histocompatibility disparities between donor and recipient (29). Not only T cells, but also other cell types can play a role in GVHD, e.g. natural killer cells. It might be that the latter cells are especially involved in the effector phase of mild to moderate GVHD (30). In the fully allogeneic BALB/c - (C57BL x CBA)F1 strain combination that we studied, purified $CD4^+$ T cells were necessary and sufficient to induce lethal GVHD, whereas purified $CD8^+$ T cells were not, suggesting a major role for the $CD4^+$ T cell subset. This was confirmed by the finding that anti-CD4 treatment given the day before reconstitution was able to prevent the development of lethal GVHD, whereas anti-CD8 treatment was not (Chapter 3.2). These results are at variance with the observation that in the spleen of recipient mice the number of $CD8^+$ T cells strongly increased preceding the development of clinically overt GVHD (Chapter 3.2). Analysis of the spleen of the recipient mice after allogeneic reconstitution and mAb treatment revealed that in anti-CD4-treated mice not only the number of $CD4^+$ T cells, but also the number of $CD8^+$ T cells was reduced. These data suggest that in this strain combination $CD4^+$ T cells are primarily involved in the induction phase of GVHD, while $CD8^+$ T cells play a role in the effector phase. The latter interpretation is supported by the observation that anti-CD8 treatment was able to improve the survival, provided this treatment was given during the ongoing GVH reaction. The timing appeared to be critical, since only anti-CD8 treatment at day 3 after reconstitution led to significantly enhanced survival (data not shown). Insight into the relative contribution of different lymphoid and non-

lymphoid cell types and the phase of GVHD in which they are involved will facilitate the development of more sophisticated therapeutic intervention.

Until now, the number of mAb available for use in humans is limited. The anti-T cell mAb OKT3 has been investigated most extensively. OKT3 is a murine-anti-human CD3 mAb of the IgG2a subclass. OKT3 treatment was shown to improve renal and cardiac allograft survival (31). The initial success of OKT3 probably is the reason why the attention for other potentially useful mAb decreased (32). OKT3 administration, however, is associated with serious side-effects which are related to the fact that OKT3 mAb induce T cell activation *in vivo*. This leads to the production of cytokines such as IL-2, IL-6, IFN-γ and TNF-α, which can cause considerable morbidity (33,34). Furthermore, OKT3 mAb often induce the production of neutralizing antibodies, which can hamper further use (35,36).

We compared the effectiveness of rat IgG2a and rat IgG2b anti-CD3 mAb for treatment of murine GVHD (Chapter 3.3). Our data show that both IgG2a and IgG2b anti-CD3 mAb decreased the morbidity and mortality of GVHD. *In vivo* administration of these mAb was not associated with clinical side-effects. This is in contrast to the results of Hirsch et al. who used the hamster-anti-mouse CD3 mAb 145-2C11. This mAb shows similar side-effects as OKT3 upon *in vivo* administration (37). Preliminary studies with the 145-2C11 mAb in our model confirms the T cell activation by this mAb and the occurence of severe morbidity (data not shown).

In addition to the anti-CD3 mAb, we compared the effectiveness of rat IgG2a and IgG2b anti-CD4 mAb (Chapter 3.3). Both mAb decreased the morbidity and mortality of GVHD considerably, although the IgG2a anti-CD4 mAb appeared to be less effective than the IgG2b mAb. Thus anti-CD4 mAb treatment might turn out to be an alternative for anti-CD3 mAb treatment. This is of practical importance, since anti-CD4 mAb treatment thus far was not found to be accompanied with side-effects (38-40).

The effectiveness of mAb as immunosuppressive agents seems to be related to the capacity of the mAb to eliminate its target cell population *in vivo* (41). Both rat anti-CD3 mAb were similarly effective in this respect. In contrast, Thierfelder et al. reported that only rat IgG2b mAb were able to prevent GVHD in contrast to rat IgG2a, IgG2c and IgM mAb (41). This was claimed to be related to the ability of IgG2b mAb to bind the C1 component of the complement cascade and was correlated with the capacity to eliminate T cells *in vivo* (42,43). Various mechanisms have been proposed for the elimination of T cells by mAb treatment, such as opsonization, complement dependent cytotoxicity, antibody dependent cellular cytotoxicity and apoptosis (44-47).

The third part of the experimental work was dedicated to the role of cytokines in GVHD. In the same model in which we demonstrated the importance of $CD4^+$ T cells in the induction of acute GVHD (Chapters 3.1 and 3.2), we investigated the role of cytokines.

Preliminary studies in humans have shown that serum levels of IFN-γ and TNF-α were elevated after allogeneic BMT, which preceded the development of clinical symptoms of GVHD (48-50). In a recent study, however, only low levels of TNF-α were found after allogeneic BMT (51). These levels were not different from those observed after autologous BMT. The differences between the data reported in the literature might be due to variation in the sensitivity of assays used for the determination of cytokines and to variation in the immunosuppression applied after BMT. Also the degree of decontamination of the intestine prior to BMT might play a role, since it has been postulated that one of the pathogenic mechanisms in GVHD might be the induction of TNF- release by bacterial LPS (52). Our study suggests that other cytokines might also play a role, in particular IL-2 and IL-6 and possibly IL-1 (Chapter 4). Studies on IL-6 in human BMT are not available, despite the fact that many studies show its role in transplant rejection (53,54). A recent study also reports on the role of IL-1 in GVHD (55). The failure to detect cytokines in serum does not exclude their involvement in GVHD, as shown for IL-2 in our model (Chapter 4.2). The fact that anti-IL-2R mAb were able to decrease GVHD in humans (28) implicates a role for IL-2 in GVHD in humans as well.

Anti-T cell as well as anti-cytokine, anti-cytokine receptor and anti-adhesion molecule mAb should be considered for future immunosuppressive treatment in human BMT. However, the administration of mAb might not only have beneficial effects, but disadvantages as well. The side-effects of OKT3 treatment were mentioned before. The administration of anti-T cell and anti-TNF-α mAb might reduce the graft-versus-leukemia effect and increase the risk of infectious disease. It will be important to establish appropriate models for evaluating the properties of mAb considered for clinical application. *In vitro* models don't seem very reliable in this respect. Murine models might be used as a first step (56). Data obtained from studies in such models will increase our insight into the mechanisms that underly the observed effects and side-effects. This will allow the development of strategies for prevention of these side-effects. Preferably, mAb that are considered for use in humans should be tested in nonhuman primates for their immunosuppressive effects, but also for their possible side-effects. A study in rhesus monkeys revealed similar effects of mAb administration as reported in humans (38). Studies are underway to evaluate the value of anti-TNF-α, anti-IL-2R and anti-LFA-1 mAb for treatment of GVHD in humans (28,57,58).

Humanization of immunosuppressive mAb by advanced molecular biological techniques will be an important step forward, since this will reduce the immunogenicity of the administered mAb and consequently the immune response to it (59). Whether this will circumvent the problem of induction of cytokine release remains doubtful (59).

The effect of combination therapies employing different mAb and/or new immunosuppressive drugs such as FK506, RS-61443, rapamycin and brequinar sodium has also to be evaluated (60,61). Studies using cyclosporin A in combination with anti-CD4 or anti-TNF-α mAb therapy showed a synergistic immunosuppressive activity (60,62). Different mAb may have synergistic as well as antagonistic properties (63).

The ultimate aim of immunosuppressive treatment is to induce long-term survival and tolerance. In our studies this could be achieved by immunosuppressive mAb treatment. Therefore, our data suggest that mAb are likely to constitute effective components of future treatment regimens in human GVHD.

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Summary

Bone marrow transplantation (BMT) is a treatment modality for patients with life-threatening diseases, such as leukemias, aplastic anemia and severe immunodeficiencies. However, after BMT serious complications can occur, such as infectious diseases. In the case of incompatibility of tissue antigens between donor and recipient, graft-versus-host disease (GVHD) can develop. GVHD is associated with considerable morbidity (e.g. skin lesions, diarrhoea, liver injury) and mortality. T lymphocytes appeared to be responsible for the development of GVHD. These T lymphocytes reside within the bone marrow graft and are transplanted into the recipient together with the hemopoietic stem cells that should reconstitute hematopoiesis and immunity. When the recipients' tissue antigens are recognized by the donor T lymphocytes as foreign, they become activated, which subsequently can lead to serious tissue damage and even death of the recipient.

This thesis describes the results of experiments aimed to improve the insight into this complex disease using a murine model. Based on previous work from our laboratory we investigated the possibilities to prevent GVHD in a specific manner, namely by pretreatment of prospective donors with a recipient-specific blood transfusion. Furthermore, we assessed the role of (subsets of) T lymphocytes and of cytokines in GVHD. Cytokines are factors that can be produced a.o. by T cells and play a central role in immune and inflammatory reactions. Recent data from the literature indicate that cytokines might be involved in the development of GVHD. We studied the therapeutic effect of *in vivo* administration of anti-T cell (subset), anti-cytokine and anti-cytokine receptor monoclonal antibodies (mAb) on GVHD.

The results presented in Chapter 2 show that pretreatment of prospective donors with a recipient-specific blood transfusion suppressed the anti-host delayed-type hypersensitivity response, which is an early event during GVHD. Moreover, donor blood transfusion delayed and decreased the morbidity and mortality caused by GVHD. White blood cells appeared to be required for induction of the blood transfusion effect. Although the mechanism of the beneficial effect of donor blood transfusion has not been elucidated yet, $CD4^+$ T cells apparently are important targets. In our model, it appeared that blood transfusions induced a state of suppression which is mediated by T cells. We hypothesize that the induced suppression is the consequence of an altered balance between the Th1 and Th2 $CD4^+$ T cell subsets. It is known from the literature that the Th1 and Th2 subsets are able to crossregulate each others activity and that the balance between these subsets affects immune responses.

Chapter 3 deals with the role of T cells and the $CD4^+$ and $CD8^+$ T cell subsets in GVHD. It appeared that GVHD in our model could be induced by purified $CD4^+$, but not by purified $CD8^+$ T cells (Chapter 3.1). The latter cell population might play a role in a later phase of the disease. *In vivo* treatment of recipient mice with anti-T cell (anti-Thy-1 or anti-CD3) or anti-T cell subset (anti-CD4) mAb reduced or even completely prevented the morbidity and mortality of GVHD (Chapter 3.2 and 3.3). The timing of mAb treatment appeared to be critical. This mAb treatment could be given just before or after reconstitution with similar effectiveness, and could even be postponed for a few days (Chapter 3.1). The effectiveness of mAb treatment *in vivo* is known to be dependent on a number of parameters, such as the specificity and the isotype of the mAb used. In Chapter 3.3 we show that anti-CD4 mAb can be equally immunosuppressive *in vivo* as anti-CD3 mAb. This might be relevant to future clinical applications of mAb, since the available anti-CD4 mAb in human don't seem to be associated with side effects, which is a major drawback of currently available anti-CD3 mAb. Our data further show that mAb of different isotypes can be equally effective for therapeutic use in murine GVHD. The finding that a rat IgG2a anti-CD4 mAb that caused only moderate T cell depletion *in vivo* was able to decrease GVHD is important, because the use of hardly or not depleting mAb likely decreases the chance of serious therapy-related complications such as infectious disease.

Chapter 4 deals with the role of cytokines in GVHD. Our data show that IL-2, IL-6, IFN-γ and TNF-α are involved in GVHD, since serum and mRNA levels of these cytokines were elevated after allogeneic, but not after syngeneic reconstitution. The observation that mRNA levels for IL-1 also differed between these two groups of mice suggests that IL-1 is also involved in the pathogenesis of GVHD. Thus far only mAb treatment directed to IL-2 or the IL-2 receptor appeared to be effective in the prevention of GVHD. This might be due to the fact that besides IL-1, IL-2 plays a critical role in the induction phase of GVHD. The other cytokines studied, might be more involved at later stages. As our study on the role of cytokines in GVHD was restricted to only some of this large family of regulatory proteins, more extensive studies in this area are required for a detailed insight into the role of cytokines in the pathogenesis of GVHD.

Together the results presented in this thesis suggest that different T cell subsets and cytokines can be involved in GVHD. It can be anticipated that in future treatment protocols mAb to T cells or T cell subsets or to cytokines and/or cytokine receptors will contribute significantly to the prevention and treatment of GVHD.

Samenvatting

Beenmergtransplantatie is een behandelingsmethode voor patiënten die lijden aan levensbedreigende ziekten, zoals leukemieën, aplastische anemieën en ernstige immunodeficiënties. Na beenmergtransplantatie kunnen echter ernstige complicaties ontstaan, zoals infecties. Wanneer de weefselantigenen tussen donor en ontvanger verschillen, kan bovendien graft-versus-host ziekte (GVHZ) optreden. Dit is een immuunreactie van het getransplanteerde beenmerg tegen de ontvanger. GVHZ gaat gepaard met ernstige ziekteverschijnselen, zoals huidafwijkingen, diarree en leverfunktiestoornissen en zelfs met sterfte. Een bepaald type witte bloedcel, de zogenaamde T lymfocyt, blijkt verantwoordelijk te zijn voor het ontstaan van GVHZ. T lymfocyten bevinden zich in het beenmergtransplantaat en worden samen met de stamcellen die de hemopoiese en de immuniteit moeten herstellen, getransplanteerd naar de ontvanger. Wanneer de weefselantigenen van de ontvanger als "vreemd" worden herkend door de donor T lymfocyten worden deze geactiveerd, wat vervolgens kan leiden tot ernstige schade aan weefsels van de ontvanger en zelfs tot de dood.

Dit proefschrift beschrijft de resultaten van experimenten die werden uitgevoerd om het inzicht in de complexe GVHZ te vergroten. Hiertoe werd gebruik gemaakt van een muizemodel. Mede op grond van eerdere resultaten van ons laboratorium onderzochten we de mogelijkheden om GVHZ te voorkomen op een specifieke wijze, namelijk door voorbehandeling van toekomstige donoren met een ontvanger-specifieke bloedtransfusie. Daarnaast besteedden we aandacht aan de rol van T cellen, T cel subpopulaties en cytokinen bij GVHZ. Cytokinen zijn factoren die o.a. kunnen worden geproduceerd door T cellen. Zij spelen een centrale rol bij immuun- en ontstekingsreacties. Recente gegevens uit de literatuur wijzen erop dat cytokinen betrokken zouden kunnen zijn bij het ontstaan van GVHZ. In ons onderzoek bestudeerden we tevens het effect van *in vivo* toediening van monoclonale antistoffen (mAs) gericht tegen T cellen, T cel subpopulaties, cytokinen en cytokine receptoren.

De resultaten die worden gepresenteerd in Hoofdstuk 2, laten zien dat voorbehandeling van toekomstige donoren met een ontvanger-specifieke bloedtransfusie de vertraagd-type overgevoeligheidsreactie tegen de ontvanger, die optreedt in de vroege fase van GVHZ, onderdrukt. Bloedtransfusie van de donor bleek bovendien het optreden van ziekteverschijnselen en sterfte door GVHZ te vertragen en te verminderen. De witte bloedcellen in het voor de transfusie gebruikte bloed bleken daarbij een essentiële rol te spelen. Hoewel het mechanisme van het gunstige effect van donor transfusie nog niet is opgehelderd, blijkt het dat bloedtransfusies in ons model met name een effect hebben op $CD4^+$ T cellen. Bloedtransfusies geven aanleiding tot

het ontstaan van suppressie, welke wordt onderhouden door $CD4^+$ T cellen. Onze hypothese is, dat deze suppressie een gevolg is van een veranderde balans tussen Th1 en Th2 $CD4^+$ T cellen. Het is bekend uit de literatuur dat de Th1 en Th2 subpopulatie elkaar wederzijds kunnen beïnvloeden en dat de balans tussen deze Th subpopulaties van invloed is op immuunreacties.

Hoofdstuk 3 gaat in op de rol van T cellen en $CD4^+$ en $CD8^+$ T cel subpopulaties bij GVHZ. Het bleek dat GVHZ in ons model kon worden geïnduceerd door gezuiverde $CD4^+$ T cellen, maar niet door gezuiverde $CD8^+$ T cellen (Hoofdstuk 3.1). De $CD8^+$ T cel populatie zou een rol kunnen spelen tijdens een latere fase van de ziekte. *In vivo* behandeling van ontvanger muizen met anti-T cel (anti-Thy-1 of anti-CD3) of anti-T cel subpopulatie (anti-CD4) mAs verminderde of voorkwam het ontstaan van ziekteverschijnselen en sterfte door GVHZ (Hoofdstuk 3.2 en 3.3). Het tijdstip van toediening van de mAs bleek belangrijk voor de effectiviteit van de behandeling. Behandeling met mAs kort voor en kort na transplantatie had een vergelijkbaar effect. De behandeling kon zelfs zonder nadelige gevolgen enkele dagen worden uitgesteld (Hoofdstuk 3.1).

Het is bekend dat de effectiviteit van mAs behandeling *in vivo* afhangt van een aantal variabelen, zoals de specificiteit en het isotype van de gebruikte mAs. Hoofdstuk 3.3 laat zien dat anti-CD4 mAs een vergelijkbare immunosuppressieve werking kunnen hebben *in vivo* als anti-CD3 mAs. Dit zou relevant kunnen zijn voor de klinische toepassing van mAs, aangezien de huidige anti-CD4 mAs bij de mens minder bijwerkingen lijken te hebben dan de anti-CD3 mAs die op dit moment beschikbaar zijn. Voorts tonen onze gegevens aan dat mAs van verschillende isotypen effectief kunnen zijn bij de preventie en behandeling van GVHZ. De bevinding dat een rat anti-CD4 mAs van het IgG2a isotype die slechts matige depletie veroorzaakt in staat was GVHZ te verminderen is belangrijk, omdat het gebruik van weinig of niet depleterende mAs waarschijnlijk de kans op ernstige therapie-gerelateerde complicaties, zoals infecties, vermindert.

Hoofdstuk 4 gaat in op de rol van cytokinen bij GVHZ. Onze gegevens laten zien dat IL-2, IL-6, IFN-γ en TNF-α betrokken zijn bij GVHZ, aangezien de serum en mRNA spiegels van deze cytokinen verhoogd waren na allogene, maar niet na syngene reconstitutie. De waarneming dat de mRNA spiegels van IL-1 tevens verschilden tussen de twee groepen, suggereert dat IL-1 ook bij de pathogenese van GVHZ betrokken is. Tot nu toe bleken alleen mAs gericht tegen IL-2 of de IL-2 receptor effectief in de preventie van GVHZ. Dit zou een gevolg kunnen zijn van het feit dat IL-2, naast IL-1, een centrale rol speelt in de inductiefase van GVHZ. Andere cytokinen spelen waarschijnlijk vooral tijdens latere fasen een rol. Omdat in ons onderzoek slechts enkele van deze grote familie van regulerende eiwitten werden bestudeerd, zijn

uitgebreidere studies nodig om een meer gedetailleerd inzicht in de rol van cytokinen in de pathogenese van GVHZ te verkrijgen.

De resultaten van ons onderzoek tonen aan dat verschillende T cel subpopulaties en cytokinen betrokken kunnen zijn bij GVHZ. Behandeling gericht tegen T cellen, T cel subpopulaties, cytokinen en/of cytokine receptoren zal in de toekomst waarschijnlijk een belangrijke bijdrage kunnen leveren aan de preventie en behandeling van GVHZ na klinische beenmergtransplantatie.

Abbreviations

Ab	:	antibody
APC	:	antigen presenting cell
ATG	:	anti-thymocyte globulin
BMT	:	bone marrow transplantation
BSS	:	balanced salt solution
C	:	complement
CsA	:	cyclosporin A
CTL	:	cytotoxic T lymphocyte
CTLp	:	cytotoxic T lymphocyte precursor
DTH	:	delayed-type hypersensitivity
EBV	:	Epstein-Barr virus
ELISA	:	enzyme-linked immunosorbent assay
$F(ab')_2$	:	divalent antigen-binding fragment
FCS	:	fetal calf serum
FITC	:	fluorescein isothiocyanate
GVH	:	graft-versus-host
GVHD	:	graft-versus-host disease
GVL	:	graft-versus-leukemia
Gy	:	Gray
H-2	:	histocompatibility-2
HLA	:	human leukocyte antigen
HVG	:	host-versus-graft
IFN	:	interferon
IL	:	interleukin
i.p.	:	intraperitoneal
IU	:	international unit
i.v.	:	intravenous
mAb	:	monoclonal antibody
MHC	:	major histocompatibility complex
MLR	:	mixed lymphocyte reaction
Mls	:	minor lymphocyte stimulating
MST	:	mean survival time
n	:	number in study or group
p	:	probability
PBS	:	phosphate-buffered saline
RNA	:	ribonucleic acid
s.c.	:	subcutaneous
SEM	:	standard error of the mean
Th cell	:	T helper cell
Thp cell	:	T helper precursor cell
TNF	:	tumor necrosis factor
U	:	unit

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André

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