STUDIES UNDERTAKEN TO DETERMINE THE MECHANISMS UNDERLYING TRANSPLANTATION TOLERANCE EMPLOYING DIFFERENT CONDITIONING REGIMENS IN A SEMIALLOGENEIC MURINE BONE MARROW TRANSPLANTATION MODEL

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Submitted in partial fulfillment of the requirements for the degree of *M Med Sci* in the Department of Surgery

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FOREWORD

I am indebted to the Catholic University of Leuven for allowing me the opportunity of working in the Transplantation Laboratory and the exposure to the basic sciences and basic science research.

To this end, I thank Professor Mark Waer for the direction and the assistance he provided so willingly. The overwhelming enthusiasm Professor Waer has for the sciences made the transition from the clinical environment to the laboratory an effortless task. It certainly helped ease the frustrations of the traditional failed experiments. His inspiration, support and motivation is testimony to this work being completed.

To Omer Rutgeerts, whose technical skills are flattering, I am indebted for his patience and his teaching skills as he passed on his wealth of technical skills to me. The success of the experiments bears testimony to his efforts, patience and guidance.

To the laboratory staff and colleagues I worked alongside, I owe you for the ease with which you accommodated me in the laboratory, and for assisting in making my transition to functioning in an alien environment that much easier. There are too many to mention and I acknowledge them all for their unwavering assistance in times of distress – both academic and social!

I must thank the Department of General Surgery for the encouragement and support they provided in allowing me to assume this post. The professional response and ease with which all of the arrangements were undertaken is truly appreciated.

To Samad Shaik, whose skills I hope one day will become legendary outside of our small community, (and I am certain that he can get there if he chooses), I am indebted for the assistance with the statistical analysis and resolving the minor nuances with the computer. A thank you underestimates the input!

Lastly, I must thank Professor AA Haffejee for his willingness to supervise the project locally and I am grateful for his perusal and criticism of the project.

PUBLICATIONS

Stable mixed chimerism induced by total lymphoid irradiation or by total body irradiation is maintained by different mechanisms and leads to different graft versus leukemia or graft rejection reactions. Sefrioui H, Moodley J, Rutgeerts O, Goebels J, Vandeputte M, Waer M. *Transplant Proc* 1997 Aug; **29(5):** 2352

Presence of intrinsic B lymphocyte tolerance in mixed but not in complete semiallogeneic bone marrow chimeras. Salam A, Moodley J, Waer M. *Transplantation* 1997 Mar 15; **63(5):** 699-704

CHAPTER 1

INTRODUCTION

Bone marrow transplantation has entrenched itself as an indispensable therapeutic modality in clinical practice. Its role in the salvage of patients with haematological malignancies following ablative cytotoxic chemotherapy is well established. More liberal application of this therapeutic modality resulted in the observation that Bone Marrow Transplantation (BMT) resulted in a lower relapse rate independent of the cytotoxic therapy establishing the entity of the Graft Versus Leukaemia (GVL) effect. This has been well documented in both laboratory experiments as well as in clinical practice.

Three important facets emerged from these observations:

- BMT allowed for more aggressive chemotherapy to effect eradication of tumour and minimise relapse
- the success of BMT raised the potential for the use of this modality to correct congenital abnormalities of the haematological system
- Its use in the context of malignancies served to consolidate the anti-tumour effect which minimised relapse and served as maintenance for tumour surveillance

However, wider use of this technique has been frustrated by three major factors:

- Engraftment failure
- the emergence of Graft Versus Host Disease (GVHD)
- The deleterious effects of the conditioning regime and the post-transplantation care

Despite these limitations, its role has been gradually extended to embrace the treatment of benign conditions as well as in the treatment of solid visceral malignancies.

DEFINITION AND HISTORY OF BONE MARROW TRANSPLANTATION

Bone Marrow Transplantation is a well-established clinical therapeutic procedure involving the transfer of pleuripotent stem cells. At present, BMT may be performed with the individual's own stem cells whence it is referred to as autologous transplantation; this also occurs when the transplantation is from an identical twin. BMT between individuals of the same species is referred to as allogeneic transplantation. Whilst Xenogeneic transplantation (between different species) has been extensively performed in laboratory experiments, only a single case has been performed in clinical practice for acquired immunodeficiency. At present, only autologous and allogeneic transplantation are routinely performed in clinical practice.

The history of BMT dates back to 1939 when a patient suffering from aplastic anaemia was treated with 18 ml of blood from his brother[1]. From 1970 to 1990 more than 10000 BMT have been performed throughout the world[2].

The best matching situation for successful BMT is identical twins. In the absence of this, HLA identical sibling donor is optimal for allogeneic transplantation. Other options for BMT include unrelated but well matched donors and related but less well-matched donors.

PRETRANSPLANT CONDITIONING USED FOR BMT

In the first half of the century it was observed that irradiation had profound suppressive effects on the immune system[3]. Based on these findings, Total Body Irradiation (TBI) was used for preparing recipients for bone marrow and organ transplantation. However, it became apparent that very high doses of irradiation were necessary to prevent allograft rejections. These high irradiation doses were associated with severe toxicity culminating in formidable morbidity and mortality rates[4]. Subsequently, irradiation protocols were modified to minimise these effects and fractionated irradiation administered loco-regionally were implemented to minimise the toxic effects. One of these fractionated protocols, Total Lymphoid Irradiation (TLI) was used for bone marrow and organ transplantation by a group of researchers at Stanford University[5, 6]. Prior to its application to effect immunosuppresion, TLI was used in the treatment of Hodgkin's Lymphoma. This led to the recognition of the various immunological alterations that occurred as a consequence of the treatment. Of significance, TLI resulted in a T cell lymphopaenia, a decrease responsiveness of the residual T cells to the mitogens Concanavalin A (Con A) and phytohaemagglutinin (PHA), and a decreased in vitro mixed lymphocyte reaction (MLR). These observations encouraged the use of TLI as an immunosuppressant to achieve tolerance to both solid organ transplantation and as a therapeutic modality for autoimmune disorders. Subsequent laboratory investigations confirmed that TLI proved to be an excellent conditioning regimen to achieve transplantation tolerance and the studies performed with BMT proved to be more exciting as the incidence of GVHD was rare[7, 8, 9]. Additionally,

secondary haematological malignancies were rare and infections, barring benign herpes zoster[10], were rare. TLI involves irradiating selected parts of the body, essentially the lymphoid system, with the other organs being shielded by lead blocks. The efficacy of the regimen can be increased by widening the TLI field or by adding 1 to 2 TBI fractions[11].

Despite the induction of donor specific tolerance as well as the activation of natural suppressor cells, there are some limitations to TLI. There is an optimum time period of about 1week after TLI during which transplantation must be performed. If transplantation is not performed within this period then the tolerance is less predictable and unclear[11, 12].

IMMUNE RESPONSE AND SELECTION OF THE IMMUNE REPERTOIRE

Normally, 2 types of immune responses have been recognised that are reactive to alloantigens and hence can play a role following BMT: The cellular response, mediated by T cells and Natural Killer/ Lymphokine Activated Killer (NK/LAK) cells and the humoral response mediated by B cells and antibodies. T cells undergo maturation and differentiation in the thymus mainly consequent upon 2 processes; positive and negative selection. During positive selection T cells acquiring T cell receptors (TCR's) with intermediate affinity for self-MHC will survive. This implies that T cells recognise foreign antigens in the context of foreign antigens (peptides) in the context of self-MHC molecules. During negative selection T cells with a high affinity for self-MHC molecules will be clonally deleted in order to circumvent autoimmune reactions. With these 2 selection processes, an effective T cell repertoire is formed intrathymically as a result of the interactions between TCR's and MHC molecules expressed on thymic epithelial

cells in positive selection and, TCR's and MHC molecules expressed on bone marrow derived Antigen Presenting Cells (APC) in negative selection. Similar selection phenomena are suggested for B cells but are not proven as yet[13]. For NK or LAK cells, no data is available with respect to their selection or their repertoire.

THE MINOR LYMPHOCYTE STIMULATING (Mls) SYSTEM

The negative selection process leading to self tolerance for T cells has been investigated using superantigens and transgenic mice. Since the Mls system is crucial to the subsequent experiments, it will be discussed in more detail.

Mls antigens are viral products that are encoded for by open reading frame (orf) in 3 long terminal repeat of either exogenous or endogenous mouse mammary tumour viruses [MMTV][14, 15]. These orf products are type II transmembrane glycoproteins that can bind to MHC class II molecules[16]. They can also function as superantigens like other infectious agents such as bacterial superantigens. In addition to their ability to react with T cells by binding with some specific β components of the variable region of αβ TCR's, they also have the capacity to bind to MHC class II molecules outside the peptide binding groove[17, 18]. This means that Mls antigens stimulate large proportions of T cells expressing specific V TCR's so vigorously that after a period of massive proliferation and lymphokine release, T cells die.

The trimolecular interaction between superantigens usually lead to apoptosis of immature T cells and proliferation followed by anergy of mature T cells bearing the relevant Vβ domains. This occurs in vivo.

IMMUNOLOGIC TOLERANCE

In the early 1950's Peter Medawar and colleagues demonstrated that when neonatal mice of strain "A" were injected with lymphohaematopoeitic cells of strain "B" they became tolerant to skin grafts from strain "B" but not for skin grafts taken from strain "C". This form of neonatally induced tolerance was donor specific and long lived[19, 20].

The term 'tolerance' is now generally accepted to as a state of non-responsiveness of the immune system to an antigenic challenge. There are three main factors that will determine whether an individual will elicit an effective immune response or develop tolerance. The factors are

- The nature, dose, route of administration and character of the antigen
- The type of antigen presenting cells
- The maturational phase of the responding lymphocyte

Self-tolerance refers to the destruction or functional inactivation of lymphocytes with potential reactivity for self-antigens. This form of tolerance is achieved mainly in the thymus for T lymphocytes and probably in the bone marrow for B lymphocytes and is therefore frequently referred to as **central tolerance**. Under certain circumstances some self-reactive lymphocytes can undergo inactivation in the peripheral lymphoid tissue and this is referred to as **peripheral tolerance**.

Allograft acceptance without immunosuppressive therapy is called **allotolerance** or **transplantation tolerance**. The achievement of the latter remains the challenge in transplantation biology. In animal models, various techniques have been developed that can achieve transplantation tolerance. As already alluded to, Medawar and Billingham showed that tolerance to foreign skin grafts could be acquired and sustained

for long periods after innoculation of donor bone marrow cells into neonatal mice[19]. After this initial achievement of neonatal tolerance, various procedures were developed to induce tolerance in adult recipients as well. Unlike neonatal tolerance, a preparative protocol is necessary in adult recipients to allow for the successful transfer of allogeneic bone marrow to circumvent host immunocompetent cells from rejecting donor cells.

MECHANISMS OF TRANSPLANTATION TOLERANCE

Four mechanisms of transplantation tolerance have been described and substantiated in the literature.

CLONAL DELETION

Clonal deletion refers to the physical elimination of specific T cell clones and this occurs mainly in the thymus. Bone marrow derived Antigen Presenting Cells (APCs) found in the thymic medulla or at the cortico-medullary junction are the most important cells that cause the appropriate reactive T cells to die by a process called apoptosis (programmed cell death). Amongst these BM derived APCs, the most important appears to be the Dendritic cells[20].

Several reports employing transgenic or minor antigen disparate models have demonstrated the presence of peripheral tolerance and the mechanisms of peripheral tolerance are multiple.

CLONAL ANERGY

This term refers to a condition in which lymphocytes become functionally inactivated without undergoing physical elimination. Although thymic epithelial cells are also

believed to use anergy for tolerance induction, this mechanism is mainly operative in the peripheral lymphoid tissues[20, 21]. Clonal anergy is usually achieved when T cell receptors are occupied by antigens in the absence of sufficient costimulatory second signals[22]. There is no single molecule or pathway that delivers this costimulation but rather a whole group of adhesion molecules and their ligands expressed on lymphocytes and antigen presenting cells[21, 22]. These include the pairs LFA-1/ICAM, CD2/LFA-3, VLA-4/VCAM-1 and CD28/CTLA4:B7 or BB1[21, 23]. The latter molecules seem especially important as B7/BB1 are expressed in high levels on dendritic cells[24], activated B cells[25], activated monocytes[26] and also on activated T cells[27]. During TCR-MHC interactions, B7:CD28/CTLA4 costimulatory molecules can be blocked e.g. by CTLA4 Ig fusion proteins[28]. Lafferty reported much earlier that costimulatory molecules were important in transplantation immunology as they determined immune responses to donor antigens[29]. In most situations, T cell anergy is reversible. This has been demonstrated in vivo by the addition of exogenous IL-2[30].

IMMUNE IGNORANCE

This term is used to describe the situation where the non-reactivity of the T cells is either as a result of antigen inaccessibility (sequestered inside cells or behind anatomical barriers) or when the affinity of the TCRs for antigens are too low (presented by non professional APCs). With regard to transplantation immunology, it is believed that donor grafts that are devoid of donor type professional APCs are accepted more readily due to T cell ignorance[31].

CLONAL DELETION

There is ample evidence that mature T cells can be clonally deleted in the periphery and this may also be imparted by Veto cells[32, 33].

Veto cells: The term Veto cells is used for cells that are able to delete or inactivate T cells only when Veto cells themselves are attacked by these T cells[34]. Miller was first to describe these cells in in vitro experiments[33]. The precise identity of Veto cells remain to be established. They are thought to effect their action by direct lysis[35] or by delivering an anergising signal[36].

ACTIVE SUPPRESSION

This term is employed to describe the phenomenon where the T cell responsiveness is diminished or abrogated either by other cell populations, soluble factors or both. These cells and soluble products are commonly termed suppressor cells and suppressor factors. Suppressor cells can be antigen or idiotypic specific or natural suppressor cells. Natural suppressor cells are believed to block or inhibit the activation of T cells in response to different antigenic challenges[37]. These cells have been demonstrated to expand after total lymphoid irradiation or after treatment with cyclophosphamide. The phenotype of these cells are not established but surface markers are similar to those described for NK cells although they do not demonstrate the same functions[37]. It is believed that some soluble factors or proteins released from NS cells down regulate immune responsiveness.

B cell tolerance: B lymphocytes are dependent on T cells for activation under most circumstances, hence humoral tolerance is frequently based on T cell tolerance. However, T independent B cell activation may also occur. The experiments performed

using transgenic mice show that the same mechanisms that allow for T cell tolerance are relevant to B cell tolerance[38].

Another mechanism of B cell tolerance entails the exclusion of B cells from the primary follicles of lymph nodes and the spleen. The fate of these excluded cells is apoptosis[39].

CHIMAERISM

Allogeneic BMT can result in chimaerism. This describes the situation where cells from the donor persist in the recipient either alone or alongside host bone marrow cells. Attaining stable long-term chimaerism requires overcoming rejection which is mediated by host type T cells and NK cells and is usually overcome by immunosuppression achieved with irradiation or cytotoxic agents[40]. Once rejection is overcome, the next problem that needs to be resolved is GVHD.

Stable chimaeras usually remain specifically tolerant for donor and host alloantigens but are capable of responding to third party antigens. Chimaeras may either be complete, where the donor bone marrow completely replaces the host marrow, or mixed, where the donor and recipient marrow coexist. Mixed chimaeras are thought to confer superior immunocompetence especially antiviral and antibody responses[41].

PROBLEMS INHERENT TO BONE MARROW TRANSPLANTATION

Bone marrow transplantation mandates a conditioning regime to free spaces or niches in the host to accommodate the transplanted stem cells. This mandates a preconditioning to "create" this space with either chemotherapy or irradiation[42]. This situation does not exist for solid visceral transplantation.

The immunocompetent cells in the transplant may be directed against antigens present on the host tissues leading to GVHD, a situation that is uncommon following solid visceral transplantation. BMT is also associated with a period of immunodeficiency.

In addition to the incompatibility of the major and minor histocompatibility antigens, haematopoeitic histocompatibility (Hh) antigens, which are present on marrow cells, are also involved in the rejection of Bone marrow grafts[43].

REJECTION OF BONE MARROW GRAFTS

This occurs when mature T cells are removed from the graft or the preconditioning regimen is inadequate and is usually consequent upon histocompatibility and or Hh antigen mediated.

Both cellular and humoral factors have been implicated in the rejection of marrow transplants. The evidence suggests that rejection is mediated mainly by 3 types of cells[44,45]:

- Cytotoxic T cells (CD3+, CD8+)
- NK/LAK cells (CD3-, NK1.1+)
- TNK cells (CD3+, CD8-, NK1.1+)

NK Cells and BM graft rejection

NK cells are mononuclear cells. Originally described as "null cells", they usually display a morphology of large granular lymphocytes (LGL). The definition of NK cells by morphology is however inadequate because all LGL's are not NK cells and vice-versa. In human peripheral blood and in mice, the spleen is the principal site where NK cells

are located[46]. In mice, NK cells are phenotypically CD3-, TCR-, sIg-, NK1+, AsialoGM+, CD16 (Fcγ111)$^{+}$ LGL's. Although NK cells do not express full CD3 molecules, they have a CD3 subunit (CD3ζ) that is incorporated within their Fc receptor structure [47]. In addition, NK cells express medium affinity IL-2 receptors [47]. Functionally, NK cells are characterised by their lytic activity against tumour and virally infected cells that are non-MHC restricted and do not require prior sensitisation[48]. They also participate in the rejection of BM grafts.

In mice, NK cells originate from and differentiate in the bone marrow. They do not require the thymus for their differentiation. The IL-2 and IL-12 are major growth factors known to be active in inducing differentiation of NK cells in both humans and in mice[49, 50, 51]. The effects of IL-2 may be indirect because the activity of IL-2 on NK cell maturation can be abrogated when IFN-γ and TNF-α are blocked by antibodies. This demonstrates that IFN-γ and and TNF-α play a role in IL-2 mediated maturation of NK cells [51, 52].

NK cells are capable of producing lymphokines such as the IFN's, IL-2, IL-3, TNF's, B cell growth factors, TGF-β, and GM-CSF and other colony stimulating factors [53, 54].

NK cells mediate cytotoxicity through their lytic machinery composed of large cytotoxic granules. A surface marker, Ly-49, is present on some subset of murine NK cells [55]. The binding of Ly-49 with MHC class I molecules can result in a negative inhibitory signal to NK cells that renders the target cell resistant to NK cell mediated killing [55].

Rejection of BM grafts cannot occur in hosts if their NK cells are depleted. Much evidence proving the role of NK cells in BM rejection has come from experiments with SCID and athymic nude mice. Such mice are able to reject parental or allogeneic BM

grafts despite being devoid of T and B cells. This suggests that NK cells are not only necessary but are sufficient for BM rejection [56].

T cells and BM graft rejection

Rejection of BM grafts as mediated by T cells with classical markers such as $CD3^+$, Thy1+, CD4+, and CD8+ differ from that mediated by both the target antigens recognised and by the kinetics of rejection [57]. With NK cells, rejection is effected via the Hh antigens and usually occur rapidly [57]. For T cells, rejection is directed against the MHC antigens and is not as rapid as for NK cells [58,59]. T cells destroy BM grafts by either a direct cytotoxic action or indirectly by secreting cytokines that that stimulate macrophages to destroy BM cells [60].

T cells with NK markers (CD3+, NK1+) and BM rejection

Although T cells and NK cells are 2 distinct cell types, they may share some common markers. Cells expressing both NK and T cell markers are known as TNK cells or T cells with NK markers [46, 61]. Several reports have indicated that such cells are important effector cells in the acute rejection of allogeneic and parental BM grafts. These cells are said to utilise both TCRα/β for the recognition of MHC antigens and NK1.1 receptors for interaction with appropriate ligands on target tissues indicating two independent pathways [57, 58].

Graft Versus Host Disease (GVHD)

GVHD is a disease due to the immune reactivity exhibited by the donor against host alloantigens. It involves a series of interactions between various cells and cytokines of the immune system. GVHD frequently occurs as a fatal complication that is still considered the principle factor limiting the clinical use of allogeneic BMT. GVHD was first observed in irradiated mice receiving allogeneic spleen cells. GVHD, initially, was thought to be caused by post-irradiation injury and bone marrow aplasia [62]. Animals with GVHD developed a syndrome consisting of alopecia, weight loss, diarrhoea, liver abnormalities and ultimately, death. The classical requirements for the development of GVHD was set forth in 1956 and are as follows [63]:

- The graft must contain immunocompetent cells
- There must be transplantation barriers between the donor and the host
- The recipient must be immunoincompetent to react immunologically against the graft

GVHD can be separated into 2 different types based on clinicohistopathological criteria; acute and chronic.

In mice **acute GVHD** is characterised by weight loss, alopecia erythroderma, hunched posture, diarrhoea and death. Amongst these, the most important parameter is weight loss. Histologically, epithelial cell necrosis of the main target organs is the sine qua non [62]. The epidermis and hair follicles are injured and necrosed. In the liver, small bile ducts and periductular epithelium are affected. The base of the intestinal crypts are damaged.

Chronic GVHD is better described by histology than by clinical manifestations. Chronic GVHD can follow acute GVHD or can occur without prior acute GVHD. The histological

features include fibrosis and atrophy of one or more target organs. In the thymus, involution of medullary epithelial cells with total effacement of the cortico-medullary junction and disappearance of Hassals corpuscles are usually seen [64]. These can affect the thymic environment markedly culminating in defective T cell maturation. Subsequently, the thymus is unable to detect and delete autoreactive T cells rendering the animal susceptible to the emergence of autoimmune diseases.

Immunopathogenesis: Initially, GVHD was considered a consequence of T cell reactivity. Recent evidence suggests that the immunopathophysiology underlying GVHD involves a series of complex interactions in which cellular and cytokine components of the immune system are involved [65]. The cellular components comprise T cells, NK/LAK cells and macrophages. Amongst these, T cells are the prime mediators for the initiation of GVHD. NK/LAK cells and macrophages are important in the effector phase. The cytokine component of the disease comprise a multiplicity of substances such as IL-1, IL-2, TNFα that are able to damage target tissues [66, 67]. Kinetic studies performed in murine BM transplantation models involving MHC identical but minor antigen disparate mice have shown that during GVHD mRNA transcripts for IL-1 and TNFα increased several hundred and 4 – 6 fold respectively in target tissues [66]. Tissue injury during the conditioning period may also result in the release of some inflammatory cytokines [68]. As a consequence of this, increased MHC expression and upregulation of adhesion molecules is seen in host tissues. The pathophysiology of GVHD is frequently regarded as a cytokine storm [69].

GVHD and minor antigens: There are some conflicting reports regarding the T cell subsets responsible for GVHD in MHC compatible but minor antigen mismatched strain

combinations. Studies where T cells were depleted suggest that CD4+ T cells are critical for the development of GVHD [70]. Other investigators stressed the role of CD8+ T cells [71]. It is now agreed that immunogenetic and environmental factors will dictate which subset is important in the pathogenesis of GVHD [72].

Prevention of GVHD:

Donor T cell depletion: The most effective way of preventing GVHD is the removal of mature T cells from the donor innocula. Two methods are used; either seperation of T cells by lectine agglutination or depletion of T cells by coupling using Mab's together with either complement or toxins such as ricin. Both these methods are associated with a higher incidence of engraftment failure and leukaemia recurrence.

In vivo activation of Th2 subsets: It is hoped that by activating Th2 cells, the activation of Th1 cells will be blocked thereby abrogating GVHD. Stimulation of the Th2 subset can be accomplished by the injection of IL-4 alone or in combination with high doses of IL-2 [65].

Blocking T cell activation: Addition of non-mitogenic antiCD3-F(ab)2 fragments in vivo allows T cell functions to be altered without physical elimination [73]. Blocking the costimulatory pathway of T cell activation may also render the T cells inactive in vivo. This can be effected by the infusion of soluble CTLA-Ig (a fusion protein). It has been reported that lethal GVHD in the murine model can be consistently diminished employing these therapeutic strategies [74].

Induction of suppressor cells: Several reports have suggested that some therapeutic manoeuvres preventing GVHD may involve the generation of suppressor cells. Sykes et al have shown that GVHD can be effectively prevented by treating the recipient with

a short course of high doses of IL-2. The underlying mechanism seems to be the generation of suppressor cells [75].

GRAFT VERSUS LEUKAEMIA EFFECT (GVL)

It was first proposed in 1956 that BM transplantation had the potential ability to eradicate leukaemia independently from the pretransplant radiation regime and chemotherapy [76]. This effect is presently designated GVL activity. In humans, BMT is now effectively used to cure patients with acute myelogenic leukaemia, acute lymphoblastic leukaemia and chronic myelogenic leukaemia [77]. These beneficial effects of BMT are often offset by the emergence of GVHD. An association between GVHD and GVL was recognised and reported by the Seattle BM transplant group. They reported that the incidence of leukaemia relapse was significantly decreased in those patients in whom acute or chronic GVHD supervened compared to those patients in whom GVHD did not occur [76, 78]. This was supported by the observation that leukaemia relapse was more common after autologous or syngeneic BMT as documented by the International Bone Marrow Registry [79].

Antitumour effector mechanisms:

T cell mediated mechanisms: This mechanism is antigen specific, MHC restricted and the most potent mechanism described to date [77 - 81]. This is best observed in MHC mismatched situations. Both subsets of T cells have been shown to participate in the GVL effect. The Th2 subset has been shown to augment the GVL effect whilst it has been suggested that the Th1 subset is mainly responsible for the GVHD effects. However, there is controversy regarding the splitting of the effects and laboratory

results have demonstrated that the effects are intimately related to the strain combinations as well as tumour models employed [82].

Natural Killer (NK) and LAK cells: In contrast to T cells, NK cells can mediate a response without prior activation and without MHC restriction. This effect may be potentiated by cytokines such as IL-2. The role of these cells have been reported. NK cells are cells that respond most rapidly following BMT. Once activated by cytokines, they are called LAK cells and have a strong potential to eradicate or suppress tumour growth [83].

Cytokines: Many cytokines are able to enhance the GVL effects directly or indirectly. Amongst these, IL-2 is thought to be the most important. It enhances the antileukaemic ability of both T cells and NK cells. TNFα and IFN can increase NK cell activity directly. Additionally, the NK cell sensitivity for low levels of IL-2 can be augmented in the presence of TNFα and IL-1. In addition to the above, macrophages and inflammatory products are also reported to play a role [84].

Disassociation of GVL and GVHD: There are various clinical and experimental reports suggesting that GVL effects are at least partially separable from GVHD and that both phenomena may be mediated by different cell populations [76, 84]. Patients with AML receiving TCD allogeneic BMT showed lower relapse rates than patients receiving syngeneic BMT although both groups were free from GVHD implying an allogeneic GVL effect in the absence of GVHD [79]. In an animal model, it was demonstrated that a short course of IL-2 could inhibit GVHD with the preservation of a GVL effect [81].

Target cells for the GVL activity: It is possible that some minor antigens may act as targets for both GVHD and the GVL effect. If the GVL effect is a separate entity, it may

be concluded that there are tumour specific antigen determinants. A fusion protein known as p210 BCR-ABL is considered to be such a leukaemic specific antigen against which a class II restricted T cell response can be generated [85]. Hence, these independent GVL effects mediated by tumour specific clones can be utilised for clinical purposes.

CHAPTER 2

AIMS

Bone Marrow Transplantation has unequivocally demonstrated the potential to resolve genetic abnormalities as well as procure transplantation tolerance beyond its currently established roles. However, the application of BMT for more benign therapies demands a measure of predictability and safety. This study endeavoured to establish the mechanisms underlying transplantation tolerance in a semiallogeneic murine model employing different irradiation conditioning regimens to

- Determine the mechanisms underlying transplantation tolerance at the cellular level
- Determine the GVL effect in the different conditioning regimes after the elimination of GVHD
- Determine the relationship of the GVL effect with respect to the presence or absence of GVHD; that is, determine whether subclinical GVHD existed that accounted for the GVL effect.
- Determine the impact of complete chimaeras with respect to B lymphocyte tolerance in a semiallogeneic bone marrow model.

CHAPTER 3

MATERIALS AND METHODS

Mice:

The following strains of mice were used in these experiments. BALB/C (H-2d, Thy1.2+, Mls1b/2a), C3H (H-2k, Thy1.2+, Mls1b/2a) were purchased from Charles River, Sulzfield, Germany. AKR mice (H-2k, Thy1.1+, Mls1a/2b) were obtained from Bomholtgard Breeding Centre Ltd., Ry, Denmark. The recipients were housed in groups of 6 - 10 mice per cage under filter caps with twice weekly cage changes. The animals were kept in solid bottom plastic cages with a plastic top fitted with a filter. Standardised pellet chow and acidified drinking water were used as routine. Antibiotic (Tylan, Eli Lilly, Brussels, Belgium) was added to the drinking water one week before, during and one week after BMT.

Induction of chimaerism

- **Irradiation**

Recipient mice received either 9.5 Gy or 10.5 Gy of Total Body Irradiation (TBI) as a single dose or Total Lymphoid Irradiation (TLI) = {10 TLI + 2 TBI, each fraction consisting of 2 Gy as a daily fraction}. The irradiation was delivered by a 60 Cobalt source at a dose rate of approximately 0.3 Gy/min. The source to skin distance was approximately 100cm for the TBI group and 80cm for the TLI group. During TLI, the skull, lungs, kidneys, tail and long bones of the animals were shielded with lead blocks thus exposing the supra- and infradiaphragmatic lymph nodes, spleen and thymus to

the irradiation field. Shieldings were focal and made of MCP. Before TLI, all mice were placed in a prone position and after being induced with enflurane, anaesthesia was then maintained using Enflurane delivered and controlled by a semi-closed circuit inhalation system. TBI groups were irradiated without anaesthesia. Six mice were irradiated at a time.

- **Preparation of chimaeras**

One day after irradiation, the recipients were reconstituted with bone marrow cells. TBI mice were reconstituted with either T cell depleted allogeneic bone marrow cells (5 x 10^6 cells) or T cell depleted syngeneic BMT (5 x 10^6 cells) + non T cell depleted allogeneic bone marrow cells (15 x 10^6 cells) to circumvent the complication of GVHD. T cell depletion was carried out in vitro using monoclonal antibodies and low toxic rabbit complement.

TLI groups were reconstituted with non T cell depleted allogeneic BMT (15 - 30 x 10^6 cells).

Bone marrow cells were prepared by flushing the shafts of donor animal's femora and tibia (6 - 8 weeks old) using heparinised RPMI 1640^R with Glutamine and antibiotics. The cells were washed twice with RPMI, counted and suspended in 0.3ml ice cold RPMI and injected into the recipient via the tail vein.

- **Scoring of Chimaerism**

Scoring of chimaerism was performed by using a flow cytometric assay that had been previously demonstrated to identify chimaerism as low as 2%. Chimaerism was confirmed in the live animals with the use of monoclonal antibodies conjugated with phtcoerythrin (PE) and flouresceinated isothiocyanate (FITC) after aspirating 0.2 ml of

blood from the heart under anaesthesia. This confirmation was undertaken after the animals had regained the pre-irradiation weight and were clinically well and demonstrated no evidence of GVHD to confirm Engraftment in the absence of GVHD.

- **Determination of $Vb6^+$ cells**

This was determined by double colour FACS analysis after confirmation of engraftment as well as clinical confirmation of the absence of GVHD.

IN VITRO ASSAYS

- **Mixed Lymphocyte Reaction (MLR)**

The MLR is an in vitro proliferative response of T cells to allogeneic cell associated antigens. It was performed by culturing responder cells with allogeneic stimulator cells (irradiated) together in a medium at a concentration of 5×10^6 cells/ml and at a final volume of 200µl per well in 96 well flat bottomed microculture plates. The MLR medium was prepared with RPMI 1640 with glutamine with added antibiotics and 10% foetal calf serum (FCS). 5×10^{-5}M 2-mercaptoethanol was added. The stimulator cells were prepared by irradiating them with 3000 rads. Cells were cultured in quadruplicate and were always accompanied by negative controls (i.e. wells with responder cells alone and with stimulator cells alone). The cultures were incubated for 96 hours at 37°C in a 5% CO_2 humidified incubator. DNA synthesis was assayed by the addition of 1µCi [methyl-3h] thymidine per well and incubated for an additional 18 hours. Thereafter, the cells were harvested onto glass filter paper and the counts per minute (cpm) determined in a liquid scintillation counter. The Stimulation Index (SI) was used to assess the MLR. It was calculated as follows:

$$SI = \frac{\text{cpm of stimulated cells - cpm of unstimulated cells}}{\text{cpm of unstimulated cells}}$$

NK/LAK CYTOTOXIC ASSAYS

Standard Chromium release assays were used to determine NK/LAK cell activity. YAC-1 and BW-5147.3 (AKR mouse lymphoma) tumour cells were used as targets for NK and LAK cell mediated cytotoxic activity respectively. After 2 washes with RPMI 1640, $1x10^6$ target cells were resuspended in 0.4 ml of media containing RPMI + 5% FCS. The cells were then labelled with 200μci ^5Cr in loosely capped 15 ml conical tubes and were incubated at 37°C for 90 min in a 5% CO_2 incubator. Thereafter, the ^{51}Cr labelled target cells were washed 3 times with RPMI. The cells were resuspended in RPMI + 10%FCS and were adjusted to a concentration of $1x10^5$ /ml. The effector cells ($5x10^6$/ml; $5x10^5$/well) were mixed ^{51}Cr labelled target cells in a final volume of 0.2 ml per well (quadruplicate) in 96 well V bottom microtitre plates at an effector: target ratio of 50: 1. The cells were incubated in a 5% CO_2 incubator for 4 hours at 37°C. Thereafter, the plate was centrifuged for 2 minutes and 100 μl of supernatant was collected into counting vials and counted in a Gamma counter. Cell lysis was evaluated by measuring the amount of radioactivity released into the supernatant.

The percentage specific lysis was measured using the following formula:

$$\%\text{age lysis} = \frac{\text{mean cpm of experimental release - mean cpm of spontaneous release}}{\text{mean cpm of maximal release - mean cpm of spontaneous release}}$$

Spontaneous release was determined by incubating target cells in medium only and the maximum release was determined by incubating the target cells with detergent (saponine). LAK cells were generated by incubating single cell suspensions harvested from the spleens of the relevant sacrificed mice and incubating them in medium consisting of RPMI 1640 + antibiotics, 10% FCS, and $5x10^{-5}$ 2-mercaptoethanol. $10x10^{6}$ cells were cultured in a single flask at a concentration of $2x10^{6}$ cells/ml of medium in the presence of mouse IL-2 [1000 U/ml] for 4 days in a 5% CO_2 incubator at 37°C. Cells were then washed and adjusted to the appropriate concentration.

Suppressor Cell Assays

Culture conditions were the same as for the MLR except that $5x10^{5}$ putative suppressor cells (irradiated with 15 Gy) were cocultured with responder and stimulator cells in a final volume of 0.3 ml/well for 120 hours before pulsing with thymidine.

Preparation of Polyclonal Antisera

Donor mice were anaesthetised with ether. The abdominal skin was scrubbed with 70% alcohol. Full thickness skin of approximately 1 cm^2 was excised and preserved immediately in ice cold PBS. Recipient mice were anaesthetised with ether. The hair was shaved off the flank. A 1 cm^2 area of skin was excised and the donor skin was engrafted and held in place with the placement of silk sutures. The grafted area was dressed with paraffin gauze, gauze, and plaster of paris. After 7 days, the bandages were removed to confirm engraftment and monitor for graft rejection. Once rejection

was observed, the recipients were immunised with thrice weekly intraperitoneal injections of $50x10^6$ irradiated allogeneic spleen cells in complete Freunds adjuvant (CFA). Ascitic fluid was collected, sterilised with filtration and purified by ammonia precipitation.

Histological examination

Histological examination of the viscera of the experimental groups were performed to clarify the presence of GVHD or tumour infiltration. Dead mice or sacrificed mice were dissected and specimens taken were the heart, lungs, liver, spleen, small bowel, large bowel, pancreas, bone marrow and brain. Harvested specimens were kept in Bouins medium for a week, thereafter, the specimens were dehydrated and then sectioned and stained.

Induction of GVHD

GVHD was induced to assess in vivo the T cell reactivity against major or minor histocompatible antigens. The following protocols were used to induce GVHD:

A single spleen cell suspension was prepared in MEM. 40×10^6 in 0.3 ml medium was used to induce GVHD. The cells were injected intravenously through the lateral tail vein that was distended using an heating lamp. The following criteria were used for the evaluation of GVHD

- Clinically: weight loss, hunched back, diarrhoea and alopecia
- By monitoring the survival curves
- By histopathological examination of the skin, intestine, liver, spleen and lymph nodes.

Histologic criteria:

- Liver: mononuclear cell infiltrate in the peripheral zone
- Spleen: depletion of lymphocytes in the white pulp and the loss of germinal centers
- Lymph nodes: aplasia of lymph nodes and absence of germinal centres and proliferation of lymphoid cells in the paracortical zone
- Skin and intestines: mononuclear cell infiltrate

Tumour cell titration assay

To determine the number of AKR tumour cells needed to cause lethal tumour development in AKR mice, BW 5147.3 tumour cells in MEM were injected intravenously into control mice. The mice were then observed daily for mortality. Different numbers of tumour cells were injected intravenously into normal control AKR, C3H and BALB/c mice. C3H and BALB/c mice remained healthy and never developed any tumour after infusion of 6×10^6 tumour cells. At the maximum dose used in this experiment, all AKR mice succumbed to tumour by three weeks.

Induction of BM chimeras (MC) were constructed by infusing $15X10^6$ C57Bl/10 BM cells into lethally irradiated (10.5 Gy of total body irradiation) F1 recipient mice. In all MC, between 15% and 49% of host-type peripheral blood leukocytes were shown to persist after BM transplantation using a sensitive flow cytometry assay (13, 14). Complete chimeras (CC) were made by infusion of only $20X10^6$ C57BL/10 BM cells. In CC, the presence of remaining host-type peripheral blood lymphocytes could not be demonstrated, despite multiple determinations. In all BM transplantation experiments, T cell depletion of the BM cells was achieved before infusion using anti-Thy monoclonal antibodies (Becton Dickinson, Mountain View, CA) and rabbit complement

(Cedarlane, Ontario, Canada). After BM transplantation, the animals were kept in specific pathogen-free conditions and were free from clinical and histological signs of graft-versus-host disease.

Immunization of chimeras. Three months after BM transplantation, chimeric and control mice were immunized intraperitoneally once a week for 3 weeks with $50X10^6$ irradiated (30 Gy) host-type (BALB/c), donor-type (C57BL/10), or third-party-type (C3H) spleen cells (four mice per group for each antigenic stimulus). Ten days later, splenocytes were taken for mixed lymphocyte reaction (MLR) tests performed with immunizing cells as stimulator cells; sera were analyzed for the presence of antibodies directed against the immunizing cells.

Detection of IgG alloantibody formation. Alloantibody detection was performed using a modification of the flow cytometry crossmatch technique, as we described previously (14). Briefly, $1X10^6$ target spleen cells in 0.1ml of saline solution were Incubated with 0.1ml of various dilutions of serum from non-immunized and immunized mice. Thereafter, the cells were washed and incubated for an additional 20 min with 0.1 ml of various dilution of serum from non-immunized and immunized mice. Thereafter, the cells were washed and incubated with 15 ul of Fluorescein Isothiocyanate-conjugated goat antimouse1gG Fc (The Binding Site, Birmingham, England) for 20 min. This second step detects only mouse 1gG and hence does not stain mouse B cells as only a negligible fraction of them express 1gG in mice. Serum titers were scored positive when they showed a significant shift in the mean fluorescein intensity by flow cytometry (FACScan, Becton Dickinson).

Detection of alloantibodies directed against MHC class I or class II expressing cells. Because murine T lymphocytes express only MHC class I antigens and not MHC class II antigens, anti-MHC class I-directed antibodies were assayed by two-color flow cytometry using anti-L_3T_4 (Becton Dickinson) Phycoerythrin-coupled monoclonal antibody (directed against the murine $CD4^+$ T lymphocyte subset) and Flourescein Isothiocyanate-conjugated anti-mouse IgG (Fc) monoclonal antibody. For assaying reactivity on class II-expressing cells, relevant anti-MHC class II-reacting anti-I-A^b, -I-A^d biotin-conjugated antibodies (PharMingen, San Diego, CA) were used.

Purification of T and B lymphocytes. T lymphocytes and B lymphocytes were purified from splenocyte suspensions using a panning technique. Rabbit anti-mouse 1g was first diluted to 10 ul per 10 ml of phosphate-buffered saline (PBS).

Petri dishes (Falcon, Becton Dickinson, Belgium) were coated with 10 ml of this solution and kept at 4° C overnight. The following morning, the solution was poured off and the Petri dishes washed three times with PBS. A single suspension of spleen cells was prepared in RPMI medium containing 10% fetal calf serum plus antibiotics. Six milliliters of medium containing $100X10^6$ cells were added to each Petri dish and kept for 30 min at room temperature. Thereafter, the Petri dishes were rotated for 30 sec. To isolate the T cell population, non-adherent cells were poured into a second antibody-coated Petri dish and kept for another 30 min at room temperature. Thereafter, the non-adherent cells were collected into a tube. To isolate the B cell-enriched populations, the Petri dishes containing the adherent B cells were first washed three times with sterile PBS to remove remaining T cells. Thereafter, 5 ml of culture medium containing 10% normal mouse serum were added and incubated at 37°

C for 1 hr in order to selectively dislodge the adherent B cells. B cells were then collected by pipetting into a tube and were washed two times with RPMI. This panning technique resulted in highly purified populations (>95% purity) of T and B lymphocytes. Where necessary, purification of donor lymphocytes from MC was done subsequently using C57BL anti-BALB/c alloantibodies and rabbit complement. After this procedure, no remaining BALB/c cells could be detected by flow cytometry.

Transfer experiments. For the transfer experiments, lethally irradiated (12 Gy) control C57BL/10 mice were given $15X10^6$ purified T cells and $15X10^6$ purified B cells intravenously from various origins. One week later, the mice were immunized intraperitoneally once a week for 2 weeks with $1X10^8$ irradiated (30 Gy) BALB/c or C3H spleen cells. A week after this, the serum was taken for alloantibody detection. Alloantibodies were scored using flow cytometry as described above.

CHAPTER 4

MECHANISMS UNDERLYING TRANSPLANTATION TOLERANCE IN MIXED BONE MARROW CHIMERAS IN A SEMIALLOGENEIC COMBINATION INDEPENDENT OF GRAFT VERSUS HOST DISEASE

INTRODUCTION

Bone Marrow Transplantation (BMT) is now an established procedure in clinical medicine. It has been successfully used to salvage patients from intensive cytoablative therapy especially for haematological malignancies. This success, especially with successful allograft transplantation, has alerted clinicians to the application of this form of therapy for a more diverse group of conditions such as immunodeficiencies, metabolic deficiencies, auto-immune disorders, benign haematological disorders, genetic abnormalities and the most recent, inducing donor specific tolerance. In addition, oncologists are now exploring the potential of BMT to salvage patients with solid tumours following more intensive cytoablative therapy.

These potential applications have not been universally adopted in clinical practice because BMT is fraught with problems. The most sinister complications limiting the use of BMT are Engraftment failure, the ravages of the conditioning regime, Graft Versus Host Disease (GVHD), and Graft Rejection. The literature attests to the magnitude and prevalence of these problems. The lack of alternative, consistently successful therapy for these conditions has prompted research into BMT to enable a more liberal clinical application.

Whilst immunosuppressive therapy has evolved to become more precise, optimum conditioning regimens, quality of the Bone Marrow Transplant and the mechanisms of Engraftment and GVHD need to be determined to allow clinicians greater access to this therapy.

AIMS

This study was undertaken to

1. Attempt to establish the mechanisms sustaining transplantation tolerance in a semiallogeneic murine model using different conditioning regimens with the elimination of GVHD
2. To determine the immunocompetence of the different regimens

MATERIALS AND METHODS

Mice: 6 - 12 week old mice were purchased. C3H/HeJ (H-2k, Thy 1.2, Mls 1b/2a) and Balb/c (H-2d, Thy 1.1, Mls 1b/2a) were purchased from Charles River (Sulzfield, Germany) and AKR/J (H-2k, Thy 1.2, Mls 1a/2b) were purchased from Bomholtgard Breeding Center Ltd. (Ry, Denmark)

Recipient mice were housed in plastic cages fitted with a filter cap, had sawdust bedding and housed in a pathogen free environment. Pellet chow and acidified drinking water was the standard diet. Antibiotic (Tylan, Eli Lilly, Brussels, Belgium) was added to the drinking water 1 week prior to BMT and was continued for a week following it.

Irradiation: Recipient mice received either 9.5 Gy Total Body Irradiation (TBI) as a single dose or Total Lymphoid Irradiation (TLI) as 12 daily fractions (10 TLI + 2 TBI) of

2 Gy each as described previously. Irradiation was delivered by a 60 Cobalt source (Gammatron, Siemens) at a low dose rate. The source to skin distance for the TBI was 100 cm and for mice receiving TLI, the distance was 80 cm. Mice receiving TLI were anaesthetised; they were induced with enflurane and then maintained on anaesthesia in a prone position using Enflurane (Abbott, s.p.a., Campoverde-LT, Italy) delivered by a semi-closed inhalation anaesthetic system. The skull, long bones, kidneys, lungs and tail were shielded with lead blocks confining irradiation to the thymus, spleen, supra- and infradiaphragmatic lymph nodes. A maximum of 6 mice were irradiated simultaneously.

BMT: To prepare stable mixed chimeras with the elimination of GVHD, the recipient mice were reconstituted with donor cells in the following manner:

TBI chimeras were prepared with 5 x 10^6 T cell depleted (TCD) allogeneic bone marrow. TCD was performed in vitro using Thy 1.1 (Serotech, Oxford, UK) or Thy 1.2 (Sigma Chemie, GmbH, Drisenhof, Germany) antibodies and low toxic rabbit complement (Cedarlane, Hornby, Ontario, Canada).

TLI chimeras were prepared with 15 x 10^6 non-TCD allogeneic bone marrow.

Bone marrow cells were harvested by flushing the shafts of the sacrificed donor animals with a solution of RPMI with antibiotics and 1% heparin. The cells were washed twice with RPMI with added antibiotics. The viable cell count was determined by staining with Trypan blue and counted in a Burkër haemocytometer. Cells were kept on ice throughout. The cells were reconstituted to the relevant concentration such that 0.25 ml was injected into the tail vein of the recipient, with the appropriate number of cells.

Determination of chimerism and Vβ6cells

Chimerism was determined in a Flourescein Activated Cell Sorter (FACS) assay using Thy 1.1 (Serotech, Oxford, UK), and Thy 1.2 (Sigma Chemie, GmbH, Drisenhof, Germany) monoclonal antibodies (mAbs) conjugated with Phycoerythrin (PE) and Flourescein Isothiocyanate (FITC) respectively. These are lymphocyte markers specifically confined to either donor cells or recipient cells depending on the direction of the transplantation.

Vβ6 cells were evaluated in the CD4 window (Caltag lab, San Francisco) with Vβ6 mAb (Pharmingen, Antwerp, Belgium) and percentages were estimated in the peripheral blood lymphocytes, thymus, splenic lymphocytes and lymph node lymphocytes.

Mixed Lymphocyte Cultures (MLC):

Responder cells: Single cell suspensions were made from the spleen/s of the responder mice by teasing them into fragments with forceps in RPMI on ice and then passed through a 100 μm cell strainer and washed twice with RPMI with added antibiotics. The viable cell count was determined. The cells (maximum of 10^8 cells) were incubated at 37°C in a 5% CO2 incubator in 2 ml of RPMI with 5% foetal calf serum (FCS) in a nylon wool syringe. This syringe was previously washed with 50 ml of the same solution and kept in the incubator for a minimum of 1 hour prior to use. The enriched T cells were eluted. This was done by simply washing the non-adherent T cells from the syringe by passing 20 ml of the medium through the syringe and collecting the fluid under sterile conditions. The eluted cells were counted and made up

to a concentration of 5 x 10^6 cells/ml in RPMI with 10% FCS and 2ME (Mercaptoethanol).

Stimulator cells: Single cell suspensions were prepared from the spleen/s of the mice as described as above. The cells were counted and made up to a concentration of 5 x 10^6 cells/ml in RPMI with 10% FCS and 2ME. These cells were irradiated with 3000 Rads and kept in the incubator until use.

Chimeric cells: Cells of donor or recipient origin form the chimeric animals were isolated from single spleen cell suspensions by incubating the spleen cells with polyclonal antibodies* in RPMI with 20% FCS. After incubation, the cells were washed twice with RPMI to remove excess unbound antibodies and the cells were then incubated with low toxic rabbit complement for 45 minutes in a 37°C water bath. The cells were washed twice with RPMI. Cell counts were determined and the cells were reconstituted to yield a concentration of 2,5 x 10^6 cells/ml in RPMI with 10% FCS and 2ME. These cells were irradiated with 1500 Rads and were added to the MLC to determine their influence on it.

Vβ6cells: These cells were isolated from single cell suspensions from the thymus of chimeric AKR mice using magnetic beads to isolate the cells and then bringing them to a concentration of 2.5 x 10^6 cells/ml.

The cells were plated into 96 well flat bottom plates; 5 x 10^5 responder cells and 5 x 10^5 stimulator cells were added to the wells in the experiments. Four experiment wells were performed and the mean values were used. Chimeric cells were added i.e. either of donor or recipient origin to determine their influence on this reaction, again performed

in 4 wells and the mean value taken. Control wells comprised cells from each group without the addition of other cells.

The cells were incubated for a period of 96 hours in an incubator. Thereafter, 10:l of [H^3] Thymidine was added to each well and the cells were incubated for an additional 16 hours after which the cells were harvested and the radioactivity measured. Results were interpreted as the mean of the experimental counts minus the sum of the mean counts of the relevant control cells.

Polyclonal antisera: Polyclonal antibodies were raised by skin grafting the mice with skin from the allogeneic mice against which antibodies were to be raised. Once rejection occurred, the grafted mice were injected repeatedly with 5 x 10^6 donor haematopoeitic cells in Complete Freunds Adjuvant intraperitoneally until the development of ascites. The ascites was harvested, centrifuged, filtered by passage through a 100μm filter and stored at -20°C. The concentration of the antibodies necessary was established by titration against a known concentration of cells.

Cotransfer experiments

Recipient mice were lethally irradiated (10,5 Gy) and injected with 40 x 10^6 splenocytes of donor origin and simultaneously with tolerant splenocytes of either donor or recipient origin from the chimeric animals also at a dose of 40 x 10^6 cells. The 5 groups were:

Group 1: Donor splenocytes alone

Group 2: Donor splenocytes + Chimeric TBI donor splenocytes

Group 3: Donor splenocytes + Chimeric TBI recipient splenocytes

Group 4: Donor splenocytes + Chimeric TLI donor splenocytes

Group 5: Donor splenocytes + Chimeric TLI recipient splenocytes

RESULTS

RESPONSE TO IRRADIATION AND TRANSPLANTATION

All mice subjected to the conditioning regimens lost significant weight following irradiation and transplantation. However, this was regained to pretransplant conditioning levels by the end of the second week confirming the absence of GVHD.

CHIMERISM

All mice subjected to BMT were confirmed via a Flouresceinated Activated Cell Sorter (FACS) assay performed on blood taken from direct cardiac puncture after the mice were anaesthetised with ether. The extent of the chimerism was a mean of 52% (44 - 60) in the TBI group and a mean of 46% (30 - 52) in the TLI group. All mice were healthy; they displayed no features of GVHD such as alopecia, hunched back or diarrhoea. The weight loss imparted by the conditioning regime was rapidly recruited following BMT by the end of 2 weeks.

CLONAL DELETION

Vβ6 cells normally comprise 9 - 11% of the CD4 population of the lymphocytes of C3H mice and are absent in AKR mice. They are normally reactive to the Mls antigen present in the AKR mice. This allowed for the Vβ6 cells to be evaluated in the separate models to determine whether clonal deletion played a role in this model of transplantation tolerance.

In the TLI mice, Vβ6 cells were deleted in both groups; i.e. they were not evident in the C3H mice who received AKR bone marrow and were not present in the AKR mice when these had received C3H marrow. This confirms that clonal deletion is an important mechanism underlying transplantation tolerance when TLI is used as a conditioning

regime and deletion occurs in both the donor marrow as well as in the recipient that is reactive to the donor cells.

When C3H marrow was transplanted into AKR mice conditioned with TBI, clonal deletion of Vβ6 cells was demonstrated. However, these mice were transplanted with TCD bone marrow and this may reflect the inability of the immature cells of donor origin to proliferate into mature T cells thereby facilitating their deletion by host cells since they are reactive to host cells. This also suggests that clonal deletion is one of the mechanisms sustaining transplantation tolerance in this model after conditioning with TBI.

In sharp contrast to the phenomenon seen in the previous groups, C3H mice transplanted with TCD bone marrow of AKR origin following TBI show a proliferation of Vβ6 cells. This was evident in all compartments i.e. they were present in increased percentages in the peripheral blood, thymus, spleen and lymph nodes (Table 1&2). The significance of this cell proliferation was determined in the in vitro assays.

Table 1

TYPE OF CHIMAERA	**Vβ6 cells**
C3H (BM) into AKR (TBI) TCD	DELETED (0%)
C3H (BM) into AKR (TLI) NTCD	DELETED (0%)
AKR (BM) into C3H (TBI) TCD	PROLIFERATION
AKR (BM) into C3H (TLI) NTCD	DELETED (0%)

Presence of Vβ6 cells in chimaeras

Table 2

	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5
PBL	22	36	35	36	20
Lymph node	6	5	11	16	17
spleen	26	19	25	26	26
thymus	34	44	50	33	21

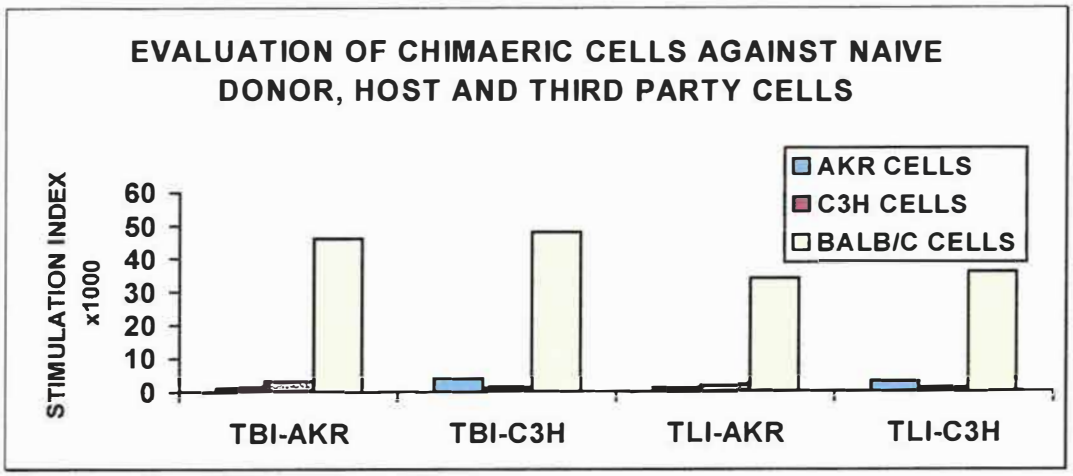
Percentage of Vβ6 cells in TBI chimaeras

IN VITRO ASSAYS

Confirmation of tolerance:

MLC done with responder cells from the chimeric animal spleen cells against irradiated spleen cells of naïve donor and recipient animals confirmed that no proliferative response was generated. This confirmed that transplantation tolerance was achieved. To establish that this did not reflect an immunosuppressive state, the chimeric cells were used as responder cells against third party antigens. Irradiated Balb/c splenocytes were used as stimulator cells and both the TLI chimeras as well as the TBI chimeras achieved significant proliferative responses against these antigens (Fig 1).

Fig. 1



Test Statistics[a,b]

	TBIAKR	TBIC3H	TLIAKR	TLILBH
Chi-Square	9.846	9.846	9.846	9.846
df	2	2	2	2
Asymp. Sig.	.007	.007	.007	.007

a. Kruskal Wallis Test

b. Grouping Variable: STRAIN

Descriptive Statistics - For Strain AKR

	N	Minimum	Maximum	Mean	Std. Deviation
TBIAKR	4	932.00	1055.00	994.0000	50.2195
TBIC3H	4	3978.00	4052.00	4004.5000	34.2394
TLIAKR	4	807.00	992.00	867.7500	83.9697
TLILBH	4	2809.00	3211.00	3002.2500	168.8735
Valid N (listwise)	4				

Descriptive Statistics - For Strain C3H

	N	Minimum	Maximum	Mean	Std. Deviation
TBIAKR	4	2824.00	3397.00	3147.2500	241.2238
TBIC3H	4	942.00	1102.00	1015.2500	77.6375
TLIAKR	4	1876.00	2234.00	2040.2500	171.7525
TLILBH	4	914.00	1067.00	977.5000	68.8985
Valid N (listwise)	4				

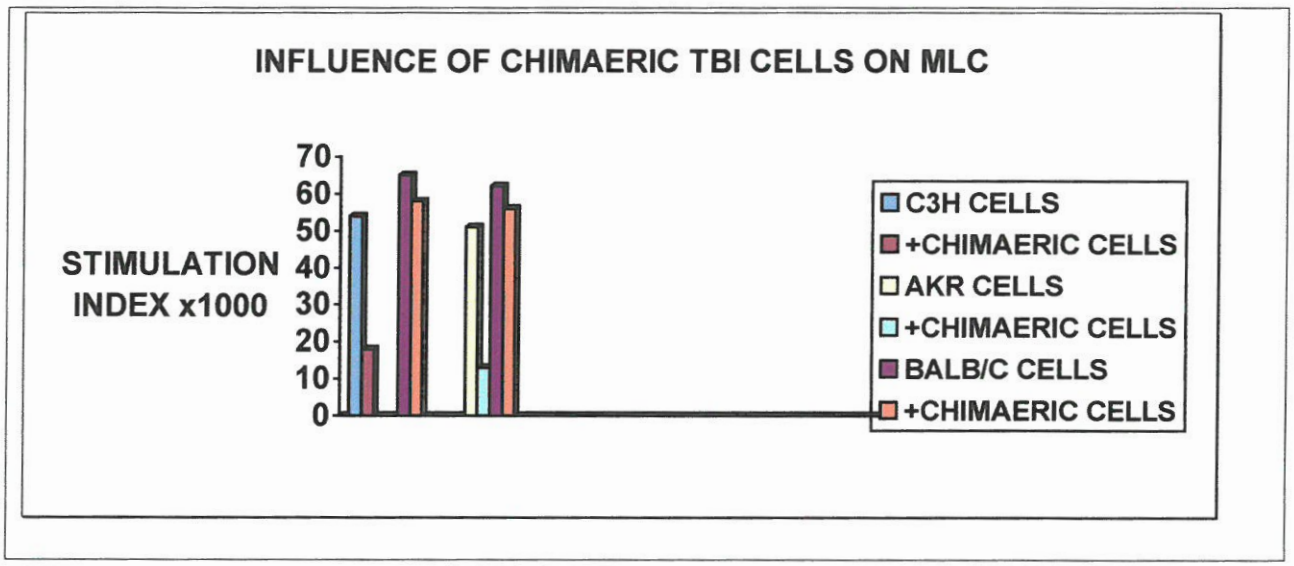
Descriptive Statistics - For Strain BALB / C

	N	Minimum	Maximum	Mean	Std. Deviation
TBIAKR	4	47550.00	48980.00	48115.00	614.9526
TBIC3H	4	47459.00	48242.00	47828.25	406.5287
TLIAKR	4	33914.00	34082.00	33998.75	80.0307
TLILBH	4	35807.00	36163.00	36007.00	148.2700
Valid N (listwise)	4				

Evaluation of the influence of the chimeric cells on the MLC of naïve donor and recipient splenocytes

When spleen cells from naïve donor animals were used in a MLC against naïve recipient cells or vice-versa, a good proliferative response was attained. However, the addition of cells of either donor or recipient origin from the TBI chimeras resulted in a significant depression in the response. This is suggestive of a suppressive mechanism also responsible for sustaining transplantation tolerance. To establish that this was specific to the mechanisms sustaining tolerance, these cells were added to a MLC against third party antigens (Balb/c splenocytes). This did not result in an abrogation of the response (fig. 2)

Fig. 2



Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
AKR	4	17907.00	18112.00	18003.50	93.1969
Valid N (listwise)	4				

Ranks

	STRAIN	N	Mean Rank	Sum of Ranks
AKR	1.00	4	5.50	22.00
	2.00	4	3.50	14.00
	Total	8		

Test Statistics[b]

	AKR
Mann-Whitney U	4.000
Wilcoxon W	14.000
Z	-1.155
Asymp. Sig. (2-tailed)	.248
Exact Sig. [2*(1-tailed Sig.)]	.343[a]

a. Not corrected for ties.

b. Grouping Variable: STRAIN

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	49886.00	50047.00	49975.00	72.8331
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	10947.00	11107.00	11019.00	70.0904
Valid N (listwise)	4				

Ranks

	STRAIN	N	Mean Rank	Sum of Ranks
C3H	3.00	4	6.50	26.00
	4.00	4	2.50	10.00
	Total	8		

Test Statistics[b]

	C3H
Mann-Whitney U	.000
Wilcoxon W	10.000
Z	-2.309
Asymp. Sig. (2-tailed)	.021
Exact Sig. [2*(1-tailed Sig.)]	.029[a]

a. Not corrected for ties.

b. Grouping Variable: STRAIN

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	61977.00	62107.00	62035.50	56.2761
AKR	4	66487.00	66600.00	66553.25	50.2154
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	54400.00	54800.00	54664.75	180.6237
AKR	4	59750.00	59837.00	59788.00	38.8072
Valid N (listwise)	4				

Ranks

	STRAIN	N	Mean Rank	Sum of Ranks
C3H	5.00	4	6.50	26.00
	6.00	4	2.50	10.00
	Total	8		
AKR	5.00	4	6.50	26.00
	6.00	4	2.50	10.00
	Total	8		

Test Statistics[b]

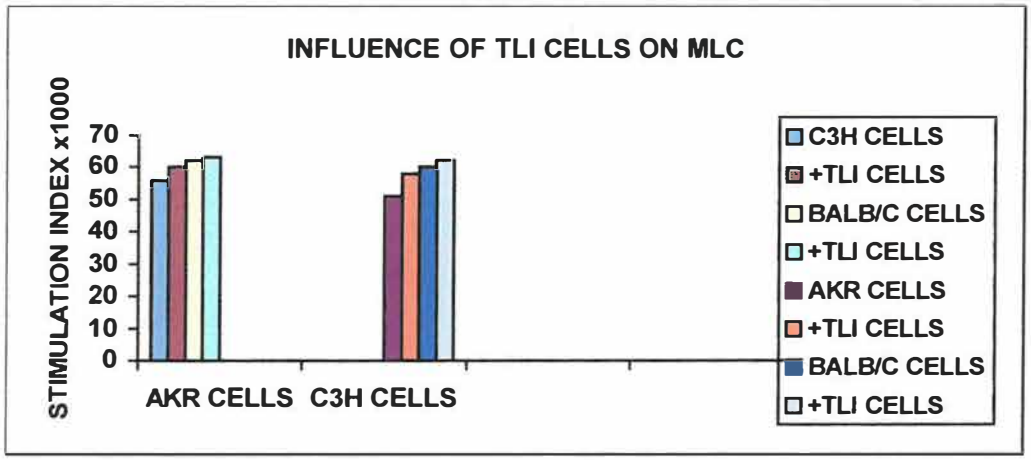
	C3H	AKR
Mann-Whitney U	.000	.000
Wilcoxon W	10.000	10.000
Z	-2.309	-2.309
Asymp. Sig. (2-tailed)	.021	.021
Exact Sig. [2*(1-tailed Sig.)]	.029[a]	.029[a]

a. Not corrected for ties.

b. Grouping Variable: STRAIN

When this experiment was performed using cells of either donor or recipient origin from the TLI chimeras the response was increased. This may be explained on the basis of better antigen presentation when the added cells are responder cells in origin or, on the basis of a greater volume of antigen if the added cells are of stimulator origin. This suggests that suppression is unlikely to play a role in the transplantation tolerance. Furthermore, tolerance is unlikely to be based on the presence of veto or suppressor cells as suggested by this MLC result (fig. 3).

Fig. 3



Ranks

	STRAIN	N	Mean Rank	Sum of Ranks
AKR	1.00	4	2.50	10.00
	2.00	4	6.50	26.00
	Total	8		

Test Statistics[b]

	AKR
Mann-Whitney U	.000
Wilcoxon W	10.000
Z	-2.309
Asymp. Sig. (2-tailed)	.021

b. Grouping Variable: STRAIN

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
AKR	4	56100.00	56247.00	56141.75	70.3438
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
AKR	4	58547.00	58696.00	58623.50	63.3535
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	50979.00	51104.00	51044.75	51.5841
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	57768.00	57971.00	57874.25	83.1560
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	50979.00	51104.00	51044.75	51.5841
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	57768.00	57971.00	57874.25	83.1560
Valid N (listwise)	4				

Test Statistics[b]

	C3H
Mann-Whitney U	.000
Wilcoxon W	10.000
Z	-2.309
Asymp. Sig. (2-tailed)	.021
Exact Sig. [2*(1-tailed Sig.)]	.029[a]

a. Not corrected for ties.

b. Grouping Variable: STRAIN

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
AKR	4	60440.00	60583.00	60492.50	66.0126
C3H	4	60729.00	60850.00	60798.50	50.5602
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
AKR	4	61700.00	61809.00	61750.25	48.5481
C3H	4	62107.00	62494.00	62345.75	171.7389
Valid N (listwise)	4				

Test Statistics[b]

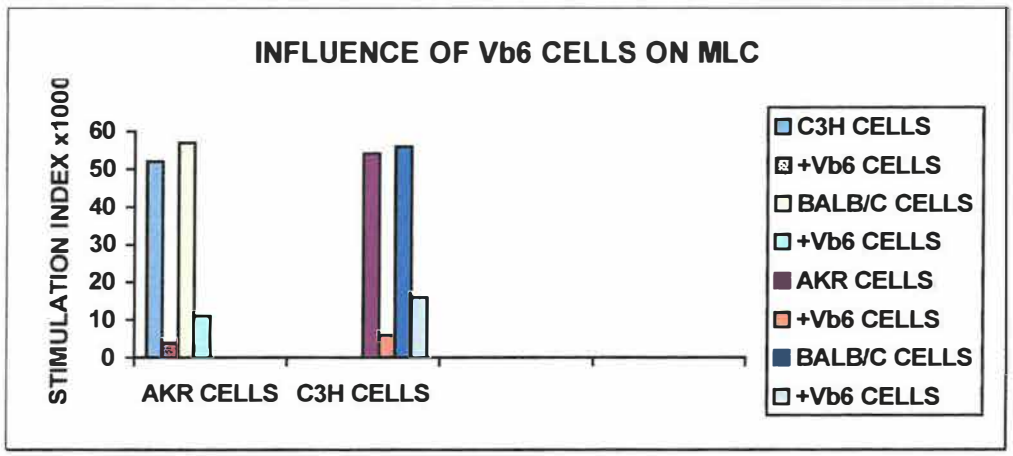
	C3H	AKR
Mann-Whitney U	.000	.000
Wilcoxon W	10.000	10.000
Z	-2.309	-2.309
Asymp. Sig. (2-tailed)	.021	.021
Exact Sig. [2*(1-tailed Sig.)]	.029[a]	.029[a]

a. Not corrected for ties.

b. Grouping Variable: STRAIN

To establish the significance of the Vβ6 cells that were expanded in the TBI chimeras, single positive (CD4) Vβ6 cells were added to the naïve response. These cells resulted in a significant depression of the response irrespective whether the responder cells were of donor or recipient origin. This suppression was found to be non-specific as it abrogated the response even when third party antigens were used (fig. 4)

Fig. 4



Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
AKR	4	50847.00	51107.00	50973.25	108.5798
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
AKR	4	443.00	643.00	558.2500	86.2839
Valid N (listwise)	4				

Ranks

	STRAIN	N	Mean Rank	Sum of Ranks
AKR	1.00	4	6.50	26.00
	2.00	4	2.50	10.00
	Total	8		

Test Statistics[b]

	AKR
Mann-Whitney U	.000
Wilcoxon W	10.000
Z	-2.309
Asymp. Sig. (2-tailed)	.021
Exact Sig. [2*(1-tailed Sig.)]	.029[a]

a. Not corrected for ties.

b. Grouping Variable: STRAIN

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	51846.00	52110.00	52003.50	127.4088
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	779.00	954.00	883.5000	74.1463
Valid N (listwise)	4				

Ranks

	STRAIN	N	Mean Rank	Sum of Ranks
C3H	3.00	4	6.50	26.00
	4.00	4	2.50	10.00
	Total	8		

Test Statistics[b]

	C3H
Mann-Whitney U	.000
Wilcoxon W	10.000
Z	-2.309
Asymp. Sig. (2-tailed)	.021
Exact Sig. [2*(1-tailed Sig.)]	.029[a]

a. Not corrected for ties.

b. Grouping Variable: STRAIN

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	53097.00	53400.00	53202.25	139.4426
AKR	4	51400.00	51797.00	51569.25	173.6076
Valid N (listwise)	4				

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
C3H	4	1350.00	1566.00	1458.0000	94.6150
AKR	4	846.00	1004.00	947.5000	72.9498
Valid N (listwise)	4				

Ranks

	STRAIN	N	Mean Rank	Sum of Ranks
C3H	5.00	4	6.50	26.00
	6.00	4	2.50	10.00
	Total	8		
AKR	5.00	4	6.50	26.00
	6.00	4	2.50	10.00
	Total	8		

Test Statistics[b]

	C3H	AKR
Mann-Whitney U	.000	.000
Wilcoxon W	10.000	10.000
Z	-2.309	-2.309
Asymp. Sig. (2-tailed)	.021	.021
Exact Sig. [2*(1-tailed Sig.)]	.029[a]	.029[a]

a. Not corrected for ties.

b. Grouping Variable: STRAIN

INFLUENCE OF ALLOGENEIC TUMOUR INNOCULATION

The suppressive response demonstrated in vitro by both the host and donor cells from the chimeric TBI chimeras was unexpected. This strongly challenged the widely held view that mixed chimeras have a superior immunocompetence to either donor or recipient individually. To establish the magnitude of this suppression, C3H TBI recipients confirmed to be chimeric and healthy were injected with tumour cells BW 5147.3. These cells are from a lymphoma/ leukaemia AKR line. Normally, this tumour innoculation has no deleterious effects on C3H mice. However, in the TBI mice of C3H origin reconstituted to mixed chimeras with AKR/J bone marrow, all the mice succumbed to tumour innoculation by 22 days (fig. 5). Two phenomena that were recognised in this experiment were engraftment and susceptibility.

Fig. 5

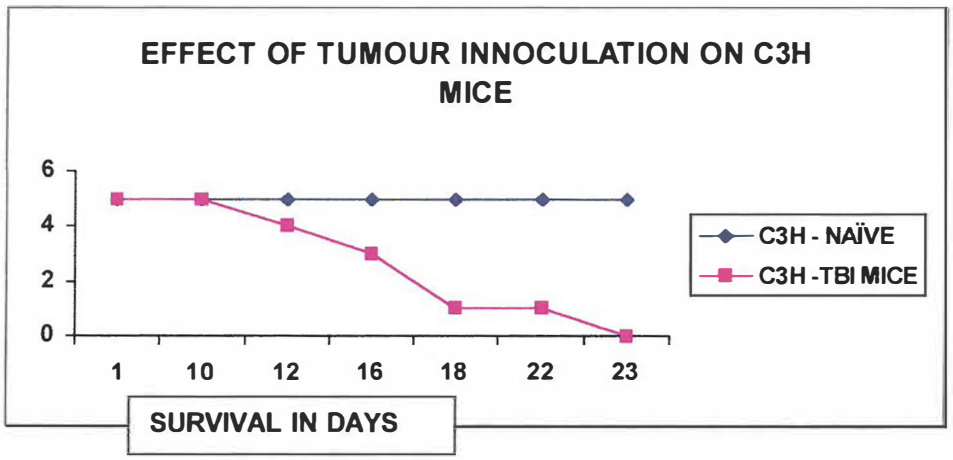
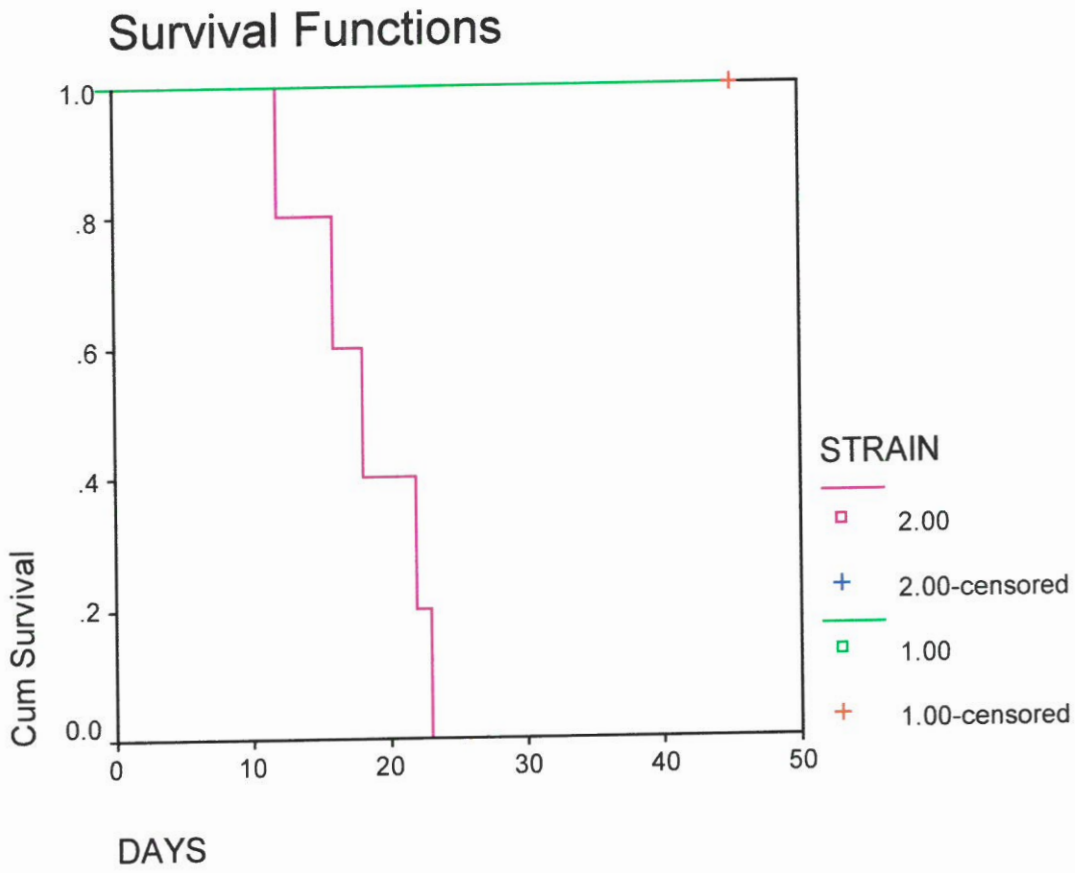


Fig. 5



	Survival Time	Standard Error	95% Confidence Interval
Mean:	18.20	2.01	(14.26, 22.14)
Median:	18.00	2.19	(13.71, 22.29)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	18.20	2.01	(14.26, 22.14)
Median:	18.00	2.19	(13.71, 22.29)

COTRANSFER OF TOLERANT CELLS - EVIDENCE FOR CELLS PROTECTIVE AGAINST GVHD

In the cotransfer experiments, 40 x 10^6 cells of naïve donor origin were injected into the lateral tail vein veins of lethally irradiated recipient mice. Simultaneously, splenocytes from the tolerant chimeras were injected intravenously as well and, were either of donor or host origin to determine the source of the cells maintaining transplantation tolerance. As anticipated, those mice injected with naïve splenocytes alone succumbed to lethal GVHD within 3 weeks of reconstitution.

In the TBI cotransfer groups, both the tolerant donor cells and the tolerant recipient cells conferred protection against the emergence of lethal GVHD. These mice remained healthy at the 2month and 5month evaluation period (fig.6). This was most likely due to the suppressive nature of these cells as evident in the in vitro experiments.

Fig. 6

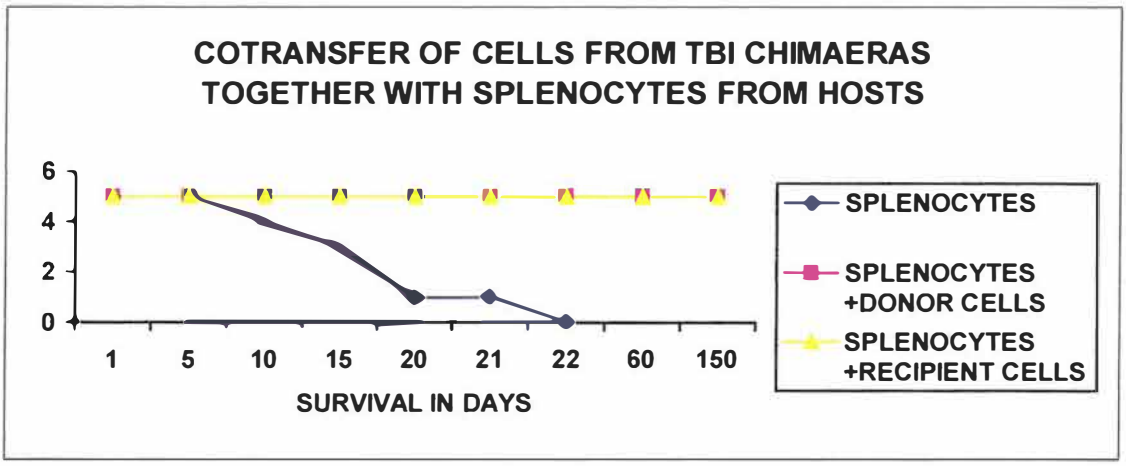
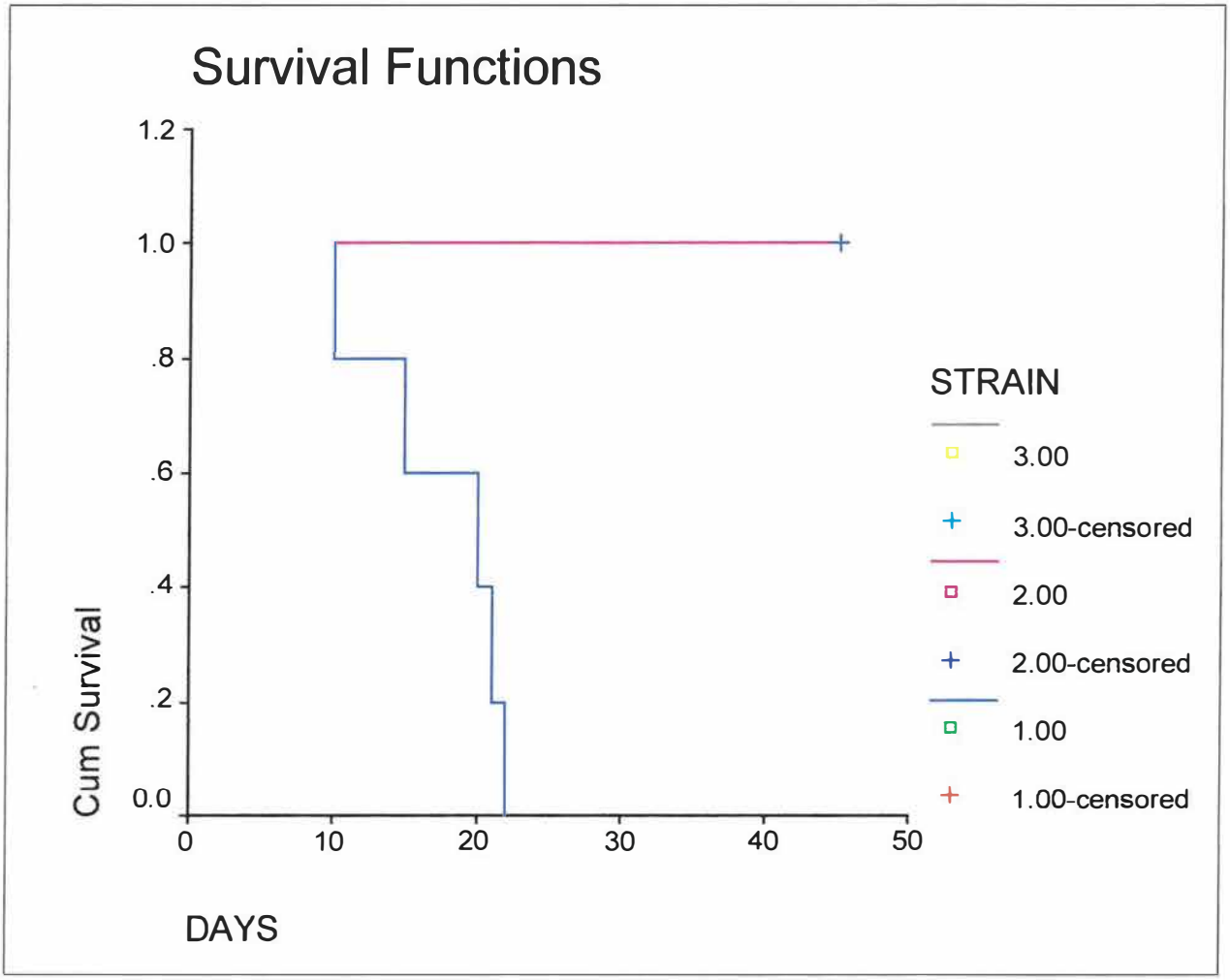


Fig. 6

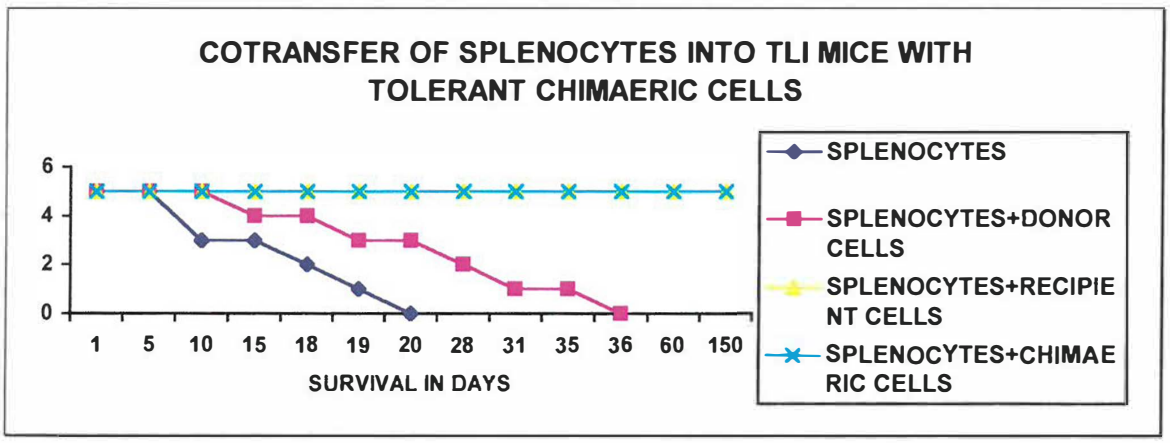


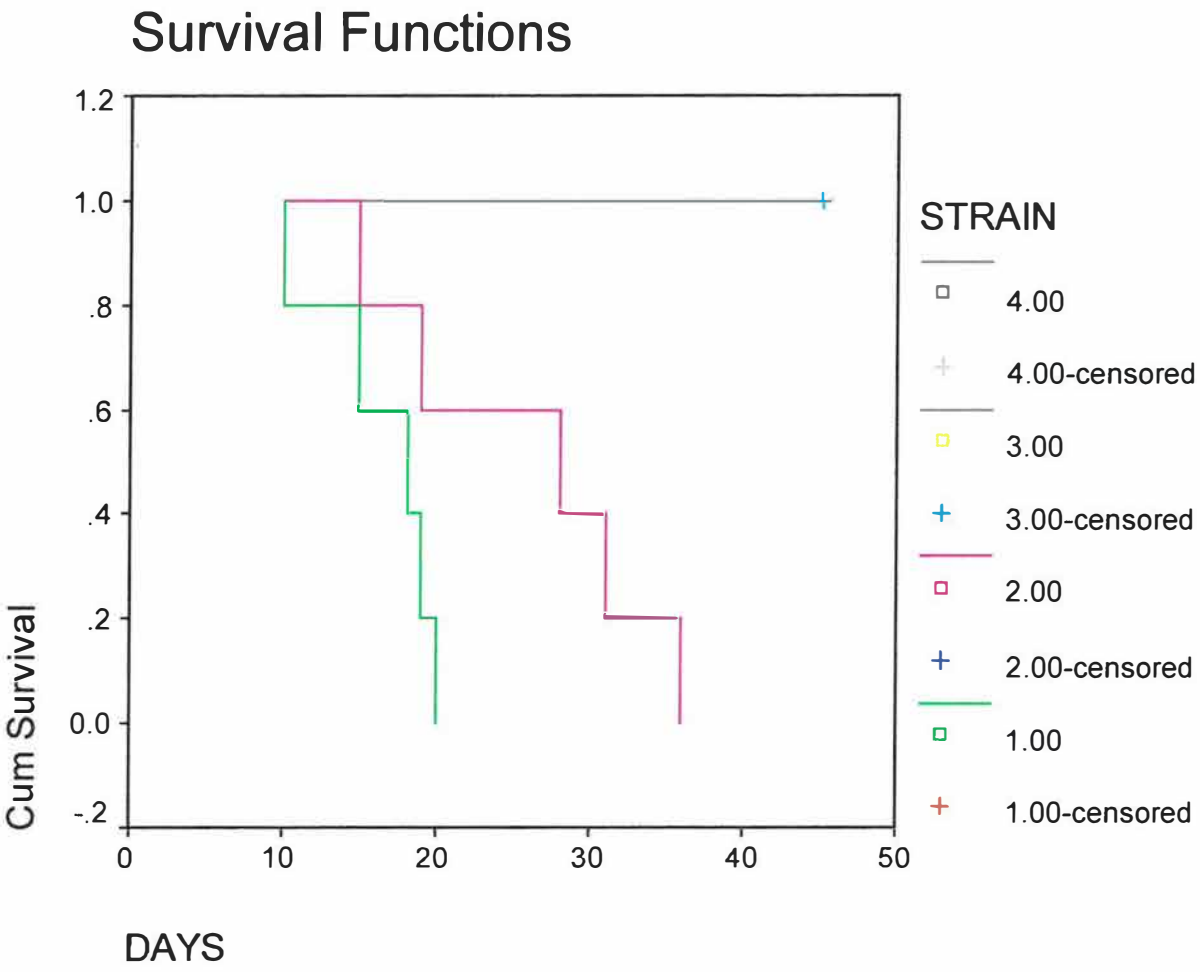
	Survival Time	Standard Error	95% Confidence Interval
Mean:	17.60	2.25	(13.19, 22.01)
Median:	20.00	5.48	(9.26, 30.74)

In the TLI group, the co-injection of tolerant donor cells conferred no significant benefit to the outcome of these mice except that there was a marginal prolongation in survival with the longest survivor demising at 34 days.

In contrast, cells of recipient origin unexpectedly circumvented the onset of GVHD but the mechanism underlying this protection is not readily apparent in this study and this protection persisted when chimeric tolerant cells from TLI chimeras were co-injected (fig 7). This would require further evaluation in subsequent studies. The suggestion from the cotransfer experiments is that, beyond clonal deletion, recipient cells also play an active role in either deleting or suppressing donor reactive cells.

Fig. 7





	Survival Time	Standard Error	95% Confidence Interval
Mean:	16.40	1.81	(12.86, 19.94)
Median:	18.00	3.29	(11.56, 24.44)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	25.80	3.87	(18.22, 33.38)
Median:	28.00	9.86	(8.68, 47.32)

DISCUSSION

The attainment of mixed chimerism with BMT is attractive owing to the theoretical potential of superior immunocompetence. There is consensus that for BMT to become more widely adopted, engraftment success must be more predictable together with the elimination of GVHD. Clinicians have attempted to achieve this by either increasing the lethality of the conditioning regime, increasing the immunosuppressive regime, the use of more specific immunosuppressive agents and the manipulation of the BMT by either T cell depletion or the transfusion of pure stem cells (CD 34) cells.

In order to identify new strategies to optimise BMT both to ensure engraftment as well as reduce the incidence of GVHD it is imperative that the mechanisms sustaining transplantation tolerance be predictable. At present, four methods have been described and confirmed in experimental models. These are **clonal deletion, immunosuppressive cells, veto cells and clonal anergy.** The mechanism by which these various strategies emerge is not predictable and at present it is postulated that this is determined by the quality of the BMT and the disparity in MHC.

The results from this study have highlighted some interesting issues that need to borne when considering BMT as a therapeutic option. The influence of the conditioning regimen is well highlighted in this study. TLI as a conditioning regimen allows for mature T cells to be transfused without imposing the risk of GVHD. The mechanism of transplantation tolerance that is subsequent to this type of transplantation is based mainly on clonal deletion. This was borne out in the in vitro studies where the MLC was unresponsive to both donor and recipient cells. However, the co-transfer experiments suggests that another probable mechanism facilitating transplantation tolerance is the

possibility of veto cells which were not evident in the in vitro experiments. This was not apparent initially in the in vitro experiments and may reflect the need to identify appropriate cells in the T cell compartment that may have undergone clonal expansion or, alternatively, identify cells that may perform this task outside of the mature T cell compartment such as NK cells. This is based on the results of the cotransfer experiments where the injection of recipient cells from the stable chimaeras of TLI origin protected against the emergence of GVHD.

In the TBI group, transplantation tolerance can be achieved by the manipulation of the donor bone marrow with TCD. This will adequately circumvent GVHD. However, the mechanism of transplantation tolerance is completely different to the TLI group. In this group, the emergence of suppressor cells was quite evident from the in vitro experiments in both the donor and recipient compartments. Furthermore, the identification of clonal expansion of Vβ6 cells was unexpected. This may suggest that expansion occurs in the most reactive compartments and due to an inability to mount an appropriate response such as a cytotoxic response, results in a suppressive response. Whether this is a consequence of the conditioning regimen or the quality of the bone marrow transplant remains to be established. The precise mechanism of action of these cells need to be determined in future studies to establish their cytokine profile and possibly, the mechanism of function.

In addition to the suppressive element of the tolerance, it is obvious that whilst this mechanism adequately depresses GVHD, it also diminishes the response to third party antigens and this may compromise the chimeric animal in responding to immunological challenges. This is substantiated in the in vitro responses where the MLC is depressed

to third party antigens upon addition of chimeric tolerant cells that were found to be expanded (the Vβ6 cells). The precise magnitude of this is further substantiated in the response of the chimeric animal to donor (allogeneic) tumour. All chimeric mice infused with donor tumour cells succumbed to the tumour by 22 days. This raises the theoretical potential that BMT under these circumstances may expose the host to the potential of developing malignancies of donor origin with the same or worse outcome than would be anticipated in the donor.

The conclusions that can be drawn from this study is that transplantation tolerance is influenced by various factors and the most important appear to be the quality of the BMT and the conditioning regimen used to prepare the recipient. Measures undertaken to minimise GVHD should be performed with caution especially in the context of malignancies as TCD used in this model resulted in suppressive mechanisms being the most prominent and this may account for the increase in relapse rates identified in recipients of TCD bone marrow in clinical transplantation.

This study illustrates that whilst in vitro studies substantiate the role of pure mechanisms sustaining tolerance, the in vivo mechanisms are more complicated and diverse. Several mechanisms coexist to support transplantation tolerance. These are influenced by the various variables and, in this in vivo model, it was difficult to establish the precise mechanism of transplantation tolerance in the TLI group. However, the clinical implications of this study suggests that the more aggressive forms of conditioning may render the host susceptible as it encourages the emergence of a suppressive mechanism of tolerance which is attended with an inferior immunocompetence of the chimeric host. An alteration in the quality of the graft, such

as the infusion of T cells runs the risk of allowing GVHD to emerge with its own attendant risks.

CONCLUSIONS

The conclusions from this study suggest that the mechanisms that support transplantation tolerance are not necessarily beneficial to the host. Tolerance may be accompanied by immuno-incompetence that places the recipient at risk of handling potentially benign conditions ineffectively. Of greater significance, there is also the risk of susceptibility to donor diseases that may be of greater risk to the recipient as a result of the depression in immuno-competence.

CHAPTER 5

THE GRAFT VERSUS LEUKAEMIA EFFECT (GVL) - EVALUATION OF THE MECHANISMS INDEPENDENT OF GRAFT VERSUS HOST DISEASE (GVHD) WITH DIFFERENT MECHANISMS OF TRANSPLANTATION TOLERANCE

INTRODUCTION

Bone Marrow Transplantation was used to salvage patients following more intensive cytoablative therapy. The principle that the transplantation regimen might be inadequate to completely eradicate leukaemic haematopoiesis and that the antileukaemic effect of infused marrow elements contributes to the ability of marrow transplantation to cure leukaemia was first proposed by Barnes et al in 1956[1]. Although their studies and the studies of subsequent investigators in murine models generally confirmed the existence of a Graft-Versus-Leukaemia (GVL) effect[2], data in humans was entirely indirect. Mathe et al[3,4] rationalised early efforts at marrow transplantation by invoking a GVL reaction, but evidence for this reaction was first provided by Weiden and the Seattle transplant team[5, 6]. They demonstrated that the likelihood of relapse was substantially lower in patients with either acute or chronic Graft-Versus-Host-Disease (GVHD) compared with unaffected patients. A higher relapse after syngeneic transplantation compared with allogeneic transplantation was shown retrospectively[7] and confirmed with a larger retrospective study[8]. Furthermore, a GVL effect was

inferred when recurrent leukaemia after marrow grafting regressed after a GVHD flare associated with the discontinuation of immunosuppression.

It appears that GVHD and GVL may be mediated by overlapping but not identical subsets of cells. If these populations can be defined, they can be exploited in an immunotherapy regimen.

Murine models of GVL support the concept that GVL and GVHD can be mediated by separate as well as identical cell populations but there may also be a leukaemia specific reaction that can give rise to GVL without GVHD[2].

AIMS

This led to the attempt to determine the mechanisms underlying the phenomenon of GVL without GVHD and identify the factors responsible for its emergence. Against this background we undertook the following study to determine

1. The reproducibility of the GVL phenomenon in TLI and TBI chimeras
2. The mechanisms underlying the effect

MATERIALS AND METHODS

Mice: 6 - 12 week old mice were purchased. C3H/HeJ (H-2k, Thy 1.2, Mls 1b/2a) and Balb/c (H-2d, Thy 1.1, Mls 1b/2a) were purchased from Charles River (Sulzfield, Germany) and AKR/J (H-2k, Thy 1.2, Mls 1a/2b) were purchased from Bomholtgard Breeding Center Ltd. (Ry, Denmark)

Recipient mice were housed in plastic cages fitted with a filter cap, had sawdust bedding and housed in a pathogen free environment. Pellet chow and acidified drinking

water was the standard diet. Antibiotic (Tylan, Eli Lilly, Brussels, Belgium) was added to the drinking water 1 week prior to BMT and was continued for a week following it.

Irradiation: Recipient mice received either 9.5 Gy Total Body Irradiation (TBI) as a single dose or Total Lymphoid Irradiation (TLI) as 12 daily fractions (10 TLI + 2 TBI) of 2 Gy each as described previously. Irradiation was delivered by a 60 Cobalt source (Gammatron, Siemens) at a low dose rate. The source to skin distance for the TBI was 100 cm and, for mice receiving TLI, was 80 cm. Mice receiving TLI were anaesthetised; they were induced with Enflurane and then maintained in a prone position using Enflurane (Abbott, s.p.a., Campoverde-LT, Italy) delivered by a semi-closed inhalation anaesthetic system. The skull, long bones, kidneys, lungs and tail were shielded with lead blocks confining irradiation to the thymus, spleen, supra- and infradiaphragmatic lymph nodes. A maximum of 6 mice were irradiated simultaneoulsy.

BMT: To prepare stable mixed chimeras with the elimination of GVHD, the recipient mice were reconstituted with donor cells in the following manner:

TBI chimeras were prepared with 5 x 10^6 T cell depleted (TCD) allogeneic bone marrow. TCD was performed in vitro using Thy 1.1 (Serotech, Oxford, UK) or Thy 1.2 (Sigma Chemie, GmbH, Drisenhof, Germany) antibodies and low toxic rabbit complement (Cedarlane, Hornby, Ontario, Canada).

TLI chimeras were prepared with 15 x 10^6 non-TCD allogeneic bone marrow.

Bone marrow cells were harvested by flushing the shafts of the sacrificed donor animals with a solution of RPMI with antibiotics and 1% heparin. The cells were washed twice with RPMI with added antibiotics. The viable cell count was determined by staining with Trypan blue and counted in a Burkër haemocytometer. Cells were kept on

ice throughout. The cells were reconstituted to the relevant concentration such that 0.25 ml was injected into the tail vein of the recipient, with the appropriate number of cells.

Determination of chimerism and Vβ6cells

Chimerism was determined in a Flourescein Activated Cell Sorter (FACS) assay using Thy 1.1 (Serotech, Oxford, UK), and Thy 1.2 (Sigma Chemie, GmbH, Drisenhof, Germany) monoclonal antibodies (mAbs) conjugated with Phycoerythrin (PE) and Flourescein Isothiocyanate (FITC) respectively. These are lymphocyte markers specifically confined to either donor cells or recipient cells.

Vβ6 cells were evaluated in the CD4 window (Caltag lab, San Francisco) with Vβ6 mAb (Pharmingen, Antwerp, Belgium) and percentages were estimated in the peripheral blood lymphocytes, thymus, splenic lymphocytes and lymph node lymphocytes.

Mixed Lymphocyte Cultures (MLC):

Responder cells: Single cell suspensions were made from the spleen/s of the responder mice by teasing them into fragments with forceps in RPMI on ice and then passed through a 100 μm cell strainer and washed twice with RPMI with added antibiotics. The viable cell count was determined. The cells (maximum of 10^8 cells) were incubated at 37°C in a 5% CO2 incubator in 2 ml of RPMI with 5% foetal calf serum (FCS) in a nylon wool syringe. This syringe was previously washed with 50 ml of the same solution and kept in the incubator for a minimum of 1 hour prior to use. The enriched T cells were eluted. This was done by simply washing the non-adherent T cells from the syringe by passing 20 ml of the medium through the syringe and collecting the fluid under sterile conditions. The eluted cells were counted and made up

to a concentration of 5 x 10^6 cells/ml in RPMI with 10% FCS and 2ME (Mercaptoethanol).

Stimulator cells: Single cell suspensions were prepared from the spleen/s of the mice as described as above. The cells were counted and made up to a concentration of 5 x 10^6 cells/ml in RPMI with 10% FCS and 2ME. These cells were irradiated with 3000 Rads and kept in the incubator until use.

Chimeric cells: Cells of donor or recipient origin from the chimeric animals were isolated from single spleen cell suspensions by incubating the spleen cells with polyclonal antibodies* in RPMI with 20% FCS. After incubation, the cells were washed twice with RPMI to remove excess unbound antibodies and the cells were then incubated with low toxic rabbit complement for 45 minutes in a 37°C water bath. The cells were washed twice with RPMI. Cell counts were determined and the cells were reconstituted to yield a concentration of 2,5 x 10^6 cells/ml in RPMI with 10% FCS and 2ME. These cells were irradiated with 1500 Rads and were added to the MLC to determine their influence on it.

Vβ6cells: These cells were isolated from single cell suspensions from the thymus of chimeric AKR mice using magnetic beads to isolate the cells and then bringing them to a concentration of 2.5 x 10^6 cells/ml.

The cells were plated into 96 well flat bottom plates; 5 x 10^5 responder cells and 5 x 10^5 stimulator cells were added to the wells in the experiments. Four experiment wells were performed and the mean values were used. Chimeric cells were added i.e. either of donor or recipient origin to determine their influence on this reaction again performed in

4 wells and the mean value taken. Control wells comprised cells from each group without the addition of other cells.

The cells were incubated for a period of 96 hours in an incubator. Thereafter, 10:l of [H^3] Thymidine was added to each well and the cells were incubated for an additional 16 hours after which the cells were harvested and the radioactivity measured. Results were interpreted as the mean of the experimental counts minus the sum of the mean counts of the relevant control cells.

Polyclonal antisera: Polyclonal antibodies were raised by skin grafting the mice with skin from the allogeneic mice against which antibodies were to be raised. Once rejection occurred, the grafted mice were injected repeatedly with 5 x 10^6 donor haematopoeitic cells in Complete Freunds Adjuvant intraperitoneally until the development of ascites. The ascites was harvested, centrifuged, filtered by passage through a 100 μm filter and stored at -20°C. The concentration of the antibodies necessary was established by titration against a known concentration of cells.

TUMOUR CELLS

The BW 5147.3 tumour cell line, a lymphoma/ leukaemia cell line of AKR/J origin was injected intravenously at varying doses in the different experimental groups and the outcome was evaluated.

COTRANSFER EXPERIMENTS

Lethally irradiated AKR mice (10.5 Gy TBI) were reconstituted with 5 x 10^6 TCD syngeneic bone marrow and the groups were given tolerant splenocytes from the stable TLI AKR BM chimaeras that were either

- not manipulated,
- not administered,
- depleted of CD4 cells,
- depleted of CD8 cells, or
- depleted of both CD4 and CD8 cells

one day following irradiation.

One day later, the mice were injected with 10^6 BW 5147.3 tumour cells and the outcome in the various groups were monitored.

Depletion was performed using magnetic beads (MACS) and the cells used in the cotransfer experimental cells were eluted by negative selection to prevent in vitro activation.

IN VIVO EXPERIMENTS

Early tumour innoculation

There were 5 experimental groups:-

- 15 x 10^6 non TCD syngeneic BMT - TBI group
- 5 x 10^6 TCD semiallogeneic BMT - TBI group
- 15 x 10^6 non TCD semiallogeneic BMT + 5 x 10^6 syngeneic BMT - TBI group
- 15 x 10^6 non TCD syngeneic BMT
- 15 x 10^6 non TCD semiallogeneic BMT

Two days later 10^6 BW 5147.3 tumour cells were injected into the lateral tail vein of the mice. The delay in tumour innoculation was deliberate to ensure engraftment of the BMT. Simultaneous innoculation with tumour resulted in engraftment failure as observed previously.

Delayed tumour innoculation

Two months after BMT, in groups similar to those described above, mixed chimaerism was confirmed in a FACS assay as already alluded to. Five animals from each group reconstituted with semiallogeneic BMT were sacrificed and the percentage composition of and the ratios of the CD4 : CD8 was determined in both the peripheral blood and the spleen. The correlation to the outcome to the groups was determined to see if a relationship existed.

At 2 months after confirmation of mixed chimaerism and clinical absence of GVHD, 3 x 10^6 BW 5147.3 tumour cells were injected into the lateral tail vein of the mice.

RESULTS

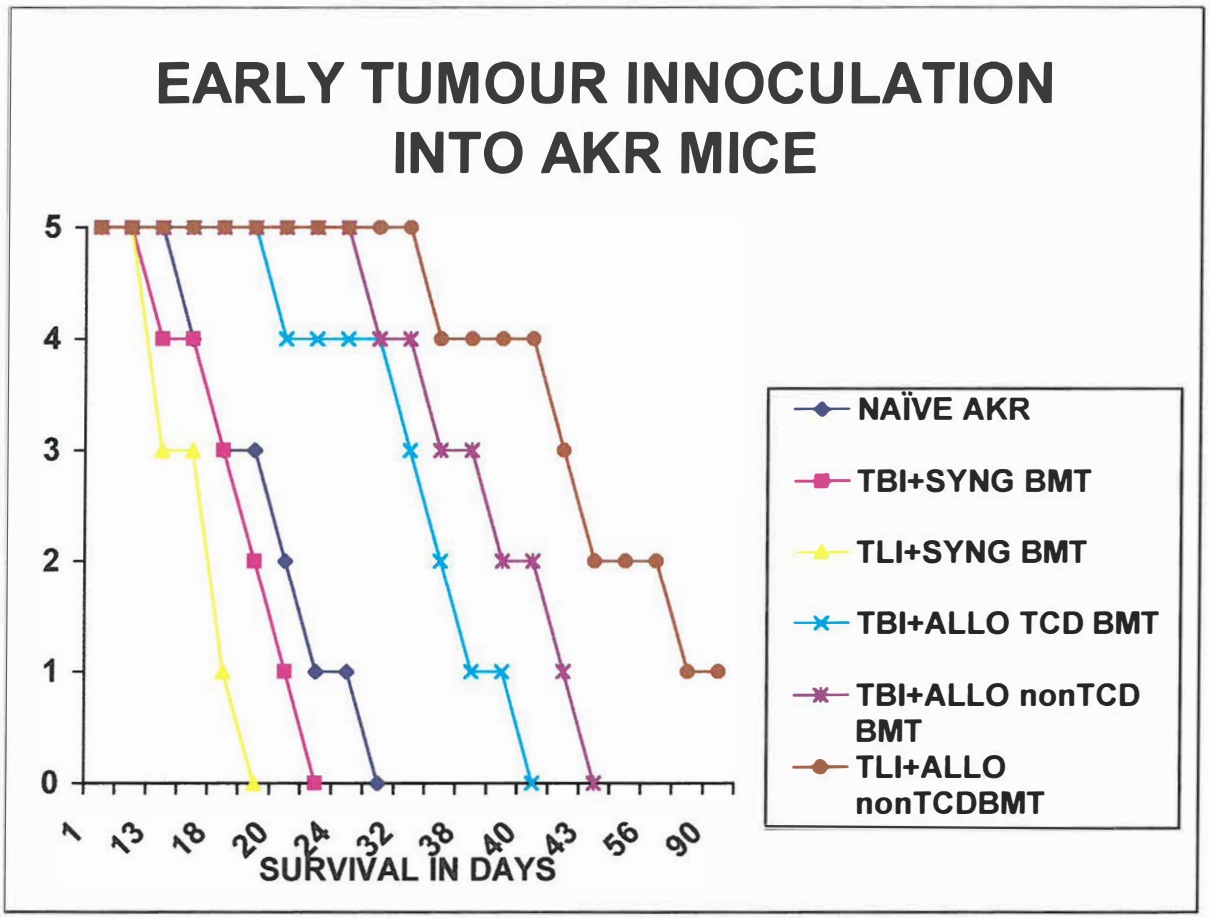
Early tumour innoculation

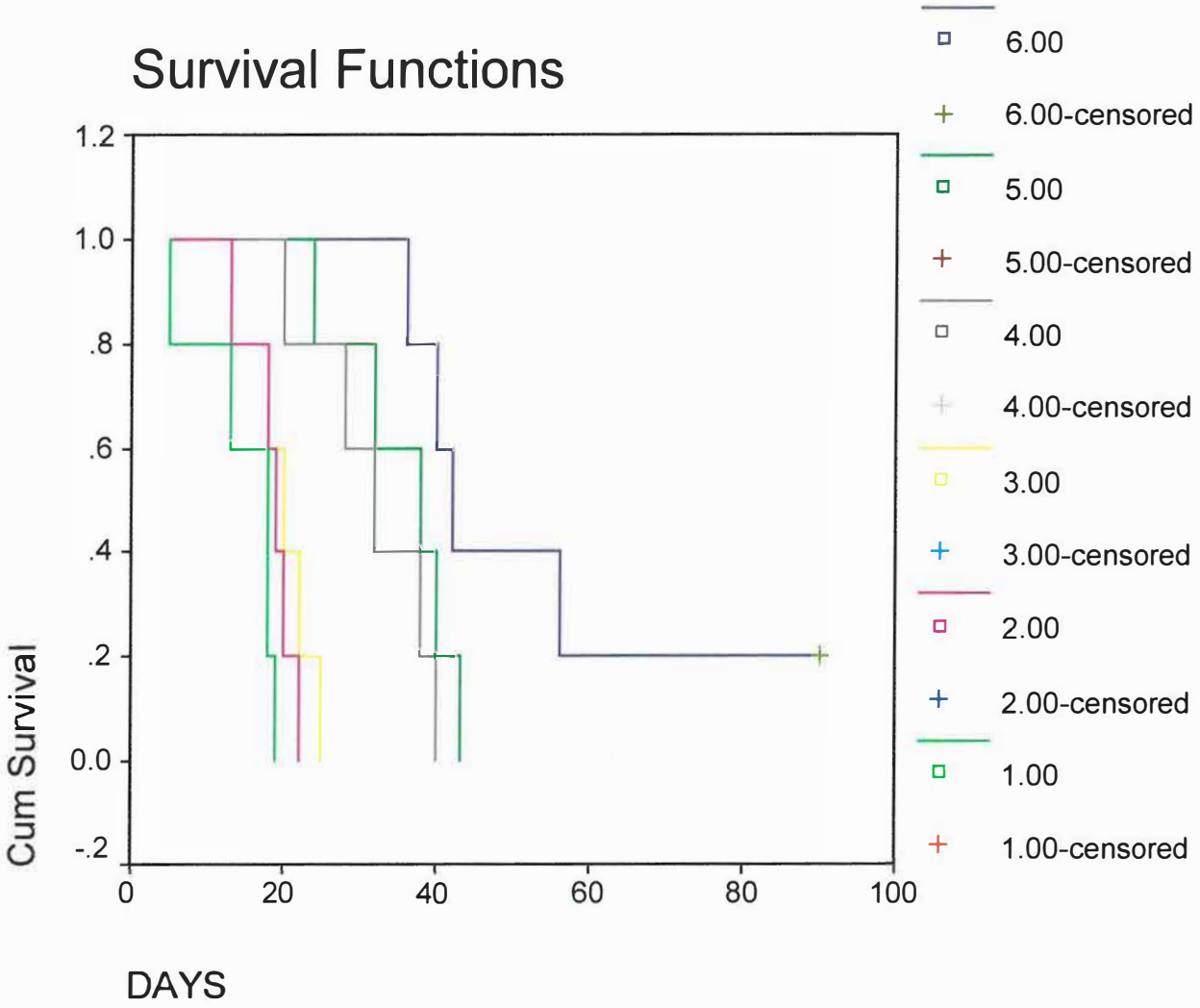
The group of mice who were reconstituted with syngeneic BM all succumbed to tumour within 3 weeks after tumour innoculation. This was confirmed by histological evaluation of the tissues taken as soon as the mouse demised. This occurred in both the groups i.e. the group conditioned with TLI and TBI.

In contrast, animals reconstituted with semiallogeneic BM fared better than the animals reconstituted with syngeneic BM as well as those animals that were not irradiated but injected with tumour.

Although both TBI groups reconstituted with semiallogeneic BM survived for significantly longer than the syngeneic groups, they in turn survived for a shorter period than the TLI group reconstituted with semiallogeneic BM and this was also statistically significant (fig 1).

Fig. 1


EARLY TUMOUR INNOCULATION INTO AKR MICE
5
4
3
2
1
0
1
13
18
20
24
32
38
40
43
56
90
SURVIVAL IN DAYS
NAÏVE AKR
TBI+SYNG BMT
TLI+SYNG BMT
TBI+ALLO TCD BMT
TBI+ALLO nonTCD BMT
TLI+ALLO nonTCDBMT



	Survival Time	Standard Error	95% Confidence Interval
Mean:	14.60	2.62	(9.47, 19.73)
Median:	18.00	2.24	(13.62, 22.38)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	18.40	1.50	(15.45, 21.35)
Median:	19.00	1.10	(16.85, 21.15)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	19.60	2.01	(15.65, 23.55)
Median:	20.00	2.19	(15.71, 24.29)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	31.60	3.60	(24.54, 38.66)
Median:	32.00	4.38	(23.41, 40.59)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	35.40	3.37	(28.79, 42.01)
Median:	38.00	6.57	(25.12, 50.88)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	52.80	8.85	(35.46, 70.14)
(Limited to	90.00)		
Median:	42.00	2.19	(37.71, 46.29)

Delayed tumour innoculation

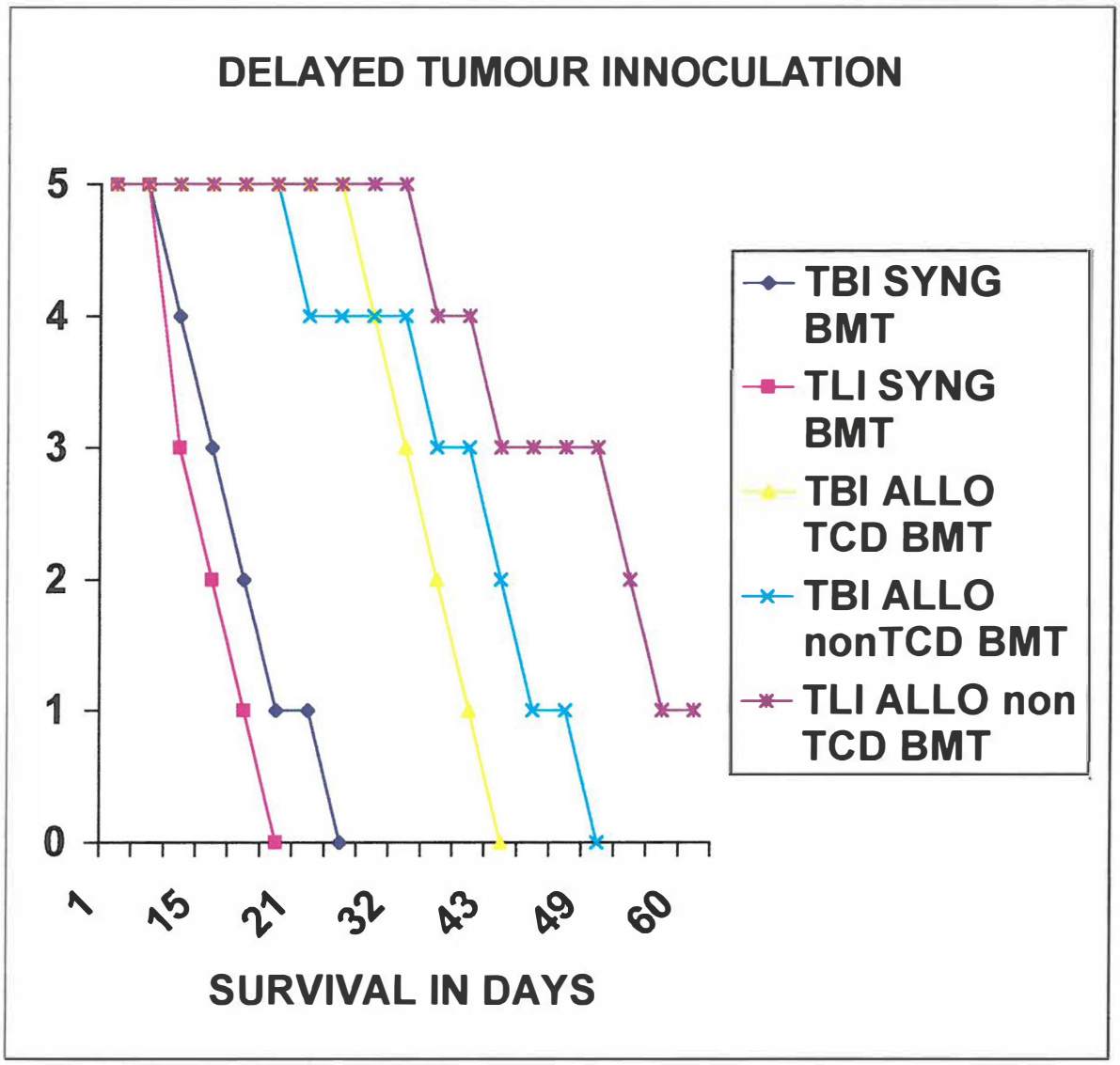
Delayed tumour innoculation allowed for the opportunity to confirm that the animals were mixed chimaeras and this was confirmed in a FACS assay of the peripheral blood using Thy 1.1 and Thy 1.2 antibodies coupled with PE and FITC respectively. All the animals reconstituted with semiallogeneic BM were found to be mixed chimaeras and the respective percentages of these are reflected below in table 1.

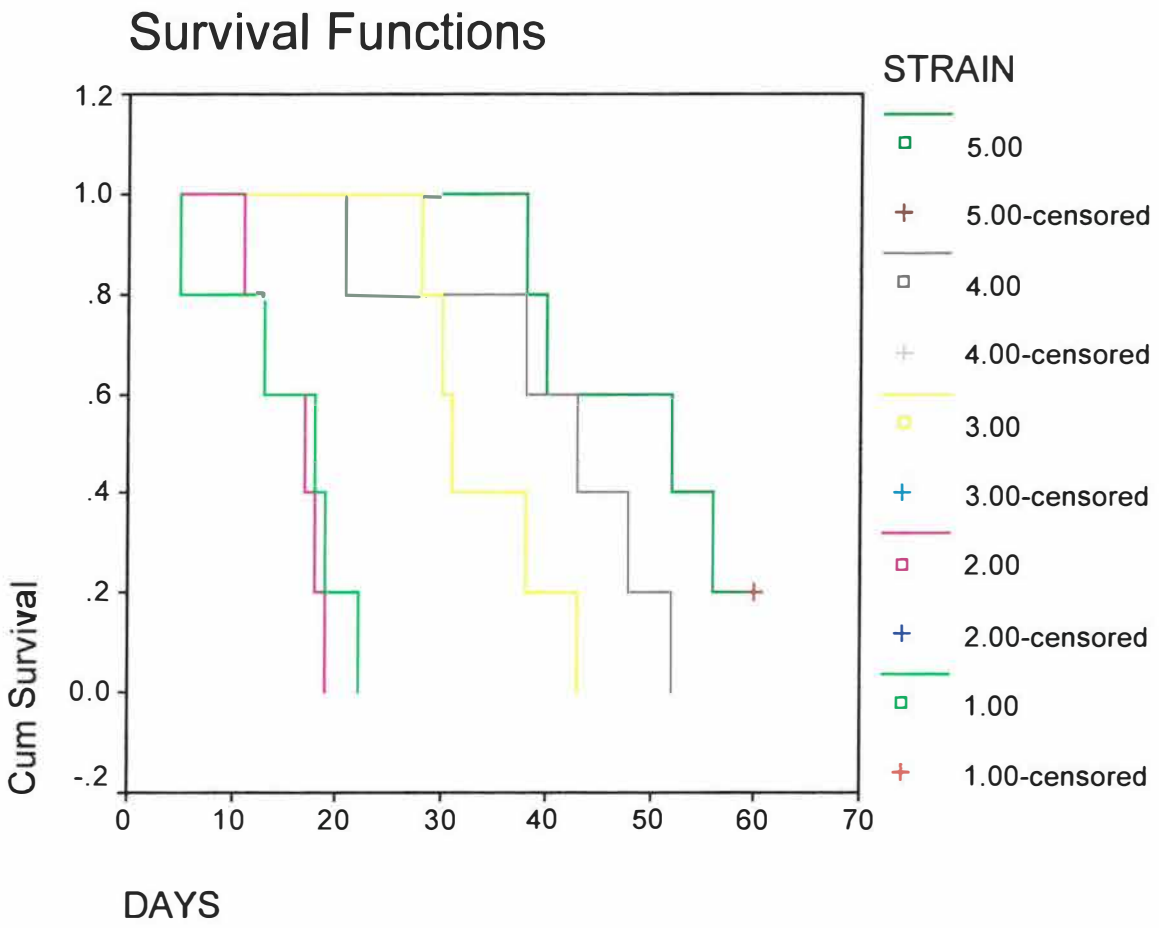
	TBI MICE	TLI MICE
%chimaerism	57,5 (48 – 62.7)	43.2 (31 – 52)

As expected, the outcome in the respective groups of mice mirrored those in the early tumour innoculation group with the semiallogeneic group surviving for significantly longer than the syngeneic reconstituted group. TLI mice survived for significantly longer than TBI mice. Additionally, TBI mice reconstituted with non-TCD BM survived

longer than those reconstituted with TCD allogeneic BM, however, this did not achieve statistical significance (fig 2).

Fig. 2





	Survival Time	Standard Error	95% Confidence Interval
Mean:	15.40	2.98	(9.57, 21.23)
Median:	8.00	5.48	(7.26, 28.74)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	15.60	1.54	(12.59, 18.61)
Median:	17.00	4.38	(8.41, 25.59)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	34.00	2.81	(28.49, 39.51)
Median:	31.00	1.10	(28.85, 33.15)

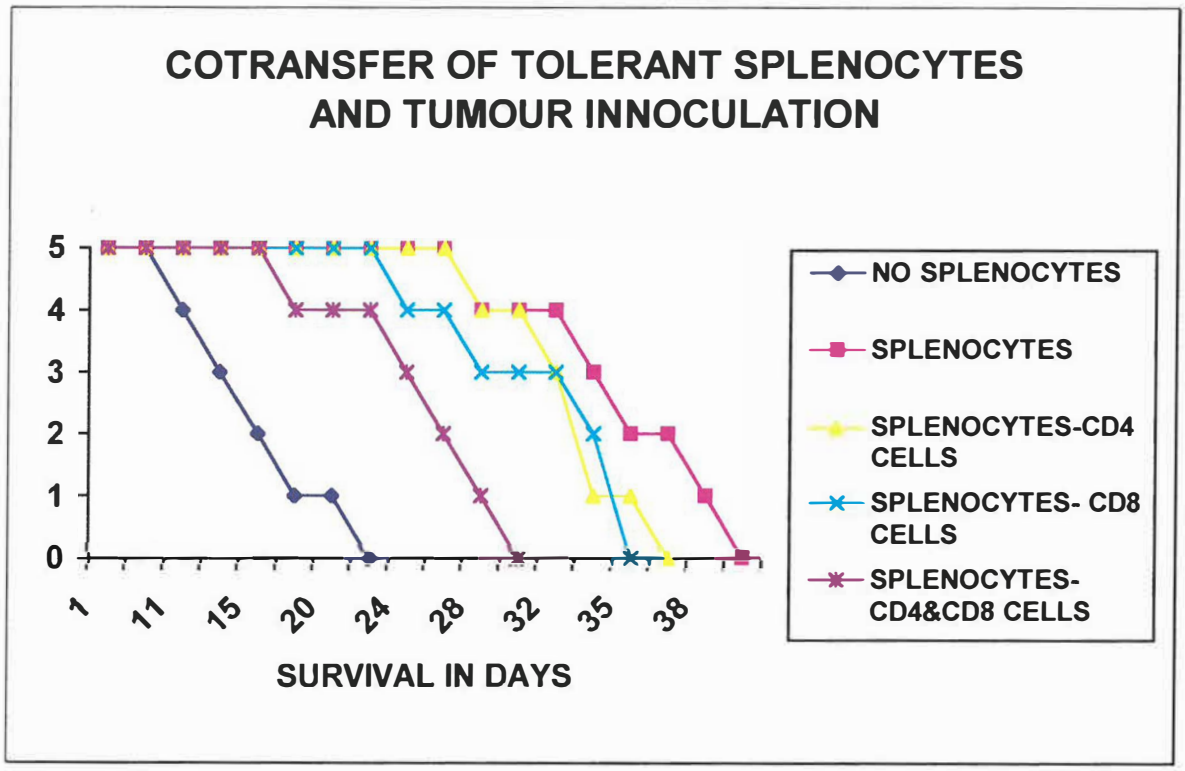
	Survival Time	Standard Error	95% Confidence Interval
Mean:	40.40	5.39	(29.83, 50.97)
Median:	43.00	5.48	(32.26, 53.74)

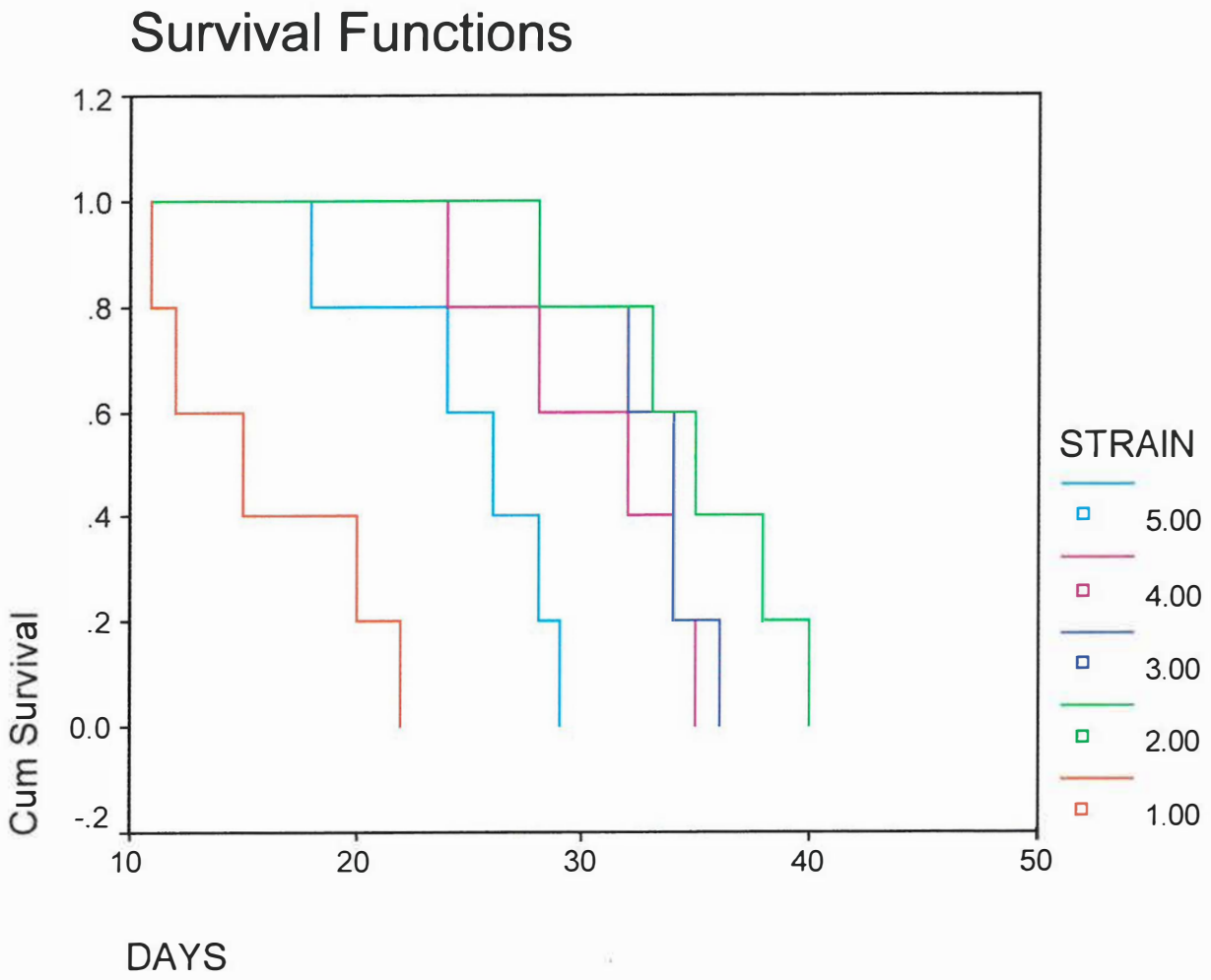
	Survival Time	Standard Error	95% Confidence Interval
Mean:	49.20	3.90	(41.55, 56.85)
(Limited to	60.00)		
Median:	52.00	13.15	(26.24, 77.76)

Cotransfer Experiments

The cotransfer experiments were designed to attempt to establish the cell types that were responsible for effecting the GVL effect. As anticipated, the group that was denied allogeneic splenocytes succumbed the fastest to the tumour innoculation. Unexpectedly, all mice reconstituted with the splenocytes survived significantly longer than the group without allogeneic splenocytes. As expected, the group given the unmanipulated splenocytes survived the longest. Those mice with the T cell subgroups did not achieve any significant differences in survival. However, the necessity of T cells to support a GVL effect can be inferred from the results in that the group that was given splenocytes devoid of CD4 and CD8 cells fared worse than the groups that were given mature T cells (Fig 3).

Fig. 3


COTRANSFER OF TOLERANT SPLENOCYTES AND TUMOUR INNOCULATION
5
4
3
2
1
0
1
11
15
20
24
28
32
35
38
SURVIVAL IN DAYS
NO SPLENOCYTES
SPLENOCYTES
SPLENOCYTES-CD4 CELLS
SPLENOCYTES- CD8 CELLS
SPLENOCYTES-CD4&CD8 CELLS



	Survival Time	Standard Error	95% Confidence Interval
Mean:	16.00	2.17	(11.75, 20.25)
Median:	15.00	3.29	(8.56, 21.44)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	34.80	2.08	(30.72, 38.88)
Median:	35.00	2.19	(30.71, 39.29)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	32.80	1.36	(30.14, 35.46)
Median:	34.00	.89	(32.25, 35.75)

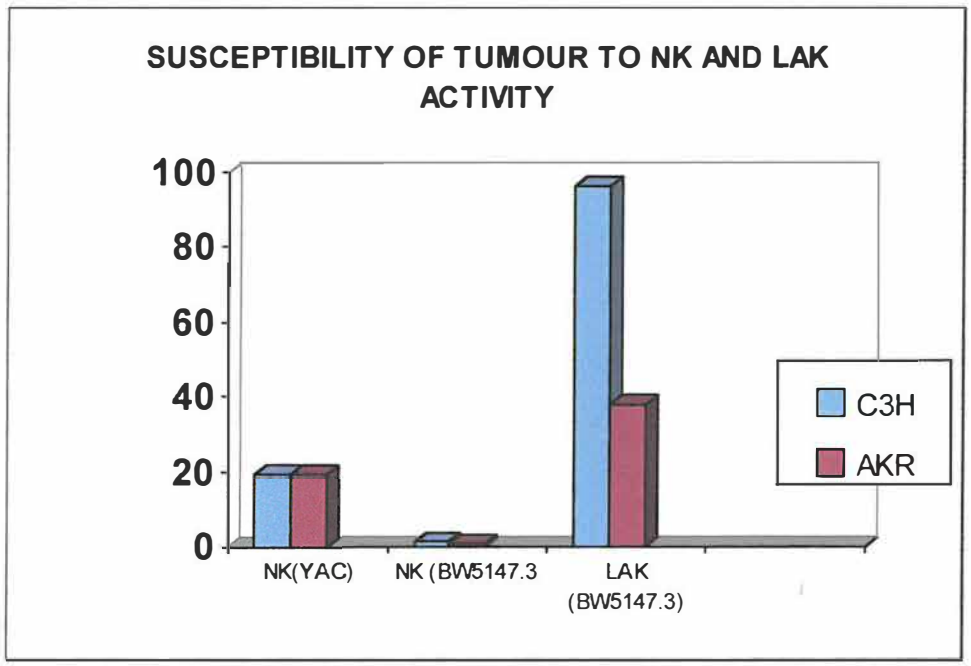
	Survival Time	Standard Error	95% Confidence Interval
Mean:	30.60	2.04	(26.60, 34.60)
Median:	32.00	4.38	(23.41, 40.59)

	Survival Time	Standard Error	95% Confidence Interval
Mean:	25.00	1.95	(21.18, 28.82)
Median:	26.00	2.19	(21.71, 30.29)

NK/LAK EVALUATION

These results confirm that there is minimal NK activity against the tumour line by the transplanted bone marrow cells. However, cells primed with IL-2 have a significant cytotoxic activity mediated against the tumour cell line in vitro (fig. 4).

Fig. 4



Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
NKACT	8	2.4000	1.1784	1.10	3.80
LAKACT	8	67.2500	31.0840	36.00	98.00
CELLTYPE	8	1.5000	.5345	1.00	2.00

Test Statistics[b]

	NKACT	LAKACT
Mann-Whitney U	.000	.000
Wilcoxon W	10.000	10.000
Z	-2.309	-2.323
Asymp. Sig. (2-tailed)	.021	.020

b. Grouping Variable: CELLTYPE

DISCUSSION

Bone Marrow Transplantation is now a necessary and important therapy in the treatment of haematological malignancies. However, all the benefits that have been recognised do not occur in a predictable fashion. Additionally, the potential for adverse effects remain in clinical practice with occasional fatal outcomes. The only plausible solution is to unravel the mechanisms responsible for the various effects so that clinical manipulation can be safe, predictable and beneficial.

This study undoubtedly confirms that BMT has an anti-tumoural effect that is allogeneic in origin. Furthermore, the mechanisms effecting the anti-tumour effect is not mandatorily accompanied by a GVHD effect.

Of significance, the mechanisms sustaining transplantation tolerance play a significant role in the response that is effected. This in turn is influenced by the conditioning regimen. In mice conditioned with TBI, the GVL effect is significantly weaker than mice that are conditioned with TLI. To eliminate the bias of TCD, the method of Sykes et al was employed to sustain tolerance without TCD and, although there was a marginal improvement in survival, this did not attain statistical significance.

The cotransfer experiments that were performed attest to the role of the T cells in playing a role in the GVL effect. However, this effect is present even in the absence of T cells but is best with mature splenocytes that were not manipulated. This suggests that the GVL effect in vivo is probably mediated in concert with a host of cells either playing direct roles or subsidary roles. The role of LAK cells may be important with T

cells perpetuating the response by facilitating the emergence of LAK cells. However, this is speculative and remains to be established.

CONCLUSIONS

The conclusions from this study are that allogeneic BMT does induce a GVL effect and can be attained without the emergence of GVHD. However, the response is influenced by both the conditioning regime and the quality of the BMT. T cells appear to be integral to the facilitation of a GVL effect. The effects however, may be to augment or induce the response rather than effect it.

CHAPTER 6

PRESENCE OF INTRINSIC B LYMPHOCYTE TOLERANCE IN MIXED BUT NOT IN COMPLETE SEMIALLOGENEIC BONE MARROW CHIMERAS[1]

The existence of intrinsic B lymphocyte transplantation tolerance was investigated in murine semiallogeneic complete and mixed bone marrow chimeras. Complete chimeras (CC), which were obtained by infusing $20x10^6$ C57BL/10xBALB/c) F1 recipients, were repopulated for 100% with P1 lymphohaematopoietic cells. In mixed chimeras (MC), which were obtained by injection of $15x10^6$ F1 BM cells, between 15% and 40% of F1 lymphohaematopoietic cells persisted after BM transplantation. Neither MC nor CC were able to develop significant T cell immunity (mixed lymphocyte reaction) or B cell immunity (1gG alloantibodies) against the mismatched host antigens (BALB/c), despite repetitive immunizations. However, after immunization with third-party cells (C3H), the IgG alloantibodies raised cross-reacted with the host-type (BALB/c) antigens in the CC but not in the MC. This suggested that intrinsic B lymphocyte tolerance for host antigens had occurred in MC but not in the MC. This was further evidenced in transfer experiments using lethally irradiated C57BL/10 mice reconstituted with purified control C57BL/10 T Lymphocytes together with purified C57BL/10 B lymphocytes isolated from CC or MC. Only the recipients reconstituted with B lymphocytes from CC, and not those from MC, produced anti-BALB/c IgG alloantibodies after immunization. These results show that intrinsic B lymphocyte

tolerance can be achieved after transplantation and that this depends on the presence of lymphohaematopoietic cells expressing the tolerogens.

T lymphocytes can develop tolerance for allogeneic transplantation antigens based on mechanisms such as clonal deletion, clonal anergy, immunoignorance, and active suppression mechanisms that are also involved in the establishment of self tolerance (1-5). The long-proposed existence of intrinsic B lymphocyte tolerance (6) has been demonstrated in recent years using mice carrying rearranged immunoglobulin heavy and/or light chains (7,8). These experiments confirm that B lymphocytes that bind certain self or neo-self antigens may be tolerant by mechanisms such as clonal anergy (7), or exclusion from the peripheral lymphoid tissues (10). However, it remains unclear whether similar B cell tolerance may also be induced after allotransplantation, a situation that is very different from the transgenic mouse experiments because it involves multiple B cell clones with different affinities for numerous antigens.

The latter question was addressed here in a model involving semiallogeneic bone marrow (BM*) transplantation in lethally irradiated mice. This model embraced a recapitulation of B lymphocyte ontogeny to see whether donor B lymphocytes could become tolerant for host-type transplantation antigens. The C57BL/10 into (C57BL/10XBALB/c) F1 semiallogeneic combination model was used because immunodeficiency does not occur, whereas it is usually observed in fully allogeneic situations as a consequence of a lack of MHC restriction (11,12). Indeed, in fully allogeneic combinations, donor T cells are restricted for host MHC during thymic

maturation, but because their antigen-presenting cells express donor MHC antigens to which T cells are not restricted, this may impair the interaction between the T lymphocytes and the antigen-presenting cells. The experiments presented here show that whereas donor T lymphocytes become tolerant for host MHC antigens in both mixed as well as complete chimeras, donor B lymphocytes are tolerized for host MHC antigens only in mixed chimeras. These experiments thus demonstrate that B lymphocyte transplantation tolerance can be attained, but this depends upon the presence of haematopoietic cells expressing the tolerogens. This finding may have implications for clinical transplantation.

MATERIALS AND METHODS

Induction of BM chimeras (MC) were constructed by infusing $15X10^6$ C57Bl/10 BM cells into lethally irradiated (10.5 Gy of total body irradiation) F1 recipient mice. In all MC, between 15% and 49% of host-type peripheral blood leukocytes were shown to persist after BM transplantation using a sensitive flow cytometry assay (13, 14). Complete chimeras (CC) were made by infusion of only $20X10^6$ C57BL/10 BM cells. In CC, the presence of remaining host-type peripheral blood lymphocytes could not be demonstrated, despite multiple determinations. In all BM transplantation experiments, T cell depletion of the BM cells was achieved before infusion using anti-Thy monoclonal antibodies (Becton Dickinson, Mountain View, CA) and rabbit complement (Cedarlane, Ontario, Canada). After BM transplantation, the animals were kept in

specific pathogen-free conditions and were free from clinical and histological signs of graft-versus-host disease.

Immunization of chimeras. Three months after BM transplantation, chimeric and control mice were immunized intraperitoneally once a week for 3 weeks with either $50X10^6$ irradiated (30 Gy) host-type (BALB/c), donor-type (C57BL/10), or third-party-type (C3H) spleen cells (four mice per group for each antigenic stimulus). Ten days later, splenocytes were taken for mixed lymphocyte reaction (MLR) tests performed with immunizing cells as stimulator cells; sera were analyzed for the presence of antibodies directed against the immunizing cells.

Mixed Lymphocyte Reaction. The MLR was performed by incubating $5X10^5$ responder spleen cells with $5X10^5$ irradiated (30 Gy) stimulator spleen cells for 96 hr at 37°C. Proliferation was assayed by measuring the incorporation of radioactive [^{3}H]-thymidine, and expressed as

$$\text{Stimulation Index} = \frac{\text{cpm of stimulated cells} - \text{cpm of unstimulated cells}}{\text{cpm of unstimulated cells}}$$

Detection of IgG alloantibody formation. Alloantibody detection was performed using a modification of the flow cytometry crossmatch technique, as we described previously (14). Briefly $1X10^6$ target spleen cells in 0.1 ml of saline solution were incubated for 20 min with 0.1ml of various dilutions of serum from non-immunized and immunized mice. Thereafter, the cells were washed and incubated with 15 ul of Fluorescein Isothiocyanate-conjugated goat antimouse1gG Fc (The Binding Site,

Birmingham, England) for 20 min. This second step detects only mouse 1gG and hence does not stain mouse B cells as only a negligible fraction of them express 1gG in mice. Serum titers were scored positive when they showed a significant shift in the mean fluorescein intensity by flow cytometry (FACScan, Becton Dickinson).

Detection of alloantibodies directed against MHC class I or class II expressing cells. Because murine T lymphocytes express only MHC class I antigens and not MHC class II antigens, anti-MHC class I-directed antibodies were assayed by two-color flow cytometry using anti-L_3T_4 (Becton Dickinson) Phycoerythrin- coupled monoclonal antibody (directed against the murine $CD4^+$ T lymphocyte subset) and Flourescein Isothiocyanate-conjugaed anti-mouse 1gG (Fc) monoclonal antibody. For assaying reactivity on class II-expressing cells, relevant anti-MHC class II-reacting anti-I-A^b, -I-A^d biotin-conjugated antibodies (PharMingen, San Diego, CA) were used.

Purification of T and B lymphocytes. T lymphocytes and B lymphocytes were purified from splenocyte suspensions using a panning technique. Rabbit anti-mouse Ig was first diluted to 10 ul per 10 ml of phosphate-buffered saline (PBS).

Petri dishes (Falcon, Becton Dickinson, Belgium) were coated with 10 ml of this solution and kept at 4° C overnight. The following morning, the solution was poured off and the Petri dishes washed three times with PBS. A single suspension of spleen cells was prepared in RPMI medium containing 10% fetal calf serum plus antibiotics. Six milliliters of medium containing $100X10^6$ cells were added to each Petri dish and kept for 30 min at room temperature. Thereafter, the Petri dishes were rotated for 30 sec. To isolate the T cell population, non-adherent cells were poured into a second

antibody-coated Petri dish and kept for another 30 min at room temperature. Thereafter, the non-adherent cells were collected into a tube. To isolate the B cell-enriched populations, the Petri dishes containing the adherent B cells were first washed three times with sterile PBS to remove remaining T cells. Thereafter, 5 ml of culture medium containing 10% normal mouse serum were added and incubated at 37° C for 1 hr in order to selectively dislodge the adherent B cells. B cells were then collected by pipetting into a tube and were washed two times with RPMI. This panning technique resulted in highly purified populations (>95% purity) of T and B lymphocytes. Where necessary, purification of donor lymphocytes from MC was done subsequently using C57BL anti-BALB/c alloantibodies and rabbit complement. After this procedure, no remaining BALB/c cells could be detected by flow cytometry.

Transfer experiments. For the transfer experiments, lethally irradiated (12 Gy) control C57BL/10 mice were given $15X10^6$ purified T cells and $15X10^6$ purified B cells intravenously from various origins. One week later, the mice were immunized intraperitoneally once a week for 2 weeks with $1X10^8$ irradiated (30 Gy) BALB/c or C3H spleen cells. A week after this, the serum was taken for alloantibody detection. Alloantibodies were scored using flow cytometry as described above.

RESULTS

Cellular (MLR) and humoral (alloantibody formation) immunity in BM chimeras after immunization with cells expressing donor (C57BL/10), host (BALM/c), or third-party (C3H) antigens. Three months after BM transplantation, both MC and CC were fully immunocompetent as they raised normal cellular (MLR, Fig. 1a) and humoral (alloantibody formation, Fig. 1b) immune reactions after immunizing with third party (C3H) antigens. Concurrently, they were tolerant for host-type (BALB/c) and donor-type (C57BL/10) antigens, as shown by the absence of a significant MLR response or alloantibody formation against these antigens.

Cross-reactivity of anti-third-party (C3H) antiserum of MC and CC against mismatched host-type (BALB/c) antigens. As mentioned above, the apparent humoral tolerance of all chimeric mice may be due to a lack of T cell help (2), because they all showed MLR non-responsiveness against donor- and host-type antigens. To circumvent this absence of T cell help, we decided to exploit the principle of "T cell cross-help". This principle is based on the notion that T and B lymphocytes recognise different parts of the same antigen, called carrier and hapten, respectively (15). B lymphocytes with reactivity against the hapten part of host antigens may therefore persist in some chimeras, but remain quiescent due to T cell tolerance for the carrier part of the same antigens. However, after immunization with third-party (C3H0 antigens, the same N lymphocytes may pick up C3H antigens, the same B lymphocytes may pick up C3H

antigens that share hapten parts with BALB/c antigens but display C3H-specific carrier parts. Subsequently, these B lymphocytes may receive help from anti-C3H-directed T lymphocytes may receive help from anti-C3H-directed T lymphocytes and synthesize immunoglobulins recognizing cross- reacting BALB/c haptens. The possibility that intrinsic B lymphocyte tolerance may be absent as a consequence of the above was explored by investigating the cross-reactivity of the anti-C3H allosera of MC and CC against donor-type (C57BL/10) or host-type (BALB/c) cells (Fig.2). As expected, anti-C3H antibodies from MC reacted strongly, as seen in a flow cytometry assay against C3H class I-expressing T cells (Fig.2c). However, cross-reactivity could be detected against neither C57BL/10 (Fig. 2, e and g) nor BALB/c (Fig.2,I and k) targets (one representative out of four similar experiments is shown). The outcome obtained with anti-C3H antibodies raised in CC was different, however. Again, as expected, this alloantiserum reacted with MHC class I-expressing (Fig. 2b) and MHC class II-expressing (Fig. 2d) C3H cells and not with C57BL/10 (Fig. 2, f and h). However, a clear cross-reactivity against BALB/c targets expressing MHC class II antigens (Fig. 21) was seen (one representative out of five similar experiments is shown). This reactivity was directed at MHC antigens, as the latter serum was also positive on target cells from B10D2 mice that have the C57BL/10 background but are congeneic for the BALB/c MHC antigens (Fig. 2m). Interestingly, the anti-C3H antiserum from the CC reacted only with MHC class II-positive host cells and not with MHC class II antigens are predominantly expressed on haematopoietic cells (16), host-type MHC class II antigens are essentially absent in the CC, whereas MHC class I expression persists on non-haematopoietic cells. Therefore, anti-host cross-reacting antibodies from CC were

probably absorbed in vivo on class I MHC antigens but not on class II MHC antigens. This hypothesis was further supported by the transfer experiments discussed below.

Transfer experiments show that B lymphocytes from MC develop intrinsic transplantation tolerance, whereas those from CC do not. To exclude the possibility that the absence of anti-host cross-reacting antibodies in the MC could have been due to absorption, because the MC host-type hemopoietic, and hence MHC class II as well as class I antigens, persist, purified donor B lymphocytes were isolated from MC and CC for transfer experiments (Fig. 3). These donor C57BL/10) B lymphocytes from normal control C57BL/10 mice into heavily irradiated (12 Gy) control C57BL/10 mice. One week later, these animals were immunized with BALB/c or C3H cells. Figure 3 shows the anti-BALB/c (Fig. 3a) and anti-C3H (Fig. 3b) alloantibody formation in C57BL/10 mice reconstituted with control C57BL/10 T cells and C57BL/10 B lymphocytes isolated from MC. Figure 3a, line C, gives the anti-C3H response, as assessed with flow cytometry against target T lymphocytes (see Materials and Methods). For each experiment, negative (A lines) and positive (B lines) control sera obtained from non-immunized and immunized control C57BL/10 mice, respectively, are shown as well. This experiment clearly shows that donor-type B lymphocytes isolated from MC are unable, even in the presence of non-tolerant control T lymphocytes, to produce alloantibodies directed at the mismatched antigens (BALB/c) of their original host (Fig. 3a, line C). Nevertheless, these B lymphocytes were immunocompetent, as shown by the significant anti-C3H response (Fig. 3b, line C) (one out of three similar experiments is shown).

The activity of the B lymphocytes isolated from CC is, however, very different, as shown in Figure 3c and Figure 3d. Indeed, mice reconstituted with B lymphocytes from CC were able to produce anti-BALB/c antibodies (Fig. 3c, line C) to the same extent as anti-C3H antibodies (Fig. 3d, line C). Again, flow cytometry profiles of negative and positive control sera are shown in lines A and B, respectively (one out of four similar experiments is shown). The absence of tolerance in the B cell compartment of CC was in contrast to the tolerance in their T cell compartment. Indeed, when in similar transfer experiments the T lymphocytes rather than the B lymphocytes from CC were transfused together with B lymphoctes from control C57BL/10 mice, the reconstituted mice were able to make anti-C3H antibodies (Fig. 3f) but not anti-BALB/c antibodies (Fig. 3f).

In summary, all previous transfer experiments thus clearly demonstrate that intrinsic B cell tolerance for host antigens had occurred in MC but not in CC. Moreover, as the flow cytometry data shown in Figure 3 were obtained from the T lymphocyte window and hence were likely directed against MHC class I (see Materials and Methods), B lymphocytes of CC are able to produce not only anti-host MHC class II alloantibodies, as previously shown in Figure 21, but also anti-host-type MHC class I alloantibodies (Fig. 3c. line C)

DISCUSSION

The existence of intrinsic B lymphocyte tolerance has recently been unequivocally demonstrated in experiments involving mice with rearranged immunoglobulin genes and was shown to be based on various mechanisms, such as clonal deletion and clonal energy (7-9). The immunoglobulin coded for by these genes was directed at one specific known antigen. Here, we were interested to see whether intrinsic B cell tolerance could also be achieved in a more complex clinical situation, such as after BM transplantation, where obviously many B lymphocyte clones, all with various affinities for numerous antigens, have to be tolerant. In these experiments it is impossible to unravel the precise mechanisms involved. Indeed, to demonstrate clonal deletion, anti-idiotypic antibodies, recognizing all the alloreactive antibodies with various affinities for the numerous alloantigens, should be available. This is obviously not feasible. A relative argument in favor of the mechanism of clonal anergy would be the progressive disappearance of the tolerance after transfer of the tolerized B lymphocytes in adoptive transfer experiments.

Yet even this would not be a definite proof for anergy as recovery of B lymphocytes from haematopoietic stem cells in the new host may occur. What can be inferred is that the mechanism involved must be a very efficient and stable one, as the B lymphocyte

tolerance of the MC could not be broken despite multiple immunizations of the original hosts and the presence of immunocompetent T cells, as shown in the transfer experiments.

A second important observation is that B lymphocyte tolerance was not achieved in CC. In the latter chimeras, host-type MHC class I antigens are expressed on epithelial cells, but host-type MHC class II antigens are constitutionally also present on thymic epithelial cells (17). Although it can be expected that the donor B cells encounter host-type antigens in secreted form, this is clearly inadequate to result in B cell tolerance in CC. Indeed, from secreted antigens it is known that they either do not induce B cell tolerance or induce anergy only (7,10), which may easily wane (18). Alternatively, the donor B lymphocytes may encounter host antigens under polyvalent membrane bound form on host-type mesenchymal or haematopoietic cells (19). As CC by definition lack host-type haematopoietic cells, the absence of B cell tolerance in CC suggests that B lymphocyte transplantation tolerance depends on the presence of haematopoietic cells expressing the tolerogens. The question that can be raised is why donor B lymphocytes are not tolerant in CC whereas T cells are. Indeed, the CC showed MLR nonresponsiveness for host-type antigens, and, after purification, their T cells were unable to provide help in the transfer experiments for control B lymphocytes after immunization with host-type cells. The most likely explanation is that T lymphocytes mature in the thymus, where they encounter host-type thymic epithelial cells. Even in CC these cells are known to express host MHC class I and II antigens (17), and as

several reports indicate that thymic epithelial cells may impart tolerance (20,21), they are likely to be responsible for inducing T cell tolerance in CC.

As discussed earlier, in order to circumvent the immune incompetence based on MHC restriction problems in CC, a semiallogeneic combination was used in the present experiments. We are currently exploring whether this simultaneous presence of allogeneic and syngeneic antigens on the tolerance-inducing F1 haematopoietic cells is a necessity for the B lymphocytes to develop transplantation tolerance.

The current study is relevant for two important points raised recently in transplantation immunology. First, it was shown that CC have poor survival rates as compared with MC (22). This may be due to their persistent capacity to make antibodies against the host when challenged with foreign antigens that share haptens with the host, as shown in our CC immunized with C3H cells. Such as scenario may manifest during infections. Under these circumstances, CC have the potential to produce antibodies that cross-react with host antigens and hence may develop chronic graft-versus-host disease. The finding of a more profound B cell tolerance in MC may be relevant for organ transplantation as well, as it has been claimed that transplant patients with the best outcomes, even after withdrawal of immunosuppressants, frequently show mixed chimerism (23). If the latter patients show better B cell transplantation tolerance, then the development of antibodies against the donor organ is likely to occur. Subsequently, these patients may be less susceptible to chronic rejection.

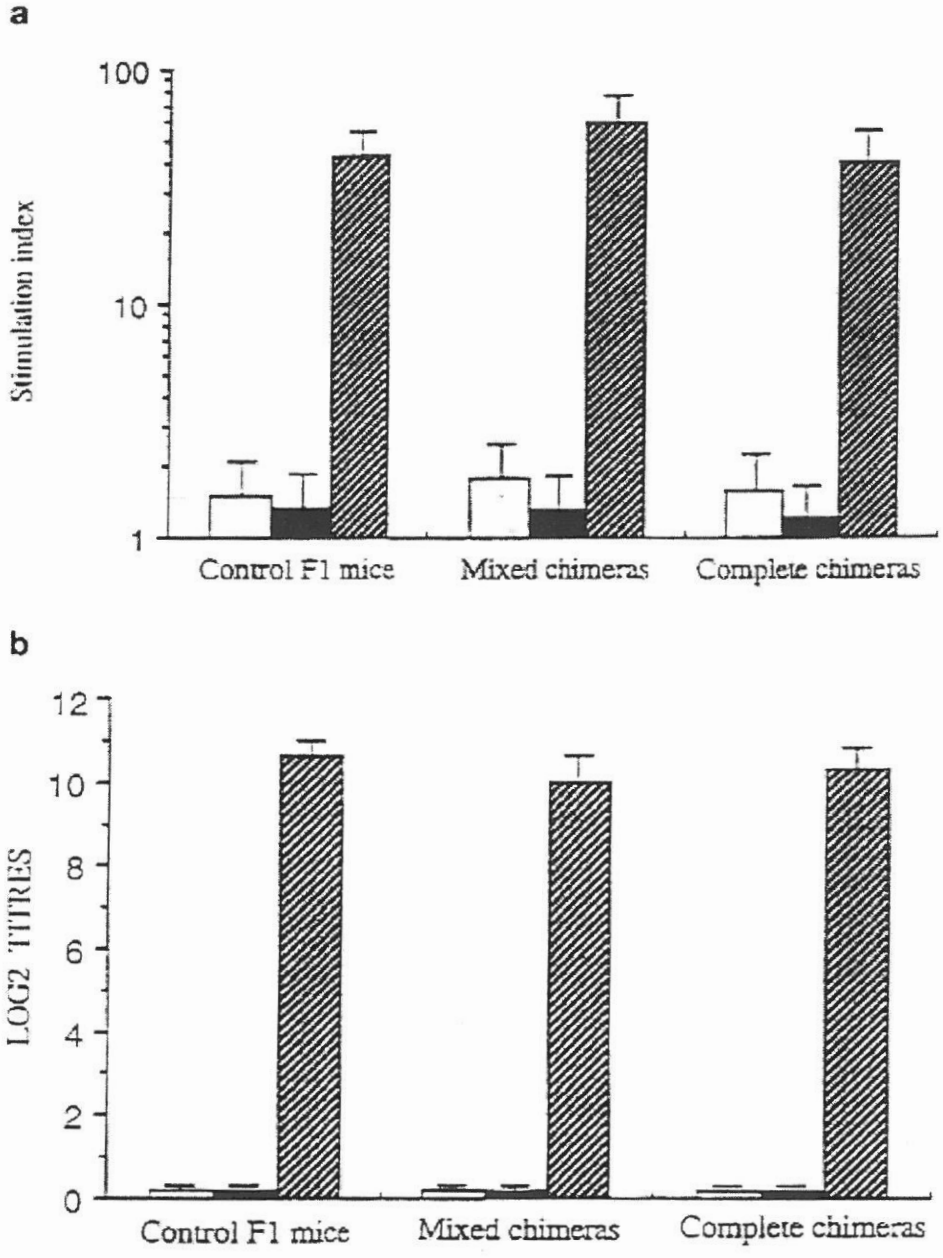


Figure 1. MLR (a) and alloantibody titer (b) of control F1 mice MC and CC 3 months after BM transplantation and immunization at three weekly intervals with host-type (BALB/c), donor type (C57BL/10), or third-party (C3H) splenocytes (four mice per group for each antigenic stimulus). Ten days later, MLRs were performed against the immunizing cells. The data are expressed as the mean ±SE of the stimulation index (a) (□, BALB/c; ■ C57BL; ▨, C3H). At the same time , the sera of the mice were screened for IgG alloantibody formation (expressed as mean log 2 titer ± SE) against the immunizing cells (b) (□, titer of anti-BALB/c allosera; ■, titer of anti C57BL allosera; ▨, titre of anti-C3H allosera).

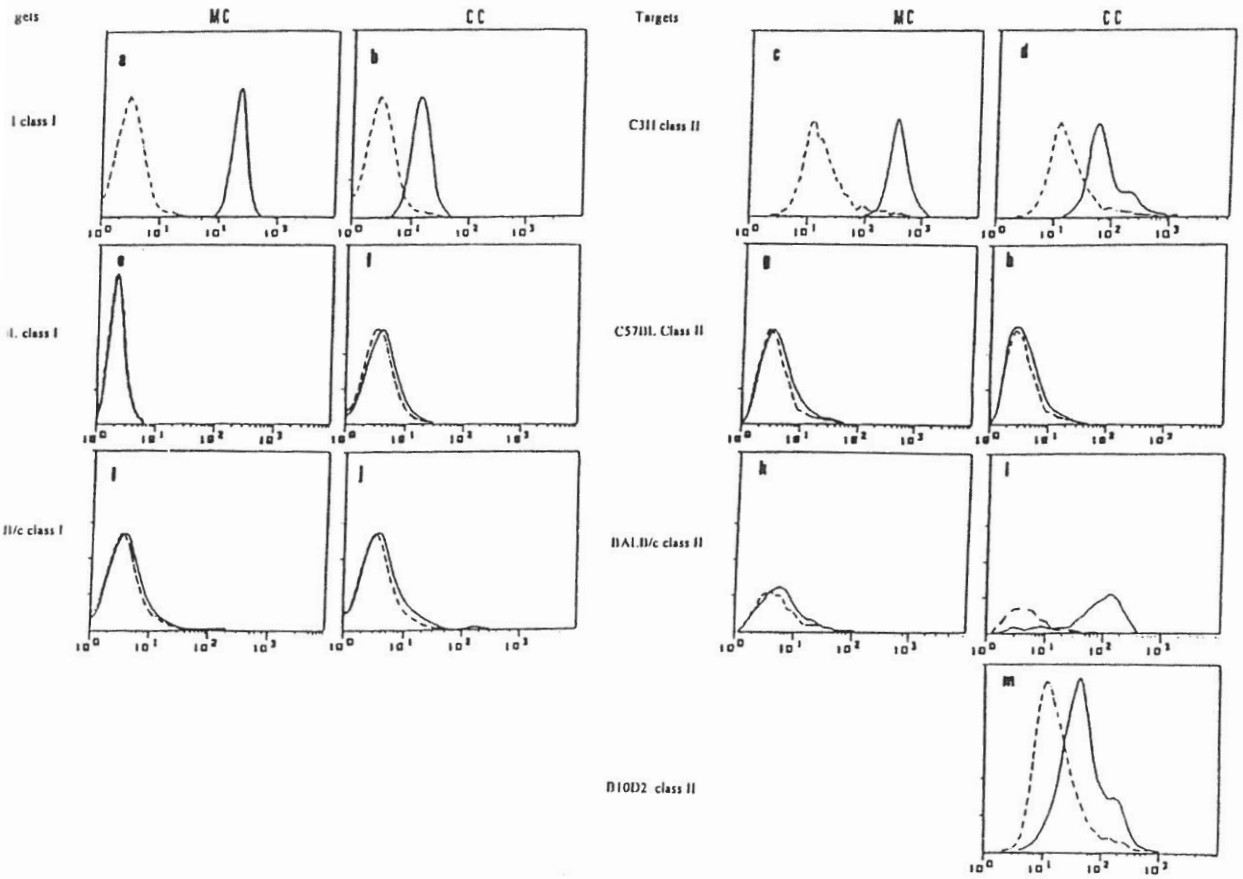


Figure 2. Cross-reactivity of anti-C3H immune sera obtained from MC or CC. The sera raised in MC or CC were assayed in a 1:10 dilution against C3H MHC class I-expressing T cells (a and b, for MC and CC, respectively), C3H MHC class II expressing cells (c, d), donor-type C57Bl/10 class I-expressing cells (e,f), donor -type C57BL/10 class II-expressing cells (g, h), host-type BALB/c class I-expressing cells (k,l). The date shown were obtained from the L3T4 (b,e,f,I,j) or I-A-positive (c, d, g, h, k, l,m) windows and given as the log of fluorescence (horizontal axis) against the number of cells (vertical axis) for sera obtained before (dashed line) and after immunization (solid line).

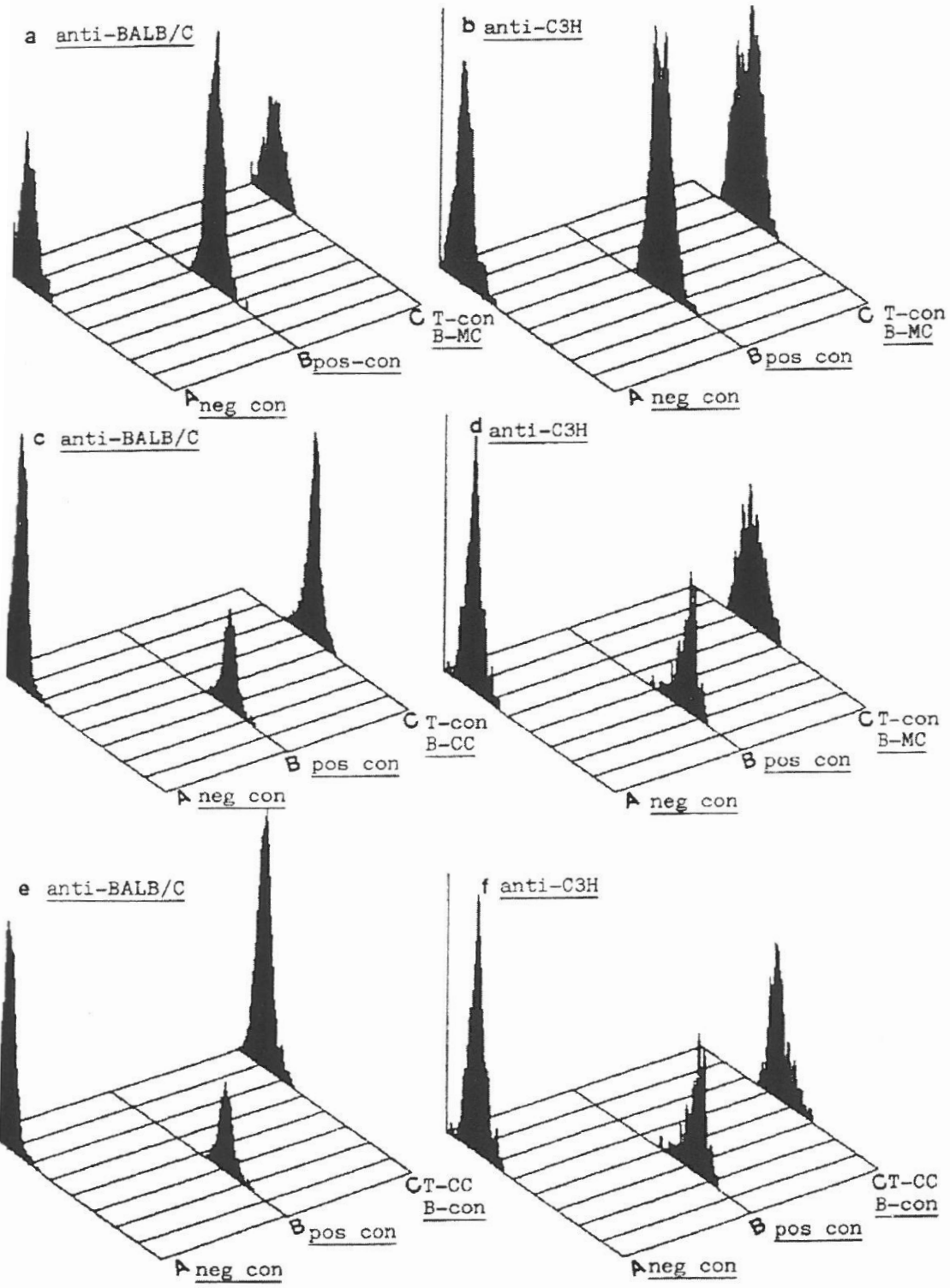


Figure 3. Alloantibody formation in lethally irradiated C57BL/10 control mice reconstituted with purified T and B lymphocytes of various origins. For each experiment, a negative control mice reconstituted with purified T and B lymphocytes of various origins. For each experiment, a negative control serum (line A, neg con) taken from non immunized C57BL/10 control mice and a positive control serum (line B, pos con) taken from control C57BL/10 mice immunized with the relevant antigen are shown. In panel a, line C shows the anti-BALB/c response of mice reconstituted with control C57BL/10 T lymphocytes (T-con)

together with C57BL/10 B lymphocytes from MC (B-MC). In panel b, line C shows the antibody formation in mice reconstituted with C57BL/10 B lymphocytes from CC (B-CC) together with control C57BL/10 T cells (T-con), respectively, after immunization with BALB/c (c, line C) and C3H (d, line C) cells. In panels e and f, the alloantibody formation of mice reconstituted with C57BL/10 T lymphocytes from CC (T-CC) together with control C57BL/10 B lymphocytes (B-con) after immunization with C3H cells (f, line C) or BALB/c cells (e, line C) is given. The results are shown as flow cytometry profiles of 1:5 diluted sera on relevant T lymphocyte targets (see *Materials and Methods*).

CHAPTER 7

SUMMARY

It has been previously demonstrated that two different conditioning regimens (TLI and TBI) are able to create a stable chimerism without signs of GVHD. In the current study, the mechanisms of tolerance that maintain chimerism and determine the behaviour of the chimeric animals against GVL and rejection reactions were investigated.

In the mixed chimeric animals induced by TBI, the tolerance was not based on the mechanisms of deletion. On the contrary, an expansion of Vβ6 positive cells of the recepient, that recognises the antibodies mlsa on the donor is present. Additionally, these cells behave as suppressor cells in the donor versus recipient and the recipient versus donor reactions and this was demonstrated in both the in vivo and in vitro experiments. This explains why the mixed chimeric animals did not lose their chimeric state after injection of splenocytes originating from the donor and were less effective in elaborating a GVL reaction after injection of tumour cells.

In contrast, the mixed chimeras that were conditioned with TLI develop a tolerance mainly based on clonal deletion, in the absence of suppressor cells. This may explain why the TLI conditioned mice were unable to resist rejection and showed a strong GVL reaction.

The mechanisms of transplantation tolerance is would be different depending on the type of conditioning regimen employed as well as the GVL effect. This partly

explains the resistance to rejection of chimeric animals and their capacity to facilitate GVL reactions.

Complete chimeras (CC), which were obtained by infusing $20x10^6$ C57BL/10xBALB/c) F1 recipients, were repopulated for 100% with P1 lymphohaematopoietic cells. In mixed chimeras (MC), which were obtained by injection of $15x10^6$ F1 BM cells, between 15% and 40% of F1 lymphohaematopoietic cells persisted after BM transplantation. Neither MC nor CC were able to develop significant T cell immunity (mixed lymphocyte reaction) or B cell immunity (1gG alloantibodies) against the mismatched host antigens (BALB/c), despite repetitive immunizations. However, after immunization with third-party cells (C3H), the IgG alloantibodies raised cross-reacted with the host-type (BALB/c) antigens in the CC but not in the MC. This suggested that intrinsic B lymphocyte tolerance for host antigens had occurred in MC but not in the MC. This was further evidenced in transfer experiments using lethally irradiated C57BL/10 mice reconstituted with purified control C57BL/10 T Lymphocytes together with purified C57BL/10 B lymphocytes isolated from CC or MC. Only the recipients reconstituted with B lymphocytes from CC, and not those from MC, produced anti-BALB/c IgG alloantibodies after immunization. These results show that intrinsic B lymphocyte tolerance can be achieved after transplantation and that this depends on the presence of lymphohaematopoietic cells expressing the tolerogens.

GENERAL PERSPECTIVES AND OPINION

Bone Marrow Transplantation is a well-established therapeutic modality in clinical practice. Currently, the indications for its use remain pertinent to life threatening malignancies and congenital defects where it has made a significant impact on survival, but more importantly, on the quality of life of the individual.

It is obvious that were it a safer procedure, its application to other less sinister conditions would be beneficial. Current clinical experience is guarded given the experience with BMT especially with respect to engraftment failure, the intensive care required during the period of engraftment as well as the later emergence of GVHD.

Experimental data would suggest that the concept of transplantation tolerance as well as immunocompetence is attainable. However, this has yet to be predictably achieved in clinical medicine. The results of experimental data must be guarded in the face of the incongruity of homogenous subjects with respect to the results of in vitro and in vivo results as illustrated in these experiments.

While mechanisms for transplantation tolerance are well-established, the mechanisms that prevail in individuals is influenced by factors beyond donor compatibility and includes the preconditioning regime and the quality of the bone marrow. This is relevant because it impacts on immunocompetence.

Furthermore, whilst T cells appear to be pivotal in the various mechanisms that have been identified, the in vivo results would suggest that other cells may play important roles and elaborating the network of interaction remains elusive.

This study clearly demonstrated that differing conditioning regimes sustain different mechanisms of tolerance when GVHD has been eliminated and this has long-term consequences.

It is hoped that the advent of newer immunosuppressive agents will allow for more predictable engraftment eliminating GVHD and, importantly, supporting transplantation tolerance that will allow for a superior immunocompetence rather than pose the risk of susceptibility to the recipient.

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