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PRODUCTION AND CHARACTERIZATION OF HEMOPOIETIC ANTIBODY-FACILITATED CHIMERAS

by

LOUIS HUGO FRANCESCUTTI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled PRODUCTION AND CHARACTERIZATION OF HEMOPOIETIC ANTIBODY-FACILITATED CHIMERAS submitted by LOUIS HUGO FRANCESCUTTI in partial fulfilment of the requirements for the degree of Doctor of Philosophy in MEDICAL SCIENCES.

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Dedication

I would like to dedicate this thesis to the memory of Stanley Amey, a close and dear friend, who provided me with inspiration and a sense of direction at just the right moment in my life.

Abstract

Complete hemopoietic takeover between semiallogeneic adults can be accomplished by the administration of antihost major histocompatibility complex (MHC) monoclonal antibody (mAb) and donor stem cells. The recipients of such treatment are termed antibody-facilitated (AF) chimeras, and they have been produced in BALB/cCR → (BALB/cCR x C3H/HeJ)F_1 and DBA/2J → (DBA/2J x C3H/HeJ)F_1 strain combinations. Donor stem cells can be derived from spleen, bone marrow, or T cell-depleted bone marrow. Engraftment by donor hemopoietic cells can be facilitated by mAbs directed toward Class I (anti-H-2K^k) or Class II (anti-H-2I-A^k) MHC antigens of the host. By monitoring isozymes of glucose phosphate isomerase, it can be shown that the establishment of donor hemopoiesis is stable, persisting for more than two years without graft-*versus*-host disease.

Examination of these AF chimeras reveals a correlation between peripheral blood chimerism and the stem cell constitution of both spleen and bone marrow. The peripheral blood chimerism also correlates with the level of chimerism in peritoneal exudate macrophages.

The development of these stable long-term AF chimeras across MHC barriers in the absence of irradiation or immunosuppressive agents may suggest a new approach to clinical bone marrow transplantation for certain conditions, such as aplasia and immunodeficiency syndromes.

Preface

Parts of the work described in this thesis have previously been published in *Transplantation Proceedings* and *Transplantation*.

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List of Abbreviations

ABO	ABO human blood group
AF	antibody-facilitated
AML	acute myelogenous leukemia
αΘBM	anti-Thy-1.2 treated bone marrow
BALB	BALB/cCR
BFU-E	burst forming unit-erythroid
BM	bone marrow
BMT	bone marrow transplantation
BU	busulfan
C	centigrade
C3H	C3H/HeJ
CBA	CBA/CAJ
CC3F$_1$	(BALB/cCR x C3H/HeJ)F$_1$
CFU-E	colony forming unit-erythroid
CFU-EOS	colony forming unit-eosinophil
CFU-GM	colony forming unit-granulocyte-macrophage
CFU-MEG	colony forming unit-megarkaryocyte-platelets
CFU-S	colony forming unit-spleen
CML	chronic myelogenous leukemia
CO_2	carbon dioxide
cpm	counts per minute
CY	cyclophosphamide
CSA	colony-stimulating-activity

CSP	cyclosporin
CC3F$_1$	(DBA/2J x C3H/HeJ)F$_1$
DBA	DBA/2J
DLA	canine leukocyte antigens
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EPO	erythropoietin
F$_1$	first progeny
FU	5-fluorouracil
GPC	guinea pig complement
Gpi	glucose phosphate isomerase enzyme
Gpi	glucose phosphate isomerase gene
GVH	graft-*versus*-host
GVHD	graft-*versus*-host disease
Hct	hematocrit
H&E	hematoxylin & eosin stain
Hh	hemopoietic histocompatibility gene
HLA	human leukocyte antigens
HU	hydroxyurea
iv	intravenbus
Ig	immunoglobulin
^{125}IUdR	^{125}I-Iododeoxyuridine
L-PAM	L-phenylalanine mustard
M	Molar
mAb(s)	monoclonal antibody(ies)
mg	milligram(s)
MHC	major histocompatibility complex

min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
MLC	mixed-lymphocyte-culture
ND	not determined
NK	natural-killer cells
P_1	parental strain
PBS	phosphate buffered saline
R	irradiation
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SC	spleen cells
SCID	severe combined immunodeficiency disease
SCM	spleen conditioned medium
TBI	total body irradiation
TLI	total lymphoid irradiation
µg	microgram(s)
µl	microliters(s)
v/v	volume/volume
w/w	weight/weight

I. Introduction

Attempts to transplant hemopoietic tissue across major histocompatibility barriers, have met with a certain degree of success. Despite extensive efforts in both the experimental and the clinical models, however, a variety of problems still exist that must be overcome before bone marrow transplantation can achieve its full potential.

The objective of this chapter is to review the literature pertaining to the history of bone marrow transplantation, its use, and its present drawbacks. In addition, a review of hemopoiesis as it applies to the transplant model and a look at some of the past, present, and future animal models of bone marrow transplantation will be presented. Together, these topics will provide an overview of the current state of the art in cellular hemopoietic transplantation.

II. Clinical Bone Marrow Transplantation

Bone marrow transplantation (BMT) has, within the last 20 years, become the treatment of choice for a variety of hematologic diseases. Mathe *et al.*, in the late 1950's, described the use of unrelated donors in the treatment of individuals accidentally exposed to radiation. Although eventual rejection of the grafts occurred, the treatment allowed for endogenous repopulation by the host hemopoietic tissue to occur (Mathe, 1959). Reports of controlled studies relating to the clinical application of transplantation, using thymic, fetal liver, and bone marrow cells, began appearing in the 1950's and 1960's (Uphoff *et al.*, 1958; Pegg 1960; Hong, 1968a; Gatti *et al.*, 1968; Cleveland *et al.*, 1968; August *et al.*, 1968). Since those initial days, a considerable degree of success has been seen in the treatment of severe aplastic anemias (Gluckman *et al.*, 1981), some leukemias (Thomas *et al.*, 1977a, 1977b, 1979), certain immunodeficiency diseases (Pahwa *et al.*, 1978), and even in genetic disorders of hemopoiesis (Thomas *et al.*, 1982). Despite these recent advances, several drawbacks still exist, including graft-*versus*-host disease (GVHD) (Storb *et al.*, 1983a; Gale, 1982), opportunistic infections (Meyers, 1982a, 1982b, 1984; Sullivan *et al.*, 1984; Winston *et al.*, 1984; and Buckner *et al.*, 1984), and the lack of complete immunological reconstitution (Witherspoon *et al.*, 1984a, 1984b). Several of these problems are a direct result of the irradiation and immunosuppressive regimens used in

the preparation of the patient prior to engraftment. It will soon become apparent, that to date, no acceptable means of host preparation prior to transplantation has been achieved. The effects of the irradiation and the cytotoxic agents used, are still far too nonspecific in their mode of action. Our results suggest a new approach to circumventing these problems. Let us now consider certain conditions in which some degree of success has been achieved.

A. Severe Combined Immunodeficiency Disease

A little over a decade and a half ago, Good and his colleagues reported the successful correction of a X-linked form of severe combined immunodeficiency disease (SCID) (Hong *et al.*, 1968a; Gatti *et al.*, 1968). Although the marrow engrafted and subsequently corrected the state of immunodeficiency, an immunologically based aplastic anemia ensued as a result of a mismatch at the HLA-A locus (Gatti *et al.*, 1971a). This was corrected by a second marrow transplantation using an HLA-matched sibling as a donor (Gatti *et al.*, 1971b).

In patients with SCID, an immunosuppressive regime is not required since these patients are already immunodeficient. Therefore, the success rate is very high if HLA-identical siblings are used as donors (Meuwissen *et al.*, 1969). A severe GVH reaction occurs if donor and recipient are not HLA-matched (Biggar, 1973; Park, 1974). Recently, Reisner *et al.* have attempted to remove or inactivate T

cells in the donor marrow responsible for the GVH reaction by means of fractionation techniques, and their results look promising (Reisner *et al.*, 1983).

B. Aplastic Anemia

A somewhat more complicated situation exists in the case of aplastic anemia. Aplastic anemia is an example of a major hypoproliferative disorder of the bone marrow. It should be noted that the marrow is not truly 'aplastic' but hypoplastic. Areas of normocellular as well as hypercellular marrow can be found in certain cases, although 2/3 of them are apparently idiopathic (Beck, 1982). Some causative agents have been associated with the onset of aplastic anemia. Most noticable among these are ionizing radiation, benzene and its derivatives, various cytostatic agents, some classes of drugs and viral infections like hepatitis-B. The onset of symptoms is usually insidious. The individual presents with anemia, thrombocytopenia, and an increased incidence of infection, as a result of neutropenia. Therapeutic modalities include supportive transfusions, steroids, stimulation of the marrow with androgens, and possible splenectomy. It has been shown that transplantation can lead to a significantly better survival rate than supportive measures alone (Camitta, 1979). Although the marrow in these patients is partially hypoplastic and, in a sense, an ideal site for donor marrow to engraft, major problems must be overcome. One must find a HLA identical

donor as defined by antigenic HLA identity and non-reactive MLC and even in such case the rejection of the donor marrow cells can still occur by the immunologically competent host. Currently the preparative immunosuppressive regime consists of the administration of cyclophosphamide (CY), 50 mg/kg for 4 days. Some centres also include 750 rad total lymphoid irradiation (TLI) in the regime (Storb and Thomas, 1983a; Ramsay *et al.*, 1980; Kersey *et al.*, 1980).

Graft rejection and secondary immunodeficiency can occur, and can be directly related to the regime of immunosuppression used in the transplantation. On the one hand it is possible to reduce the rate of graft rejection by increasing the severity of the immunosuppressive regime, while on the other hand this will probably increase the risk of iatrogenic complications such as infections.

Several factors have been identified which enhance graft rejection. For instance, multiple-transfusion and hence sensitization of the patients prior to grafting; a low number of transplanted marrow cells ($<3 \times 10^8$/kg); and marrow grafts from male donors (Storb, 1977, Storb *et al.*, 1983b). The Seattle Marrow Transplant Team has performed 39 transplants in patients who had not previously been transfused. Thus far, 83 % of their patients have survived between 2 and 9.5 years post grafting (Storb *et al.*, 1980).

In patients who had previously received multiple-transfusions, the transplant protocol included the addition of donor buffy coat cells. This modification

improved the 2 - 6 year survival rate from 45 % in patients not receiving buffy coat treatment to 75 % survival in those receiving these additional cells. It was postulated that these findings were analagous to the enhancement of allogeneic marrow engraftment seen experimentally in mice and dogs. One must bear in mind that this modification also increases the incidence of chronic GVHD, while not affecting the mortality rate (Storb *et al.*, 1982a).

Several recent studies indicate that the use of Cyclosporin-A (CSA) can reverse transfusion-related sensitization and lower the likelihood of the marrow graft being rejected (Storb *et al.*, 1982b; Hows *et al.*, 1982). Attempts to prevent sensitization by ABO-and HLA-typing the patient and trying to find suitably matched donors has been instituted in some centres. Ideally, patients identified as being likely candidates for BMT should be transplanted as soon as possible in light of the success of the Seattle Marrow Transplant Team and other centres around the world that use this principle (Storb, 1980).

Finally, Ildstad and Sachs (1984) have used an animal model to show that reconstitution of irradiated hosts with T cell depleted bone marrow, containing both syngeneic (host) as well as allogeneic (donor) cells, leads to long-term survival of the reconstituted animals as well as prolongation of donor skin grafts. Interestingly, these recipients are able to mount normal immune responses to third party antigens and show no signs of GVHD.

C. Acute Leukemia

Bone marrow transplantation in leukemic patients is somewhat similar to the situation in aplastic anemia, although graft rejection is rarer. This is no doubt due to the use of high doses of total body irradiation (TBI) to destroy the leukemic cells. It has been noted that the occurence of GVHD also exhibits a peculiar graft-*versus*-leukemia effect, to be discussed later.

Hobbs (1983) and Bortin (Bortin *et al.*, 1982) reported that BMT is the treatment of choice for acute myelogenous leukemia (AML) as compared to chemotherapy alone, providing the patient is under 40 years of age, is engrafted with an adequate number of cells (>2 x 10^8 nucleated cells/kg body weight) from a compatible sibling donor while in first complete remission, and before he has been multiple-transfused (<22 times). In children, a long-term survival rate of 70 % has been the experience of Good (Good *et al.*, 1983). In acute nonlymphocytic leukemia in first remission, Thomas reports survival rates in unmaintained first remission of 55 %; similar findings are also reported in other centres, where rates of apparent cure range from 35 % to 65 %, depending on the age group (Thomas *et al.*, 1982; Thomas *et al.*, 1984; Forman *et al.*, 1983; Blume *et al.*, 1980). Patients presenting with chronic granulocytic leukemia in blast crisis and undergoing marrow grafting show survival rates of approximately 30 % (Thomas *et al.*, 1984).

D. Other Hematogenous and Genetic Disorders

Recent articles summarize the many other diseases in which BMT can be used as an effective therapeutic modality. These include Wiskott-Aldrich syndrome, Blackfan-Diamond syndrome, Fanconi anemia, severe neutrophil dysfunction, chronic granulomatous disease of childhood, severe (Kostman-type) agranulocytosis, infantile osteopetrosis, Burkitt's lymphoma, Hodgkin's disease, myelofibrosis, preleukemia, and multiple myeloma (Good *et al.*, 1983; Thomas *et al.*, 1984; Appelbaum *et al.*, 1984; Iriondo *et al.*, 1984). However, Thomas notes the need for more organized studies before the full potential of BMT can be realized in these patients.

Recently, bone marrow transplantation has been used with some success in treating the twitcher mouse, the animal model of galactosylceramidase deficiency (Krabbe's disease). Prolonged survival and gradual repair of demyelination in peripheral nerves is seen, although improvements in the CNS were not noted. The latter observation is most likely related to the involvement of the blood-brain barrier, which may be acting as an impervious barrier (Yeager *et al.*, 1984).

An exciting recent development has been the advent of gene transfection and its potential application to defective stem cell therapy. This subject will be considered later in the **Conclusion.**

Despite the advances in BMT for the treatment of the various disorders just reviewed, several problems still exist and will be considered next.

E. Complications of Bone Marrow Transplantation

Deeg *et al.*, (1984a) have recently reviewed the delayed complications of BMT, such as problems associated with engraftment, like acute or chronic GVHD, immunodeficiencies and associated infections. They also discuss the inherent complications of chemotherapy and irradiation when these are used as immunosuppressive preparatory regimes, for example pulmonary fibrosis and occlusive disease of the liver. One also finds neurologic complications, endocrine dysfunction and infertility, the appearance of cataracts, and the development of secondary malignancies. Reoccurrence of the disease being treated is also a frequent complication. The overall theme of this review is one of caution. BMT has significantly improved the chances of surviving a variety of disorders but the preparative regime is still far too toxic for more widespread use. As one of the principal complications is GVHD, I now consider it in more detail.

III. Graft-versus-Host Disease

The occurrence of either acute or chronic graft-*versus*-host disease (GVHD) post-transplantation has been attributed to donor T lymphocytes in the marrow graft. These T cells recognize host histocompatibility antigens as being foreign and subsequently mount an immune based assault (Sprent and Miller, 1972; van Bekkum, 1980; Storb, 1984; Tsoi *et al.*, 1982, 1983; van Bekkum *et al.*, 1979; Pals *et al.*, 1984a, 1984b; Micklen and Loutit, 1966; Elkins, 1971; von Boehmer *et al.*, 1975a). GVHD is seen primarily in situations where the host is unable to reject the grafted donor cells. These situations exist in immunologically immature individuals, in immunodeficient patients, and in those who have undergone an immunosuppressive treatment (for example TBI or cytotoxic drugs).

GVHD has a preference for certain target organs: in both animals and humans, the skin, gastrointestinal tract, and liver seem to be particularly susceptible. In addition, some studies have indicated that in dogs, the involvement of the bronchial glands, the salivary glands, and the prostate is evident (Thomas *et al.*, 1975; van Bekkum and de Vries, 1967; Glucksberg *et al.*, 1974; Grebe and Streilein, 1976; van Bekkum *et al.*, 1979).

A. Acute GVHD

The cause of acute GVHD has been assumed to be the presence of immunocompetent T cells in the marrow graft (Grebe and Streilein, 1976; Sprent and Miller, 1972). This has been borne out by studies in which immunosuppressive agents, such as xenogeneic anti-lymphocyte serum, anti-theta monoclonal antibodies, methotrexate, cyclophosphamide, and cyclosporin, have been shown to prevent or reduce the severity of the GVHD (Rodt, 1974, 1981; Korngold, 1983; Prentice, 1982; Filipovich, 1982; Storb, 1980). The presence of these cells varies between species, being few in number in mouse bone marrow, intermediate in the dog bone marrow, and abundant in monkey and human bone marrow. The degree of MHC mismatching between donor and recipient has been shown to be directly related to the degree of severity of the ensuing acute GVHD (Storb *et al.* 1973; 1976). Pritchard and others have also suggested that the host's genetic background, for example, non-MHC genes and/or antigens, may play an important role in modifying the severity of a graft-*versus*-host (GVH) reaction (Cantrell and Hildemann, 1972; Pritchard and Halle-Pannenko, 1981; Halle-Panneko *et al.*, 1980). Indeed, Pritchard and her colleagues now have direct evidence for the involvement of the MLS locus in death from GVHD in mice (Pritchard *et al.*, personal communication 1984). In the dog model, Storb has shown that the probability of survival of marrow grafts between DLA-identical littermates is far superior to

DLA-haploidentical matching. Similar findings have been reported in man, where HLA-identical siblings show a higher rate of successful engraftment when compared to the haploidentical situation (Storb *et al.*, 1983b).

The clinical appearance of acute GVHD in man manifests itself as an initial involvement of the skin, with subsequent involvement of the liver and intestines. Acute GVHD has been classified into four different stages, depending on the severity of involvement of the target organ systems. In addition, Thomas *et al.*, (1975) has proposed a clinical grading scale from 0 to IV, which takes the staging classification a step further (Glucksberg *et al.*, 1974). The histopathology associated with acute GVHD has been extensively reviewed (Thomas *et al.*, 1975; van Bekkum and de Vries, 1967; Sale *et al.*, 1977, 1979; Simonsen, 1962; Hansen *et al.*, 1981; Beschorner *et al.*, 1982; Gleichmann *et al.*, 1976,1982; Rolnink *et al.*, 1982, 1983a, 1983b; Rappard-van der Veen *et al.*, 1983). Skin changes can include basal vacuolar degeneration or necrosis; localized dermal-epidermal separation; infiltration and damage by eosinophils and other types of cells. Lymphocytic infiltration may occur in a variety of areas, such as joints or salivary glands. A full blown Sjögrens syndrome may appear. A number of other disease-like syndromes can occur, resembling such things as scleroderma, periarteritis, and systemic lupus erythematosus with immune complex glomerulonephritis. Lymphoproliferative syndromes may occur

and can encompass the spectrum from benign lymphoid hyperplasia to malignant lymphoma. An alternative may be the appearance of a lymphoid hypoplastic syndrome with thymic aplasia, pancytopenia, and disappearance of plasma cells from the gut.

One can readily appreciate that while all these pathologic events are occurring, the likelihood of the recipient succumbing to opportunistic infection is very high. In a clinical review, Thomas found that in the first 3 to 4 months post-transplantation, recipients were prone to a variety of bacterial, viral, and fungal organisms (Thomas *et al.*, 1975). Management of the bacterial and fungal infections involved the use of appropriate antibiotics, including amphotericin B, and when deemed necessary, granulocytic transfusions were administered. Pneumonia due to *pneumocystis carinii*, a previously serious post-transplantation complication, has recently been effectively prevented by the prophylatic use of trimethoprim-sulfamethoxazole. Interstitial pneumonias, appearing one to three months post-transplantation, remains a major problem.

Meyers and Storb separately reported that the use of cyclophosphamide as compared to TBI reduces the incidence of interstitial pneumonia from 40 - 50 % to 16 % (Meyers and Thomas, 1982a; Storb *et al.*, 1982b). Approximately 60 % of the pneumonias are associated with cytomegalovirus involvement; of these, 50 % are fatal. However, O'Reilley

and Meyers have recently reported that the use of cytomegalovirus hyperimmune globulin can influence the outcome of these highly fatal viral pneumonias (O'Reilley *et al.*, 1983; Meyers *et al.*, 1983). The Seattle Marrow Transplant Team has found that decontamination of the normal flora of the recipient in conjunction with the use of laminar air flow rooms has decreased the incidence of GVHD and increased the survival rate (Storb and Thomas, 1983a; Deeg, 1983; Thomas *et al.* 1975; Solberg, 1971).

The occurence of GVHD can be predicted neither on the basis of sex-matching the donor and recipient nor on the number of marrow cells or buffy coat cells infused. Vallera *et al.*, (1983) have attempted to remove T cells from the donor inoculum using ricin linked to a variety of mAbs specific for differentiation antigens on the surface of the T cells lymphocytes. They found that by using mixtures of these immunotoxins, they could significantly kill T lymphocytes. Current therapy for established acute GVHD consists of the use of methylprednisone, (Kanojia *et al.*, 1984), anti-thymocyte globulin, and, recently, murine monoclonal anti-T-antibodies (Remlinger *et al.*, 1984). Using a new monoclonal rat anti-human lymphocyte antibody *in vitro*, Waldmann *et al.*, (1984) were able to prevent the development of GVHD in 11 high-risk patients who had received treated marrow from HLA-matched siblings. T cell levels post-depletion, as measured by both erythrocyte-rosette formation and immunofluoresence with a

monoclonal anti-T cell antibody, were less than 0.5%. Martin *et al.*, (1984) were not as successful with their protocol, which involved using murine monoclonal anti-T cell antibodies without complement; graft-versus-host disease remained a problem in their studies.

The immunological recovery of patients in which cyclosporin (CSP), in contrast to methotrexate, is used for the prevention of GVHD, has been compared (Witherspoon *et al.*, 1984a). Evaluation of various immunological parameters, such as Ig levels and cell mediated immunity, suggests that recovery is comparable between the two treatments. Although CSP is useful in reducing the incidence and severity of GVHD, it has deleterious side-effects such as hypertrichosis, gum hypertrophy, a rare but serious "capillary leak" syndrome, and serious neurological illnesses, such as motor spinal cord syndrome, a cerebellar-like syndrome, and mental confusion (Atkinson *et al.*, 1984; Witherspoon *et al.*, 1984a; Morris, 1984; Deeg *et al.*, 1984b; Harper *et al.*, 1984). It should be noted that the neurological disorders were successfully controlled by either reducing the dosage or eliminating the CSP from therapy. Let us now look at another form of GVHD.

B. Chronic GVHD

Even if HLA-identical marrow is used in BMT, chronic GVHD still occurs in some cases for unknown reasons, presumably related to minor histocompatibility mismatching

(Storb, 1983). Of 130 patients transplanted with HLA identical grafts for aplastic anemia, 45 % developed chronic GVHD between 3 to 15 months post-transplantation. Reports from several centers indicate that the main clinical findings include epidermal disease, obliterative bronchiolitis, severe buccal mucositis, keratoconjunctivitis, gastrointestinal involvement, and liver dysfunction, among others (Glucksberg, 1974; Saurat *et al.*, 1975; Gleichman *et al.*, 1984; Graze and Gale 1979; Sullivan *et al.*, 1981; Weiden *et al.*, 1980; Ralph *et al.*, 1984; Shulman *et al.*, 1980). The likelihood of developing chronic GVHD is related to whether the patient previously manifested acute GVHD. An increase in the severity of acute GVHD is directly correlated with the occurence of chronic GVHD (Storb, 1983c). The incidence of chronic GVHD is higher with increasing age. It has been suggested that this is related to thymic epithelial function.

Chronic GVHD can be controlled in a third of all cases by the administration of prednisone, either alone or in combination with procarbazine, cyclophosphamide, or azathioprine (Storb, 1984). Efforts to prevent the development of chronic GVHD by using steroids have yielded conflicting results (Forman *et al.*, 1982; Ringden *et al.*, 1982).

Considering the morbid complications associated with GVHD, it soon becomes obvious that protocols must be developed which will prevent or reduce the likelihood of the

occurrence of GVHD.

In contradiction to what has just been said, there is some speculation that a certain degree of GVHD may be beneficial in the promotion of hemopoietic activity post-transplantation. Equally interesting is the finding that GVHD may, in some unique way, also effect an anti-leukemic response , our next topic of discussion.

C. Graft-versus-Leukemia Effect

Barnes and Louitt (1956) and Bortin (1979, 1981) reported the observation that marrow cells transplanted into irradiated mice could destroy any radioresistent leukemic cells. A similar situation has been noticed in humans, where Weiden (Weiden *et al.*, 1979; 1980) has found that both acute and chronic GVHD exert an antileukemic effect. Analysis of 163 recipients with acute nonlymphoblastic leukemia or acute lymphoblastic leukemia transplanted while in remission or relapse reveals that the likelihood of achieving and/or maintaining a remission is greatest in patients who had either acute or chronic GVHD and lowest in those without any evidence of GVHD. The survival rate also increases, with those having undergone chronic GVHD showing an 80 % survival rate, 50-55 % for those with acute or acute and chronic GVHD and 25-30 % for those patients without GVHD. These results have stimulated a great deal of interest in the graft-*versus*-leukemic effect. Studies aimed at modifying the effects of GVHD to maximize its antileukemic effect are in

progress.

We will now consider the role of hemopoiesis as it relates to the transplant model.

IV. Hemopoiesis

Bone marrow transplantation *per se* is a relatively simple and straightforward procedure. The inherent problems associated with it are post-transplantation GVHD, possible incomplete reconstitution of the immune system, development of subsequent opportunistic infection, and the failure of the marrow to engraft. The requirements for hemopoiesis play a key role in engraftment.

A. Fetal Hemopoiesis

In the mouse it is known that blood islands arising from mesenchymal cells during the mesoblastic period of hemopoiesis are the site of erythropoiesis in the yolk sac (Metcalf and Moore, 1971). These cells, incidentally, have been successfully transplanted into lethally irradiated animal recipients. In the human, the seeding of the liver occurs at six weeks and that organ then becomes the chief site of hemopoiesis in the embryo. As time progresses, the hemopoietic potential shifts to the spleen and finally to the bone marrow (Zucker-Franklin, 1981).

B. Hemopoietic Concepts

Circulating and fixed blood cells such as platelets, fixed and free macrophages, erythrocytes, granulocytes, and lymphocytes are constantly turning over, hence the requirement for a constant renewal and replenishment of their numbers. Renewal is achieved by means of a pluripotent

stem cell compartment which responds to the demands for replenishment of the various cell lineages. This can best be simplified into a three-tiered structure where the ultimate stem cell, as yet not identified, is a totipotent cell capable of giving rise to a variety of pluripotent cells as well as having the unique potential of self-renewal. The second level represents the progenitor cell compartment: it is here that we start to see differentiation into the various lineages. A common lymphoid progenitor is believed to give rise to either B or T cell precursors. A pluripotent myeloid stem cell can differentiate into BFU-E, burst-forming unit, erythroid; CFU-E, colony-forming unit, erythroid; CFU-EOS, colony-forming unit, eosinophil; CFU-GM, colony-forming unit, granulocyte-macrophage; or CFU-MEG, colony-forming unit, megagaryocyte-platelets. Finally, the last tier represents the mature or so-called effector cells such as polymorphonuclear cells, monocytes-macrophages, erythrocytes, platelets, eosinophils, basophils, various T lymphocytes subsets and B-lymphocytes.

Two important concepts have been established with regards to the first two tiers. Microenvironmental factors play a key role in determining the commitment of multipotential stem cells to a particular differential pathway, and the proliferation of the progenitor cells is greatly influenced by specific humoral factors, for instance the effect of erythropoietin on erythropoiesis, or CSF-GM on granulopoiesis.

C. Hemopoietic Microenvironment

Undoubtedly, one of the most important and interesting aspects of hemopoietic transplantation is the concept of the "microenvironment", the proposed site of stem cell induction. Unfortunately, it is very difficult to study the microenvironment *in vivo* and until recently, no assay existed which would allow one to better understand exactly what goes on at this unique site of cellular interaction. Keller (1979) in an elegant set of *in vivo* experiments showed that both granulocytic and erythrocytic colonies could develop on the chick embryo chorioallantoic membrane. He showed, quite conclusively, that a stromal cell population of the chick embryo spleen, represented the hemopoietic microenvironment. In a set of *in vitro* experiments, Dexter and Moore (1977a) demonstrated the direct involvement of this so-called microenvironment in the maintenance of hemopoiesis. They established, in long-term bone marrow cultures, an adherent environmental monolayer consisting of phagocytic mononuclear cells, flattened epitheloid-like cells, endothelial cells, reticular cells, and giant fat-containing cells. (Incidentally, Spooncer *et al.* (1981) showed that the presence of certain glycosaminoglycans such as heparin sulphate, chondroitin sulfate, and hyaluronic acid were important factors that contributed to the growth of these cultures). Using the anemic W/Wv and Sl/Sld mouse strains, which have, respectively, either defective stem cells (and a competent

environment) or a defective environment (and competent stem cells), Dexter showed that adherent layers derived from the W/Wv strain bone marrow could support stem cell activity of the Sl/Sld strain, while the Sl/Sld adherent layer failed to develop. This strongly suggested the importance of cellular interaction between the stem cells and the microenvironmental compartment, in this case represented by the monolayer of adherent cells.

Moore and his colleagues then extended this concept to see if adherent layers of a particular genetic strain could indeed support the growth of allogeneic hemopoietic cells. Using semiallogeneic or allogeneic bone marrow combinations CBA/Cum (H-2k) → C57Bl/6 (H-2b) or in the opposite direction; CBA/Cum → (CBA/Cum x C57Bl/6)F_1; C57Bl/6 → (CBA/Cum x C57Bl/6)F_1; CBA/Cum → CBA/Cum and C57Bl/6 → C57Bl/6, they reported that bone-marrow derived adherent cells could stimulate the growth of both syngeneic and allogeneic hemopoietic cells. In their summary, they clearly indicate that the cells responsible *in vivo* for transplantation resistance and GVHD may simply not exist in the *in vitro* culture conditions and that these results should not be overinterpreted. The question as to whether the marrow stroma is a transplantable element is addressed in the **Discussion.**

Schofield (1978) talked of a hemopoietic concept known as "niches", which represented a clustering of various microenvironmental support cells in association with stem

cells. This proposed model of a close interaction network would allow for cell-cell communication to occur and allow short-range factors to be effective. This concept has been supported by studies which have shown that hemopoietic tissue is not a randomly distributed arrangement of a mixture of various cells but in fact the opposite (Trentin 1970, 1978). Examination of the morphological arrangements of cells within the mouse femur have shown that an orderly pattern does indeed exist, quite similar to the "niche" concept (Weinbeck, 1938; Lord and Hendry, 1972; Frassoni *et al.*, 1982; Hendry and Lord, 1982; Xu and Hendry, 1981).

The implication of thymus-derived cells in the promotion and maintenance of hemopoietic activity has been proposed by several groups (Lord and Schofield, 1973; Goodman and Shinpock, 1968, 1972; Dexter *et al.*, 1973; Vallera *et al.*, 1982). These findings are of particular interest given the extensive efforts being made to remove T cells from the donor innoculum, in an attempt to reduce the incidence of GVHD. This interesting observation suggests that T cells may not be required, despite reports of their involvement in hemopoiesis.

D. Hemopoietic Assays

Since the initial CFU-S assay described by Till and McCulloch (1961), a variety of equally useful *in vitro* and *in vivo* culture systems have proven to be extremely beneficial in determining the origin of various cell

lineages post-transplantation. As mentioned previously, Dexter's introduction of *in vitro* long-term bone marrow cultures allowed for the investigation of not only the stem cell (nonadherent) population but more importantly the microenvironmental (adherent) cell population as well (Dexter *et al.*, 1973, 1977b).

This culture system allows for both the establishment, *in vitro*, of an environment which promotes self-renewal of the stem cell population, as well as commitment of progenitor cells to occur. *In vitro* assays which allowed the committed cells to be identified, provided the much needed link to hemopoietic investigation of various cell lineages containing the major cell types (Wolf and Trentin, 1968; Fauser and Messner, 1979; Pluznik and Sachs, 1965; Bradley and Metcalf, 1966; Metcalf, 1977; Metcalf and Johnson, 1978; Metcalf *et al.*, 1979; Dorshkind and Phillips, 1983). By using these assay systems, it is also now possible to investigate the origin of related marrow derived cell lineages. Examples include Langerhans' cells in the epidermis, osteocytes in the bone, and a variety of tissue macrophages.

The rapid recent development in these hemopoietic assays has been directly associated with the discovery of various humoral factors that promote the *in vitro* growth of hemopoietic cells. The most extensively studied factor, colony-stimulating-activity (CSA), is made by conditioning medium with peripheral blood leucocytes in the presence of

phytohemagglutin (Fausner and Messner, 1979).

These assay systems have been used extensively in this thesis to show that once engraftment has been established in our model, it remains a permanent phenomena.

Our earlier discussion of BMT concerned itself primarily with various aspects of the clinical model. Let us now review the production of animal bone marrow chimeras by means of irradiation, cytotoxic drugs, a combination of both, and by a novel approach that utilizes anti-host monoclonal antibodies (mAbs).

V. Experimental Bone Marrow Transplantation

A. Radiation Chimera Model

Earlier studies on radiation-induced bone marrow transplantation were extended by von Boehmer *et al.*, (1975a, 1975b), who realized the potential of this *in vivo* model for studying T-B cell interaction. They successfully reduced the detrimental effect of the graft-versus-host reaction by eliminating mature peripheral blood T cells, utilizing anti-Thy 1 antibody and complement. This does not prevent normal stem cell development in the irradiated recipient. The basic model employed was to repopulate 800-900 rad irradiated $(P_1 \times P_2)F_1$ hybrids with either P_1 and/or P_2 donor cells. Subsequent development of either P_1 or P_2 donor stem cells in the chimeric environment rendered them mutually tolerant of each other.

Several groups then reported that chimeric T cells of P_1 origin which had differentiated in the $(P_1 \times P_2)F_1$ environment could cooperate equally well with B cells originating from either P_1 or P_2: the same applied to the P_2 cells differentiating in $(P_1 \times P_2)F_1$ environment (Waldmann *et al.*, 1978; Kappler and Marrack, 1978; Sprent and von Boehmer, 1979; Singer *et al.*, 1979; Zinkernagel *et al.*, 1978; Santos *et al.*, 1979; Bevan, 1977; Longo and Davis, 1983). Restriction specificity therefore, appears to be learned during the antigen-independent differentiation of T cells in the F_1 environment (Tada, 1984).

Kappler and Marrack (1978) and Longo *et al.*, (1981) used the radiation chimera model to study whether T cell precursors of non-responder type could respond to antigen presented by antigen-presenting cells of the responder type. They found that when non-responder T cells, which had matured in an (responder x nonresponder)F_1 environment and which were tolerant of responder MHC antigens, were then primed in the presence of (responder x nonresponder)F_1 antigen-presenting cells, they responded. Paul (1984) summarizes these findings by stating that: "..... unresponsiveness to antigen is not an innate property of T cells from a non-responder animal; in the proper setting, such T cells can respond - but what they respond to is the pair of antigen and responder Class II molecules."

In terms of the hemopoietic makeup of radiation chimeras, Cudkowicz and Stimpfling (1964), and Lotzová and Cudkowicz (1973) found resistance and deficient growth of bone marrow grafts. This resistance has been referred to as "hybrid-resistance", a topic we will now consider. Many reports have appeared with respect to the ability of various strain combinations to successfully engraft following irradiation. Daley and Nakamura (1984) showed that the natural resistance of H-2b/d and H-2b/k F_1 hybrid mice against parental H-2b bone marrow grafts was mediated by effectors restricted by the *H-2Db/Hh-1b* gene(s). This confirmed that the natural-killer (NK)-like effector cells, thought to mediate natural resistance in the P → F_1

irradiation model, are restricted, and this contrasts with reports of unrestricted NK cell activity against a wide range of tumors and normal cells. This restriction was previously postulated as being under the control of a set of unique noncodominant genes denoted as *Hh* (hemopoietic histocompatibility) (Snell, 1976).

Lotzová and her colleagues have shown that natural-killer (NK) cells are actively involved in the mechanism of allogeneic bone marrow graft rejection (Lotzová and Savary, 1977; Lotzová and Gutterman, 1979; Lotzová, 1980; Lotzová *et al.*, 1983). They showed that the *in vivo* treatment with an antiserum specific against the NK-cell surface antigen NK 1.1, as previously described by Pollack *et al.*, successfully prevented the rejection of allogeneic bone marrow transplants (Pollack *et al.*, 1979).

Using a cloned cell line with NK activity, Warner and Dennert (1982) were able to show that this line, when injected into syngeneic NK-deficient mice had an effect *in vivo*. The ability of the NK-deficient mice to reject allogeneic bone marrow was shown to be specific, in that identity at the H-2 locus precluded rejection while H-2 disparity was associated with rejection. Using congenics and recombinants they were able to narrow the target antigens to the H-2D region primarily. Further implications of the role that NK cells play in hemopoiesis and allograft marrow transplantation comes from studies by Hansson *et al.*, 1982. They reported that human NK cells have the ability to

inhibit *in vitro* granulopoiesis of autologous and allogeneic bone marrow cells. This strongly suggests that NK cells may possibly play a role *in vivo* as a regulatory of certain aspects of hemopoiesis.

Using various congenics, recombinants, and F_1 hybrids, Drizlikh *et al.*, (1984) report that both Class I (K and D) and Class II (I-A and I-E) MHC genes, rather than hypothetical *Hh* genes, are actually responsible for the phenomenon of hemopoietic resistance. They examined the ability of irradiated (H-2b x H-2k)F_1 and (H-2b x H-2d)F_1 recipients to prevent the growth of H-2b parental BM cells, without impeding the engraftment of non-H-2b parents. The authors explain that the *Hh* restriction phenomenon is only seen in certain strain combinations because "... there exist parental determinants that are not formed in some F_1 hybrids due to preferential association of either Ia alpha chains with allogeneic beta chains or of Class I antigens with allogeneic or hybrid Class II restriction elements." Their results cannot be accepted as conclusive, since their use of congenics and recombinants does not necessarily prove that *Hh* genes do not exist.

Carlson and Wegmann (1977) found that by monitoring the elimination and metastatic potential of ^{131}I-iododeoxyuridine prelabeled H-2^d leukemia cells, they could study *in vivo* models of hybrid and allogeneic resistance. Using an ingenious technique of whole-body gamma counting along with colonization of the spleen, they were

able to describe that although all nonirradiated mice showed early recognition and elimination of cells which were not H-2 identical with self, as one would expect; irradiation had varying effects on different strains of mice in their ability to reject the labelled leukemic cells. They concluded that although dissimilarities at the H-2 locus provided the targets for recognition, non-H-2 genes played a role in the susceptibility of the rejection process to irradiation.

When the role of irradiation was previously considered as a means of preparing patients prior to BMT, its drawbacks were not discussed quite adequately. Van Bekkum (1980) showed quite clearly that GVHD is enhanced by TBI, but this has not been confirmed by others. In addition Deeg *et al.*, (1983) have found that canine radiation chimeras prepared with TBI have an estimated relative risk of developing a malignancy that was 5-fold higher than in control dogs.

In light of these difficulties, new methods were introduced which attempted to address these problems. Slavin and Strober, in animal models, selectively irradiated areas that were rich in lymphoid tissue while shielding the rest of the body (Slavin *et al.*, 1979a; Slavin and Strober, 1979b). They delivered the radiation by means of multiple small fractionated doses, for example, 200 rad x 17. Using this approach, they found that they were capable of transplanting across allogeneic barriers without any sign of GVHD. As well, they showed that immunological tolerance was

induced and that both nonspecific and specific suppressor T cells appeared.

Slavin and Seidel, (1982) examined the hemopoietic status of these TLI bone marrow chimeras and found the following. TLI did not cause irreversible damage to the marrow. In fact, the procedure allowed a larger total cumulative radiation dosage to be administered. This advantage of TLI has successfully been used in the clinical situation, in radiotherapy prior to bone marrow and organ transplantation, and also in attempts to treat various autoimmune disorders (Strober *et al.*, 1979; Slavin *et al.*, 1979b).

It has been reported that no neoplasms were detected in an ongoing study of drug-induced chimeras prepared with cyclophosphamide or dimethyl busulfan, suggesting that the drug regime may be a better preparatory protocol than irradiation (Deeg *et al.*, 1983).

Several classes of drugs may be used in the preparation of a recipient prior to BMT. The following are representative agents.

B. Drug-Induced Chimera Models

Alkylating agents, such as cyclophosphamide (CY) and busulfan (BU), as well as other drugs such as 5-fluorouracil (FU), hydroxyurea (HU), and L-phenylalanine mustard (L-PAM) have been studied in terms of their short- and long-term effects on hemopoietic tissue (Fried and Johnson, 1968;

Fried *et al.*, 1977; Hays *et al.*, 1982; Santos and Owens, 1969, Santos and Tutschka, 1974; Tutschka and Santos, 1977; Morley and Blake, 1974; Boggs and Boggs, 1980; Sensenbrenner *et al.*, 1968; Botnick *et al.*, 1978; Brown and Carbone, 1971; Van Zant, 1984; Hodgson *et al.* 1975, 1982).

The majority of these reagents can be classified as antineoplastic, chemotherapeutic drugs, and therefore we should be aware of their potential to cause late complications in therapy. They have been shown to have severe adverse effects on the stem cell compartment if not administered in therapeutic doses. It is this very effect, on the hemopoietic tissue, that has stimulated their use in host preparation, as a means of replacing or reducing the requirements for irradiation.

Busulfan (BU) Treatment

BU is used almost exclusively for the treatment of myeloproliferative disorders, especially chronic myelogenous leukemia (CML). Its use requires judicious care since, if overprescribed, it can lead to irreversible bone marrow failure. Busulfan was originally discovered when the effects of sulphonic acid esters on tumor growth were being evaluated. At low doses, BU acts by selectively depressing granulocytopoiesis. In slightly higher doses, platelet and erythroid elements may be affected, with a resultant pancytopenia developing.

Udupa *et al.*, (1972), using a murine model suggested that the target cell populations of BU treatment are noncycling cells, thus possibly explaining the observation of a slow recovery rate of CFU-S after BU administration. Morley *et al.* (1976) concluded from their studies that the effect of BU is primarily on the CFU-S and not the stromal cells. When BU was given repeatedly at short intervals for a limited time, it resulted in marrow aplasia and subsequent peripheral blood pancytopenia several months later. Hays and her colleagues (Hays *et al.*, 1982) reported that BU treatment causes a "prolonged and apparently permanent injury to the bone marrow". In addition, they found a moderate to marked reduction in marrow CFU-S and CFU-GM that remained for as long as 18 months post treatment. Morley *et al.*, (1975) and Hellman *et al.*, (1978) concluded that BU affects stem cells directly, as evidenced by failure of CFU-S from BU-treated mice to self-renew and reconstitute hemopoiesis in irradiated normal recipients. Interestingly, however, irradiated BU-treated mice would not allow normal hemopoietic cells to fully engraft, suggesting damage to the microenvironment. Hays and colleagues postulated that the repopulation of irradiated BU-treated mice, evidenced by Morley, was due in part to the outgrowth of stromal elements within the donor inoculum. Research in this area is still ongoing. Side effects of BU treatment include increased risk of cataract formation, pulmonary fibrosis, and premature graying as a consequence of damage to melanocytes (Botnick

et al., 1978).

Cyclophosphamide (CY) Treatment

CY (5 mg/kg), if given intermittently, appears to cause damage to hemopoietic stromal function to the extent that there is no significant recovery for as long as six weeks post-administration (Fried *et al.*, 1977). CFU-S potential in these same hosts, after an initial decline in numbers, returns to pretreatment levels by six weeks. The major problem with this study is the unrealistically high dosage used when compared to the typical human dose of 60-120 mg/kg of body weight. In the latter, no evidence of cumulative effects on stromal elements was found. However, Buckner *et al.* (1974) reported of adverse effects of CY on thrombopoiesis.

5-Fluorouracil (FU) Treatment

FU appears to selectively kill rapidly dividing cells in various hemopoietic lineages until the surviving cells are those with a pluripotent potential and hence slower turnover. This view, advocated by Hodgson *et al.*, (1982), is supported by Van Zant (1984), who proposes that the reason for "... the delay in spleen colony growth caused by FU-treated cells is that primitive stem cells surviving FU are unable to directly initiate colony growth. Rather, the population undergoes developmental maturation, giving rise to a cohort of cells capable of spleen colonization and

growth". Van Zant then provides evidence that this is, indeed, correct. FU, it appears, is selectively more toxic to a population of older stem cells, than to younger ones. The sparing effect on the more primitive subpopulations is evidenced indirectly by a 100-fold increase in CFU-S colonies derived from FU-treated marrow cells. This effect on stem cells was shown to be dose dependent, both *in vitro* and *in vivo*.

Hydroxyurea (HU) Treatment

Studies by Hodgson *et al.*, (1975), support an initial observation by Sinclair, (1967) that HU affects cycling CFU-S, by acting as a synchronizing agent. This allows CFU-S in the S (synthesis) phase of their growth cycle to be collected *in vivo*. Hodgson assumes that HU kills cells in S phase by inhibiting DNA synthesis while permitting cells in other phases to continue in cycle and accumulate at the G_1/S boundary. When the HU concentrations drop, the accumulated cells move into S.

The drugs cited in the above section, therefore, have a variety of effects on the proliferative capacity of various stem cell lineages and stromal microenvironments. Recently, the clinical use of alkylating agents has been associated with tumor induction (Botnick *et al.*, 1978).

So far we have examined two different means by which one can establish hemopoietic engraftment across MHC barriers. The first, the radiation model, although

successful under certain conditions, still is attended by a variety of complications. Most notable is the fact that TBI increases the incidence of GVHD and also gives rise to the subsequent development of malignancies. Secondly, the cytotoxic drug approach is far too nonspecific in its mode of action, inflicting, in some situations, irreversible damage to the hemopoietic environment.

Perhaps the simplest approach to solving or reducing the complications inherent in the above mentioned models, comes from studies which will be considered next.

C. Parabiont Model

Drell and Wegmann (Drell *et al.*, 1979a,1979b; Wegmann *et al.*, 1980) describe, in some detail, their findings of a parabiotic model of hemopoietic engraftment. Basically, these authors confirmed and extended an initial observation by Rubin (1959), of an unexpected 50% survival rate in parabiosed mice differing in their MHC haplotypes. They showed that in the H-2 incompatible strain combination of DBA mice, parabiosed to (DBA x C3H)F_1 hybrid mice, after an initial chimeric state, both parental and F_1 hybrid partners displayed a complete takeover of their red and white blood cell compartments by cells of the DBA phenotype. Interestingly, if spleen cells from either of the parabionts are removed and reexposed to C3H or third party alloantigens, either *in vitro* or upon adoptive transfer *in vivo*, they show virtually normal reactivity (Shaw *et al.*,

1974; Drell *et al.*, 1979a, 1979b). Later, these investigators found that an antibody present in the DBA mice parabiosed, to the (DBA x C3H)F_1 hybrid, was specific for antigens present on cells of some hemopoietic lineages. This antibody was cytotoxic *in vivo* and *in vitro* and reacted with an antigen encoded by a gene(s) linked to the left hand (K-I) end of the H-$2K^k$ haplotype (Drell *et al.*, 1979a). These observations are reminiscent of a particular set of earlier experiments. Fox *et al.*, (1962) reported on a chromosome marker study that suggested that 10^8 C57BL spleen cells injected into (C57BL x CBA(T6))F_1 hybrids repopulated the spleen completely, and remained for a period of at least 60 days. In contrast, chimerism in the marrow was only temporary. Of further interest was the finding that if the CBA(T6) partner was used as the source of donor cells, no splenic takeover was seen. Later, Batchelor and Howard (1965) found that hemopoietic chimerism could be achieved by the pretreatment of (C57BL x A)F_1 hybrids with uncharacterized C57BL anti-A antibody followed by 10^8 C57BL spleen cells. Taken together, these findings support the possibility that GVHD could possibly be avoided by somehow selecting the proper combination of not only the donor cells but also the anti-host antibody.

D. Suppressor Cell Involvement in the Maintenance of Tolerance

In their parabiont studies, Drell *et al.*, (1979c) could not find any evidence for the presence of suppressor cells.

However, other investigators using allogeneic bone marrow chimeras prepared with total lymphoid irradiation have shown these chimeras to be specifically unresponsive when subsequently tested against both recipient and donor strains in an MLR assay (Slavin *et al.*, 1977). They postulated and went on to show the presence of antigen-specific donor-type suppressor cells that were able to inhibit the MLR of normal donor-type spleen cells against recipient-type and third-party stimulator cells (Okada *et al.*, 1983). Waer *et al.*, (1984) reported of an apparent association between TLI-irradiation protocols that gave rise to the successful engraftment of allogeneic bone marrow and the presence of nonspecific suppressor cells in the spleen. It must be mentioned, that it would be difficult to draw any conclusions between these studies and Drells' work, since in the latter situation no irradiation was used. Nonetheless, further evidence existes which describes a naturally occurring suppressor cell population in the bone marrow of normal adult mice (Bennett *et al.*, 1978; Bennett and Mitchell, 1980). An interesting observation that Strober (1984) made with regards to this population of suppressor cells, is that they have also been identified in other sites of "intense hematopoiesis in the rodent", for example in spleens of adult mice which had received TLI, Sr^{89}, BCG, or cyclophosphamide. Incidently, these suppressor cells are not found in the spleens of normal adult mice, unless the mouse has undergone one of the above mentioned hemopoietic

stimulating treatments (Oseroff *et al.*, 1984).

Strober (1984) in a recent review, proposed that a population of non-specific suppressor cells in the spleens of adult mice given TLI before marrow transplantation are capable of preventing the development of cytotoxic T cells, while allowing the emergence of a population of antigen-specific suppressor T cells after marrow transplantation. These latter suppressor cells, of host origin, are presumed to block the generation of cytolytic T cells directed against host cells. In a similar manner, donor-derived antigen-specific suppressor cells may possibly prevent graft-*versus*-host disease by preventing the development of cytolytic cells directed against the host.

Let us now consider the research project.

E. Antibody-Facilitated Model: Introduction to The Research Project

AF chimeras are a natural extension of the ideas which were generated out of the parabiont studies of Drell and Wegmann (Drell *et al.*, 1979a, 1979b; Wegmann *et al.*, 1980). Their discovery of complete hemopoietic takeover of both parabiont partners, and the subsequent appearance and persistence of a circulating anti-host antibody, suggested that this could be simulated by the injection of F_1 hybrids with parental donor spleen cells along with a monoclonal anti-host antibody. Preliminary results did indeed reveal this hypothesis to be correct.

This project involved determining the optimal cellular and mAb requirements for obtaining a successful hemopoietic engraftment of the recipient, while avoiding or minimizing the effects of GVHD. The cellular requirements involved determining both the cell type, bone marrow or spleen, and cell numbers, while the mAb requirements concentrated on amount, class, and specificity. Equally important was the establishment of a protocol for the administration of cells and mAb.

The lack of adequate quantitation of hemopoietic takeover by means of an existing qualitative Gpi electrophoretic assay was resolved with the introduction of a far more sensitive, rapid and reliable method.

Analysis of the effects of the mAbs on the recipients hemopoietic tissue were addressed in a variety of both *in vitro* and *in vivo* assays. Similarly, hemopoietic reconstitution of long-term AF chimeras was examined using both *in vitro* long-term bone marrow cultures and methyl cellulose colony cultures and *in vivo* by means of the CFU-S assay.

The findings, that complete hemopoietic takeover between semiallogeneic adults can be accomplished by the administration of antihost MHC monoclonal antibody and donor stem cells, as well as the chimeric characteristics of these animals once engrafted will now be presented.

VI. Materials and Methods

Mice

BALB/cCR(BALB, H-2^d,*Gpi-1a*), DBA/2J (DBA, H-2^d,*Gpi-1a*), C3H/HeJ (C3H, H-2^k, *Gpi-1b*), CBA/CAJ (CBA, H-2^k, *Gpi-1b*), (BALB/cCR x C3H/HeJ)F_1, ((BALB x C3H)F_1,H-$2^{d/k}$,*Gpi-1a/b*) and (DBA/2J x C3H/HeJ)F_1, ((DBA x C3H)F_1, H-$2^{d/k}$,*Gpi-a/b*) were originally obtained from Jackson Laboratory, Bar Harbor, MAINE, and maintained as pedigree lines at the Laboratory Animal Breeding Unit of the University of Alberta (Ellerslie, Alberta, Canada.) The H-2 haplotypes of these strains are shown schematically in Table 1.

Monoclonal Antibodies

Anti-H-$2K^k$ (11-4.1) and anti-H-2I-A^k (10-3.6) hybridomas were obtained from the Salk Cell Distribution Centre, La Jolla, CALIFORNIA, and from American Type Culture Collection, Rockville, MARYLAND. These hybridomas originated in the laboratory of Dr. L. A. Herzenberg (Oi *et al*, 1978). Monoclonal antibodies produced are of the IgG2a subclass and were produced as ascites fluid from hydridoma-bearing BALB mice. Each batch of mAb-containing ascites fluid was tested in an *in vitro* competitive binding assay with the appropriate ^{125}I-labeled antibody. ^{125}I-mAb (10^5 cpm) was mixed with graded amounts of the ascites and added to 2 x 10^7 C3H (target) and/or BALB (control) spleen cells (Raghupathy *et al.*, 1981). The 50% inhibiting dose of the

Table 1

H-2 and *Gpi* genotypes of mouse strains and the regions of the H-2 major histocompatibility complex recognized by the monoclonal antibodies used in these studies.

STRAIN[1]	K	I-A	I-E	D	*Gpi*
DBA/2J	d/d	d/d	d/d	d/d	a/a
BALB/cCR	d/d	d/d	d/d	d/d	a/a
CBA/CAJ	k/k	k/k	k/k	k/k	b/b
C3H/HeJ	k/k	k/k	k/k	k/k	b/b
(DBA/2J x C3H/HeJ)F_1	d/k	d/k	d/k	d/k	a/b
(BALB/cCR x C3H/HeJ)F_1	d/k	d/k	d/k	d/k	a/b
MONOCLONAL ANTIBODIES[2]					
Anti-H-2K^k (11-4.1)	k	-	-	-	
Anti-H-2I-A^k (10-3.6)	-	k	-	-	

[1]Reference: Klein, 1982.
[2]IgG2a subclass.

ascites was compared with a reference batch of ascites of known activity.

The procedure used to determine the amount of specific mAb necessary to produce AF chimeras is as follows: Ascites fluid was put through a Protein A-Sepharose column, and the eluted antibody was labeled with ^{125}I, and then purified by adsorption and elution from glutaraldehyde-fixed target C3H spleen cells. Labeling resulted in 600 μg of specific IgG containing 3 x 10^7 cpm. In competitive inhibition studies, the addition of 0.3 microliter (μl) of ascites resulted in 50% inhibition of binding of 5 x 10^4 cpm of the ^{125}I-mAb preparation on target C3H spleen cells. Therefore, 0.3 μl of ascites equals 0.5 μg of specific IgG. This was related back to the amount (0.25 ml) of ascites used to produce AF chimeras. The subsequent estimate of specific IgG injected is 416 μg/mouse.

Radioiodination of Immunoglobulins

The antibodies used in the competitive binding radioimmunoassay were ^{125}I-labeled by the chloramine T method of Greenwood et al (1963). Labeling was performed at a molar ratio of one iodine atom to one IgG molecule. 5-millicuries of radiolabeled sodium iodide (New England Nuclear, Lachine, QUEBEC) was added to a 5 milligram (mg) solution of the immunoglobulins in PBS. 50 μl of chloramine T (2 mg/ml) was subsequently added to this mixture and the

oxidation reaction allowed to continue for two minutes (min) at room temperature. To prevent further oxidation, the mixture was neutralized with 60 µl of sodium metabisulfite (6 mg/ml). 10 µl of 1% potassium iodide was then added and the solution dialyzed against saline to remove free iodine.

Ammonium Sulphate Precipitation

MAbs used in the preparation of AF chimeras in later experiments were precipitated with ammonium sulphate. 200 ml of raw ascites was added to 100 ml of cold phosphate buffered saline (PBS), mixed, and allowed to stand on ice. 300 ml of a cold, supersaturated solution of ammonium sulphate (Fisher Scientific Co. Cat. No. A-7023, Fair Lawn, NEW JERSEY) was then slowly added. The complete mixture was allowed to stand undisturbed on ice for a minimum of 1 hr.

The cooled mixture was spun for 30 min in 250 ml polycarbonate bottles at 7500 - 9000 rpm in a Sorval Superspeed Model RC2-B refrigerated centrifuge. The supernatant was discarded and the pellet resuspended in 60 ml PBS. Exactly 40 ml of a 100% saturated ammonium sulphate solution was then added slowly with continuous stirring and the mixture allowed to stand on ice for a minimum of one hour. This mixture was then centrifuged at 7500 - 9500 rpm for 30 min. The supernatant was discarded and the pellet resuspended completely in 50 ml normal saline. This was then dialyzed in three changes of normal saline, spun at 9,000 -

10,000 rpm to remove fibrin clots, aliquoted and stored at -70° C. Specific activity was tested in an *in vitro* competitive binding radioimmunoassay, described above.

Preparation of Antibody-Facilitated (AF) Chimeras

AF chimeras were prepared by the intravenous (iv) administration of donor parental stem cells accompanied by antihost mAb into otherwise untreated F_1 recipients (Figure 1). (Wegmann *et al*, 1980). Adult BALB or DBA mice provided donor stem cells, derived from either spleen, bone marrow (BM), or T cell-depleted BM. Spleens were excised, pressed through a stainless steel screen, and washed in Leibovitz medium. BM cells were obtained by flushing Leibovitz media through tibias and femurs of donor mice.

To obtain T cell-depleted BM cells ($\alpha\Theta$BM), single cell suspensions from the BM were treated for a one hour period in a two-step cytotoxicity assay with a 1:5000 dilution of monoclonal anti-Thy 1.2 (New England Nuclear, Boston, MASSACHUSETTS), followed by a 1:6 dilution of agarose-adsorbed guinea pig complement (GPC) (Flow Laboratories, McLean, VIRGINIA).

AF chimeras were produced by the injection of donor stem cells (spleen, BM, or $\alpha\Theta$BM) accompanied by anti-H-$2K^k$ or anti-H-2I-A^k ascitic mAb (approximately 416 μg of specific IgG). Depending on the specificity of mAb, AF chimeras are referred to as anti-H-$2K^k$ AF chimeras or

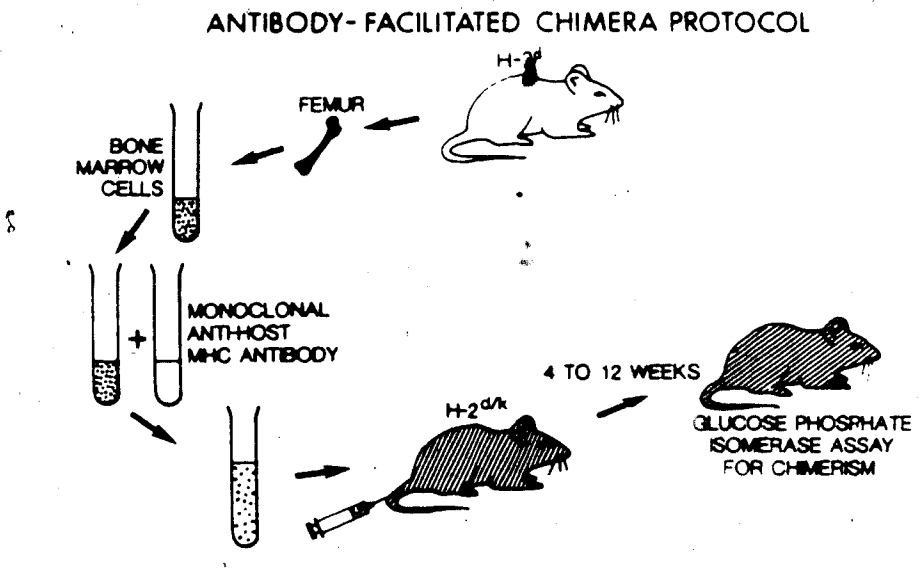

Figure 1. Antibody-Facilitated Chimeras protocol.

anti-H-2I-A^k AF chimeras. AF chimeras were maintained on 325 mg/liter Terramycin (Pfizer Canada, Montreal, QUEBEC) in their drinking water.

Hematocrit determinations

Mice were placed under light anesthesia with Penthrane (Methoxy fluorane, N.F., Abbott Laboratories, Montreal, QUEBEC) and bled from the retro-orbital sinus through heparinized microhematocrit tubes (Dade B4415-30, Miami, FLORIDA). These tubes were then plugged at one end with Critoseal (Clay-Adams, NEW JERSEY) and spun in a hematocrit centrifuge for 10 min (Model MB, International Equipment Co., Needham, MASSACHUSETTS). The percent hematocrit was determined using a microhematocrit capillary tube reader (Lancer, St. Louis, MISSOURI).

Quantitation of Chimerism by the Gpi assay

Heparinized blood, collected from normal P_1 and F_1 mice, was spun at 300 g for 10 minutes, and the cell pellets resuspended in phosphate buffered saline (PBS) and the cells washed three times in PBS. Artificial mixtures were prepared by mixing varying numbers of P_1 and F_1 cells. Cell lysates were run in starch gel electrophoresis and GPI activity visualized using the nitrocellulose overlay staining technique of Peterson (1978). GPI is a dimeric molecule and is expressed in inbred strains of mice in either of two

electrophoretically distinct forms: a slower migrating protein encoded by the glucose phosphate isomerase - 1a (*Gpi-1a*) gene and a faster migrating protein encoded by the (*Gpi-1b*) gene. Genetic hybrids between these two types (*Gpi-1a/b*) express both parental forms of the enzyme as well as a heterodimer protein with intermediate mobility (Figure 2). Donor GPI content of the artificial cell mixtures was measured by scanning the nitrocellulose filters with a densitometer (Camag, Muttenz, SWITZERLAND.). A Hewlett-Packard Model 3390A Reporting Integrator (Palo Alto, CA) was used to obtain a quantitative value for each sample, which was converted to a ratio of fast band to slow band activity. These values were plotted to generate a standard curve (see Figure 3). that was used to determine the donor GPI content of unknown samples. Using this protocol, as few as 5% P_1 cells can be detected in an artificial mixture of P_1 and F_1 cells.

Separation of Peripheral Red and White Blood Cells

Studies have indicated that red and white cell chimerism in the peripheral blood are essentially equivalent to each other (Gambel *et al.*, 1984). Therefore, in most cases, the percentage of chimerism reported reflects unfractionated peripheral blood sampling. In addition, peripheral red and white cell chimerism was monitored separately in some groups. A murine white cell density gradient (specific gravity 1.09) was prepared using Percoll

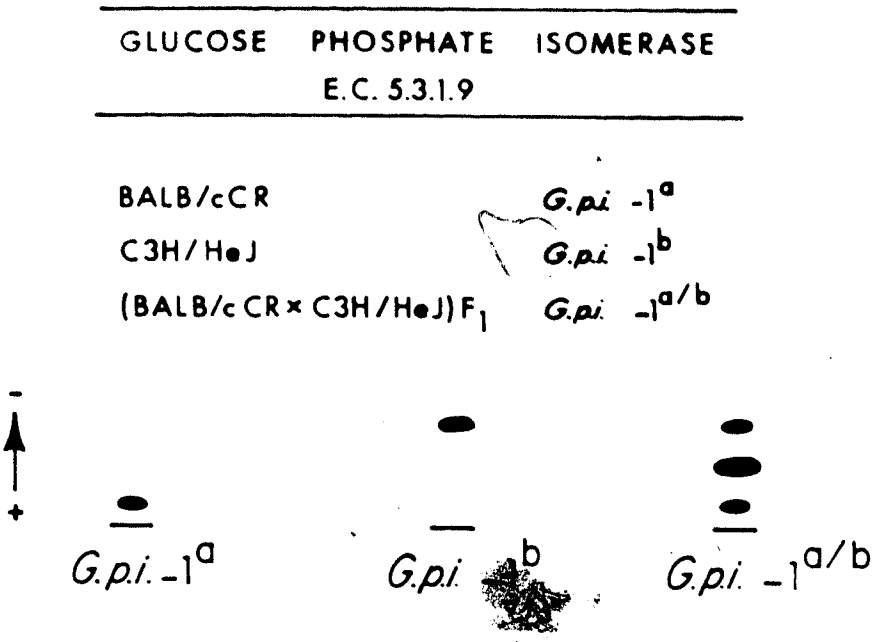

Figure 2. Electrophoretic migration patterns of glucose phosphate isomerase isozymes.

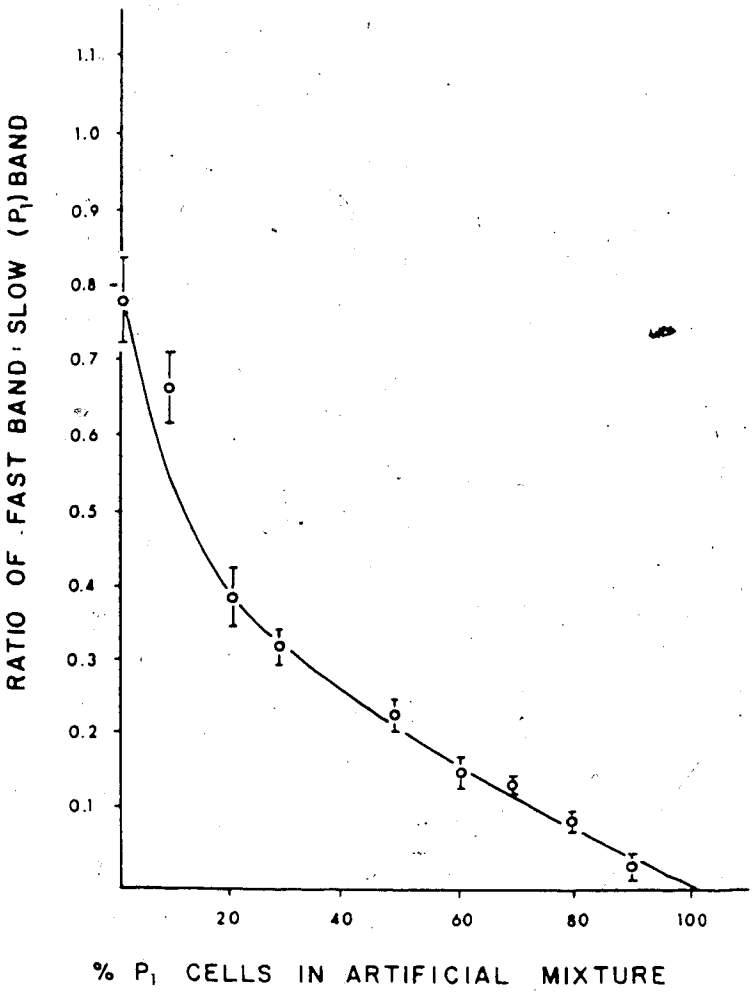

Figure 3. Gpi standard curve . Comparison of % P_1 cells in an artificial mixture of P_1 and F_1 cells using the ratio of fast to slow band. For details of the Gpi assay see Materials & Methods. Results are given as mean ± standard deviations. 5 different samples of each mixture.

(Pharmacia, Uppsala, SWEDEN). Peripheral blood was obtained from the tail vein and collected into heparin-containing PBS (1% v/v). The cell suspension was then washed twice in PBS and layered on the Percoll gradient in 15 ml conical tubes centrifuged at 300 g for 30 min at room temperature. The white cells at the interface and the red cells in the pellet were collected independently of one another, washed, and prepared for GPI isozyme analysis.

Effects of mAbs on Pluripotential Stem Cells

The spleen colony forming unit (CFU-S) assay of Till and McCulloch (1961) was utilized to determine the effects of anti-H-2K^k and anti-H-2I-A^k mAbs on pluripotential stem cells. Briefly, recipient mice were irradiated with 950 R (Cesium^{137} source, Gamma Cell 40, Atomic Energy of Canada, Ottawa, CANADA) on day -1, and injected iv with 10^5 syngeneic donor BM cells on day 0. Donor BM cells were pretreated with mAbs either *in vitro* or *in vivo* before injection into syngeneic, irradiated recipients, as described below. CFU-S were counted as macroscopic spleen colonies 11-12 days later.

For *in vitro* pretreatment, 10^7 BM cells from BALB, C3H or (BALB x C3H)F$_1$ mice were incubated with a 1:10 dilution of either anti-H-2K^k, anti-H-2I-A^k, or control P1.1 mAb , an IgG2a mAb of no known specificity, for 60 min at 4° C. The cells were washed and then incubated with a 1:6 dilution of GPC for 30 min at 37° C and finally washed twice.

For *in vivo* pretreatment, BALB, C3H or (BALB x C3H)F$_1$ mice were injected iv with anti-H-2K^k, anti-H-2I-A^k or control P1.1 mAb. The amount of mAb injected was equivalent to that necessary for the production of AF chimeras. After 48 hours (hrs), single cell suspensions were prepared from the BM of each treatment group and then 10^5 BM cells were injected into irradiated syngeneic recipients and CFU-S were counted as described above.

Bone Marrow Fractionation

Femurs from long-term AF chimeras were fractionated to yield three distinct subpopulations of bone marrow cells (Allalunis *et al*, 1983) as shown in Figure 4. Femurs were excised and subsequently treated as follows: initially, they were flushed with 3 ml of Leibovitz medium twice and the resultant cells, representing fraction I (medullary cavity), were collected and stored on ice. Crude collagenase Type 1A (Sigma Cat. No. C-9891, St. Louis, MISSOURI) was then added to the medium (1 mg/ml vol) and the femur flushed continuously for 10 - 12 min; the resultant cells, fraction II (endosteal fraction) were then resuspended and stored on ice. Fraction III or the compact fraction was obtained by breaking the femur into little pieces, less than 2 millimeters (mm) in size, in a petri dish containing media with collagenase. The mixture was vigorously agitated every 2 min over a 15 min period. The bone fragments were allowed

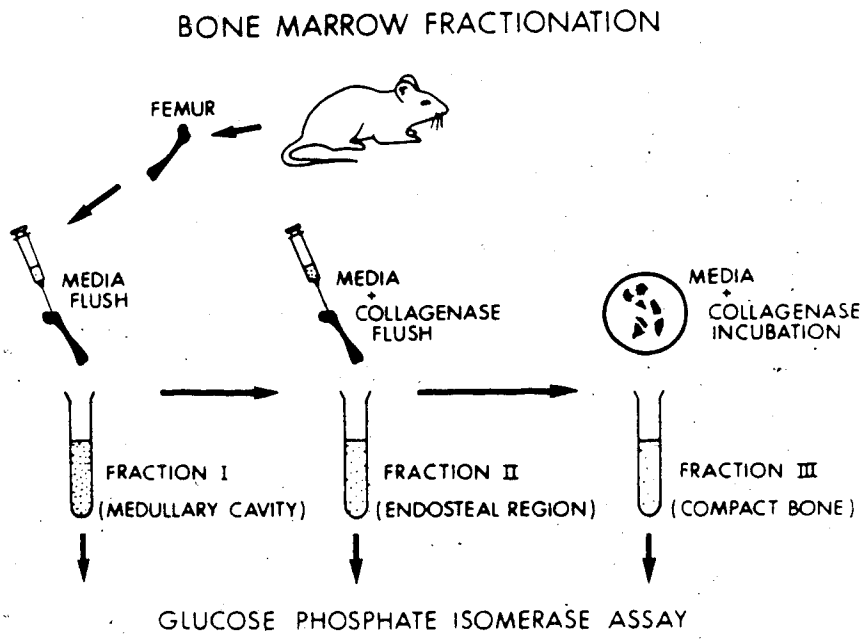

Figure 4. Bone marrow fractionation protocol.

to settle and the supernatant transferred to a tube and centrifuged. The resultant cells were saved. Cells of all three fractions were then analyzed in the Gpi assay to determine their origin.

Long-Term Bone Marrow Cultures

Long-term bone marrow cultures were established using the method described by Dexter *et al.*, (1977b) and modified by Greenberger (1978). The contents of a single femur were flushed with 10 ml of RPMI 1640 containing 20% horse serum (Flow Laboratories, McLean, VA), 10^{-7} M hydrocortisone sodium succinate (Upjohn Company, Don Mills, ONT.), and penicillin plus streptomycin (1ml/L v/v; Gibco Laboratories, Grand Island, NY). Cultures were maintained in 25 cm^2 tissue culture flasks (Corning Glass, Corning, NY) at 33° C in 5% CO_2. They were fed weekly by replacing 5 ml of old media with 5 ml of fresh media. The non-adherent cell population was collected by centrifuging the supernatants of the culture flasks; the adherent cell layer was recovered after a 5 minute incubation of the contents of the flask at 37° C with stock trypsin ethylenediamine tetraacetic acid (EDTA)(Gibco). Adherent and nonadherent cells were then analyzed in the Gpi assay see Figure 5.

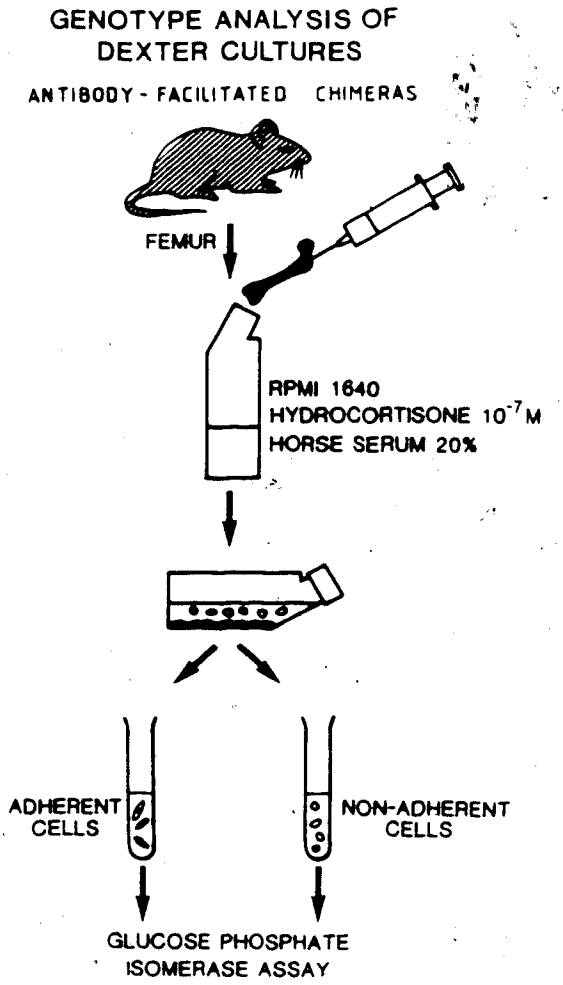

Figure 5. Gpi analysis of long-term bone marrow culture adherent and nonadherent cells.

Methyl Cellulose Cultures

Stem and progenitor cell activity of BM or spleen cells was tested in a methyl cellulose colony assay (Worton, 1969). Briefly, 7.5 x 10^4 bone marrow (BM) cells/ml or 10^4 spleen cells were suspended in a final volume of 1 ml containing 0.4 ml of 2.1% stock methyl cellulose (4000 Centipoises; Sigma Chemicals, St. Louis, MO), 0.3 ml of stock fetal calf serum (Flow), 0.2 ml Alpha medium (Gibco) or Iscove's modified Dulbecco's medium (Gibco)and 0.1 ml pokeweed mitogen-stimulated spleen conditioned medium (SCM), a source of colony stimulating factor (Johnson, 1977). Erythropoietin (EPO), prepared as described in **Erythropoietin Production**, was added to each culture at a concentration of 20 µl/dish. The cell suspension was layered in 35 x 10 mm culture dishes (Lux Scientific, Catalog No. 5221-R, Miles Laboratories, Naperville, IL) at 1 ml/dish and incubated at 37° C in 10% CO_2 for 8 to 12 days. Colonies were individually picked by micropipette, frozen overnight at -20° C and loaded directly onto a starch gel and analyzed in the Gpi assay to determine their Gpi phenotype and hence P_1 or F_1 derivation; 60-90 randomly picked colonies were analyzed from each culture of spleen or bone marrow cells in order that chimeric ratios could be calculated, see Figure 6.

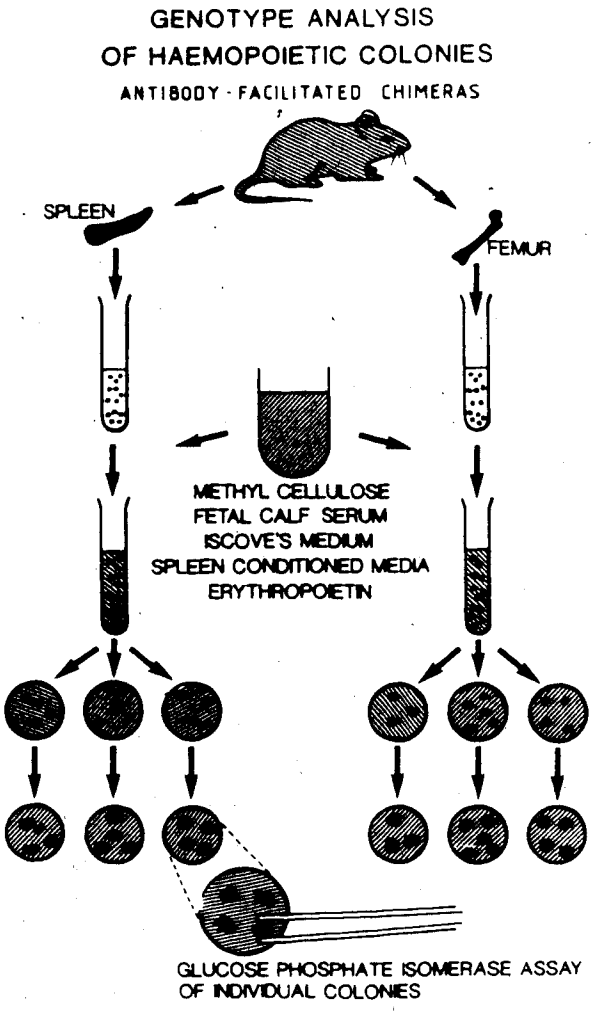

Figure 6. Technique for genotyping individual *in vitro* methyl cellulose bone marrow/spleen hemopoietic colonies.

2.1% Methyl Cellulose W/W

Stock methyl cellulose was prepared as follows: 84 gm of methyl cellulose was weighed and autoclaved in a six liter flask. A large four inch magnetic stirring bar and 2.5 L of double distilled water were also autoclaved. The water was heated to 90° C before use. To the 84 gm of autoclaved methyl cellulose powder 1.958 L of autoclaved double distilled water was added. The flask was then placed over the burner and allowed to boil until all the powder dissolved. This mixture was then stirred for approximately three hours or until it had cooled to room temperature. At that time 1.958 L of double strength alpha medium at room temperature was added. The flask was sealed and inverted several times to ensure good mixing, then placed in the cold room and immersed in ice. Stirring was continued overnight or until the solution was clear. The solution was then aliquoted into 100 ml bottles and allowed to sit at room temperature for eight days. Contaminated bottles were discarded and the remainder frozen at -20° C.

Spleen Conditioned Medium

SCM used in the methyl cellulose assay was prepared as follows: 2 x 10' spleen cells from 10 week old (BALB x C3H)F_1 mice were washed three times in RPMI 1640 media and added to 900 ml RPMI 1640 containing Penicillin-Streptomycin antibiotics, 50 ml of a 1:15 dilution of pokeweed mitogen

(Gibco Laboratories, Grand Island, NEW YORK) and 50 ml heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NEW YORK). The cultures were placed in large 1 l glass flasks and incubated at 37° C in 10% CO_2 for one week, after which time the supernatant was harvested and concentrated ten-fold through an Amicon Diaflo YM10 76mm ultrafiltration membrane (Amicon, Oakville, ONTARIO). The SCM was then aliquoted and frozen at -70° C. Prior to use in the colony assay the SCM was filtered through a 45 μ Millex-HA ultracleaning filter unit (Millipore Corporation, Bedford, MASSACHUSETTS) and diluted to a final working concentration of 1:1.

Erythropoietin Production

EPO used in the methyl cellulose assay was either produced in our laboratory or purchased from The Terry Fox Cancer Research Institute, Vancouver, BRITISH COLUMBIA. It was produced in our laboratory by irradiating 10-16 week old (BALB x C3H)F_1 mice on day -1 with 700 rads and injecting them intraperitoneally on day 0 with 60 mg/kg body weight of phenylhydrazine hydrochloride (Sigma P-7126, St. Louis, MISSOURI) in saline. Blood was collected, in heparinized tubes on day 10 and the plasma aliquoted and frozen at -70° C. Prior to use it was passed through a 45 μ Millex-HA ultracleaning filter unit and titrated to determine which concentration best supported erythroid colony growth.

Peritoneal Macrophages

Macrophages were harvested from the peritoneal cavity of AF chimeras similarly to previously described techniques (van Furth, 1968, 1970; Thompson, 1970). Mice were killed by gentle cervical dislocation in such a manner as to prevent contamination of the peritoneal cavity with blood. Three ml of Puck's saline were injected into the peritoneal cavity using a syringe. The abdomen was gently massaged to ensure adequate lavage of the peritoneum. A tiny hole was cut in the peritoneal wall and the fluid removed with a Pasteur pipette and centrifuged at 450 g for 10 minutes at 4°C. The resulting pellet was resuspended in 5 ml of Dulbecco's medium (Gibco) containing 0.5 ml of a lymphokine solution and 3 μg of lipopolysaccharide (Difco). (The lymphokine solution was prepared by culturing popliteal lymph node cell suspensions (2×10^6 cells/ml) with protein purified derivative (100 μg/ml; Difco); the lymph node cells were recovered from mice that had been injected in the footpads 7-9 days previously with complete Freunds adjuvant (Difco) emulsified 1:1 in saline.) This mixture was transferred to 100 mm culture plates (Corning) and incubated at 37°C in 10% CO_2 for 6 to 8 hours. After the nonadherent cells were removed, the adherent cells were recovered by vigorous pipetting of the plate with PBS-EDTA (Gibco). The harvested cells were lysed by freeze/thawing and subsequently analyzed in the Gpi assay.

VII. Results

A. Production of Antibody-Facilitated Chimeras

Production of anti-H-2K^k Antibody-Facilitated Chimeras With Spleen Cells or Bone Marrow Cells or Both

DBA → (DBA x C3H)F$_1$ Hybrid Strain Combination

Previously it had been shown that semiallogeneic chimeras could be produced by the injection of 0.25 ml of anti-H-2K^k mAb one day before the injection of 10^8 untreated DBA/2J spleen cells into (DBA x C3H)F$_1$ hybrid mice (Wegmann *et al.* 1980). Injection of irrelevant antibody or of cells alone did not lead to any takeover.

In an attempt to reduce the cell dosage required for takeover, we decided to use bone marrow as a source of stem cells, since the hemopoietic potential of bone marrow tissue is much greater than that of the spleen (Lord, 1983). The simultaneous injection of 2 x 10^7 untreated DBA BM cells along with 0.25 ml anti-H-2K^k mAb into age- and sex-matched (DBA x C3H)F$_1$ hybrids leads to > 95% takeover by donor cells, at eight weeks, as measured in a Gpi assay of the peripheral blood (Table 2). As before, injection of cells alone did not give rise to takeover.

To examine if any synergistic or additive effect would be seen, a combination of 2 x 10^7 DBA/2J BM cells plus 10^8 DBA/2J spleen cells were administered

Table 2

Production of Anti-H-2K^k Antibody-Facilitated Chimeras with Spleen and/or Bone Marrow Cells. DBA/2J → D2C3F$_1$.

DONOR	CELL SOURCE[1]	mAb[2]	RECIPIENT[3]	N	%DONOR CELLS[4]
DBA/2J	10^8 SC	αK^k	D2C3F$_1$	6	>95
DBA/2J	10^8 SC	-	D2C3F$_1$	3	0
DBA/2J	2 x 10^7BMC	αK^k	D2C3F$_1$	3	>95
DBA/2J	2 x 10^7BMC	-	D2C3F$_1$	3	0
DBA/2J	10^8SC + 2 x 10^7BMC	αK^k	D2C3F$_1$	25	>95
DBA/2J	10^8SC + 2 x 10^7BMC	-	D2C3F$_1$	5	0

[1]Spleen cells (SC) or bone marrow cells (BMC).
[2]416 μg of anti-H-2K^k (αK^k) (11-4.1) monoclonal antibody (mAb).
[3]D2C3F$_1$ = (DBA/2J x C3H/HeJ)F$_1$.
[4]Percentage of DBA/2J donor cells in D3C3F$_1$ host peripheral blood as determined by the glucose phosphate isomerase assay. Determinations were made 8 weeks postinjection.

simultaneously with the mAb. The kinetics of takeover showed no difference as compared to groups which received spleen cells and mAb or BM cells and mAb.

BALB → (BALB x C3H)F_1 Hybrid Strain Combination

After having worked out the minimal cellular requirements necessary for takeover, we decided to extend the model to another strain combination. The BALB → (BALB x C3H)F_1 combination was chosen, since attempts to parabiose these strains leads to parabiosis intoxication and death (Drell, 1979a).

5 x 10^7 spleen cells or 2 x 10^7 BM cells from BALB mice were injected along with anti-H-$2K^k$ mAb into (BALB x C3H)F_1 recipients (Table 3). The time course of donor engraftment was similar to that seen in the DBA/2J → (DBA/2J x C3H/HeJ)F_1 model. This time course was also comparable to the takeover seen in control radiation chimeras. Once again, injection of cells alone did not lead to any chimerism.

Production of anti-H-$2K^k$ and anti-H-2I-A^k Antibody-Facilitated Chimeras with anti-Thy 1.2 Treated Bone Marrow

To determine the optimal dosage of anti-Thy 1.2 treated BM ($\alpha\Theta$BM) cells needed for producing donor chimerism in the host, various numbers of BALB $\alpha\Theta$BM cells and a constant amount (approximately 416 μg of specific IgG, subclass IgG2a) of anti-H-$2K^k$ ascitic mAb were injected into (BALB x C3H)F_1 recipients.

Table 3

Production of Anti-H-2K^k Antibody-Facilitated Chimeras with Spleen and/or Bone Marrow Cells. BALB → CC3F$_1$.

DONOR	CELL SOURCE[1]	mAb[2]	RECIPIENT[3]	N	%DONOR CELLS[4]
BALB/cCR	5 x 10^7 SC	αK^k	CC3F$_1$	5	>95
BALB/cCR	5 x 10^7 SC	-	CC3F$_1$	5	0
BALB/cCR	2 x 10^7 BMC	αK^k	CC3F$_1$	5	>95
BALB/cCR	2 x 10^7 BMC	-	CC3F$_1$	5	0

[1]Spleen cells (SC) or bone marrow cells (BMC).
[2]416 μg of anti-H-2K^k (αK^k) (11-4.1) monoclonal antibody.
[3]CC3F$_1$ = (BALB/cCR x C3H/HeJ)F$_1$.
[4]Percentage of BALB/cCR donor cells in CC3F$_1$ host peripheral blood as determined by the glucose phosphate isomerase assay. Determinations were made 8 weeks postinjection.

2 x 10^7 αΘBM cells injected along with anti-H-$2K^k$ mAb leads to > 95% takeover by 10 weeks (Table 4). The injection of 3 x 10^6 αΘBM cells and antibody could produce only a stable, partial chimerism. The use of 10^6 αΘBM cells plus antibody, 2 x 10^7 αΘBM cells alone, or with a non-specific mAb (P1.1) did not lead to any detectable chimerism.

In contrast to anti-H-$2K^k$ AF chimeras , anti-H-2I-A^k AF chimeras exhibit only a partial chimeric state when a similar αΘBM cell dose of 2 x 10^7 is used (Table 5). Doubling the donor cell inoculum to 4 x 10^7 αΘBM cells, gave a comparable > 95% takeover pattern as seen with our anti-class I mAb. Increasing further the donor cell numbers to 6 x 10^7 αΘBM cells did not alter the degree of takeover, as monitored at 10 weeks. If αΘBM donor cells are injected alone or with control non-specific P 1.1 mAb no chimerism could be detected.

Time Frame for Donor Inoculum

Donor cells and antihost mAb do not need to be injected simultaneously to produce AF chimeras. In an effort to establish a time frame for the donor inoculum, we injected cells either two or five days before the mAb (day 0), or mAb five or seven days before cells (Figure 7). In these experiments (BALB x C3H)F_1 recipients were injected with 10^8 spleen cells plus 2 x 10^7 BM cells from BALB donors together with anti-H-$2K^k$ mAb. The reason that spleen cells were used, is related to the fact that initially AF chimeras were

Table 4

Production of Anti-H-2K^k Antibody-Facilitated Chimeras with anti-Thy 1.2 Bone Marrow ($\alpha\Theta$BM) Cells.

TREATMENT		% DONOR CELLS/WEEKS[1]		
$\alpha\Theta$BM[2]	mAb[2]	4	8	10
1 x 10^6	αK^k	0	0	0
3 x 10^6	αK^k	54(10)	63(15)	69(13)
2 x 10^7	αK^k	75(10)	86(5)	>94
4 x 10^7	αK^k	82(16)	>95	>95
2 x 10^7	None	0	0	0
2 x 10^7	P1.1	0	0	0

[1]Percentage of BALB/cCR donor cells in (BALB/cCR x C3H/HeJ)F_1 host peripheral blood as determined by the glucose phosphate isomerase assay. Each value represents the mean (standard deviation) of 5 mice.
[2]BALB/cCR bone marrow cells treated with anti-Thy 1.2+ complement.
[3]CC3F_1 = (BALB/cCR x C3H/HeJ)F_1.
[4]416 μg of anti-H-2K^k (αK^k) (11-4.1) monoclonal antibody.

Table 5

Production of Anti-H-2I-A^k Antibody-Facilitated Chimeras with anti-Thy 1.2 Bone Marrow ($\alpha\Theta$BM) Cells.

TREATMENT		% DONOR CELLS/WEEKS[1]		
$\alpha\Theta$BM[2]	mAb[3]	4	8	10
2 x 10^7	αI-A^k	16(13)	58(6)	83(7)
4 x 10^7	αI-A^k	58(11)	>95	>95
6 x 10^7	αI-A^k	73(14)	>95	>95
2 x 10^7	None	0	0	0
2 x 10^7	P1.1	0	0	0

[1]Percentage of BALB/cCR donor cells in (BALB/cCR x C3H/HeJ)F_1 host peripheral blood as determined by the glucose phosphate isomerase assay. Each value represents the mean (standard deviation) of 5 mice.
[2]BALB/cCR bone marrow cells treated with anti-Thy 1.2 + complement.
[3]4-16 μg of anti-H-2I-A^k (αI-A^k) (10-3.6) monoclonal antibody.

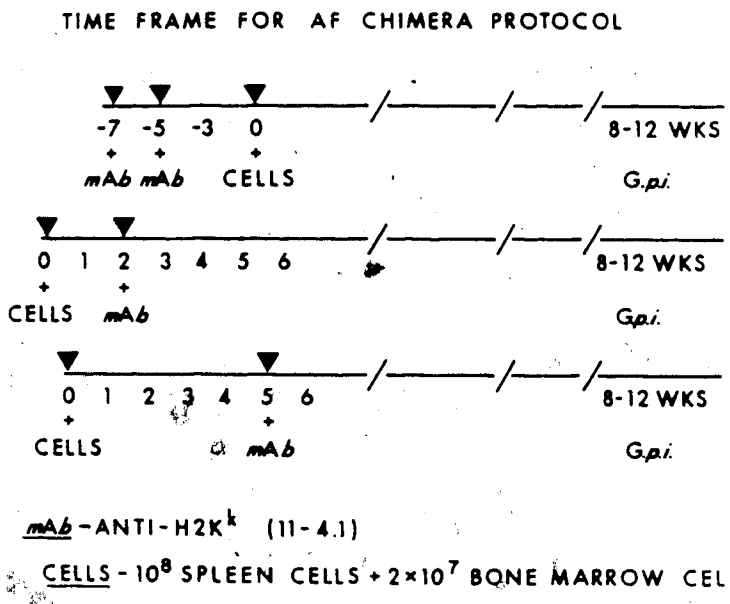

Figure 7. Variation in the time frame for the establishment of Antibody-Facilitated Chimeras.

produced using spleen cells alone. Donor takeover was seen by 8 weeks post-injection of the cells, irrespective of whether the mAb was injected before or after the donor cell inoculum. The percentage of takeover as measured by the Gpi assay was greater than 95% in all mice (10 per group) tested.

B. *in vivo* and *in vitro* Effects of mAbs on Hemopoietic Tissue

Effects on Hematocrits and Buffy Coats

The effect of the intravenous administration of an anti-Class II (anti-H-2I-A^k) mAb into untreated recipients of various H-2 haplotypes is shown in Figure 8. Strains bearing the appropriate target antigens, namely the H-2^k haplotype, undergo a transient drop in their buffy coat levels. These levels return to normal by about 7-8 weeks. Their hematocrit levels also experience a transient decline with full recovery by 8 weeks. F_1 hybrids do not undergo as marked a drop in levels. BALB mice are not affected. Examination of mice prepared by the AF chimeras protocol, in this case using an anti-Class I mAb (anti-H-$2K^k$), reveals that a similar period of transient anemia exists, with recovery to normal pre-injection hematocrit levels by 6 weeks (Figure 9).

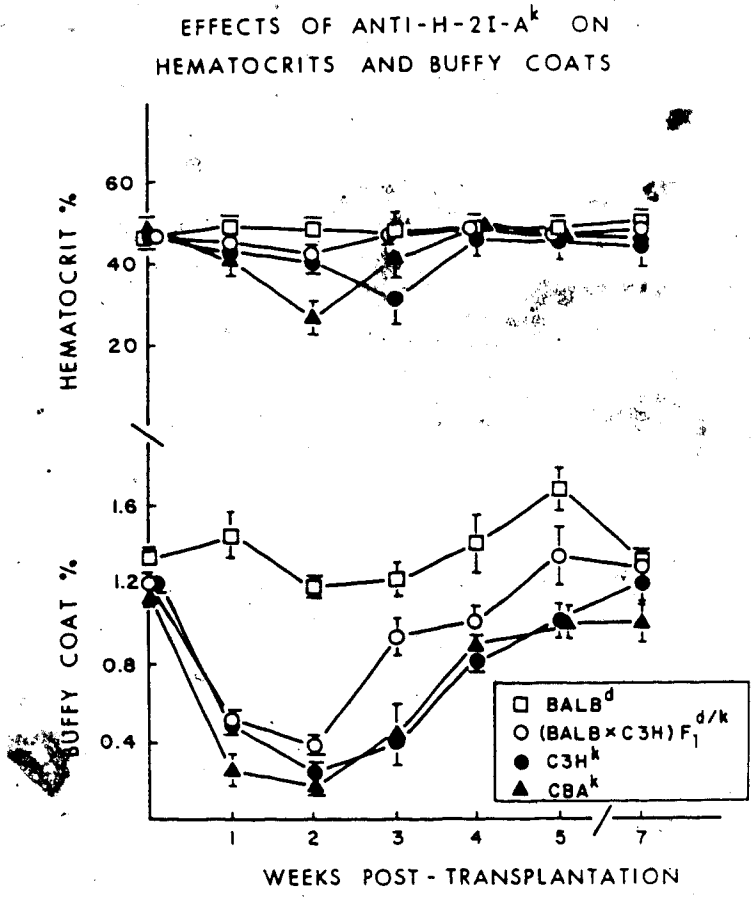

Figure 8. Effects of *in vivo* intravenous administration of anti-H-2I-A^k (10-3.6) monoclonal antibody on hematocrits and buffy coats of various strains. Values represent mean ± standard deviation of 10 mice/group. BALB = BALB/cCR; C3H = C3H/HeJ; CBA = CBA/CAJ.

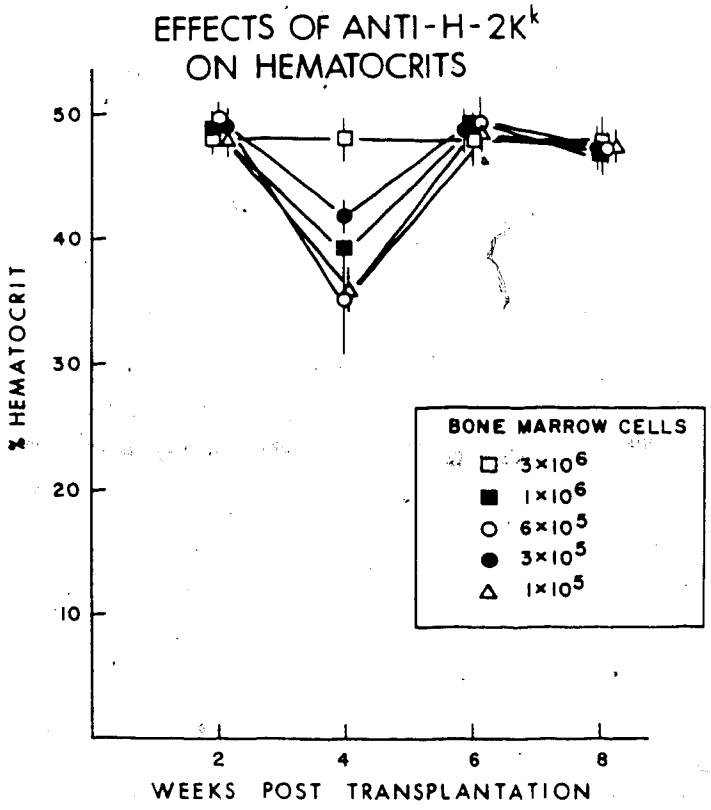

Figure 9. Hematocrit determinations of AF Chimeras (BALB x C3H)F_1 . 2, 4, 6 and 8 weeks post-injection of anti-H-2K^k (11-4.1) mAb plus varying numbers of BALB donor BM cells. Each point represents mean ± SD of 5 mice /group.

Effects on the CFU-S Assay

in vitro and *in vivo* Effects With anti-H-2K^k and anti-H-2I-A^k mAbs

Since anti-H-2K^k and anti-H-2I-A^k mAbs assist donor cell repopulation, their effects on pluripotential stem cells were evaluated by the CFU-S assay. The presence of class I and class II molecules on CFU-S was examined directly by incubating BALB, C3H, or (BALB x C3H)F$_1$ BM cells with mAb and complement *in vitro*, then using the treated cells to repopulate irradiated syngeneic mice. Repopulation was attempted with 10^5 treated or control cells (Table 6). Compared with control mAb (P1.1) anti-H-2K^k mAb pretreatment but not anti-H-2I-A^k mAb pretreatment reduced the number of CFU-S in recipient spleens. To determine the *in vivo* effects of these mAbs, BALB, C3H, or (BALB x C3H)F$_1$ mice were inoculated iv with anti-H-2K^k or anti-H-2I-A^k mAb at a dose equivalent to that required for AF chimeras production. After 48 hr, 10^5 BM cells from the treated mice, which had received the mAb, were harvested and transfered to irradiated syngeneic recipients (Table 7). Both control mAb (P1.1)-treated and untreated mice served as control donor BM cell sources. In support of the *in vitro* findings, anti-H-2K^k mAb *in vivo* could almost completely inhibit the CFU-S potential of target BM from C3H or (BALB x C3H)F$_1$ mice. In contrast to the anti-H-2I-A^k mAb pretreatment *in vitro*, the *in vivo* administration of

Table 6

In Vitro Effects of Anti-H-2K^k and Anti-H-2I-A^k on CFU-S.

Target	CFU-S COLONIES/TREATMENT GROUP Untreated	P1.1	αK^k	$\alpha I\text{-}A^k$
BALB/cCR	21(3)	19(3)	20(3)	21(4)
C3H/HeJ	15(3)	15(2)	2(1)	13(2)
(BALB/cCR x C3H/HeJ)F_1	18(2)	20(3)	3(1)	17(2)

10^5 bone marrow cells were pretreated *in vitro* with 1:10 dilution of IgG2a monoclonal antibody (untreated, P1.1, anti-H-2K^k (αK^k) or anti-H-2I-A^k ($\alpha I\text{-}A^k$) and complement and then were injected into syngeneic irradiated (950 rad) recipients (e.g. BALB/cCR BM → BALB/cCR 950 rad). Macroscopic spleen cell colonies (CFU-S) were counted 12 days later. Each value represents the mean (standard deviation) of 10 mice.

Table 7

In Vivo Effects of Anti-H-2K^k and Anti-H-2I-A^k on CFU-S.

Target	CFU-S COLONIES/TREATMENT GROUP Untreated	P1.1	αK^k	$\alpha I\text{-}A^k$
BALB/cCR	29(1)	29(1)	29(3)	20(1)
C3H/HeJ	15(1)	14(1)	2(2)	2(1)
(BALB/cCR x C3H/HeJ)F$_1$	21(2)	22(2)	1(1)	11(1)

10^5 bone marrow cells pretreated (48 hours) with 416 μg IgG2a monoclonal antibody (untreated, P1.1, anti-H-2K^k (αK^k) or anti-H-2I-A^k ($\alpha I\text{-}A^k$) were injected into syngeneic irradiated (950 rad) recipients (e.g. BALB/cCR BM → BALB/cCR 950 rad). Macroscopic spleen cell colonies (CFU-S) were counted 12 days later. Each value represents the mean (standard deviation) of 10 mice.

anti-H-2I-A^k mAb resulted in a marked decrease in the CFU-S capacity of relevant C3H or (BALB x C3H)F_1 mice. In all cases, the CFU-S capability of control BALB BM cells was not inhibited *in vitro* or *in vivo* by either mAb, which would be expected, because this is a strain which does not react with the mAb.

Ability of Long-Term Antibody-Facilitated Chimeras Sera to Inhibit CFU-S Appearance

Initial parabiont studies showed the presence of circulating cytotoxic antibodies directed against one of the parental haplotypes (Wegmann *et al.*, 1980). Therefore, we decided to examine the serum of established AF chimeras to determine if a similar antibody was present. The serum of (DBA x C3H)F_1 AF chimeras, 56 weeks post-transplantation of DBA BM cells along with anti-H-2K^k mAb, was used in an attempt to inhibit CFU-S colonies. Unlike the situation in the parabiont model, these sera had no effect on the reduction of CFU-S colonies when compared to the control anti-H-2K^k mAb (Table 8).

C. Hemopoietic Analysis and Genotyping of Antibody-Facilitated Chimeras

Red and White Blood Cell Chimerism

Examination of peripheral red and white cell chimerism shows that both cell classes are equivalent in numbers. This was evaluated in several [illegible] chimeras by separating

Table 8

Ability of Long-Term Antibody-Facilitated Chimeras Sera to Inhibit CFU-S Appearance.

TREATMENT OF C3H/HeJ BMC	CFU-S[2]
AF Chimeras' sera (pooled)	14.0(3.4)
Anti-H-2K^k mAb	2.2(1.1)
DBA/2J normal mouse serum	15.4(1.2)

[1]DBA/2J →(DBA/2J x C3H/HeJ)F$_1$ AF chimeras at 56 wks post-transplantation.
[2]Mean number of colonies at day 12; n=12; mean (SD).

peripheral red and white blood cells on a Percoll gradient. AF chimeras produced using either anti-H-2K^k or anti-H-2I-A^k, as well as radiation chimeras, exhibited > 95% donor isozymes in both red and white cell fractions by 90 days postinjection of donor αΘBM cells (Table 9).

Long-Term Bone Marrow Cultures

Primary, long-term, bone marrow cultures were established from AF chimeras showing greater than 95% donor cells in their peripheral blood and bone marrow. These cultures were not reseeded with fresh bone marrow. Eight weeks after the initiation of culture, the adherent and non-adherent cells were analyzed by the GPI assay. As seen in Table 10, the nonadherent cells were greater than 95 % donor type, while the adherent cells were between 73 - 77 % of donor origin. This was unexpected, since the initial bone marrow inoculum used to start these cultures had been typed as being greater than 95 % donor. The most likely explanation is that a differential outgrowth of host connective tissue cells in the marrow is responsible for the appearance of the residual host population.

Genotyping of Peritoneal Macrophages

Macrophages derived from the peritoneal exudate of AF chimeras showing greater than 95 % peripheral donor blood cells were typed for GPI. It can be seen from Table 10 that the peritoneal exudate cells were approximately 90% donor.

Table 9

Peripheral Red and White Blood Cell Chimerism in Antibody-Facilitated and Radiation Chimeras.

TREATMENT			PERCENTAGE OF DONOR CELLS[1]	
αΘBM[2]	mAb[3]	IRRADIATION	RED	WHITE
5 x 10^7	αK^k	None	>95	>95
4 x 10^7	$\alpha I\text{-}A^k$	None	>95	>95
2 x 10^7	None	900 rad	>95	>95

[1]Percentage of BALB/cCR donor cells in (BALB/cCR x C3H/HeJ)F_1 host peripheral blood separated on a percoll density gradient as determined by the glucose phosphate isomerase assay. Each value represents 5 mice examined >90 days post-injection of αΘBM cells.
[2]BALB/cCR bone marrow cells treated with anti-Thy 1.2 + complement.
[3]416 μg of anti-H-$2K^k$ (αK^k) (11-4.1) or anti-H-$2IA^k$ ($\alpha I\text{-}A^k$) (10-3.6) monoclonal antibody.

Table 10

Percent Donor Phenotype of Adherent and Nonadherent Cells in Long-term Marrow Cultures and of Peritoneal Macrophages in Antibody-Facilitated Chimeras.

	% DONOR TAKEOVER OF ANTIBODY-FACILITATED CHIMERAS[1,2]				
#	PERIPHERAL BLOOD	BONE MARROW CELLS	PERITONEAL MACROPHAGES	NONADHERENT[3] CELLS	ADHERENT[3] CELLS
1	>95[4] (10)[5]	>95(10)	ND[6]	>95(10)	75±2(10)
2	>95 (7)	>95(7)	90±9(7)	ND	ND

[1]Mice at 16 months post-transplantation.
[2]Using the Gpi assay.
[3]From 8 week long-term bone marrow cultures.
[4]Mean ± standard deviation.
[5]Number of mice/experiment.
[6]Not determined.

Contamination by host fibroblasts could account for the residual host component.

Residual Host Spleen, Lymph Node and Thymus Cell Populations

Gpi analysis of AF chimeras, 8 weeks to two years post-transplantation, revealed > 95 % donor takeover of both the peripheral blood (red and white cells) and bone marrow hemopoietic tissue. In contrast to these findings, examination of AF chimeric spleen, lymph node and thmymic tissue revealed the existence of a substantial residual host cell population. Residual host cells constituted as much as 55 % of the spleen, 45 % of lymph nodes and 60 % of the thymus. These values are in sharp contrast to findings in radiation chimeras , which showed > 95 % takeover in the spleen and lymph nodes (Table 11). These studies were done in collaboration with SA McCarthy, Ph.D..

Correlation Between Proportions of Donor Cells in the Peripheral Blood and the Bone Marrow

Long-term (1-2 yrs) stable AF chimeras were analyzed for hemopoietic takeover. A comparison of the percent donor cells in the peripheral blood to the percent donor cells in bone marrow plugs shows a close, linear correlation (r value = 0.97 see Figure 10). This includes AF chimeras in the process of takeover, and those which did not achieve complete takeover as a result of the use of suboptimal treatment with mAb.

Table 11

Donor Cell Contributions to Spleen, Lymph Nodes, and Thymus.

	% BALB Cells[3]			
AF Chimeras[1]	Peripheral Blood	Spleen	Lymph Nodes	Thymus
1	>95	75	nd[4]	80
2	>95	70	nd	80
3	>95	45	55	65
4	>95	70	60	40
RAD Chimeras[2]				
1	nd	>95	>95	nd
2	nd	>95	>95	nd
3	nd	>95	>95	nd
4	nd	>95	>95	nd

[1]Individual BALB → (BALB x C3H)F$_1$ AF Chimeras.
[2]Individual BALB → (BALB x C3H)F$_1$ Radiation (RAD) Chimeras.
[3]Calculated from Gpi isozyme ratios, as described in Materials and Methods.
[4]Not done.

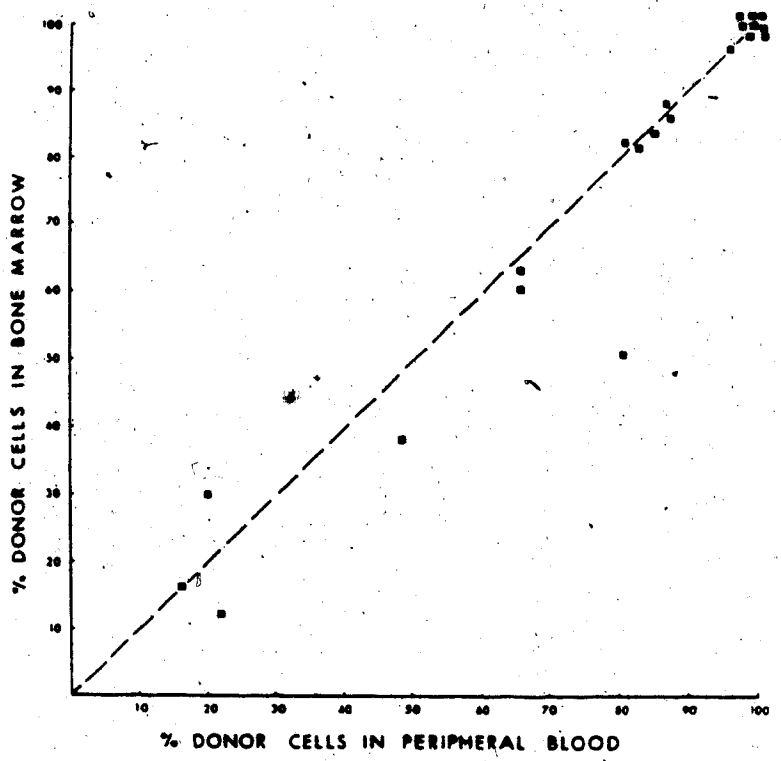

Figure 10. Comparison of the percent of donor cells in peripheral blood and in the bone marrow of AF chimeras at various times post-transplantation. Each point represents values for individual animals.

Comparison of the Percentage Donor Cells in Peripheral Blood With the Percentage Donor Bone Marrow and Spleen-Derived Methyl Cellulose Colonies

Having established that a correlation exists between the percentage of donor cells in the peripheral blood and whole bone marrow of AF chimeras, we examined the chimerism of bone marrow colonies grown in methyl cellulose cultures. This was done in order to test the possibility that a residual population of host hemopoietic cells may exist, but at levels too low to be detected in a direct assay of bone marrow or spleen. The test is especially important in view of the existence of a substantial residual host population in the spleen of AF chimeras with greater than 95% donor cells in peripheral blood (McCarthy *et al.*, 1985). Between 60 and 90 colonies per AF chimera, containing granulocyte-monocyte, erythroid, megagaryocyte, and mixed lineages, were randomly picked 7 to 9 days after the initiation of culture and individually analyzed in the Gpi assay. The results are presented in Figure 11. Analysis reveals a direct correlation between the percent of donor-derived colonies and the percent of donor cells in the peripheral blood. Spleen cells were also cultured in methyl cellulose. The spleen-derived colonies showed a similar correlation with the chimerism of the peripheral blood cells (Figure 11; spleen colonies r value = 0.93; bone marrow colonies r value = 0.98).

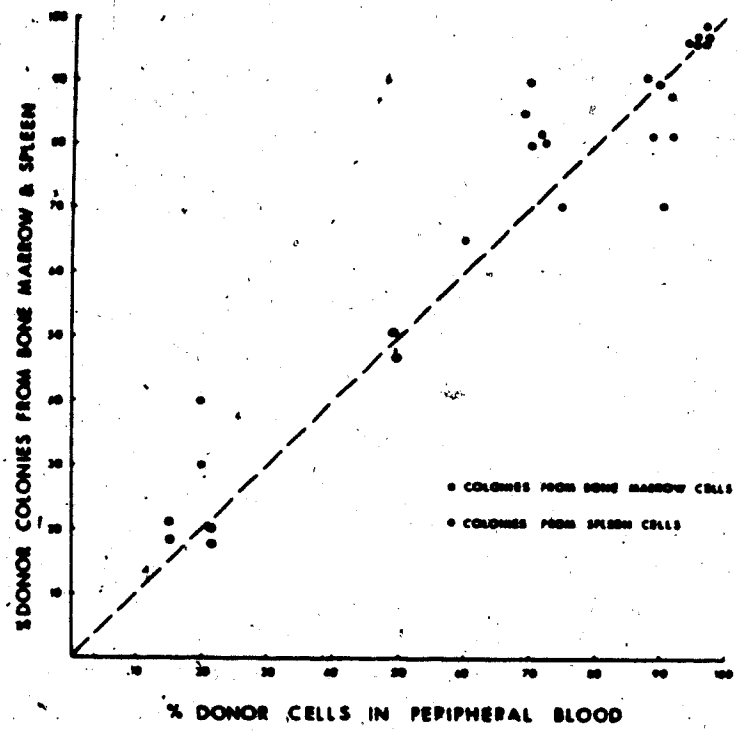

Figure 11. Comparison of the percent of donor cells in peripheral blood and bone marrow-derived or spleen-derived methyl cellulose colonies. Each point represents the average percent donor chimerism of approximately 70-90 colonies of an AF chimera at various times post-transplantation. Determination by the Gpi assay.

Bone Marrow Fractionation

When the bone marrow of these AF chimeras was fractionated and analysed in the Gpi assay, > 95 % donor takeover was found in all three compartments. Fraction I, representative of the medullary cavity contents, GPI typed as > 95 % donor type. Fractions II and III, the endosteal and compact compartments respectively, were also found to be > 95 % of donor origin. These results are comparable to the complete repopulation seen in radiation chimeras (Table 12).

D. Allogeneic Antibody-Facilitated Chimeras Production

Using anti-H-2K^k mAb

Attempts to produce fully allogeneic AF chimeras (BALB → C3H) by administering various numbers of BALB αΘBM cells together with anti-H-2K^k mAb failed (Table 13). After 8 weeks (the time by which donor cell engraftment in radiation chimeras or P → F_1 AF chimeras could be detected), there was no evidence of donor cells in the allogeneic host's peripheral blood, even when the number of donor cells used was increased to 6 x 10^7. Increasing the amount of mAb given, while maintaining a constant number of donor BALB αΘBM cells, also did not give rise to chimerism (Table 14).

Using anti-H-2K^k and anti-H-2I-A^k mAbs

Even when anti-H-2K^k and anti-H-2I-A^k mAbs were used simultaneously, chimerism could not be established across

Table 12

GPI Analysis of Peripheral Blood and Bone Marrow Fractions I, II, and III of AF Chimeras and Radiation Chimeras.

Exp	Treatment	FRACTION TESTED Blood	I Medullary	II Endosteal	III Compact
1	anti-H-2K^k[1]	>95[4]	>95	>95	>95
2	anti-H-2I-A^k[2]	>95	>95	>95	>95
3	900 rad[3]	>95	>95	>95	>95

[1] 2 x 10^7 BALB BM cells into (BALB x C3H)F$_1$.
[2] 4 x 10^7 BALB BM cells into CC3F$_1$ along with anti-H-2I-A^k mAb.
[3] 2 x 10^7 BALB BM cells into CC3F$_1$ (900 rad).
[4] Values represent percentage of BALB donor cells in CC3F$_1$ recipients as determined by the Gpi assay. Each value represents analyses as performed on 10 animals.

Table 13

Attempts to Produce Fully Allogeneic Antibody-Facilitated Chimeras with Bone Marrow Cells and anti-H-2K^k Monoclonal Antibody.

CELL SOURCE[1]	mAb[2]	RECIPIENT[3]	N	%DONOR CELLS[4]
2 x 10'BMC	αK^k	CC3F,	10	>95
2 x 10'BMC	None	CC3F,(900R)	5	>95
2 x 10'BMC	None	C3H(900R)	5	>95
2 x 10'BMC	αK^k	C3H	5	0
6 x 10'BMC	αK^k	C3H	5	0
10'SC + 2 x 10'BMC	αK^k	C3H	5	0

[1]BALB spleen cells (SC) or bone marrow cells (BMC).
[2]416 μg of anti-H-2K^k (αK^k) (11-4.1) monoclonal antibody.
[3]CC3F = (BALB/cCR x C3H/HeJ)F.
[4]Percentage of BALB donor cells in CC3F, host peripheral blood as determined by the glucose phosphate isomerase assay. Determinations were made 8 weeks postinjection.

Table 14

Attempts to Produce Completely Allogeneic Antibody-Facilitated Chimeras. (BALB/cCR → C3H/HeJ).

TREATMENT			% OF DONOR CELLS/WEEK[3]		
BM[1]	mAb[2]	RECIPIENT	6	8	12
4 x 10^7	.6ml αK^k	C3H	0	0	0
4 x 10^7	.45ml αK^k	C3H	0	0	0
4 x 10^7	.3ml αK^k	C3H	0	0	0
4 x 10^7	900 rad	C3H	>95	>95	>95

[1]BALB bone marrow cells treated with anti-thy-1.2 + complement.
[2]anti-H-2K^k (αK^k) (11-4.1) monoclonal antibody.
[3]Percentage of BALB donor cells in C3H host peripheral blood as determined by the glucose phosphate isomerase assay. Each value represents the mean (standard deviation) of 10 mice per group.

fully allogeneic barriers. 8 x 10⁷ BALB cBM cells, along with both anti-H-2K^k and anti-H-2I-A^k mAbs, did not produce any detectable level of chimerism (Table 15).

E. Health Status of Antibody-Facilitated Chimeras

Thus far, stable AF chimeras have been maintained in good health for long periods (> 2 years) without mortality. In no instance have any of the AF chimeras exhibited overt clinical signs of GVHD, such as weight loss, diarrhea, skin lesions, or ruffled or dull fur. The histopathologic status of two groups of long-term AF chimeras was evaluated. Group 1 (BALB x C3H)F$_1$ received 2.5 x 10⁷ BALB BM cells plus anti-H-2K^k mAb, and were examined > 90 days later. Group 2 (DBA x C3H)F$_1$ received 10⁸ spleen cells and 2.5 x 10⁷ BM cells from DBA donors plus anti-H-2K^k mAb and were examined more than a year later. Both of these AF chimeric groups were compared with normal, age-matched controls. All groups consisted of five mice.

No pathological changes associated with acute or chronic GVHD were discernible in AF chimeras that were produced with mAb and untreated BM cells alone (Group 1). Recipients' kidneys did not show signs of chronic, progressive glomerulonephritis, the hallmark of GVHD (Plate 1). In addition, the liver (Plate 2) and lungs (Plate 3) appeared quite normal.

In contrast to group 1, group 2 mice exhibited some evidence of localized tissue injury, particularly to the

Table 15

Attempts to Produce Fully Allogeneic AF Chimeras with Bone Marrow Cells and Anti-H-2K^b and Anti-H-2I-A^{b}mAbs.

CELL SOURCE[1]	mAb[2]	RECIPIENT[3]	%DONOR CELLS[4]
2 x 10^7 BMC	αK^b	CC3F$_1$	>95
4 x 10^7 BMC	$\alpha I\text{-}A^b$	CC3F$_1$	>95
4 x 10^7 BMC	$\alpha I\text{-}A^b$	C3H/HeJ	0
8 x 10^7 BMC	$\alpha I\text{-}A^b$	C3H/HeJ	0
4 x 10^7 BMC	$\alpha K^b + \alpha I\text{-}A^b$	C3H/HeJ	0
8 x 10^7 BMC	$\alpha K^b + \alpha I\text{-}A^b$	C3H/HeJ	0
2 x 10^7 BMC	None	C3H/HeJ(900 rad)	>95

[1]BMC - BALB bone marrow cells.
[2]αK^b - anti-H-2K^b (11-4.1) monoclonal antibody; $\alpha I\text{-}A^b$ - anti-H-2I-A^b (10-3.6) monoclonal antibody
[3]CC3F$_1$ - (BALB/cCR x C3H/HeJ)F$_1$
[4]Percentage of BALB/cCR donor cells in CC3F$_1$ and C3H/HeJ host peripheral blood as determined by the glucose phosphate isomerase assay, determinations made 8 weeks post-injection. Each value represents the mean (standard deviation) of 10 mice per group.

Plate 1. Photomicrograph of kidney from long-term AF chimera. Tissue structure appears normal, no evidence of GVHD (H&E X200).

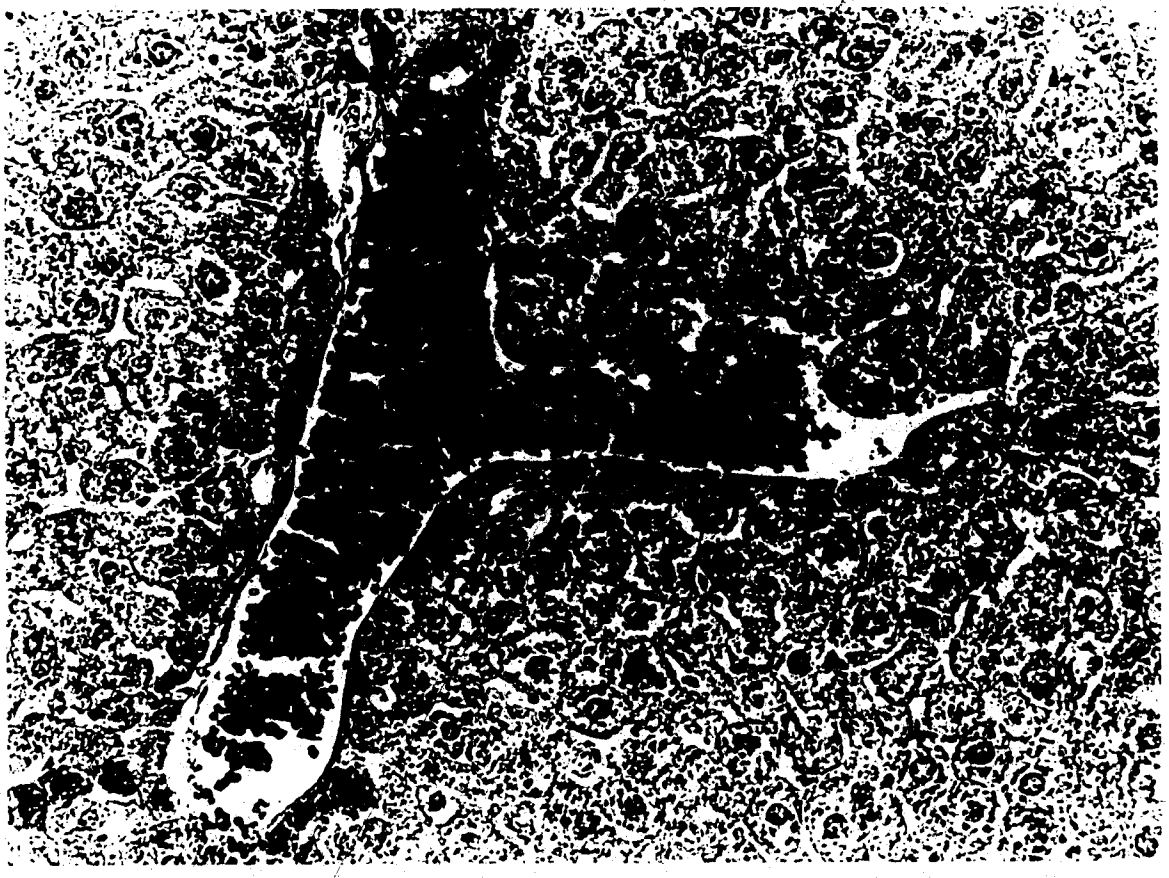

Plate 2. Photomicrograph of liver from long-term AF chimera. Tissue structure appears normal, no evidence of GVHD (H&E X200).

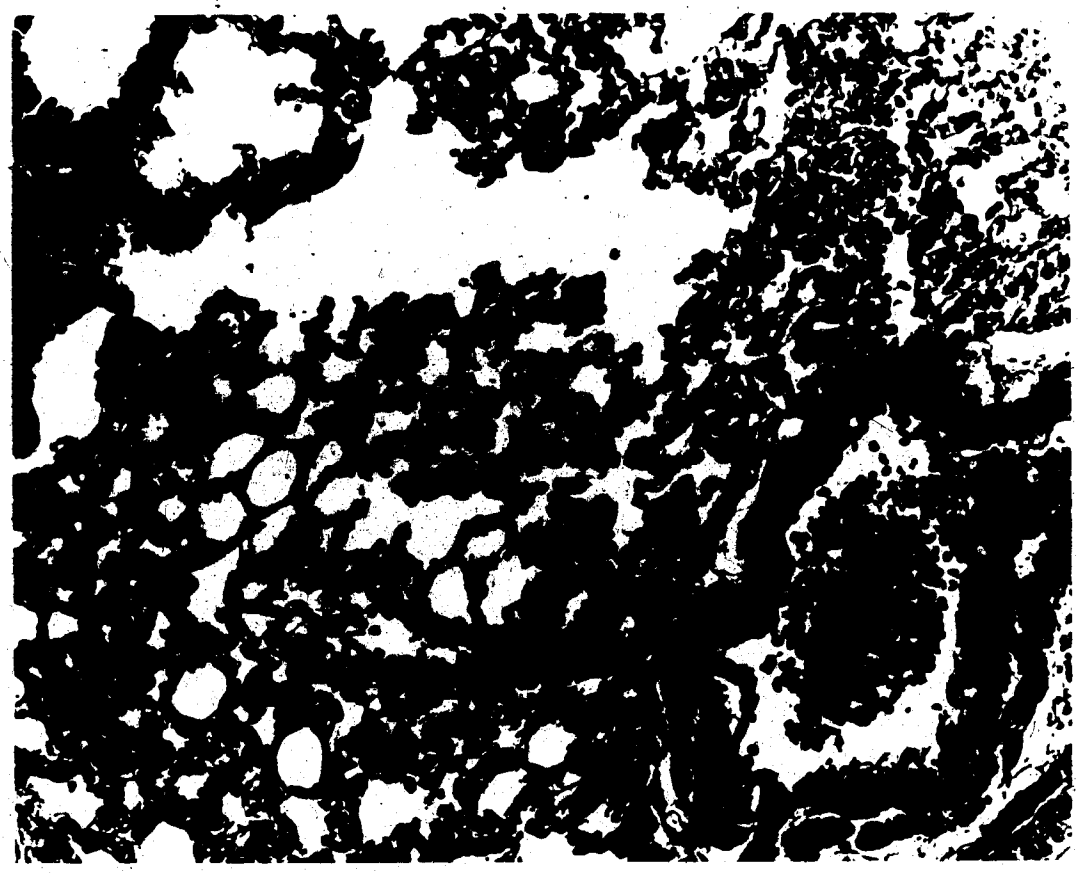

Plate 3. Photomicrograph of lung from long-term AF chimera. Tissue structure appears normäl, no evidence of GVHD (H&E X200).

lymphoid tissue. The lymph nodes and thymii were markedly atrophied and could not be clearly defined. The spleens were smaller than those of control mice and, in some instances, nodular. The spleen cell count (87 ±40 x 10^6) was approximately one-half that of controls (179 ±14 x 10^6). The most striking feature was granulomatus panniculitis leading to the pronounced thickening of the subcutis. In contrast, the kidney (Plate 4), liver (Plate 5), and lung (Plate 6) appeared normal except for occasional lymphocytic infiltrations around vessels. This infiltration was also seen in the mice which received bone marrow cells and mAb but to a lesser extent.

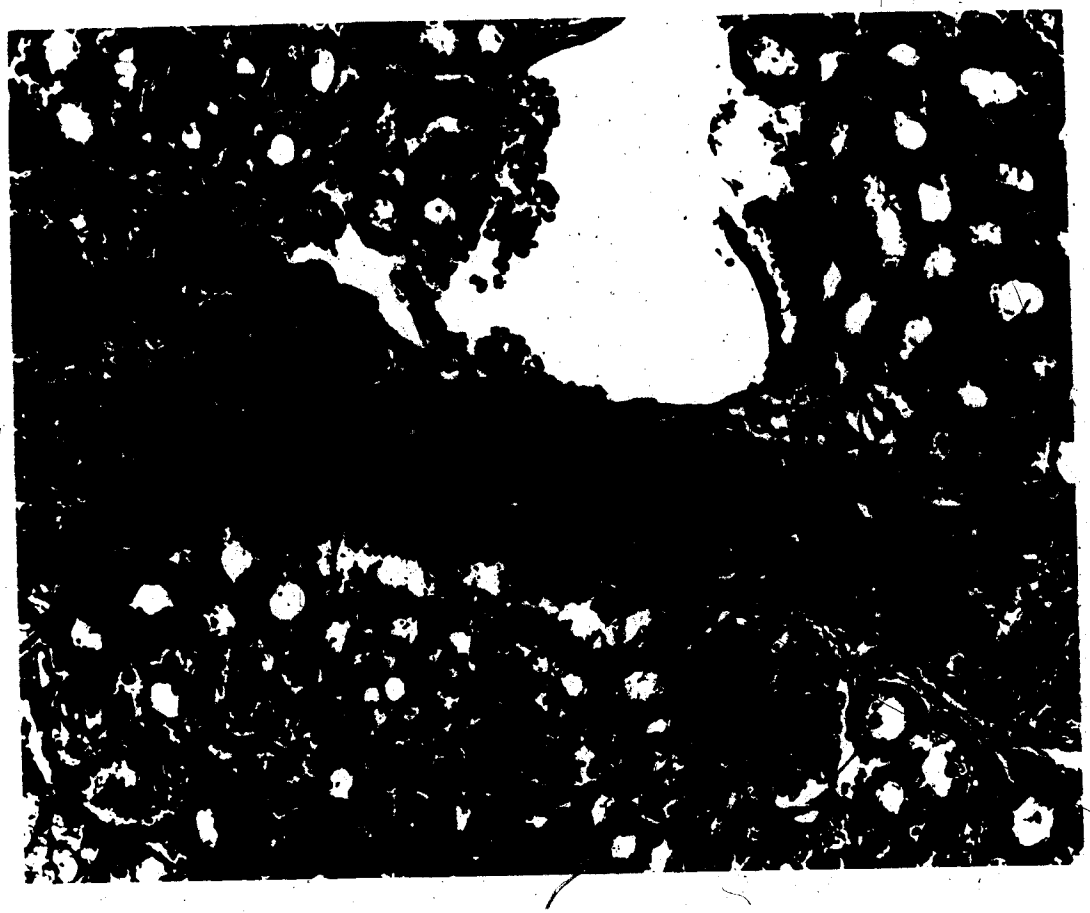

Plate 4. Photomicrograph of section of kidney from a Group 2, anti-H-$2K^k$ AF Chimera. Some evidence of lymphocyte infiltration is seen (H&E X200).

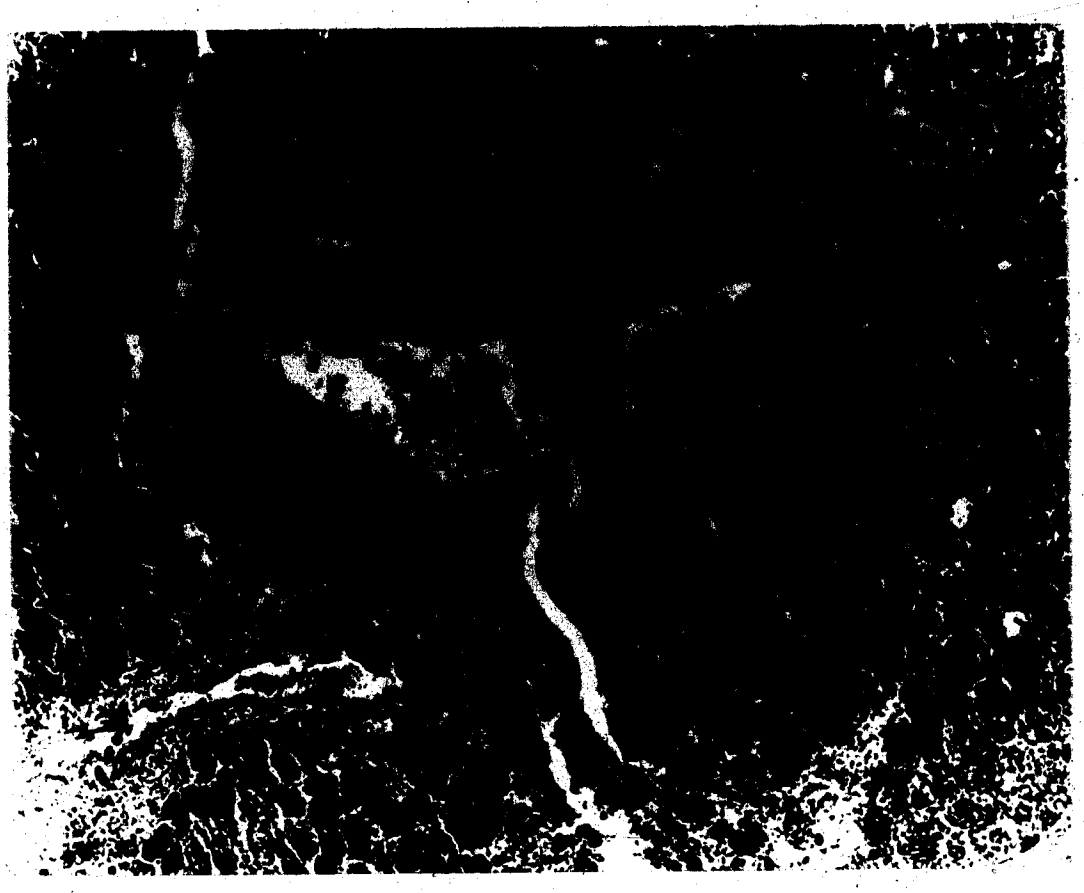

Plate 5. Photomicrograph of section of liver from a Group 2, anti-H-$2K^k$ AF Chimera. Some evidence of lymphocyte infiltration is seen (H&E X200).

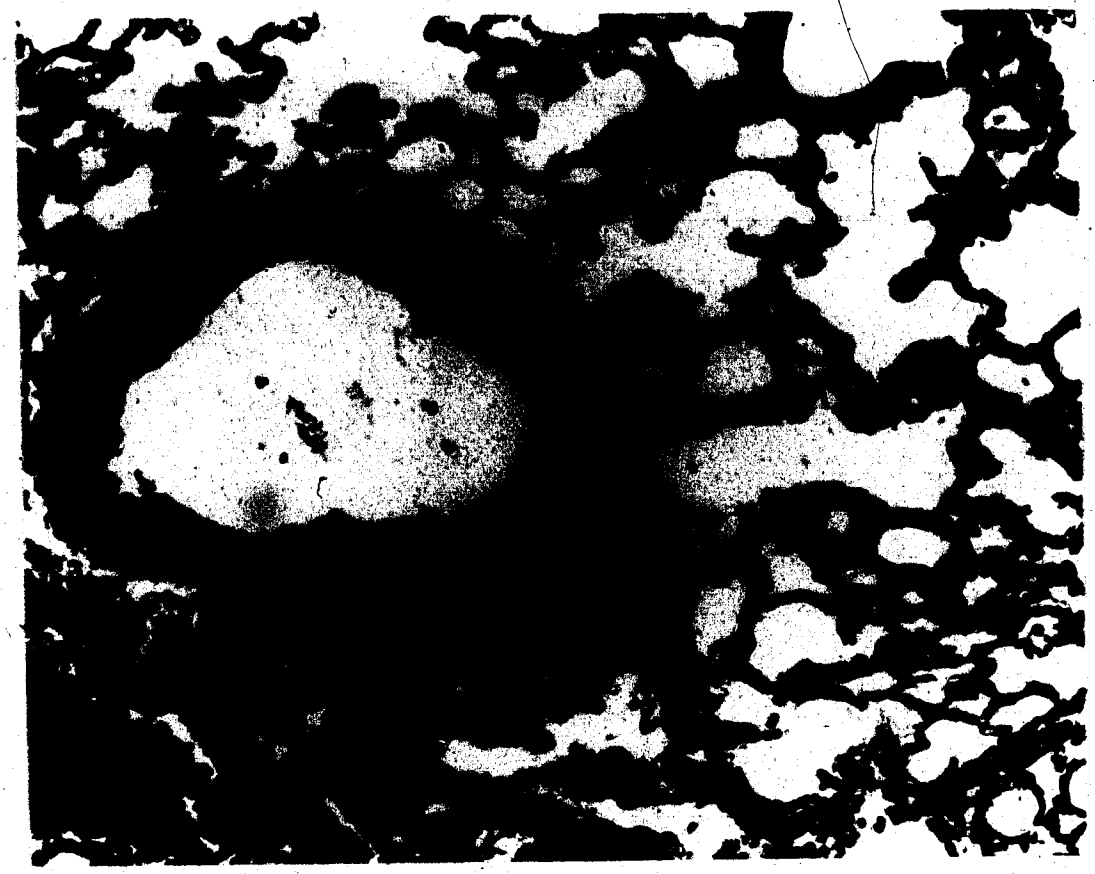

Plate 6. Photomicrograph of section of lung from a Group 2, anti-H-2K^k AF Chimera. Some evidence of lymphocyte infiltration is seen (H&E X200).

Discussion

Antibody-facilitated (AF) chimeras demonstrate that the administration of anti-MHC mAbs can give rise to the engraftment and subsequent generation of donor-type hemopoietic cells in a host environment. These chimeras are established by repopulating F_1 hybrid recipients with parental hemopoietic cells derived from bone marrow or spleen, or both, in conjunction with an IgG2a mAb directed against host Class I or Class II MHC antigens. Under appropriate conditions, this can lead to complete host hemopoietic takeover by donor cells in a semiallogeneic model [DBA → (DBA x C3H)F_1 or BALB → (BALB x C3H)F_1] (Francescutti *et al.*, 1983). Electrophoretic analysis of Gpi isozymes indicates that once takeover has been established, usually by 8 to 10 weeks, it is stable and persists for more than 24 months post-transplantation without any signs of GVHD.

The role of the mAb isotype, IgG2a (complement binding), that is used in the AF chimera model is of importance and warrants further discussion. Carlson (Carlson and Terres, 1976; personal communication) have shown that this particular subclass of immunoglobulin has marked *in vivo* effects as compared to other immunoglobulin subclasses tested. Recently, Wegmann (data not shown) has found that he was unable to facilitated hemopoietic engraftment in a P → F_1 model if he used an antibody of IgG1 (non-complement binding) subclass. These findings suggest that complement

may possibly be involved in the establishment of a successful marrow transplant.

Initially, AF chimeras were prepared utilizing the spleen as a source of stem cells. In an attempt to reduce our donor cell inoculum, we decided to use bone marrow instead. BM cell dose-response studies suggested that a threshold number of donor cells is required to achieve engraftment and another, larger, threshold number is required for complete takeover. It is interesting to note, that the donor cellular requirements for engraftment using the anti-Class II, anti-H-2I-A^k, mAb are double that needed if the mAb was instead directed against Class I MHC target antigens. This finding may be related to the role that the various mAbs play in their preparation of the host prior to engraftment. The anti-Class I mAb may simply be more effective in providing "space" for donor stem cells to differentiate in, whereas the kinetics in the case of anti-Class II reconstitution necessitates an increase in the number of donor stem cells required. The donor cell inoculum used as a source of stem cells can be derived from anti-Thy-1.2-treated or untreated BM cells. MAbs directed against both Class I or Class II MHC antigens are able to facilitate hemopoietic reconstitution of the recipients by donor stem cells.

Waldor *et al.*, 1984 have shown that the injection of anti-I-A monoclonal antibody (anti-H-2I-A^k(10-3.6)) into SJL/J mice rapidly depletes B cell levels. They noted that

one day after the mice were treated with the mAb, a significant drop in splenic IgM positive, IgD positive B cells was observed. Recovery to relatively normal levels of B cells took approximately 33 days. These values correlate with the transient drop in the buffy coat values that we found in the treatment of our chimeras.

The *in vitro* treatment by anti-Class II mAb may be causing the destruction of committed progenitors which have been shown to bear appropriate Class II antigens while lacking an effect on pluripotential stem cells. *In vitro* hemopoiesis is not dependent on viable Ia positive cells. In contrast, *in vivo* treatment by anti-Class II mAb may be inducing aplasia by the killing of committed stem cells and/or by possibly having an effect on monocytes, IL-1 or GM-CSF production? The ability to induce immune suppression in the hosts by these mAb's has never been addressed directly. Although one can envisage that this induction of immunosuppression may actually be occurring in the $P \rightarrow F_1$ model, in contrast to the $P_1 \rightarrow P_2$ model where resistance to immunosuppression has prevented us from achieving any degree of chimerism.

Class I molecules are thought to be present on almost all somatic cells, albeit at varying concentrations (O'Neill, 1984; Parr and Kirby, 1979; Daar *et al.*, 1984). However, Class II molecules are characteristic of cells of the immune system but not necessarily of all hemopoietic progenitor cells, although these determinants may be

expressed on bone marrow stromal cells (Moore *et al.*, 1980; Fitchen *et al.*, 1981). These stromal cells are thought to be intimately involved in the self-renewal and differentiation of stem cells (Dexter, 1982a). Reticular or related, branched stromal cells, in addition to macrophages and lymphocytes, are closely involved with the maintenance of hemopoietic activity (Weiss, 1980), Van Ewijk (1980), has shown that these accessory or supportive cells bear both Class I and Class II MHC antigens.

Our findings that the anti-Class I mAb (anti-H-$2K^k$) was able to significantly reduce the numbers of CFU-S in appropriate target mice are in agreement with those of Fitchen *et al.*, 1982. In addition they went on to show that the anti-H-$2K^k$ mAb also inhibited the development of myeloid (CFU-C) and erythroid (CFU-E) stem cells as well. They used fresh frozen normal rabbit serum as a source of complement in the treatment of the marrow. They also reported that the addition of thymocytes to the marrow cells, after they had been antibody treated, did not overcome the inhibitory effects of the antibody treatment.

CFU-S are inhibited by anti-H-$2K^k$ mAb pretreatment *in vitro* but not by anti-H-2I-A^k mAb pretreatment. The *in vivo* administration of anti-H-$2K^k$ or anti-H-2I-A^k mAbs results in a marked decrease in the CFU-S capacity of relevant C3H and (BALB x C3H)F_1 bone marrow. Therefore, it is likely that the action of anti-Class II mAb *in vivo* is to interfere with the maintenance of hemopoiesis, while anti-Class I mAb *in vivo*

possibly affects stem cells and/or regulatory cells. Taken together, these observations suggest that interference with host regulatory cells can lead to the elimination of host stem cells and the replacement of the hemopoietic system by donor cells. The action of our anti-host MHC mAbs may facilitate donor engraftment by suppressing host resistance to marrow grafts, as described by Cudkowicz (1978) for rabbit anti-mouse bone marrow or thymocyte serum, and by Pollack and colleagues in which they successfully prevented the rejection of allogeneic bone marrow transplants by the *in vivo* pretreatment of the host with an antiserum specific against the NK-cell surface antigen NK 1.1 (Pollack *et al.*, 1979).

Other aspects of these AF chimera studies further implicate the important regulatory role of the local cellular microenvironment for stem cell growth. Our dose-response studies indicate that the conditions favoring donor cell repopulation depend on the number of infused cells and on the type of mAb treatment. The establishment and the extent of donor cell engraftment probably reflects the availability of stem cell niches and competition between donor and host hemopoietic cells for such sites, a concept introduced by Schofield (1978). Success in establishing semiallogeneic AF chimeras and the inability to facilitate engraftment in the fully allogeneic model reflects the necessity for compatibility between donor cells and the host microenvironment. Possibly, this reflects inadequate

immunosuppression by mAb of the fully allogeneic recipient particularly in view of the demonstration by Dexter (1977c, 1978) that long-term bone marrow cultures do not require histocompatible adherent cell layers.

It is of interest to note that donor cells and antihost mAb do not need to be injected simultaneously to produce AF chimeras. We found that cells could be administered either 2 or 5 days prior to the mAb (day 0) or the mAb may be given 5 or 7 days prior to the cells. This suggests that the donor cells can be successfully maintained in the untreated recipient prior to the administration of the mAb at day 0. Likewise, the effects of the mAb on the host remain stable over a short period of time in that a subsequent injection of stem cells 5 or 7 days post mAb treatment still permits donor takeover of the recipient to occur. Bone marrow or spleen stem cell colonies grown in methyl cellulose cultures were examined to determine if any host hemopoietic precursor cells were still present in fully (>95%) or partially (<95%) repopulated AF chimeras. It was felt that this *in vitro* assay would allow the detection of host precursor cells, even if an *in vivo* regulatory control prevented their differentiation. The sensitivity of the Gpi assay is such that colonies as small as a few hundred cells can be quickly and accurately phenotyped. We found that close correlations exist between the degree of donor colonies derived from either spleen or bone marrow and the level of donor cells in the peripheral blood (Francescutti *et al.*, in press, 1985).

These findings are of particular interest in view of the fact that residual host cell population among differentiated cells of the spleen, thymus and lymph nodes are of substantial size in AF chimeras that have complete donor engraftment of peripheral blood, bone marrow and spleen stem cell compartments (McCarthy *et al.*, 1985). This demonstrates that although the hemopoietic stem cell compartment of the spleen is comparable to that of the bone marrow and is reflected in the red and white cells of the peripheral blood, an independent population of long-lived host cells remains in secondary lymphoid organs. McCarthy speculates that this could possibly be due to the differential expression of target antigens on leukocytes recognized by the mAb's; also, residual cells may be long-lived memory cells that have somehow escaped the effects of the mAb treatment. Alternatively, the microanatomical location within the organs in question may protect the host cells from antibody. These possibilities are the subject of current investigations in Wegmann's laboratory.

Conflicting reports exist as to whether the marrow stroma is a transplantable element. Keating *et al.* (1982) have shown that the hemopoietic microenvironment following marrow transplantation in man is of donor origin. In contrast Bentley (1982) could not find chromosomally-marked donor cells in long-term cultured adherent cell layers derived from the bone marrow of repopulated irradiated mice. Dexter (1982b) notes that the two models differ in that

cyclophosphamide was used in addition to the irradiation treatment in the human study. He postulates that cyclophosphamide possibly creates "space" in the hemopoietic tissue, thereby allowing donor stromal cell precursors an opportunity to establish themselves. The mAb treatment used in establishing the AF chimeras may be acting in a similar fashion. Examination of the stem-cell enriched non-adherent layer arising from long-term bone marrow cultures indicates that it is at least 95% donor derived. On the other hand, the microenvironment-associated adherent layer, which consists of macrophage-like, fibroblastoid, epithelioid and fat-containing cells, is only 72 - 77% donor origin. The host cells in this particular population may be an outgrowth of fibroblast cells from the original bone marrow plug used to innoculate the cultures, a finding similiar to that reported by Freidenstein *et al.* (1978) and Golde *et al.* (1980) in their respective studies. Gpi analysis of the AF chimera bone marrow plug prior to the long-term bone marrow culture indicates that the cells are >95% of donor origin. A minority (<5%) host cell population with the potential to repopulate as much as 25% of the adherent layer in long-term bone marow cultures therefore seems to persist in AF chimeras. The functional significance of this cell population remains to be determined. The findings with the AF chimeras are similar to those of Marshall *et al.*, (1984) who report that reciprocal bone marrow transplantation between irradiated CBA/H(T6) Gpi congenics yields a similar

70 or so percent takeover of the adherent layer of long-term bone marrow cultures by donor cells.

It has been shown that peritoneal exudate macrophages originate from bone marrow stem cells (Thompson, 1970). The extent of donor takeover in peritoneal exudate macrophages was examined and found to be similar to the level of takeover in both the peripheral blood and marrow stem cell compartments, with a small amount of residual host cell contamination.

Hemopoietic analysis therefore shows that the amount of donor engraftment seen in the peripheral blood and peritoneal macrophages of the AF chimeras is a true reflection of the degree of hemopoietic stem cell reconstitution. These findings also indicate that once hemopoietic takeover by donor cells has occured in AF chimeras, it is permanent and that the level of chimerism in the peripheral blood is an accurate indication of the hemopoietic stem cell mixture in both the bone marrow and the spleen.

The immunological competence of our AF chimeras has been tested throughly by Wegmann's group (McCarthy *et al.*, submitted). Immunocompetence of T and B lymphocytes was examined in both proliferative and effector cell assays. The AF chimeras showed normal responses to a variety of mitogenic and antigenic challenges, and were capable of mounting normal cell-mediated immune responses. In addition, splenic lymphocytes (P_1 cells in a (P_1 x P_2)F_1 host) were

found to be hyporesponsive or nonresponsive to host antigens of parental donor haplotype in *in vitro* assays of cell-mediated immunity. As in the earlier parabiosis model, no direct *in vitro* evidence for active suppression of the anti-host cytotoxic response was found. Further, no detectable anti-P_2 antibodies could be found in the serum of the AF chimeras, as tested by both a CFU-S assay and a competitive radioimmunoassay.

AF chimeras are healthy mice that can survive for more than 24 months post-transplantation. Both male and female AF chimeras demonstrate normal reproductive functions (Gambel *et al.*, 1985). Long-term AF chimeras, when compared to normal controls, do not show any increased incidence of malignancies. When BM is used as a source of donor cells, there is no clinical evidence of any tissue injury characteristic of acute or chronic GVHD. When spleen and BM cells are used in the donor inoculum, the histopathologic picture of these long-term AF chimeras (> 1 year) indicates some minimal signs of chronic but nonlethal GVHD. In particular, there is no evidence for glomerulonephritis or advanced scleroderma. However, atrophy of lymphoid tissue and granulomatous panniculitis is quite evident. This destruction of lymphoid tissue leads to generalized immunosuppression. The thickening of the subcutis has been associated with a phase of repair in chronic GVHD, as described by Elkins (1971).

Two requirements for induction of the symptoms of chronic GVHD are the presence of immunocompetent T cells in the donor inoculum and the ability of these cells to react to an incompatible recipient (Rappaport, 1979). However, GVHD-inducing capacities of different mouse strains vary. The poor capability of DBA cells to induce lethal GVHD has been described for parabionts and radiation chimeras (Drell *et al.*, 1979b; Van Elven *et al.*, 1981). BALB mice, however, differ from their DBA counterparts in their stronger ability to induce GVHD.

In summary, these findings demonstrate an important new concept in the production of hemopoietic chimeras: immunosuppressive agents and irradiation are not always necessary for a successful hemopoietic engraftment, at least in the particular strain combinations that we have tested to date. We hope these studies will play a role in the search for a new and better means of performing clinical bone marrow transplantation appropriate for certain hematologic disorders.

Future Directions

Bone marrow transplantation has the potential of becoming a cornerstone modality of therapy for a great variety of hemopoietic and genetically inherited disorders. One only has to imagine the spectrum of genetic disorders that hold promise of being amendable to genetic engineering. As mentioned in the introduction, the defects in the thalassemias, the hemoglobinopathies, the anemias, Lesch-Nyhan disease, purine nucleoside phosphorylase deficiency leading to a severe immunodeficiency disease, and adenosine deaminase deficiency which causes a severe combined immunodeficiency disease (Sondel *et al.*, 1983; Anderson, 1984), can all theoretically be repaired via sophisticated gene therapy (Good *et al.*, 1984). There are currently four broad groups of techniques for transferring cloned genes into cells. First, there is the use of viruses as agents of gene delivery. These can be either DNA or RNA viruses (retroviruses) (Joyner *et al.*, 1983; Risser *et al.*, 1983). Secondly, Wigler *et al.*, (1977) describe a modified version of calcium phosphate-mediated DNA uptake originally reported by Graham and van der Eb (1973). A third means is by the fusion of DNA-loaded membranous vesicles to cells (Anderson, 1984). Finally, physical means, such as microinjection (Anderson *et al.*, 1980a) and electroporation, or the transfer of DNA directly across the cell membrane using an electric current (Neumann *et al.*, 1982), can be exploited. Experiments in various animal models strongly

suggest that gene insertion can sucessfully be done (Hammer *et al.*, 1984; Mercola *et al.*, 1980a, 1980b; Skow *et al.*, 1983; Cline *et al.*, 1980; Miller *et al.*, 1984; Williams *et al.*, 1984).

Proper host preparation will consist primarily of the removal of the defective cell populations in a selective fashion, ensuring that the supportive structural stromal environment is not destroyed and, finally, successfully reintroducing the corrected stem cell population back into the recipient. A common factor in these requirements is the specificity of cell destruction and replacement. Specificity is stressed, since present modalities of host preparation and selective cell removal are nonspecific, including the use of total body irradiation and various cytotoxic drugs, such as alkylating agents. These agents have been implicated in a variety of unwanted side effects, including the increased incidence of subsequent malignancy and the possibility of permanent damage to hemopoietic tissue.

The specificity inherent in our AF chimera model is a major step towards eliminating these undesirable side effects. One can envisage extensions of our protocol, for instance, utilizing a wider spectrum or combination of mAbs with varying specificities directed at unique target antigens. These targets may involve abnormal hemopoietic cells, leukemic cells, or any cell population bearing appropriate markers. This method of selective removal would create "space" in a manner likely to encourage subsequent

engraftment by the corrected stem cells. As indicated by our time frame studies, it may be feasible to create a "window effect", thus allowing us a certain time frame in which we could control the hemopoietic reconstitution of the hosts.

Anderson (1980b; 1984) thoroughly reviews the prospects for human gene therapy in a recent article. He states quite emphatically and logically that "...it should be shown in animal studies that (i) the new gene can be put into the correct target cells and will remain there long enough to be effective; (ii) the new gene will be expressed in the cell at appropriate levels and (iii) the new gene will not harm the cell or, by extension, the animal."

Syngeneic studies with these goals in mind are the subject of current investigations by Wegmann's group.

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