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THE UNIVERSITY OF ALBERTA

REGULATION OF HEMATOPOIESIS AND AUTOIMMUNITY BY BONE MARROW NATURAL SUPPRESSION

by

MICHEL W. J. SADELAIN

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of

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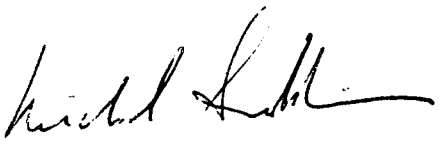

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BY BONE MARROW NATURAL SUPPRESSION

submitted by Michel W. J. Sadelain
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy
in Medical Sciences (Immunology)

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Supervisor

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Date:

DEDICATION

To Anne Sadelain, for her trust and her support, for honestly sharing her doubts and her enthusiasm, and for teaching me the respect of mankind.

A Jean Sadelain, qui m'a montre la vertu de la tenacite et de la persistance, et les extravagances de l'entetement.

To Christophe and Carole Sadelain, and to Alice and Sonja Doskoch, for all their help throughout these five years spent in Edmonton, which they made so enjoyable and so unique.

A mon Cher Maitre, Gabriel Richet, qui m'a suggere un matin, assis au Bureau de l'Anaphylaxie, d'etudier un peu l'Immunologie. Il est et sera mon modele d'integrite professionelle et intellectuelle.

A Anne-Marie Gervais, pour tous ses encouragements.

ABSTRACT

Natural suppressor activity (NSA) is defined by the non-specific, non-MHC-restricted suppression of *in vitro* immunological assays, and is mediated by cells found at sites of hemopoiesis. The physiological role of NSA is not known. The experiments described in this thesis implicate NSA in resistance to hemopoietic engraftment and suggest a role in the control of autoimmunity in the non-obese diabetic (NOD) mouse.

Using anti-host antibody instead of irradiation as a conditioning regimen, I found that parental marrow cells administered to an untreated F1 hybrid host, which apparently failed to engraft, in fact seeded the host marrow where they remained quiescent. The relative resistance to engraftment in adult recipients pretreated with complete Freund's adjuvant (CFA) and in neonatal recipients suggested NSA as a possible inhibitory mechanism because host NSA is increased in both cases. Conversely, we found that administration of facilitating antibody reduced host NSA. These correletions were extended to engraftment kinetics, which we followed using a sensitive isozyme assay permitting us to track individual donor progenitor cells within the host marrow cavity. NSA in the adult marrow tissue was mediated by Thy1-negative, CD4- and CD8-negative, large, non-adherent, radiosensitive cells. The adoptive transfer of Thy1-negative cells displaying NSA decreased engraftment kinetics. These findings were generalized to other transplantation models, where we observed that only cytotoxic drugs and irradiation doses which decreased host NSA induced chimerism. Based on these observations, I have proposed that hemopoietic engraftment may be controlled by host marrow inhibitory functions rather than cytotoxic rejection mechanisms or the availability of "space". In this scheme, a quiescent reservoir of stem cells, whether donor or host, is downregulated by cycling hemopoietic cells which generate NSA.

In another set of experiments, we observed that a single administration of CFA to young NOD mice prevented the development of diabetes. Increased NSA was suggested to

play a role since diabetes is T cell-dependent and T cells are suppressed by NSA. Moreover, the low level of NSA in our NOD mice may account for the elevated incidence of diabetes in the colony. These observations suggest an important relationship between hemopoiesis and immunological tolerance.

PREFACE

J'ai toujours cherche a montrer que les troubles divers de l'organisme ne sauraient etre bien compris qu'a la condition d'avoir acquis des notions precises sur le mechanisme normal des fonctions physiologiques qui leur correspondent.(...)

Aussi loin que vont nos connaissances sur la fonction physiologique, aussi loin vont nos donnees scientifiques sur la nature et la production du phenomene pathologique: aussitot que les premieres se heurtent momentanement contre une inconnue, les secondes sont obligees, si nous ne savons nous resigner a une sage reserve, de se perdre dans le domaine des hypotheses et des theories preconcues. Chaque progres fait dans la physiologie de la fonction glycogenique est suivi d'un progres parallele dans la connaissance scientifique du diabete. (...)

Nous devons ici, je n'hesite pas a le dire, considerer la physiologie comme la base de la medecine toute entiere, car nous etudions a la fois, sous le nom de physiologie, et les phenomenes de l'organisme normal, et ceux que presente l'organisme sous l'influence de causes modificatrices, soit physiologiques, soit morbides, soit toxiques. La physiologie est donc pour nous l'etude de l'organisme dans ses etats normaux et anormaux, dans ses reactions viv-a-vis des causes excitantes normales, ou morbides, ou toxiques. Nous devons, et c'est ce que nous avons toujours fait jusqu'ici, etudier chaque question au triple point de vue physiologique, pathologique et therapeutique.

Claude Bernard, Lecons sur le diabete et la glycogenese animale, Paris, 1877.

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I wish to thank the many people who contributed in one way or another to the realization of the work presented in this thesis.

Dr. Thomas G. Wegmann, who warmly accepted me in his laboratory on very short notice, has given me great independence to explore the antibody-facilitation model and take my first steps in Science. Having partaken of his enthusiasm for Experimental Medicine, I hope I retain his intuitive and positive disposition. Dr. Bhagirath Singh provided me an example of rigor and dedication and was always available for discussion. I greatly thank him for associating me with his work on diabetes mellitus. Dr. Douglas R. Green was an irreplaceable committee member for his everlasting willingness to discuss all hypotheses and experiments (without restriction!). His creativity and ability to relate distant observations will not be forgotten. Dr. Erwin Diener has shown me a vision of Science which certainly will exert its influence. Dr. Alexander Rabinovitch introduced me to diabetology and was most supportive in advising experimental designs as well as career choices.

Dr. S. K. Singhal's interest in this work and his comments prior to the redaction of this thesis were very much appreciated.

Ann Burrell and Jana Lauzon were always ready to help with laboratory manipulations and figure drafting, and were key players in several stages of this work. Karen Hobbs and Kevin Atchison were occasionally involved and are thanked as well. The entire staff of the Department of Immunology and the graduate students and post-doctoral fellows were always cooperative and thereby contributed to making this Department a very friendly environment to work in.

Special thanks go to Michael Voralia, for innumerable late night conversations on the control of stem cell engraftment and the meaning of life, as well as his repeated attempts at deciphering my esoteric prose.

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CHAPTER I: a review of the *in vitro* natural suppressor phenomenon.

1. Specific vs. natural immune response mechanisms.

The immune response is the result of a complex interaction of different specialized cell populations. Functionally, these cellular subsets may be divided into three categories: antigen-presenting cells present antigen complexed to cell surface major histocompatibility complex (MHC)-encoded molecules; effector cells engulf particles, release effector molecules or kill target cells upon encountering antigen and subsequent activation; regulatory cells amplify or inhibit different events leading to the immune response. All these cell populations, except for a number of antigen-presenting cells, are derived from a common hemopoietic stem cell. From a point of view of specificity of recognition, the different cell types making up the immune system may be separated into two categories. One includes cells bearing clonotypic receptors, produced following a somatic DNA rearrangement event unique to each clonotype, thus conferring a unique specificity of molecular recognition (1,2). This category includes surface immunoglobulin-positive and CD3-positive lymphocytes. The other includes cells which do not bear clonotypic receptors and therefore are not limited to antigen-specific reactions, and which may be grouped, despite their great diversity, under the label of natural systems (3). Hence, the primary characteristic of natural systems is the lack of an antigen-specific receptor. Because antigen recognition is not mediated by a structure comparable to the T cell heterodimeric antigen receptor, the activation of natural systems is not MHC-restricted (4,5). More importantly, the clonal selection theory (6), which implies clonal expansion of the very low frequency antigen-responsive clone prior to generating a specific immune response, does not apply to natural systems. The prerequisite clonal expansion, where applicable, leads to a delay in the

response. The constitutive cells of natural systems, on the other hand, engage in their functions without requiring prior antigenic priming, although they may do so more efficiently once preactivated by cytokines or adjuvants. Cytokine-mediated activation may eventually depend on an antigen-specific response. Natural systems include polymorphonuclear cells, basophils, eosinophils, cells of the monocyte lineage, natural killer cells, and natural suppressor cells. For the two latter populations, as well as for the related lymphokine activated killer (LAK) cells and natural cytotoxic (NC) cells, the existence of a receptor for antigen and the exact lineage remain unknown. Transient antigen-specificity, or preference, may be imparted to most of these cells by secreted B cell receptors, via binding of immunoglobulin to cell surface Fc receptors on the effector cell (7).

Specific and natural immunity, however, do not coexist independently. The immune response in vivo is the result of very complex and intricate cascades of cellular interactions. Antigen-specific and non-specific cells can act on each other at different levels. For example, the T cell-derived lymphokines interleukin-2 and interleukin-3 affect NK cell activation and production (8,9). Reciprocally, NK cells may affect lymphocyte function (10). This crosstalk is also operant at the earliest stages of ontogeny of these various cell types, and affects their production in the primary lymphoid organs as well as hemopoiesis in general. For example, both T lymphocytes and NK cells have been shown to enhance or downregulate hemopoietic stem cells and lineage-specific progenitor cells (11-13). This type of regulatory intervention could, hypothetically, allow for central hemopoietic production to adapt to the current immune status via feedback from peripheral cells on primary stem cells and their microenvironment. Thus, some cells of natural systems, NK cells for example, can affect the specific immune response as well as hemopoiesis, including erythropoiesis, myelopoiesis and maybe lymphopoiesis. Mechanistically, these

regulatory effects fall into one of two categories: help or amplification on the one hand, and inhibition on the other. The latter may result from downregulation mediated by cytotoxicity (an irreversible process) or by suppression (a reversible process).

Natural immunity was originally referred to as the occurrence of immunity in the absence of overt stimulation, and was thought to encompass the same principles and mechanisms as acquired immunity. It was not until the 1970's that natural resistance phenomena were investigated at the cellular level and were found to differ from adoptive immunity with respect to ontogeny, kinetics, recognition mechanisms, and regulatory influences (3). Natural resistance phenomena were uncovered *in vivo* and studied in the context of hemopoietic stem cell engraftment, tumor-host relationships, and infection immunity. Natural resistance systems or natural defence mechanisms are distinguished from acquired immunity as non-induced, non-thymus-dependent and non-MHC-restricted immune responses, although endowed with some degree of specificity. The study of natural resistance mechanisms soon revealed that their role extend beyond resistance to grafts, tumors and infectious agents to essential physiologic roles in homeostasis by regulating cell renewal and differentiation. This lead to a broadened definition applying to hemopoietic transplantation by G. Cudkowicz in 1976 (14): "Natural resistance is a cytostatic effect by the host environment on transplanted cells after the latter have settled into sites of hemopoiesis. In strongly resistant hosts such cells fail to proliferate and differentiate; in weakly resistant hosts the proliferation is deficient." A form of natural cytotoxicity part-taking in the down-regulation of donor hemopoietic cells was suggested by their apparent elimination: "For a short period after transplantation, two or three days, viable transplanted cells can be recovered from spleens of susceptible and strongly resistant recipients. Later on, the transplanted cells disappear from the hemopoietic sites of the resistant mice."(14).

In clinical transplantation, the term "marrow graft resistance" is used to describe failure of engraftment or marrow graft rejection unrelated to previous allo-immunization (15). In murine models, graft resistance has usually been assessed by measuring cellular proliferation in the spleen or lymph nodes, early after transplantation, in two situations: "hybrid resistance" is observed in irradiated F_1 hybrid recipients given small parental marrow inocula (reviewed in 16); "allogeneic resistance" refers to very early natural cytotoxicity directed against allogeneic lympho-hemopoietic cells (reviewed in 17). In both situations, the mechanism of marrow graft resistance is distinct from conventional allograft responses. Hybrid resistance is mediated by bone marrow-derived (18), thymus-independent (19), radioresistant (19) cells. Destruction of grafted hemopoietic cells occurs rapidly without prior sensitization or priming by donor antigens. Several lines of evidence have directly implicated NK cells in marrow graft resistance. *In vitro* , target cells susceptible to NK activity include, amongst tumor cells, leukemias and lymphomas, i.e. neoplasms of hemopoietic origin, as well as some sarcomas and carcinomas, and, amongst normal cells, bone marrow cells, macrophages after adherence for 18 hours, and thymus cells (20). *In vivo* administration of cloned NK cells restored allorejection in beige mice, which otherwise do not manifest marrow graft resistance and are NK-deficient (21). Treatment of recipients with an antiserum specific for NK cells ablated marrow graft resistance (22). A phenomenon distinct of hybrid resistance, early allogeneic marrow graft rejection refers to the early elimination of radiolabelled donor cells, within 24 hours of administration, thus before allo-immunization could develop. After intravenous injection into mice, the distribution of viable ^{51}Cr-labelled allogeneic and syngeneic lymphocytes in lymph nodes and spleen is broadly similar over the first 48 hours. But a careful analysis of the distribution of the radiolabelled cells reveals a constant and reproducible discrimination against the allogeneic cells after 18-24 hours in the lymph nodes and to a lesser degree in

spleen, an effect given the name of natural immunity (23). Involving the rapid killing of allogeneic lymphocytes by unsensitized hosts, it has features in common with other natural resitance systems, and has also been compared to the allograft rejection which most invertebrate animals are capable of (24). This natural cell-mediated response is indeed thymus-independent and radioresistant, but appears to be directed to the serologically determined K/D antigens (24). As for hybrid resistance again, other cell types than NK cells have been proposed to account for the target elimination, including macrophages (25) and bone marrow-derived effector cells termed M cells (26).

Natural immunity has been postulated early on as a function of the immune system engaged in the early elimination of neoplasias, referred to as immune surveillance (27). The original hypothesis, proposing thymus-dependent cells as the effectors, was eventually contradicted by experimental data, and has evolved to accomodate the wide variety of effector mechanisms proposed by different investigators (28). A large number of reports have implicated macrophages, based on their capacity to lyse tumor cell lines *in vitro* , the effect of their non-specific depletion *in vivo* on tumor incidence and metastatic spread, and adoptive transfer experiments. Comparably, NK cells are able to lyse tumor cells *in vitro* , including freshly harvested tumor cells, and may play a role *in vivo* , as suggested by the increased tumor incidence (primarily lymphomas) in NK-deficient mice (29) and in humans presenting with Chediak-Higashi syndrome (30) or drug-induced immunosuppression (31). In the NK-deficient mice, the adoptive transfer of a clone of cultured lymphoid cells with NK-like activity protected against development of pulmonary and liver matastases (21). As well, natural cytotoxic (NC) cells, which differ from NK cells with respect to some phenotypic markers and some functional characteristics, have been implicated, based on their lytic properties *in vitro* (32).

Lastly, natural resistance against infectious agents is suggested in a number of animal models. The argumentation is somewhat similar to that supporting a role in anti-neoplasic immune surveillance. As an example, it has been shown that natural killer cells may play a role in resistance against cytomegalovirus in mice, based on the *in vitro* susceptibility to NK lysis of virus-infected cells, and the increased *in vivo* susceptibility to infection of NK-deficient animals (33).

Thus, the existence of "natural immunity" is well established, as is its involvement in host resistance to hemopoietic engraftment, chiefly involving NK cells, macrophages and NC cells. But marrow graft resistance mediated by cytotoxic mechanisms did not appear to be the cause of graft failure we observed in unconditioned, normal adult recipients. The administration of a large marrow inoculum to unconditioned hosts in the BALB/c→(BALB/c x C3H/HeJ)F_1 hybrid donor-host combination did not result in any significant short or long term chimerism in bone marrow or peripheral blood. To evaluate whether donor precursor cells survive in the host and whether enough cells are left to completely reconstitute the host, one needs either to retrieve the donor cells, *ex vivo* , and examine their hemopoietic potential, or use a conditioning regimen for engraftment which spares donor stem cell within the host. This is not permitted by irradiation or cytotoxic drugs, but can be achieved in a $P_1 \rightarrow P_1xP_2$ transplantation where conditioning selectively targets the P_1xP_2 cells. This is possible by using anti-H-2 antibody directed against P_2 determinants, based on a method developed in Dr. T.G. Wegmann's laboratory.

This method, whereby host conditioning consists of a single injection of anti-host H-2K monoclonal antibody, was the adaptation to a transplant situation of earlier findings in parabiotic mice. In the latter studies, it was observed that a constant fraction of parabiosed adult parental and F_1 hybrid mice do not die of parabiosis intoxication (graft-vs-host disease). The surviving pairs were completely of parental origin in peripheral blood,

indicating that a spontaneous bone marrow transplantation had occurred between the parabiosed animals. Circulating anti-H-2 antibodies were detected in their serum, suggesting antibody as a possible means to create conditions for parental engraftment in the F_1 hybrid. This was succesfully adapted to a transplantation model where anti-host H-2K antibody and donor marrow are co-injected into an otherwise untreated recipient (34-36). As described in chapter II, we found that delayed administration of antibody, up tp 30 days after donor marrow infusion, was still able to induce complete parental engraftment. The resistance to engraftment could therefore not be explained by the complete elimination of donor cells by natural cytotoxic mechanisms. These results suggested that marrow engraftment was inhibited and that donor hemopoietic cells were suppressed rather than rejected. Other observations described in chapter III suggested that natural immunity may still account for the cytostatic effect responsible for apparent graft failure, but mediated by natural suppressor activity rather than natural cytotoxicity.

2. Natural suppressor activity in bone marrow.

Natural suppressor (NS) activity was described in the late 1970's, in the context of analyzing immunoregulatory activities in the bone marrow. Particular attention was given to the bone marrow because of a number of unique features: it is the primary hemopoietic organ in adult life, supports B cell lymphopoiesis and generates the CD4-negative, CD8-negative T cell precursors which migrate to the thymus. Moreover, it contains a small but significant number of mature T and B lymphocytes. Therefore, the presence of numerous immunoregulatory functions in the bone marrow is not unexpected.

The phenomenon of natural suppressor (NS) activity was discovered by addition of bone marrow cells to *in vitro* immunological assays and observing what effect these cells

had on the response of stimulated syngeneic splenocytes. The original description of NS activity in each assay, namely the plaque forming cell (PFC) assay, the mixed leukocyte reaction (MLR) and mixed leukocyte culture (MLC)-generated cytotoxic T lymphocyte (CTL) assay will be reviewed first.

In 1979, A.K. Duwe and S.K. Singhal described immunoregulatory cells present in bone marrow that reduce the generation of the antibody response in the PFC assay *in vitro* (37). In this assay, spleen cells are cultured for five days in the presence of sheep erythrocytes, after what plaques formed by antibody-secreting cells in the presence of fresh erythrocytes and complement are enumerated. The addition of bone marrow cells resulted in significantly decreased responses to the T-dependent antigen as well as to the T-independent antigens DNP-polymerized flagellin and lipopolysaccharide. Bone marrow cells were inhibitory only when added within 24 hours of initiating the primary sensitizing culture. This inhibition was dependent on the dose of bone marrow cells added and was not due to a shift in kinetics of the PFC response. As an example to illustrate the order of magnitude of the PFC inhibition, addition of $2x10^6$ bone marrow cells to $15x10^6$ syngeneic responding spleen cells decreased the response from 6100 PFC/culture to 1800 PFC/culture (37).

Characterization of the regulatory cells indicated that plaque inhibition was mediated by cells present in nude animals and resistant to antithymocyte serum and complement (38). Depletion of macrophages by carbonyl-iron uptake did not decrease the activity. Velocity sedimentation on a bovine serum albumin gradient indicated that the regulatory cells were medium to large cells. In these experiments, the regulatory cells formed rosettes with anti-sheep erythrocyte antibody-coated sheep erythrocytes. It was therefore concluded that the bone marrow immunoregulatory cells were "Fc receptor-positive null lymphocytes, possibly precursor cells, capable of inhibiting the response of mature lymphocytes".

Table 2. Chimerism level in individual secondary hosts.

conditioning[a]	bone marrow[b]	PBL[b]	RBC[b]	thymus[b]
9Gy	0	0	0	0
9Gy	0	0	0	0
9Gy	0	0	0	0
9Gy + mAb	80	74	70	38
9Gy + mAb	66	68	60	60
9Gy + mAb	72	62	55	35
9Gy + mAb	40	38	35	5

[a] Secondary $CC3F_1$ hosts were given 3-5 million bone marrow cells harvested from primary untreated $CC3F_1$ hosts which were transplanted 5 days earlier with 15 million T cell-depleted BALB/c marrow cells. Prior to transplantation, secondary hosts were exposed to 9Gy irradiation only or irradiated then given 500μg of anti-H-$2K^k$ monoclonal antibody (mAb) i.p. 7 days thereafter.

[b] Chimerism in each tissue is expressed as the percentage of parental representation 3 months after transplantation. Each line represents one mouse, randomly selected from each group (n=10).

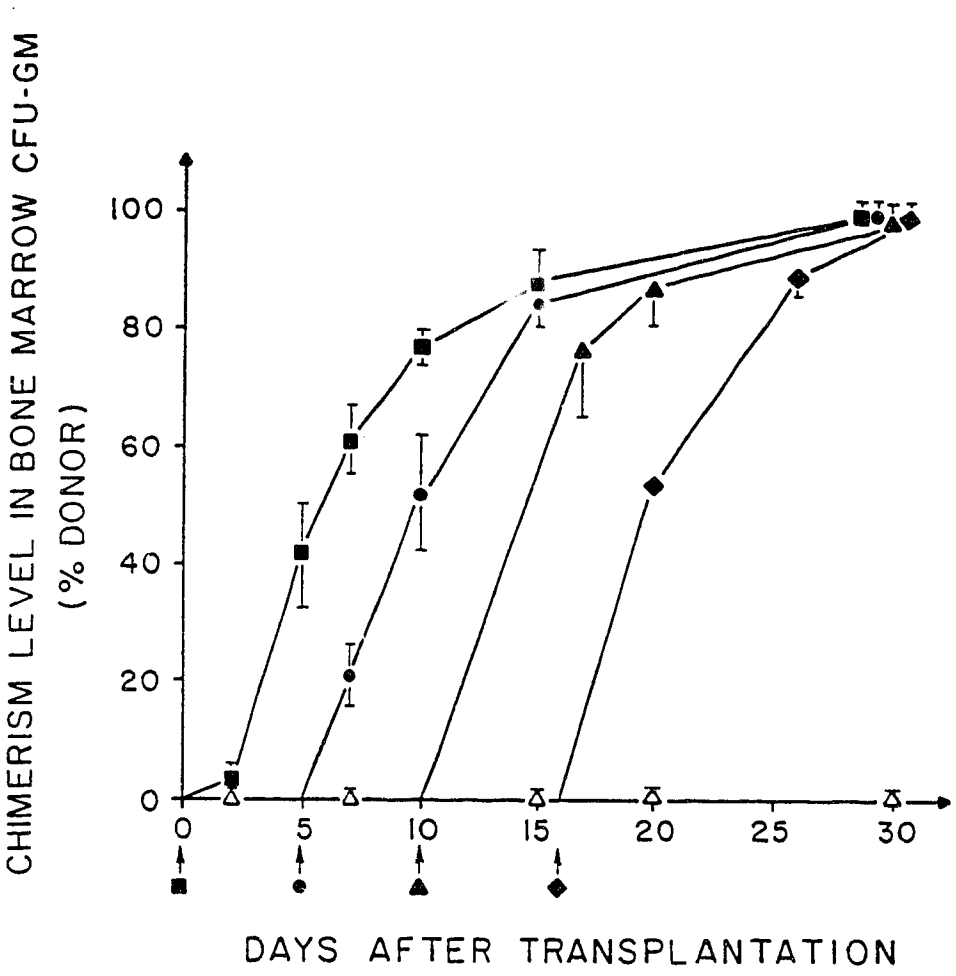

Figure 1. Medullary engraftment of donor-type CFU-GM is triggered upon delayed administration of anti-host monoclonal antibody. Adult $CC3F_1$ were transplanted with 15 million T cell-depleted BALB/c bone marrow cells on day 0. Host conditioning consisted of 500μg anti H-2K^k monoclonal antibody given either on day 0 (■), day 5 (●), day 10 (▲) or day 16 (◆), or no antibody at all (Δ). Bone marrow CFU-GM content at various time points after transplantation is plotted as the percentage of total CFU-GM that are of donor origin. Each point represents 3 to 5 mice, compiled from three different experiments. Data are expressed as mean ± s.d.

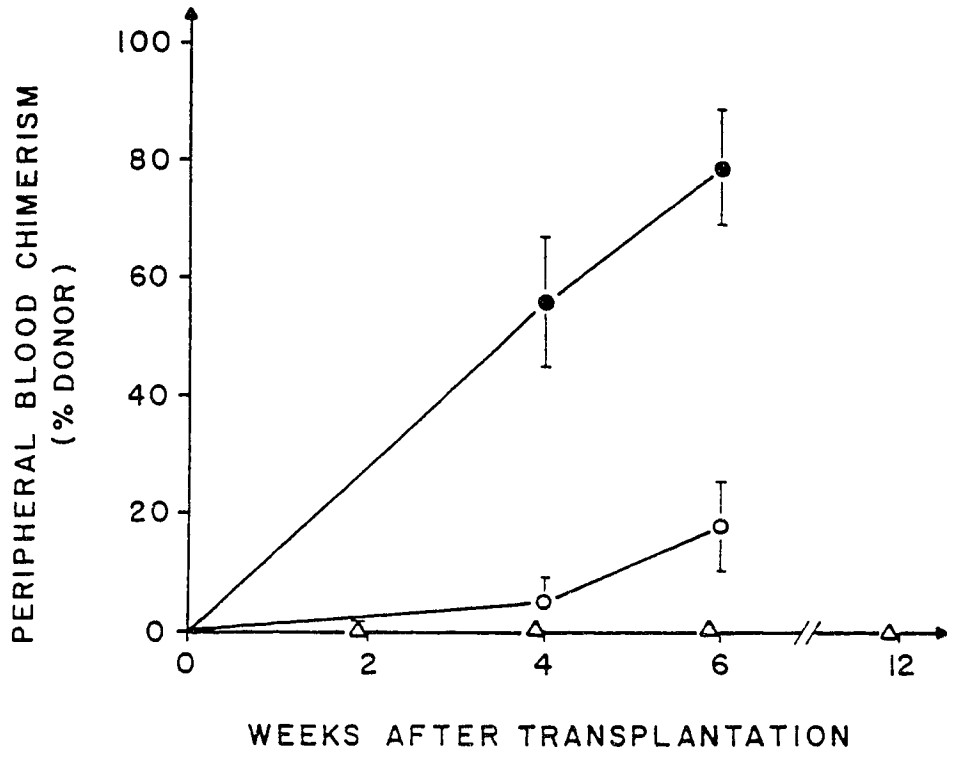

Figure 2. Peripheral blood chimerism in secondary recipients. Four hours after lethal irradiation (9Gy), $CC3F_1$ hybrids were given 3-5 million bone marrow cells from primary F_1 recipients given parental marrow 5 days earlier. One group was given only untreated bone marrow cells (Δ), another anti-H-$2K^k$ antibody-coated bone marrow cells (o), and, in the third group, recipients were administered 500μg of anti-H-$2K^k$ antibody 7 days after infusion of unmanipulated marrow. Peripheral blood chimerism is indicated at various time points after transplantation. Data are expressed as mean ± s.d. (10 mice per group).

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CHAPTER III: the role of natural suppressor and natural killer activities in resistance to hemopoietic engraftment in unirradiated recipient mice.

One of the key issues in bone marrow transplantation is overcoming host resistance to marrow engraftment. To date the most prevalent conditioning agent has been host irradiation, with or without the addition of cytotoxic drugs (1). While effective and convenient, conditioning regimens based on lethal irradiation are limited by the broad and non-specific damage they bring about, including potentiation of graft-versus-host disease (2). In order to develop conservative protocols that specifically target the effectors of host resistance to engraftment, consideration must be given to the different categories of host resistance, which can be divided into two groups. One is apparent only in allogeneic donor-host combinations and is mediated by well-defined effector mechanisms, including both humoral and cell-mediated immunity (3). The other, termed natural resistance (4), becomes apparent when one attempts to engraft host animals with genetically marked, but otherwise syngeneic bone marrow. Even though very large numbers of cells are infused, the untreated host remains resistant, allowing little or no long term engraftment (5,6). This resistance is largely radiosensitive, since lethally irradiated hosts are readily reconstituted by donor-derived cells in syngeneic and $P \rightarrow F_1$ donor-host combinations. In the latter situation, radioresistant rejection mechanisms, termed hybrid resistance, can be demonstrated when very small marrow doses are administered (7). It has been postulated that irradiation "creates space" in the bone marrow, a necessary condition for maximal engraftment (8). An alternative proposal is that a minor bone marrow cell population, perhaps distinct from stem cells, controls engraftment of incoming donor stem cells by regulatory signals. The results presented in this paper support a regulatory hypothesis, without excluding the space hypothesis.

Since irradiation non-specifically affects stem cells and many other radiosensitive functions, it is important to introduce a model which is more selective in its effects. Such is the antibody-facilitated bone marrow engraftment model applied to P→F_1 and syngeneic grafting. The basis of this model is that anti-host major histocompatibility complex (MHC) class I antibody abrogates resistance to bone marrow transplantation in both P→F_1 and syngeneic donor-host combinations (9-11). The results described herein indicate that conditions associated with an increase in host natural suppressor activity strongly correlate with increased resistance to marrow transplantation in this system. Conversely, conditions which decrease host natural suppressor activity lead to an increased level of engraftment. Natural killer activity, on the other hand, correlates poorly with resistance or susceptibility to marrow engraftment. These results suggest that stem cell engraftment is controlled by a regulatory cell population.

MATERIALS AND METHODS

Mice. Adult BALB/cCr, C3H/HeJ and (BALB/cCr x C3H/HeJ)F_1 mice were purchased from the University of Alberta animal breeding facility and C57BL/6J mice from Jackson Laboratories, Bar Harbor, ME. (BALB/cCr x C3H/HeJ)F_1 neonatal recipients, (BALB/cCr x C57BL/6J)F_1, (C3H/HeJ x C57BL/6J)F_1, (C3H/HeJ x C57BL/6J-*Gpi-1a*)F_1 hybrids were bred in our animal facility. C57BL/6J-*Gpi-1a* mice possess an allelic form of the glucose-phosphate isomerase (*Gpi*) isozyme gene substituted onto the C57BL/6J background and are syngeneic to C57BL/6J mice with respect to bone marrow transplantation, as previously described (11). Mice aged 8-12 weeks were used unless

otherwise stated. All mice were maintained under standard housing conditions.

Monoclonal antibodies. The murine hybridomas 16-3-1N (12) and 28-8-6S (13) were obtained from the American Type Culture Collection, Rockville, MD. These hybridomas produce IgG2a antibodies reactive to H-2K^k and H-2K^b molecules, respectively. Hybridoma B8-24-3 was a gift of Dr. G. Kohler and produces an anti-H-2K^b IgG1 (14). These monoclonal antibodies were produced as mouse ascites fluid and used in that form in these studies after filtration through 0.22 μm filters. The amount of specific antibody was determined for a reference batch of each antibody by high pressure liquid chromatography on hydroxylapatite columns (Bio-Rad, Missisauga, ON), as previously described (11). Different batches were compared for antibody content by competitive inhibition in a cellular radio-immunoassay, as previously described (10).

Bone marrow transplantation. After sacrificing donor mice by cervical dislocation, bone marrow was obtained by flushing balanced salt solution through excised tibias and femurs with a 26-gauge needle. Marrow cells were suspended by pipetting through a 18-gauge needle and washed once. Nucleated cells were counted in trypan blue and diluted appropriately. Viability was always greater than 95%. Twenty million cells were administered, either intraperitoneally (i.p.) in recipients 5 days old or younger, or intravenously (i.v.) via the lateral tail vein in others. In adult hosts, donors were age- and sex-matched. Recipient mice were administered a single injection of anti-host H-2K mAb-containing ascites, one week prior to bone marrow in syngeneic transplants or along with the bone marrow inoculum in P$\rightarrow$F$_1$ transplants, either i.p. or i.v. as specified for each

experiment. Injection of about 400 μg specific anti-H-2K^k antibody with 20 million BALB/cCr bone marrow results in complete engraftment in most adult (BALB/cCr x C3H/HeJ)F_1 recipients, as previously reported (10). Control mice that receive the equivalent amount of irrelevant antibody or the same volume of saline engraft minimally, depending on the inoculated cell number and the strain combination (10,11).Complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MC) was given i.p. at a constant dose of 100 μl and usually one week prior to bone marrow.

Measurement of engraftment. Long term engraftment (6-12 months post-transplant) was determined by a semi-quantitative isozyme assay, as previously described (15). Briefly, peripheral blood lysates were separated on starch gel electrophoresis, followed by transfer to nitrocellulose filters and staining; isozyme bands were measured by quantitative densitometry and compared to reference artificial mixes of blood of donor and host origin. The sensitivity of the assay depends on the isozyme combination and the level of chimerism : it is about 1% in fast band (B) : slow band (A) mixes and about 4% in (A) or (B) : (AB) mixes (A,B and AB bands are formed in heterozygotes because the enzyme is a dimer). *Gpi* isozyme distribution is as follows : BALB/cCr and C57BL/6J-*Gpi-1a* mice express *Gpi-1a* , and C3H/HeJ and C57BL/6J *Gpi-1b* . Hence, in figure 1, engraftment without antibody in the P→F_1 combination is not significantly different from zero. Peripheral blood chimerism in antibody-facilitated chimeras has been shown to closely correlate with bone marrow CFU-GM chimerism and to remain stable after 2 months post-transplant (15).

Plaque forming cell assay . Spleen cells were first washed twice and cultured in 24-well plates (Flow Laboratories, McLean, VA), each well containing 10 million nucleated cells in 1 ml medium. Twenty million sheep red blood cells (SRBC) were added to each well at the beginning of the culture. Medium contained 10% of a prescreened fetal calf serum (Flow Laboratories) in RPMI 1640, supplemented with 2 mM L-glutamine (Flow Laboratories), 1 mM sodium pyruvate (Gibco Laboratories, Chagrin Falls, OH) and $5x10^{-5}$M 2-mercaptoethanol (Sigma, Saint Louis, MO). Syngeneic spleen or bone marrow cells were added in different numbers to test their suppressive capacity, which is measured by the reduction of plaque forming cell generation. After 5 days in a humidified incubator at 37ºC in a 5% CO_2 atmosphere, the cells were resuspended in BSS and mixed with appropriately diluted SRBC and guinea pig complement (Gibco, Grand Island, NY). Plaques were read in Cunningham slides after 15-30 minutes at 37ºC. All cultures were done in triplicate. C57BL/6J adult females were generally used as the responders, except for the experiments described in figure 8, where (BALB/cCr x C57BL/6J)F_1 hybrids ($H\text{-}2^{dxb}$) were used.

Natural killer cell assay. This assay is slightly modified from the standard assay (16). Exponentially growing YAC-1 cells were labelled with ^{51}Cr to serve as targets. Spleen cells from adult C57BL/6J females were obtained by flushing warm BSS through punctured spleens. Single cell suspensions were then exposed to tris-ammonium chloride for 3 minutes at room temperature to lyse erythrocytes, followed by addition of half the volume of fetal calf serum to block further lysis. Spleen cells were washed twice, counted and resuspended in medium containing 5% fetal calf serum. Spleen cells and targets were incubated for 4 hours at 37'C in a 5% CO_2 atmosphere, mixed in V-bottom 96-well Linbro

plates in a final volume of 200 µl per well. Effector to target cell ratios were 100:1, 50:1, 25:1, and each data point was averaged from the values obtained from quadruplate wells. In some experiments, spleen cell suspensions were separated on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden). The NK-enriched fraction (density 1.055-1.065) was used at 50:1, 25:1 and 12.5:1 effector:target cell ratios. Lysis was measured by counting Y emission in 100 µl of supernatant from each well in a LKB gamma counter. Specific lysis was calculated using the formula (counts in test well - background release) x (maximum release - background release)$^{-1}$, where background release is the activity spontaneously released from 10^4 labelled target cells incubated in medium alone and maximum release is obtained in the presence of 1% Nonidet P-40 (Sigma, Saint Louis, MO).

RESULTS

Pretreatment with complete Freund's adjuvant of semiallogeneic and syngeneic hosts increases resistance to antibody-facilitated bone marrow engraftment.

Our previous work had indicated that both semiallogeneic and syngeneic bone marrow engraftment can be dramatically enhanced by conditioning the recipient with anti-host MHC class I antibody. In syngeneic combinations the antibody is given a week prior to engraftment to minimize anti-donor stem cell reactivity (11). Engraftment is generally measured by sampling chimerism in the peripheral blood three months or more after the time of transplantation, by which point chimerism is stable. Host animals given 100 µl of complete Freund's adjuvant (CFA) intraperitoneally (i.p.) a week before transplantation display a significant reduction in engraftment in both semi-allogeneic ($P \rightarrow F_1$) and syngeneic ($F_1 \rightarrow F_1$) donor-host combinations (figure 1). In order to ascertain that whole

blood chimerism (mainly erythroid) reflects stem cell engraftment, peripheral white blood cells from five animals in these experiments were separated on a Percoll density gradient and analyzed separately. The level of chimerism was comparable in the white cells, within a 3 % margin (data not shown), indicating that reduction of engraftment occurred at the level of a common erythroid-myeloid precursor. In order to investigate the kinetics of this effect of CFA in increasing resistance to engraftment, the adjuvant was injected at various times relative to antibody and bone marrow infusion in the P→F1 model. As shown in figure 2, maximum resistance to engraftment is obtained when CFA is administered from 7 days before to 4 days after bone marrow transplantation. It should be noted that CFA can be given after grafting but this has to be done prior to day +8 to have an effect. This result is consistent with medullary engraftment kinetics which indicate that engraftment is nearly complete eight days after marrow infusion (M.S and T.W., manuscript in preparation). These results suggest that CFA activates resident host populations, independent of those mediating allorecognition, which can mediate resistance to marrow engraftment. In order to better understand the nature of these cells we examined a number of host regulatory activities that could lead to such effects.

Natural suppressor activity but not natural killer activity is increased following adjuvant treatment.

Both natural killer (NK) and natural suppressor (NS) activities are associated with hemopoiesis, do not require allorecognition to trigger their activity, are not H-2-restricted and are not antigen-specific (7,17-21). We therefore decided to investigate whether these activities are altered in mice treated with adjuvant. NK activity is operationally defined by the killing of YAC-1 target cells in a ^{51}Cr release assay. NS activity is measured here by its ability to suppress the in vitro plaque forming cell response to sheep red blood cells (18).

One hundred ul of CFA was administered i.p. to adult C57BL/6J mice and spleen cells were assessed for both NS and NK activity at various times after adjuvant injection. Figure 3A shows that there is a significant increase in splenic NS activity following CFA administration, reaching a maximum between 5 and 15 days after injection. The degree of suppression was calculated by comparison to the number of plaques obtained by mixing an equal number of spleen cells from control mice that received 100 µl of saline i.p. instead of CFA. NK activity was measured at the same time points, both in whole spleen and in the low density, NK cell-enriched Percoll fraction (figure 3B). In both instances, CFA treatment decreased the amount of splenic NK activity, reaching a low point approximately 10 days after CFA administration. We also examined NS activity in the bone marrow. It is increased from day 2 to 10 following CFA administration (figure 4). This increase represents an approximate doubling of NS activity (cf figure 8). A slight increase in total bone marrow cellularity was induced by CFA administration, which reached a maximum at day 10 (figure 4). However, it was not statistically significant (117 ± 10% at day 10 compared to 100 ± 11% in untreated donors).These observations suggest a correlation between host NS activity and resistance to engraftment. Since NK activity is reduced rather than increased by CFA administration this would tend to rule out a role for NK cells in mediating marrow resistance in the model described here. On the other hand, loss of NK function could be responsible for resistance to engraftment. In order to further evaluate this correlation, a completely independent situation was studied in which the effect of these two populations on resistance to engraftment could be evaluated.

Neonatal F1 hybrid hosts are resistant to antibody-facilitated engraftment of parental bone marrow.

Elevated NS activity and absent NK activity in the spleen of neonatal mice are

well documented (22-26). We therefore explored whether these animals would be resistant to antibody-facilitated marrow engraftment. Newborn CC3F_1 hybrid mice were given 20 million BALB/c bone marrow cells i.p. along with 50 ul of monoclonal antibody specific for H-2K molecules of the host (150 μg of anti-K^k mAb per mouse or 125 μg per gram body weight). As shown in figure 5, there was no engraftment whether or not antibody was given to these animals. Antibody had a variable facilitating effect when administered on day 5 and an improved efficiency when administered on day 10 postnatally. Control animals at 8 weeks of age showed complete engraftment, despite receiving less antibody per gram body weight than younger recipients (107, 81, 40 μg mAb/g body weight given i.p. to 5, 10, 60 day old animals, respectively). Thus the newborn animal is resistant to antibody-facilitated bone marrow engraftment despite receiving a greater amount of antibody than necessary for complete engraftment in the adult, as also seen in the adjuvant-treated adult host. Nevertheless, it is possible to engraft neonatal hosts. This was achieved by including adult spleen cells in the donor inoculum (figure 6). We found that the addition of adult spleen cells syngeneic to the neonatal F_1 hybrid hosts resulted in increased long term donor engraftment. The chimerism levels achieved in two day old neonates given 25 million syngeneic CC3 F_1 adult spleen cells and facilitating antibody in addition to 15 million parental marrow cells was greatly enhanced in comparison to that seen in marrow recipients given either spleen cells or facilitating antibody. This indicates that the resistance of neonatal hosts to bone marrow engraftment is not due to faulty cell delivery or an inadequate environment for adult marrow cells.

Splenic natural suppressor activity declines postnatally along with resistance to antibody-facilitated stem cell engraftment.

Since neonatal animals are resistant to antibody-facilitated engraftment and this resistance wanes with increasing age, we decided to test whether this resistance correlated with host splenic natural suppressor activity. As expected, NS activity is maximal in the neonate and progessively declines in the days following birth (figure 7A). The reduction closely parallels the decline in resistance to antibody-facilitated engraftment over the same period. NK activity, on the other hand, is virtually undetectable in the first 10 days of life (figure 5B) and only appears later, as reported by others (27). Thus in the newborn, as in the CFA-treated adult, there is a close correlation between host NS activity and resistance to antibody-mediated engraftment. Resistance in the newborn occurs in the absence of NK activity and postnatal resistance decays while host NK activity remains constant, further suggesting that NK activity is not as relevant to resistance to engraftment as is NS activity under these conditions. Since NS activity exists in the normal adult, in which it is confined to the bone marrow cavity (18-21), and because graft resistance is increased in the presence of elevated host NS activity, we next asked whether administration of facilitating antibody would reduce host bone marrow NS activity.

Bone marrow natural suppressor activity is decreased following injection of facilitating antibody and increased by administration of complete Freund's adjuvant.

To test whether NS activity is affected by in vivo administration of facilitating antibody, the bone marrow of $CB6F_1$ hybrid mice was studied one week after i.v. injection of 900 μg of specific anti-host anti-H-$2K^b$ monoclonal antibody (either IgG1 or IgG2) or control monoclonal antibody of irrelevant specificity (anti-H-$2K^k$ IgG2), at which time

reduction of NS activity is maximal (manuscript in preparation). Irrelevant antibody did not modify NS activity as compared to untreated animals (data not shown). Treatment with specific anti-H-2K^b of the IgG2 isotype greatly reduced bone marrow NS activity, whereas injection of anti-H-2K^b IgG1 did not affect NS levels (figure 8A). The number of bone marrow cells necessary to reduce the control response by 50% can be estimated to be 10^5 cells in marrow from untreated, IgG1 anti-K^b or IgG2 anti-K^k-injected donors, and about 10exp6 cells in marrow from IgG2 anti-K^b-injected donors. These results are thus in agreement with those observed in transplantation studies : the same IgG2 antibody facilitates engraftment whereas the IgG1 does not (11). Conversely, CFA treatment a week prior to bone marrow harvest leads to an approximate doubling of bone marrow NS activity (about $6x10^4$ bone marrow cells required for 50% suppression) (figure 8A). We also analyzed the effect of anti-class I antibody treatment on splenic NK activity : a week after antibody injection, splenic activity is reduced to about 55% of the normal level (figure 8B).

These data thus establish that the antibody that promotes engraftment also reduces host bone marrow NS activity (this observation has been confirmed in another mouse strain using another facilitating antibody; data not shown) while control antibodies that fail to facilitate engraftment do not reduce this regulatory activity. Conversely, adjuvant treatment, which increases resistance to engraftment, augments bone marrow NS activity.

DISCUSSION

In this report we have examined the mechanisms of natural resistance to bone marrow transplantation in unirradiated syngeneic and semi-allogeneic hosts. In antibody-conditioned recipients, we find that resistance to engraftment varies with the immune status of the host at the time of transplantation. Two independent situations where such resistance

is greater than in the normal adult are described, the adjuvant-treated adult and the neonate. By comparing immune functions associated with control of hemopoiesis, and making use of a sensitive chimerism assay, we have established a strong correlation between host NS activity and resistance to engraftment. One situation associated with increased resistance to engraftment is the transplantation of normal adults pretreated with complete Freund's adjuvant. A significant reduction of chimerism in both the syngeneic and semi-allogeneic combinations was seen after CFA administration. Adjuvant treatment given before or after antibody administration was effective in reducing engraftment within a two week period. Thus, altered antibody clearance or biodistribution is unlikely to account for resistance, since CFA given up to 4 days after antibody can still reduce the eventual chimerism level. In addition, the antibody doses used were more than double the minimal requirement for complete engraftment. Our goal then was to investigate possible host regulatory activities which could account for reduced engraftment

Although NS activity has not previously been implicated in mediating resistance to bone marrow engraftment, we focused on that activity for the following reasons : (a) it is present in the bone marrow of normal adult hosts, and there only (18-21); (b) it does not require priming and is not H-2-restricted (19-20), and thus it can account for resistance to syngeneic targets; (c) it is activated in adults by diverse adjuvants (28-30), prompting us to examine NS activity after CFA treatment; (d) NS activity has been suggested to play a role in regulating the proliferation of hemopoietic tissue, as it is also found in the neonatal spleen (22-26), in the spleen after total lymphoid irradiation (31,32,20) and in chronic graft-versus-host disease (21), and after $^{89}Sr^{2+}$ (33) or cyclophosphamide treatment (34). We made the following observations : (a) natural suppressor activity is induced in the adult spleen and increased in the bone marrow after adjuvant treatment; (b) the kinetics of NS activity in the spleen reach a peak by day 10, and are similar to kinetics reported by others

following BCG treatment (30); (c) there is a parallel between resistance to engraftment and the evolution of NS activity in the host as a function of time. These findings are summarized in figure 9, and indicate a linear relationship between long term engraftment levels and host NS activity at the time of transplantation.

The second situation where we find a correlation between resistance to engraftment and levels of host NS activity is shown when applying antibody conditioning to neonatal hosts. The following observations were made : (a) the facilitation of engraftment achieved in the adult is not seen in the newborn host and resistance to engraftment progressively wanes in the post-natal period; (b) a spontaneous NS activity, absent in the untreated adult, is present in the neonatal spleen (22-26); (c) there is a progressive weakening of that activity throughout normal ontogeny, as previously reported by others (35,20); (d) there is a linear relationship between the decline in resistance to engraftment and the ontogenic decline in NS activity in the postnatal period, as shown in figure 9.

The findings presented here lead to the question of whether natural suppression in the host is causally related to hemopoietic stem cell regulation and resistance to stem cell engraftment in unirradiated recipients. The fact that natural suppressor cells are found only in the bone marrow in the normal adult is compatible with this hypothesis. One would then predict that other conditions allowing for marrow engraftment would reduce host NS activity. Such conditions are likely to be met in general, as lethal total body irradiation and certain cytotoxic drugs can preclude the expansion of that activity *in vivo* (32,30). Specific conditions associated with elevated NS activity nonetheless permit for donor engraftment (20,36). Under those circumstances, where recipients are conditioned by irradiation, satisfactory chimerism can be achieved in the presence of elevated splenic NS activity. This however is observed in allogeneic donor-host combinations, where suppressor activity would be expected to enhance engraftment by down-regulating rejection mechanisms,

whilst irradiation drastically alters the marrow cavity in favor of incoming donor stem cells Thus these data are not directly conflicting with ours.

We have previously suggested that the mechanism of action of the facilitating antibody cannot only be the direct reduction of the host stem cell pool and present further evidence for this elsewhere (11; and manuscript in preparation), as CFU-S and CFU-GM are only partially and temporarily reduced following antibody injection, and facilitated engraftment is observed even after stem cell levels have returned to normal levels. Increased cellularity in the CFA-treated adult and the neonatal hemopoietic compartments is not a likely explanation for resistance to engraftment under the current circumstances for the following reasons : in the CFA-treated adult, we found no significant increase in total marrow cellularity (which does not preclude changes in specific subpopulations); in the neonatal host, additional modifications of the conditioning regimen, which would not be expected to reduce cell numbers in the recipient, increased donor chimerism, thus establishing that neonatal hosts can be engrafted despite a possible inherent hypercellularity. While the mechanism by which such a treatment may be effective is unknown, it has been reported that addition of adult syngeneic macrophages to neonatal hosts results in a decrease in neonatal natural suppressor activity (37). This suggests a further, albeit indirect, correlation between host NS activity and resistance to engraftment. The hypothesis that engraftment of donor stem cells is subjected to host regulatory activities is therefore an attractive alternative explanation. The data presented here lead us to propose that cells exhibiting NS activity may be the cells responsible for regulating stem cell engraftment, since their function is decreased by facilitating antibody, while increased NS activity is associated with increased resistance to engraftment. The mode of action of NS cells in vivo is yet a matter of speculation. It has been suggested that their pronounced anti-proliferative activity could contribute to the protective effect that certain NS cells exert on GVHD (38). Their effect on

hemopoietic activity is presently unknown. There are several levels at which limitation of engraftment in our model could occur : inhibition of stem cell seeding and/or proliferation, placing any incoming donor stem cells at a relative disadvantage; protection of host stem cells from cytotoxic events leading to their elimination; and possible interference with other regulatory events induced by antibody treatment and necessary for engraftment. An effect on T cell-mediated GVH reactions is unlikely as CFA induces resistance to engraftment in recipients of syngeneic marrow and further because $P \rightarrow F_1$ transplantation is unaffected by T cell depletion of the graft (manuscript in preparation).

We also examined NK activity levels in the host and made the following observations : (a) NK activity is decreased following CFA injection, as is the case following *Corynebacterium parvum* injection (39) and (b) it is absent in the newborn, as reported by others (27). Hence in two independent situations, increased resistance to engraftment occurred in the context of lowered NK activity. In the neonatal mouse, increasing susceptibility to antibody-facilitated engraftment developed in the absence of any change in host NK activity, lending weight to the suggestion that host NK cells are not necessary for antibody-facilitation. This is further supported by the finding that bone marrow engraftment in (C57BL/6J-*bg* x C3H/HeJ-*bg2*) NK-deficient F_1 hybrids is not significantly less than in their normal (C57BL/6J x C3H/HeJ)F_1 counterparts, when identically antibody-conditioned (M.S., unpublished observation). The relevance to our transplantation model of hybrid resistance mechanisms (7) is therefore uncertain.

We are currently investigating how the reduction in bone marrow NS activity takes place and the relation of this activity to ongoing hemopoietic activity. Another objective is to define the phenotype of these cells (in adult bone marrow, neonatal spleen and CFA-treated adult spleen) and compare them to other known categories of cells with natural suppressor activity; preliminary data indicate they are Thy-1 negative, plastic non-adherent,

radiosensitive cells. Adoptive transfer experiments have confirmed that spleen cells from CFA-treated donors contain cells capable of adversely affecting antibody-facilitated engraftment (manuscript in preparation). Our ultimate goal is to identify the cell population(s) that mediate resistance to engraftment in unirradiated hosts and eventually specifically target them in vivo in order to reduce the toxicity of the conditioning regimen for bone marrow transplantation.

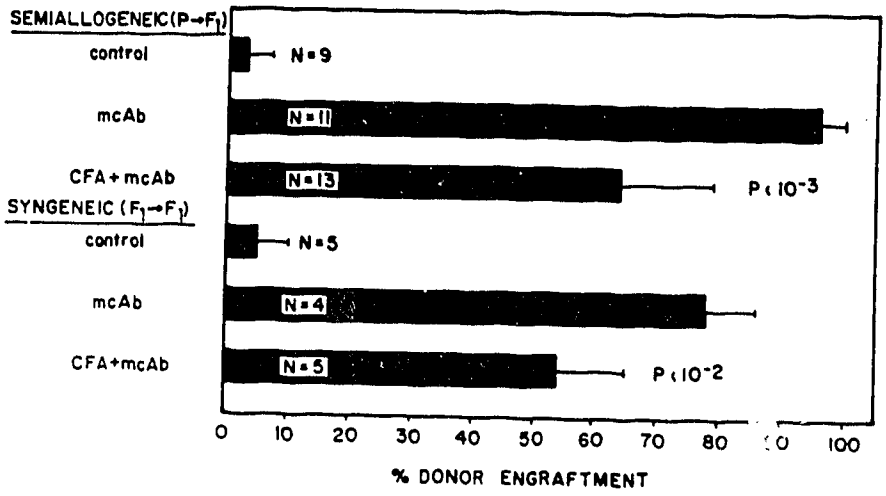

Figure 1. Host pretreatment with CFA reduces antibody-facilitated engraftment in semiallogeneic and syngeneic donor-host combinations. Unirradiated, adult (BALB/cCr x C3H/HeJ) and (C3H/HeJ x C57BL/6J) F1 hybrids receiving 20 million bone marrow cells from age-matched, sex-matched BALB/cCr and (C3H/HeJ x C57BL/6J-*Gpi1a*) F1 donors, respectively, are only marginally engrafted (3%±4 and 5%±5, respectively). Anti-host monoclonal antibody treatment with 900 and 3000 ug anti-Kk monoclonal antibody, respectively, dramatically enhances engraftment, which is significantly reduced in mice given 100 ul CFA one week prior to marrow infusion. Engraftment is represented by the percentage donor GPI isozyme in peripheral blood cells one year after transplantation. Error bars represent one standard deviation, N the number of mice per group (pooled from two experiments comprising all groups).

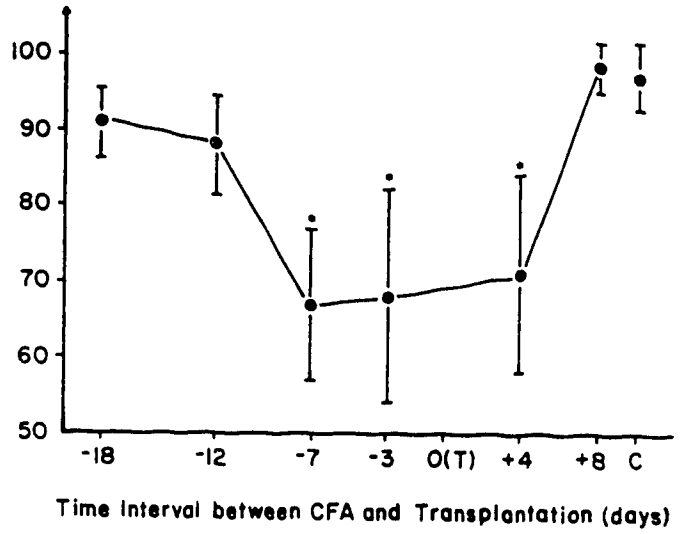

Figure 2. Effect of time interval between CFA injection and transplantation on long term engraftment. All hosts were adult (BALB/cCr x C3H/HeJ) F_1 hybrids and received 20 million BALB/cCr bone marrow cells and 900 ug anti-K^k monoclonal antibody on day 0, day of transplantation (T). One hundred ul of CFA were given intraperitoneally at different time points in 5 to 7 mice for each point. Donor peripheral blood chimerism is shown 6 months after transplantation, and compared to control engraftment achieved in mice given 100 ul saline intraperitoneally (C). Error bars represent one standard deviation, * indicates $p<0.02$.

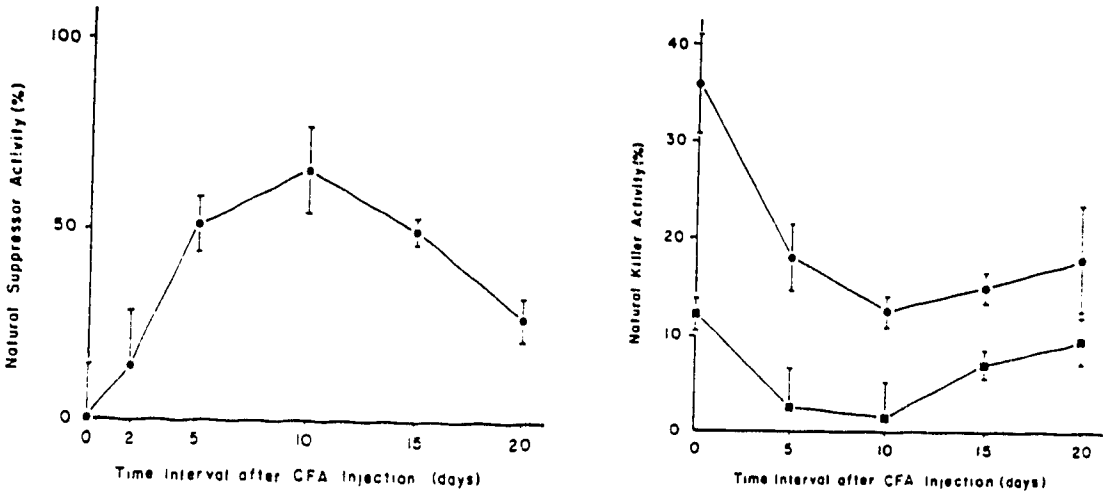

Figure 3. *In vivo* effect of CFA administration on NS and NK activities as a function of time. C57BL/6J adult females were tested for splenic NS (panel A) and NK (panel B) activities at various time intervals after intrapertoneal injection of 100 ul of CFA. Readout for NS activity is the PFC response obtained when mixing spleen cells from CFA-treated animals at 1:5, compared to control mixing with spleen cells from saline-treated animals. Results are expressed as the percentage of control where 100% represents 590 PFC per million cells. Readout for NK activity is the specific ^{51}Cr release of labelled YAC-1 cells mixed at 100:1 effector:target ratio for unseparated spleen cells (■), 50:1 for the NK-enriched low density spleen cells (●). Data are plotted as mean±s.d., representing at least three independent experiments for each data point.

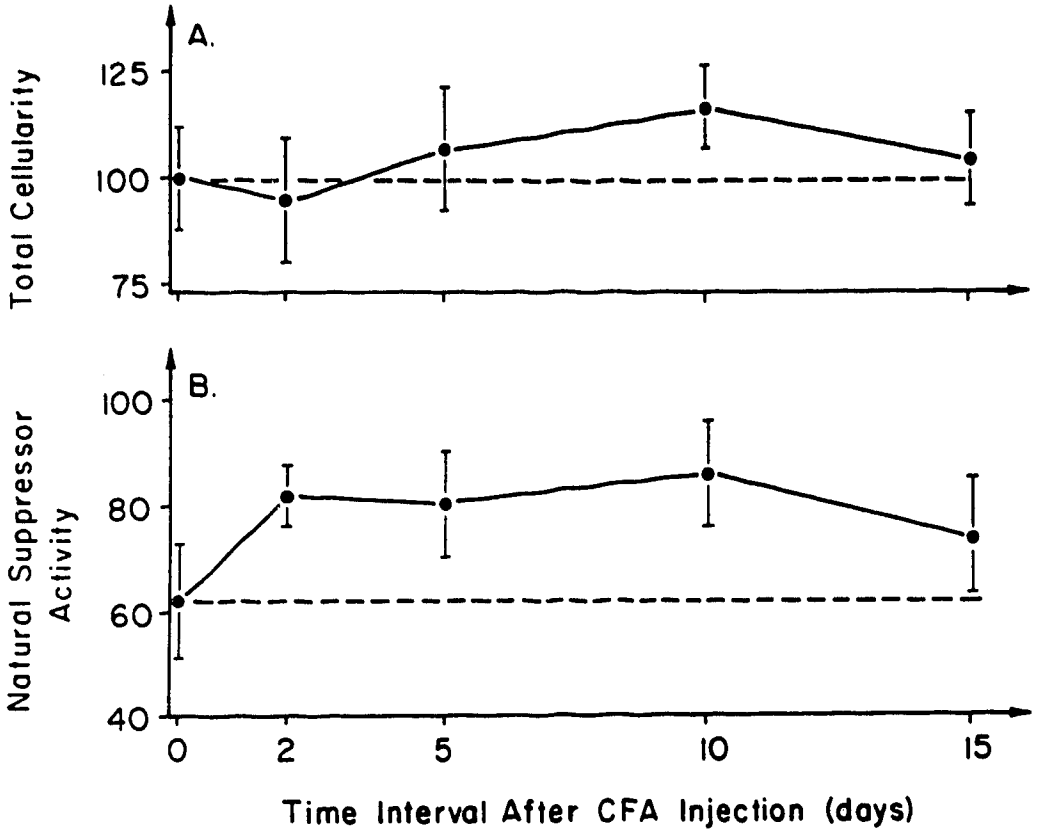

Figure 4. Effect of CFA administration on total marrow cellularity (A) and bone marrow NS activity (B). Adult females were sacrificed at various time points after i.p. administration of 100 ul CFA on day 0 (3 to 6 mice per time point). Total marrow cellularity is expressed relative to counts from untreated animals (100% represents 38 million nucleated cells per animal). Bone marrow NS activity is represented by the percentage suppression of control cultures (without bone marrow) at 1:62.5 bone marrow : spleen cell ratio. Results are expressed as mean ± standard deviation.

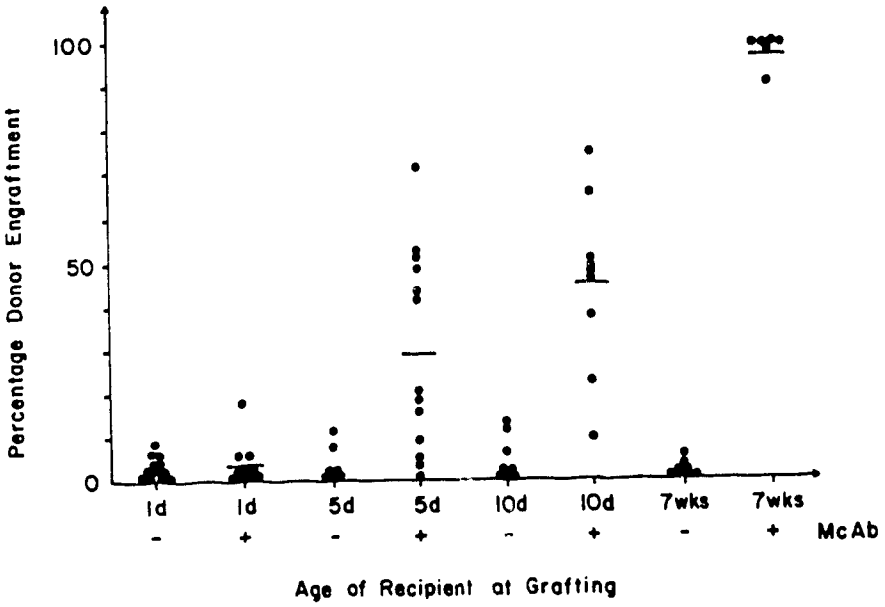

Figure 5. Antibody-facilitated engraftment is reduced in neonatal hosts. (BALB/cCr x C3H/HeJ) F_1 mice within 24 hours of birth, or on day 5 or 10, received 20 million BALB/cCr bone marrow cells as well as anti-H-2 K^k monoclonal antibody (McAb +) or saline (McAb -). The level of engraftment is represented by the percentage of donor cells in the peripheral blood one year after transplantation. Each data point represents the engrafted value for one mouse. Horizontal bars represent the mean engraftment in each group.

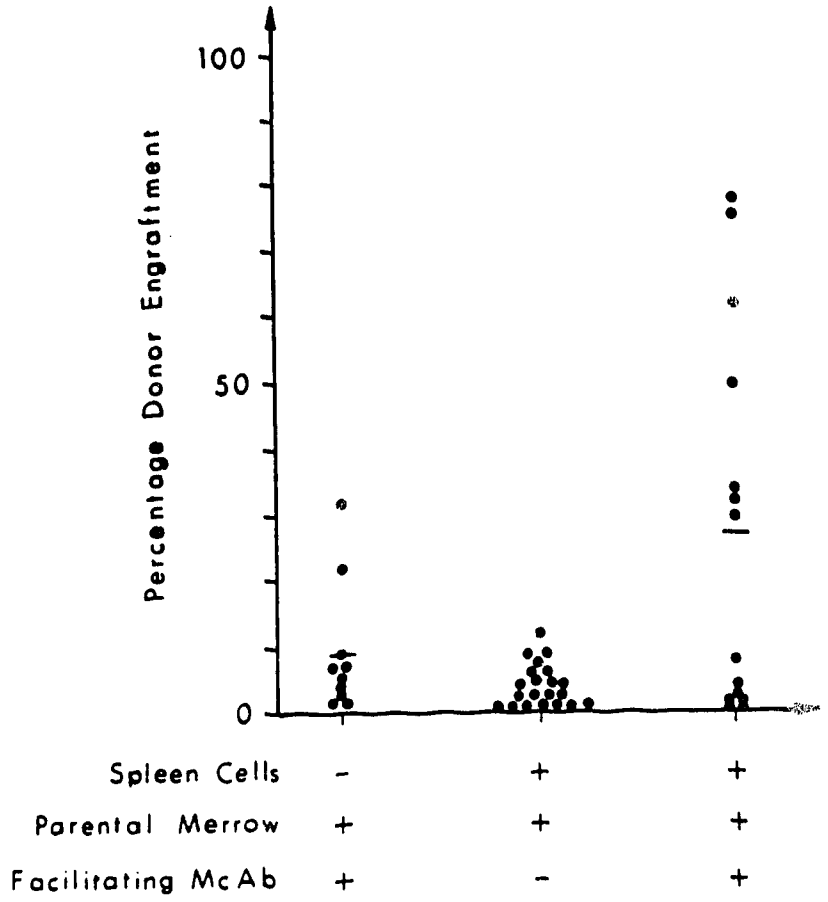

Figure 6. Antibody-facilitated engraftment in neonatal hosts is enhanced by administration of adult spleen cells. (BALB/cCr x C3H/HeJ)F1 mice were given 15 million BALB/cCr bone marrow cells on day 2 after birth. In addition, they were given either 25 million (BALB/cCr x C3H/HeJ)F1 spleen cells from 2 month old females, or 900 ug anti-H-2Kk monoclonal antibody on day 3, or both. Peripheral blood chimerism one year after transplantation is shown, each data point representing one recipient. Horizontal bars represent the mean engraftment level in each group.

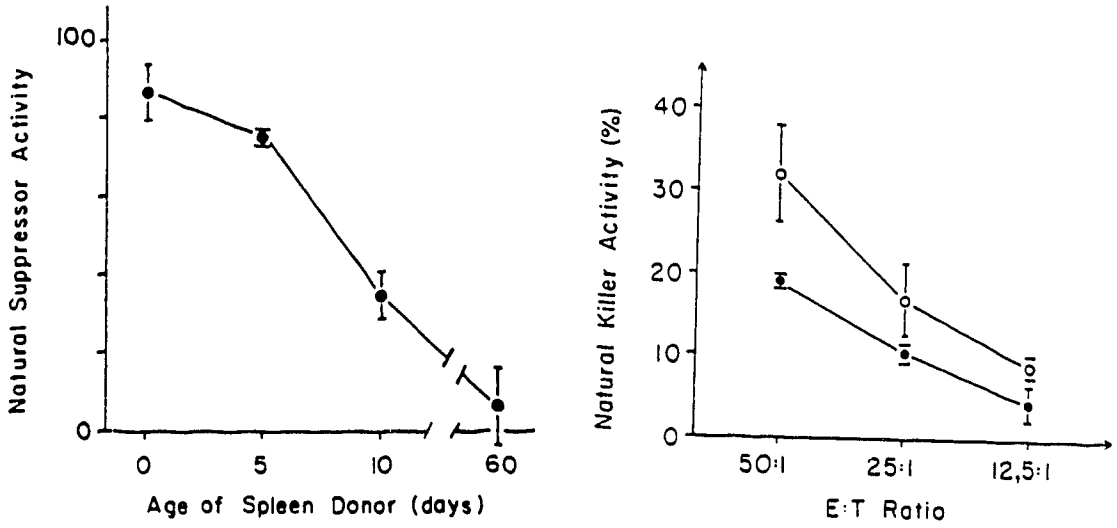

Figure 7. Progressive decline of NS activity in the spleen after birth (panel A) and absence of NK activity (panel B). Results are expressed as in figure 3. The data are plotted as the mean±s.d. of three independent experiments, each representing data from pooled spleens of one litter.

In 1980, K. Dorschkind, G.R. Klimpel and C. Rosse described "natural regulatory cells in bone marrow" using the *in vitro* primary MLR and CTL generation assay (39). In these assays, normal adult spleen cells were co-cultured with irradiated allogeneic spleen cells, then assayed for proliferation, measured by the cellular incorporation of tritiated thymidine, or for alloantigen-specific cytotoxicity, in a standard ^{51}Cr-release assay. Addition of bone marrow fractionated on a sucrose gradient resulted in marked inhibition of both alloantigen-induced proliferation and CTL generation by one fraction, in a dose-dependent manner. As an example to illustrate the order of magnitude of the suppression of the alloproliferative and allocytotoxic responses, addition of bone marrow to individual cultures in a 1:1 ratio of bone marrow cells to responding syngeneic spleen cells decreased the alloproliferation from 276593 ± 34625cpm (without added bone marrow) to 38270 ± 4257cpm (in the presence of bone marrow cells) for a background syngeneic MLR yielding 6488 ± 2049cpm, and decreased the % specific ^{51}Cr release from 31% to 2% for a background killing of 3% (39). Different cell populations were added under the same conditions to control for crowding of the culture wells. Addition of red blood cells, thymocytes or irradiated spleen cells syngeneic to the responder splenocytes resulted in far lesser inhibition (about 10% at the 1:5 bone marrow : responder spleen ratio and 45% at the 1:1 ratio when adding irradiated spleen cells, compared to 45% and 100% inhibition when adding bone marrow cells at the same ratios, respectively).

Bone marrow cells had to be added at the onset of culture to observe significant inhibition, as most of this effect disappeared if cells were added more than 24 hours later. Inhibition of immune function was not MHC-restricted in that it operated across major and minor histocompatibility barriers. This function was cortisone-resistant and not affected by doses of γ-radiation up to 10Gy. Phenotypic characterization showed that the inhibition was mediated by cells negative for surface immunoglobulin (sIg), Ia, Thy1, asialo-GM1

and Fc receptor (FcR) (40). The cells were larger than small lymphocytes, heterogeneous in density and adherence to plastic. The authors therefore concluded thet these "natural regulatory cells" are likely to be "immature marrow cells of undetermined lineage".

This bone marrow immunosuppressive activity was subsequently confirmed by other groups using either the PFC assay (41-43), the MLR (44,45), and, for one group, the in vitro inhibition of macrophage migration, which is thought to reflect delayed-type hypersensitivity *in vivo* (46). The phenotypes ascribed to these bone marrow immunoregulatory cells vary between different investigators (table 1). Importantly, it was established that the cell populations with inhibitory activity lacked NK activity as defined by the killing of the YAC-1 lymphoma target cells and NC activity as defined by the killing of the Meth A target (45). The absence of detectable lytic properties suggested that inhibition might be mediated by a suppressor mechanism. Hence, a consensus emerges with respect to the phenotypic definition of the NS effector cells. One cell type, referred to as NS cells, is Thy1-negative, CD4- and CD8-negative, sIg-negative, Ia-negative; it is a large cell which does not adhere to plastic and does not lyse YAC-1 target cells. The other appears to belong to the monocytic lineage and is referred to as a suppressor macrophage by some authors. These cells are also Thy1-negative and sIg-negative, but are Ia-positive, Mac1-positive, and adhere to plastic. Moreover, the inhibitory function of these cells is overcome by indomethacin.

3. Natural suppressor activity in neonatal spleen.

A neonatal natural suppressor cell has been studied by many investigators over the past 15 years. The existence and role of neonatal suppressor cells was addressed in an attempt to explain the relative immunological unresponsiveness of the newborn and the ease

of induction of immunological tolerance to foreign antigen shortly after birth (47,48). More specifically, investigators sought to distinguish between immunological immaturity and active suppression on the one hand, and to determine the role of suppression in inducing and maintaining tolerance on the other.

Utilizing inhibition of the *in vitro* PFC assay or MLR as a readout to monitor suppressor activity, several groups reported the existence of a splenic neonatal suppressor cell which progressively wanes in the weeks following birth (49-60). These neonatal suppressor cells are not antigen-specific and not H-2-restricted, and, as for bone marrow, different phenotypes have been ascribed to the effector cell (table 2). Two groups described cloned neonatal spleen cells which mediated non-specific inhibition of the MLR. Strober et al. reported an interleukin-2-dependent, Thy1-positive, CD4- and CD8-negative, sIg-negative, Ia-negative, Mac1- and F4/80-negative, asialoGM1-positive clone which does not kill YAC-1 targets (59). The description of cloned cells without detectable lytic properties suggested a mechanism of inhibition akin to suppression. Jadus et al described interleukin-3-dependent, Thy1-negative, CD4- and CD8-negative, Ia-negative, Mac1-negative, asialoGM1-weak, Ly5- and J11d-positive non-specific suppressor cells which killed WEHI-164 but not YAC-1 target cells (60).

Overall, the data in neonatal spleen are relatively more confusing than the reports on bone marrow-associated natural suppressor cells. This is perhaps due to the fact that the spleen, although a site of hemopoiesis in the post-natal period, is also a secondary lymphoid organ, thus containing additional lymphocytic cell types, some of which may contribute to the inhibitory effect measured *in vitro* . The overview in table 2 does suggest, however, the coexistence of at least two cell types: one defined as Thy1-negative, sIg-negative, Ia-negative, non-adherent and without NK activity, and the other belonging to the

monocyte lineage. Interestingly, a comparable activity was described in fetal liver, the site of hemopoiesis in late fetal stages.

4. The molecular basis of natural suppressor activity.

The lack of a precise definition of the physiological NS effector cells has not deterred certain investigators from addressing the molecular basis of suppression, which we will briefly review here. Four groups investigating the mechanism of natural suppression have reported that the activity could be, at least in part, mediated by molecules released by the effector cells. Two groups have attempted to characterize the molecule(s) prepared from fresh cells with NS activity, while the other two analyzed clones with NS activity. Singhal and colleagues have described a low molecular weight (10kD) moiety, termed bone marrow-derived suppressor factor (BDSF), which suppresses the PFC response *in vitro* and mitogen responses (61,62). Cleveland et al. investigated splenic NS activity during neonatal development and in graft-vs-host disease and irradiation recovery (63). When placed in culture, all three sources of spleen cells spontaneously produced interferon-ß. NS activity in the MLR was partially reverted by anti-interferon antibody. Bone marrow-associated NS activity was not examined. From an interleukin-2-dependent large granular lymphocyte(LGL)-like cloned cell from neonatal spleen, Strober's group described a 90 kD glycoprotein which mediated suppression in the primary allogeneic *in vitro* MLR (personal communication). That group also reported that the supernatant from that clone was never as inhibitory as the addition of the cloned cells directly into the culture wells during the assay, suggesting that the inhibitory effect may be due to more than just soluble mediators (59). Pope and collegues reported that their interleukin-3-dependent, LGL-like cell line M1-A5, derived from the spleen of a fibrosarcoma-bearing mouse,

secretes two protein factors referred to as suppressor cell-inducing factor (SIF) (64,65). SIF- had a M.W. of 43kDa and SIF-ß a M.W. of 6kDa. The SIF containing supernatant suppresses the PFC response *in vitro* , but not the mitogen-induced proliferative response. In addition, the M1-A5 cell line posseses killer activity, killing the NC-sensitive target WEHI-164 but not the NK-sensitive target YAC-1.

All these purifications are in progress, and the biochemical and functional characterization await further studies. It is nonetheless worth noting that four different groups have described at least four distinct molecules.

5. How many different "NS cells"?

The adult bone marrow, neonatal spleen, and fetal liver all have NS activity *in vitro* . While the most important question is the role of this activity *in vivo* , two more immediate questions about the effector cells come to mind. First, do the same cells mediate suppression in both the PFC assay and the proliferative assays. Second, what is the nature and lineage of the effector cells in each tissue in which they are found. Despite the various descriptions, three observations suggest that all these cells are related, if not the same. Firstly, the tissue distribution of NS activity is identical, irrelevant of the assay used. Secondly, the variable tissue distribution during ontogeny reflects the presence of active hemopoiesis. Thirdly, the cellular phenotypes reported by different groups share common, recurrent features: a consensus yields a sIg-negative, CD4- and CD8-negative, Ia-negative, plastic non-adherent cell without NK activity, distinct from an adherent macrophage-like cell, the suppressive activity of which is indomethacin-sensitive.

However, the picture may be more complex. In comparing data from different assays, the ratio of bone marrow cells or neonatal spleen cells to responding spleen cells is

very different in the PFC assay vs the MLR, the latter requiring about ten times more mixed cells to achieve comparable levels of suppression. One explanation for this differential requirement is that different cell types are operating in each assay. The phenotypic discrepancies should perhaps not be lighlty overlooked: the NS cells are Thy1-negative for some authors, -positive for others; asialoGM1-negative for some, -positive for others; Fc receptor-negative for some, -positive for others, radiosensitive (10Gy) for some, radioresistant for others. It is therefore not surprising that different investigators have drawn very divergent conclusions about the lineage of the effector cell: a T cell, a B cell, a NK-related cell, a NC-related cell, a monocytic precursor cell, an undefined early hemopoietic stem cell. The comparative evaluation is complicated by the fact that each group has usually focused on one tissue and one *in vitro* assay, thereby not permitting a true comparison.

In the end, these questions should be resolved by clonal analysis, by thorough examination of the genetic makeup of cloned cells derived from different NS-containing tissues and by comparison of their functional properties, and the molecular basis thereof, in every available *in vitro* and *in vivo* assay. The generation of NS clones is also very useful for *in vivo* studies, permitting adoptive transfer experiments. The capacity of such a clone to carry on some immunosuppressive function in an adoptive transfer system in addition to the perturbation of *in vitro* artificial responses does not, however, prove that such a clone is a physiological natural suppressor cell. So in the meantime, one should bear in mind certain reservations regarding clones with NS activity. Different groups arbitrarily included different growth factors in their cloning procedure. Surprisingly, each group came up with a clone with NS activity. And not surprisingly, each clone has unique and distinctive properties. Given our limited understanding of the physiological role of NS activity, it is difficult to appreciate which characteristics are artefactual and which are not. It is perhaps

not unexpected that a myriad of different cell types have been isolated from bone marrow, since it contains totipotent hemopoietic stem cells.

Progress in this field is therefore dependent on a better appreciation of the functional relevance of NS activity *in vivo* , which in turn requires first the development of animal models in which NS activity can be analyzed. Such is the goal of the work presented here, the objectives of which are listed in the next section. The understansing of NS activity *in vivo* will hopefully suggest more discriminating assays than non-specific suppression to monitor the activity *in vitro* , as well as help to define conditions under which the effector cells may be propagated *in vitro* with minimal transformation.

6. Objectives. *In vivo veritas* , a shared TGW/MS leitmotiv.

The main objective of the experiments presented in this thesis is to understand the function(s) of natural suppressor (NS) activity *in vivo* . As a prerequisite to exploring these functions, it is necessary to establish a dependable *in vitro* assay system to monitor NS activity, find ways of controlling levels of NS activity in the live animal, and develop model systems in which NS activity is relevant.

Bone marrow-associated NS activity is a reproducible inhibitory immunoregulatory phenomenon, described *in vitro* . It can be distinguished from other *in vitro* immunological functions as an antigen non-specific, non-MHC-restricted inhibitory activity which does not involve a killing mechanism and is not blocked by cyclo-oxygenase inhibitors. Its selective physiological tissue distribution throughout ontogeny, being always associated with hemopoiesis but absent from thymus and secondary lymphoid organs, suggests it is more than a reproducible *in vitro* artefact which disrupts culture systems in a trivial manner. This activity can be monitored in a number of immunological assays, including the

primary plaque forming cell (PFC) response, the proliferative responses to alloantigen or mitogen, and the primary mixed leukocyte (MLC)-generated cytotoxic T cell (CTL) generation.

Despite the abundance of *in vitro* descriptions, our understanding of the physiological significance of natural suppression is very limited. This is in part due to the difficulty in isolating the effector cells, largely the consequence of the absence of any specific cell surface marker. Hence, it is difficult to establish cellular clones which are beyond doubt valid representatives of the relevant effector cells *in vivo* . Some investigators have nonetheless applied standard cloning techniques to isolate NS cells and have managed to propagate cells that meet the above definition of NS activity. While this approach to elucidating the nature and function of NS activity may be useful, we consider this approach, given our current knowledge, very uncertain, chiefly because the only available assays to monitor the activity are essentially the non-specific disruption of an artificial immune response. To address the physiological significance of natural suppression, we have followed a different principle, based on experimentation in the live animal. In the following studies, the rationale is therefore to relate variations in NS activity, whether spontaneous or induced, measured *ex vivo* , to other better known and measurable functions, measured either *in vivo* or *ex vivo* . Thus, the data will be of a correlative nature, at least in the beginning.

Two fundamental aspects of the NS phenomenon are suggestive of possible functions *in vivo* . Firstly, the basis of the *in vitro* assay in which it is monitored is non-specific immunosuppression. Although this behaviour of the NS effector cell may have little to do with its true function, a role in immunological unresponsiveness is suggested, possibly in the context of immunological tolerance and/or immunodeficiency states. Secondly, because of the close association with hemopoiesis, suggested by the tissue

distribution of NS activity throughout ontogeny, a role in hemopoietic regulation and/or hematologic disorders, and by extension in bone marrow transplantation, may be anticipated.

The experimental basis of the present work follows these two lines of thought and is based on two seminal observations. Firstly, in a murine bone marrow transplantation model, we observed that donor hemopoietic engraftment was reduced in genetically histocompatible recipients displaying elevated NS activity. This finding was used to investigate the relationship between the level of NS activity in the host at the time of transplantation and the outcome of the transplantation, measured as the resulting degree of chimerism. Secondly, in a murine model of autoimmune diabetes, we observed that experimentally-induced increment of NS activity was associated with the prevention of diabetes in non-obese diabetic (NOD) mice. This finding was investigated in order to determine a possible relationship between NS activity and tolerance to self antigens. Hence, the immediate objectives of this thesis, pursuant to setting up an *in vitro* NS assay and finding ways of increasing or decreasing NS activity *in vivo* , which are described in appendices I and II, were to develop proper animal models in which the function of NS activity could be studied.

TABLE 1: Phenotype of bone marrow natural suppressor cells.

Reference	Assay	Thy1	CD4,8	sIg	Ia	other characteristics
41	PFC	-	N.D.	-	N.D.	radiosensitive, weakly adherent. present in nu/nu
42	PFC	-	-	-	N.D.	G10-adherent, large cells, 2 types (Mac1- and Mac1+) 50% radiosensitivity = 15Gy
43	PFC	-	-	-	-	nylon wool-non-adherent, Mac1-, H-2K+, no NK activity.
44	CTL	-	N.D.	-	N.D.	NW-adherent, silicaresistant, BCG-induced in bonemarrow, spleen, but not thymus.
45	MLR	-	N.D.	-	-	aGM1-, 1,063<density<1,075, WGA+, no NK or NC activity

TABLE 2: Phenotype of neonatal spleen natural suppressor cell.

Reference	Assay	Thy1	CD4,8	sIg	Ia	other characteristics:
49-51	MLR	+	N.D.	N.D.	N.D.	Ly1+, I-J+, mitomycinC and cortisone-resistant, absent from thymus.
52	PFC	-	N.D.	N.D.	N.D.	nylon wool-non-adherent absent from thymus.
53	CTL	+	N.D.	N.D.	N.D.	nylon wool-non-adherent.
54-55	MLR	-	N.D.	-	-	1,055<∂<1,067, mast cell?
56	PFC	-	N.D.	-	N.D.	partially plastic-adherent, becomesmacrophageinculture.
57	inducible sIa expression	-	N.D.	N.D.	N.D.	radiosensitive, present in BM, poorly NW-adherent, phagocyteinculture,aspirin-sens.
58	MLR	-	N.D.	-	N.D.	plastic-non-adherent, Mac1-, F4/80-, no NK activity.
59	MLR	+	-	-	-	Mac1-, F4/80-, aGM1+, IL2-dependent.
60	MLR, mitogen	-	-	-	-	aGM1weak, H-2K+, Ly5+, J11d+, Mac1+, IL3-dependent, NC but no NK.

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CHAPTER II: Delayed activation of donor quiescent hemopoietic stem cells in the host marrow cavity by anti-host monoclonal antibody.

Bone marrow transplantation in untreated, normal adult recipient mice usually fails. This resistance to hemopoietic stem cell engraftment is effectively overcome by non-specific conditioning agents such as irradiation and cytotoxic drugs (1-4). These conventional conditioning agents, administered to the host prior to donor marrow infusion, serve several functions, most importantly immunosuppression and myeloablation (1-6). In syngeneic or semiallogeneic recipients, donor marrow engraftment can be facilitated by the administration of anti-host major histocompatibility complex (MHC)[1] class I antibody (7-9). In the latter procedure, the conditioning regimen consists of a single injection of anti-H-2K monoclonal antibody given one week prior to or along with the marrow inoculum. This form of conditioning presents two major advantages: one is to avoid the numerous undesirable radiation- and drug-related side-effects, and the other is to selectively target allogeneic or semi-allogeneic host cells in the presence of donor cells. In the absence of any of the above conditioning regimens, transplantation of T cell-depleted parental marrow into adult F_1 hybrid recipients does not result in significant peripheral blood chimerism (refs. 7,8 and this paper).

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[1] Abbreviations used: CFU-GM, colony forming unit - granulocyte/macrophage; GPI, glucose-6-phosphate-isomerase; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBL, peripheral blood leukocytes; RBC, red blood cells.

In the present communication, we have studied the fate of parental donor stem cells in unconditioned F_1 hosts in which the hemopoietic graft fails. This *in vivo* analysis was made possible by administering facilitating antibody at various time points after transplantation. The specificity of antibody-conditioning, which induces chimerism while sparing donor cells, permits us to detect the persistance of donor cells in the host at the time of antibody administration. We find that complete donor engraftment can still be triggered a month after marrow infusion, thus establishing that donor cells are not eliminated, but remain functional, albeit in a resting state. The progression of donor engraftment within the host marrow cavity after antibody administration was assessed by measuring the speed of engraftment at different time points, through the use of a very sensitive isozyme assay which permits the phenotyping of individual donor bone marrow progenitor cells. We found comparable engraftment kinetics when antibody was administered 5, 10 or 16 days after transplantation, suggesting that the status of the parental cells remained relatively stable over that period. This system, in which isozyme-marked parental cells are quiescent in the marrow and may be electively activated by anti-host antibody conditioning and tracked in the marrow cavity, may serve as a useful model to study hemopoietic regulation in the unirradiated animal.

MATERIALS AND METHODS

Mice. Adult BALB/cCr and (BALB/cCr x C3H/HeJ)F_1 hybrid ($CC3F_1$) mice were bred at the University of Alberta in our animal breeding facility. Two to three month old mice were used in all experiments and were maintained under standard housing conditions.

Bone Marrow Transplantation. The antibody-facilitation protocol has been extensively described (7-10), particularly in the BALB/cCr→$CC3F_1$ combination (8,10). Briefly, twenty million nucleated bone marrow cells from BALB/cCr donors were administered intravenously to unirradiated age-matched, sex-matched F_1 hybrid hosts. Conditioning for transplantation consisted of a single injection of 500μg of the anti-host MHC monoclonal antibody 16-3-1N (anti-H-$2K^k$ mouse IgG2a; ref. 11). Preparation of the antibody from ascites and its quantitation has been described (9,10). Administration of an equal amount of irrelevant antibody or saline has been shown not to facilitate engraftment, or affect bone marrow cellularity (8,9). Parental grafts were depleted of mature T cells by complement-mediated antibody depletion. Marrow cells were incubated at a concentration of 10^7 nucleated cells/ml in balanced salt solution with Thy1.2 monoclonal antibody (New England Nuclear, Boston, MA) diluted at 1:500 and kept on ice for 40min. After pelleting, bone marrow cells were resuspended in a 1:8 dilution of rabbit complement (Low Tox, Cedarlane, Hornby, ON) in balanced salt solution and incubated 40min in a waterbath set at 37°C. Cells were washed twice before counting and injection. This treatment completely abrogated the *in vitro* proliferative response of spleen cells and bone marrow cells to Concanavalin A (data not shown). In marrow transfers to secondary hosts, unmanipulated marrow cells were harvested from exsanguinated primary F_1 hosts 5 or 10 days after transplantation, washed once, and administered intravenously to secondary F_1 hosts exposed to 9Gy (80cGy/mn) (Atomic Energy of Canada, Ottawa, ON).

Chimerism assays. The determination of chimerism based on glucose-6-phosphate isomerase (GPI) isozyme analysis has been described (10). Briefly, peripheral blood samples were lysed and subjected to starch gel electrophoresis. The ratio of donor to host isozymes was determined by a colorimetric reaction quantified via densitometry

scanning. Chimerism levels were read from a standard curve constructed from isozyme measurements of artificial mixes of varying donor-host composition. The sensitivity of this method has been described, estimated to be ±4% for the lower values of GPI A : GPI AB mixes and ±1% for higher values (10). The small amount of GPI isozyme necessary for electrophoretic separation and staining of the different bands allows the correct phenotyping of as few as 30 cells, such as would be found in single bone marrow colonies grown *in vitro* in semi-solid media. This assay has been used to show that peripheral blood chimerism is accurately reflected in the bone marrow in steady-state chimeras (12) and to analyze engraftment kinetics within the marrow cavity (13). To determine the donor or host origin of CFU-GM progenitor cells in the marrow at different times after transplantation, bone marrow cells were cultured in a methyl-cellulose medium (Fisher Scientific, Ottawa, ON) containing 20% fetal bovine serum (Flow Laboratories, McLean, VA), 20% IMDM (Gibco Laboratories, Grand Island, NY), 10% WEHI-3 supernatant-conditioned medium (kindly provided by D. Branch) and $5x10^{-5}$M 2-mercaptoethanol. One hundred thousand bone marrow cells per ml were cultured at 37ºC in a humidified 5% CO_2 atmosphere for 6-8 days at 1 ml per plate in Lux-R plates (Miles Scientific, Naperville, IL). Colonies were individually picked under the microscope, lysed by freeze-thawing and individually loaded onto the gel for electrophoresis. About 50 colonies per animal were phenotyped, thus allowing an accurate measurement of the degree of donor representation within the granulocyte-macrophage progenitor pool at any given time point after transplantation. In radiation chimeras, chimerism was examined three months after transplantation in different lineages: erythroid (by analyzing peripheral red blood cells), lymphoid and myeloid (by analyzing thymocytes, splenocytes and peripheral blood leukocytes) and whole marrow tissue. Erythrocytes were eliminated from the latter by two consecutive tris-ammonium chloride-mediated lyses of single cell suspensions and peripheral blood buffy coats.

RESULTS

Administration of anti-host facilitating antibody after transplantation triggers delayed donor hemopoietic engraftment.

In the absence of any host conditioning, administration of T cell-depleted parental BALB/c bone marrow to adult $CC3F_1$ recipients does not result in any detectable peripheral blood chimerism, as shown in Table 1. To examine the fate of donor hemopoietic stem cells within the host, we attempted to induce chimerism by delaying the administration of the specific anti-host conditioning after donor marrow infusion. Host cells were selectively targetted *in vivo* by administering anti-H-$2K^k$ monoclonal antibody, which reacts against F_1 (H-2^{dxk}) but not donor (H-2^d) cells. As shown previously (8,10) and reiterated in Table 1, a single injection of 500μg of anti-H-$2K^k$ antibody given along with parental marrow induces complete, long term peripheral blood chimerism. Recipient F_1 mice given the facilitating antibody two weeks after the infusion of 15 million T cell-depleted parental marrow cells were equally well engrafted 6 months after transplantation. This observation establishes that donor hemopoietic cells survived in the host for at least 14 days. Thus, the apparent graft failure in the unconditioned host cannot be accounted for by the elimination of all donor cells within that time period.

To understand what happens to donor cells in the untreated adult host, we examined chimerism within the marrow cavity in the days following transplantation. To do this we used a recently described assay which accurately quantitates medullary chimerism (12,13). Individual CFU-GM progenitor cells were phenotyped for their host or donor origin at any given time, using a sensitive isozyme assay. As shown in Figure 1, donor representation in the CGU-GM pool reached 88 ± 6% 15 days after transplantation in

recipient mice given facilitating antibody along with donor marrow, whereas in untreated recipients, 0 to2% of the CFU-GM were of donor origin between 2 and 30 days after transplantation. When antibody was given 5, 10 or 16 days after transplantation, donor representation rose to about 50% of the CFU-GM pool within 4 days of antibody administration in all cases (Figure 1). The engraftment kinetics were similar for each of these time points, and, if anything, slightly faster than in F_1 hosts given donor marrow and antibody together on day 0, in which 50% chimerism was achieved after 6 days. The CFU-GM pool was completely of donor origin by 30 days after transplantation in all groups. In one experiment where engraftment kinetics were not examined, we found that antibody administration 32 days after marrow infusion resulted in complete donor chimerism as determined a month later. Since no engraftment is detected in the absence of antibody treatment, but donor hemopoietic cells nevertheless survive for a month within the host, we next asked whether donor cells are able to seed the host marrow cavity.

Quiescent, donor hemopoietic stem cells are present in the host marrow.

To examine whether donor parental hemopoietic cells seed the host marrow, bone marrow cells from primary F_1 hosts were transferred to lethally irradiated secondary F_1 mice. Bone marrow cells were harvested from unconditioned $CC3F_1$ recipients 5 days after they had been given 15 million T cell-depleted BALB/c bone marrow cells. Three to five million marrow cells were immediately administered to the secondary $CC3F_1$ hosts 4 hours after exposure to 9Gy whole body irradiation. To enhance any engraftment by parental cells putatively present in the marrow inoculum, one group of secondary recipients received marrow cells incubated *in vitro* with anti-H-$2K^k$ monoclonal antibody prior to infusion; another group was given 500μg of the antibody intraperitoneally a week after the transfer.

As shown in figure 2, chimeric hemopoietic reconstitution was observed in some recipients, thus indicating that parental precursor cells were present in the primary F_1 host marrow cavity. Peripheral blood chimerism was not observed, however, in mice that did not receive facilitating antibody. This absence of engraftment is unlikely to be due to elimination of parental cells within 7 days of the transfer, as chimerism could be induced by administration of facilitating antibody after a week. Coating *in vitro* of transferred F_1 cells with facilitating antibody was less efficient to induce chimerism. Because chimerism was sustained 3 months after the transfer and because equal reconstitution in the erythroid and myeloid compartments was observed in the secondary hosts, it appears that primitive stem cells were transferred (table 2). This equal reconstitution in these blood compartments favors hemopoietic reconstitution by common precursor cells rather than reconstitution by equal numbers of active committed progenitor cells of each lineage. This was supported by finding mixed chimerism in the thymus as well (table 2), the lower values probably reflecting a slower turnover of the bone marrow-derived lymphoid precursor that peripheral blood cells. A similar reconstitution, as assessed by peripheral blood analysis in long-term secondary host, was achieved by transferring primary F_1 host marrow harvested 10 days after parental marrow infusion (unpublished observation). Our results therefore support the idea that quiescent primitive stem cells of donor origin were present in the host marrow cavity, suggesting that they had seeded the host marrow, but failed to proliferate or differentiate as suggested by their absence from the marrow CFU-GM pool (figure 1) and the absence of any progeny in the peripheral blood (table 1). Furthermore, these cells failed to enter the active hemopoietic pool upon transfer to secondary hosts, unless anti-host antibody was administered.

DISCUSSION

In this report, we show that parental hemopoietic cells which apparently fail to engraft F_1 hybrids in the absence of host conditioning prior to transplantation do in fact seed the host marrow cavity, but remain quiescent. Their survival and ability to function up to at least 32 days after transplantation, was demonstrated by selective targetting *in vivo* of the host bone marrow cells with an anti-MHC class I monoclonal antibody, which spares donor cells. Following this delayed, specific host conditioning, engraftment was induced, resulting eventually in complete donor chimerism. By a new assay which allows us to follow the kinetics of engraftment within the marrow cavity (13), we found that donor-derived CFU-GM promptly took over the CFU-GM pool following the administration of antibody at 4, 10 or 16 days after marrow infusion. The engraftment kinetics were similar regardless of the time of antibody administration, suggesting that the donor reservoir was stable over that period. The level of engraftment was the same in at least two distinct lineages, myeloid (as represented by the CFU-GM) and erythroid (as represented by the peripheral red blood cells). Based on transfer experiments to secondary hosts, the bone marrow appeared to be a reservoir for parental precursor cells. The presence of parental totipotent hemopoietic stem cells amongst the transferred cells was suggested by the comparable level of chimerism found in different blood compartments, while, conversely, no split chimerism was observed in animals not administered antibody-conditioning.

Assuming that the failure of parental engraftment in the lethally irradiated secondary host is not caused by the lack of "space" or growth factors, which could result in selecting against the smaller pool of parental cells present in the inoculum, the absence of any degree of chimerism in the irradiated recipients which are not given facilitating antibody is intriguing. This lack of parental hemopoietic activity is suggestive of a regulatory

mechanism controlling parental quiescent stem cells. Since the transfer is set up in a P→F_1 radiation model, conditions for hybrid resistance mechanisms to operate are met (14). However, the delayed rescue of donor engraftment by administration of antibody 7 days after cell transfer argues against the elimination of parental hemopoietic cells by host natural killer activity. The delayed activation of parental hemopoiesis suggests, rather, that parental precursors were inhibited rather than eliminated, thus supporting a regulatory control of engraftment, as we proposed earlier (10,13,15). In this hypothesis, the parental hemopoietic stem cells are capable of seeding the host marrow, but are subjected to inhibitory hemopoietic regulation. This regulatory control persists under the conditions of the adoptive transfer where sufficient hemopoietic activity by cycling F_1 hemopoietic cells prevents the activation of quiescent stem cells, including those of parental origin. This interpretation is supported by the finding that the adoptive transfer of antibody-coated F_1 cells is associated with increased parental engraftment (figure 2), suggesting that the parental engraftment is indeed limited by inoculated proliferating cells rather than radioresistant functions present in the recipients.

This lack of detectable parental engraftment in the primary or secondary F_1 hosts in the absence of facilitating antibody suggests some cautions regarding the definition of hemopoietic graft failure. Based on this analysis within the marrow cavity, it appears that one can distinguish between the early elimination of donor cells, which would result in failure of seeding and evokes a rejection process, and the failure of blood production following succesful seeding, suggestive of reversible downregulation of donor hemopoietic cells. In the context of endogenous marrow repopulation, one might similarly be able to distinguish between delayed elimination of the donor cells or their downregulation. Since quiescent, isozyme-marked, donor stem cells can be selectively activated in the live animal, this model may be useful to study hemopoietic regulation within the marrow cavity in an

unirradiated microenvironment. For example, the effect of various hemopoietic stimuli, including the administration of pure hemopoietic growth factors or inhibitors, may be evaluated on the recruitment of the quiescent precursors into the active hemopoietic pool. Such a model may also be useful to study the relationship between the bone marrow microenvironment and tumor cells, of hemopoietic origin or otherwise, that are present in the marrow cavity but remain in a quiescent state over a prolonged period.

Table 1. Administration of facilitating antibody 2 weeks after marrow infusion induces complete chimerism.

conditioning[a]	number of mice[b]	chimerism[c]
none	12	<4%
mAb on day 0	10	98 ± 3%
mAb on day 14	12	97 ± 3%

[a] Adult $CC3F_1$ hosts were given 15 million T cell-depleted parental marrow cells i.v. on day 0, along with or 2 weeks prior to i.v. administration of 500μg of anti-H-$2K^k$ monoclonal antibody (mAb).

[b] Pooled from two separate experiments.

[c] Chimerism was determined in each mouse by isozyme analysis of peripheral blood lysates three months after transplantation and is expressed as mean ± s.d. for each group.

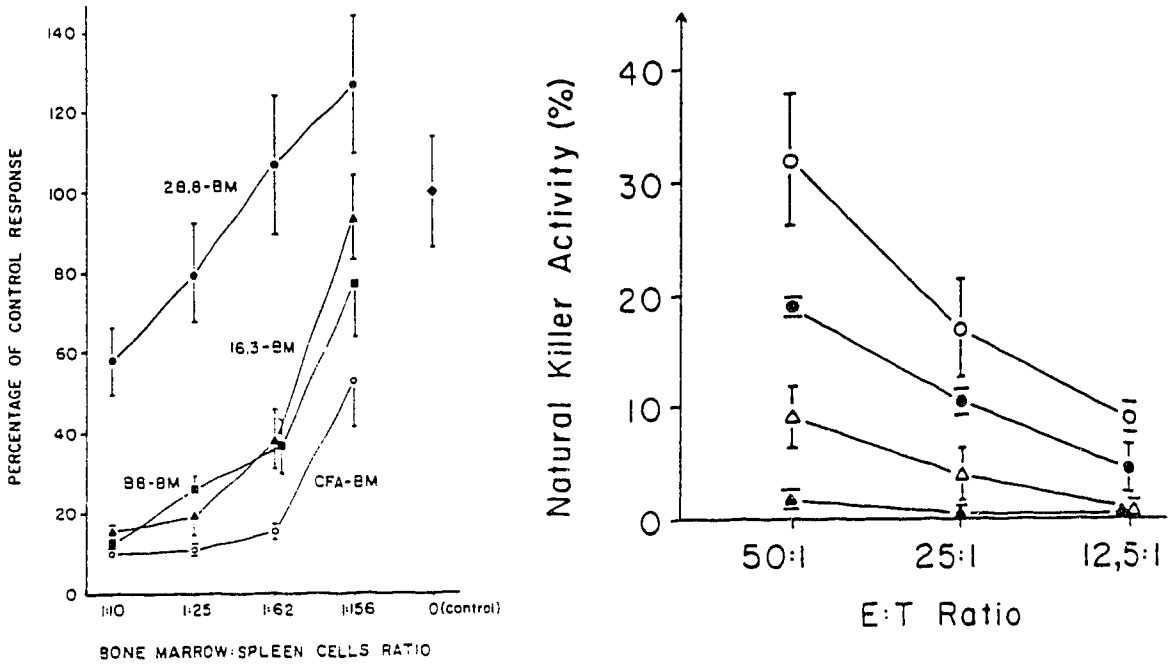

Figure 8. Panel A : effect of in vivo antibody and CFA treatment on adult bone marrow NS activity. Bone marrow cells from (BALB/cCr x C57BL/6J) F_1 adult females were titrated in cultures of syngeneic spleen from untreated animals. Bone marrow was harvested one week after intravenous injection of 900 µg of anti-H-2K^b IgG2a (28.8-BM), anti-H-2K^b IgG1 (B8-BM) or anti-H-2K^k IgG2a (16.3-BM) antibody, or intraperitoneal injection of 100 µl of CFA (CFA-BM). Data are plotted as mean±s.d., calculated from triplicate cultures, of one representative experiment of four. The control response (100%± 13) represents 315±41 PFC per million spleen cells. Panel B : effect of *in vivo* antibody treatment on splenic NK activity. Data represent mean±s.d. of three independent experiments, each consisting of two pooled spleens per group, and expressed as in figure 3B. C57BL/6J spleens were removed one week after intravenous injection of 900 ug anti-Kk (O) or anti-Kb (●) monoclonal antibody.

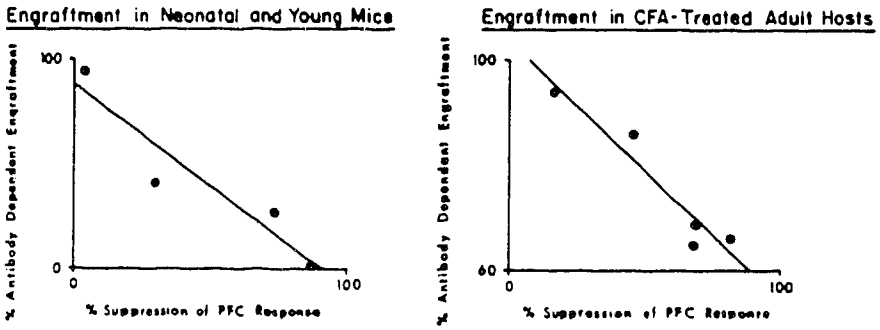

Figure 9. Correlation between level of engraftment and host NS activity. The data are derived from figures 2 and 3A for CFA-treated adults and figures 4 and 5A for postnatal hosts. Antibody-dependent engraftment is the engraftment level with facilitating antibody minus the background engraftment achieved in saline-treated mice. The suppression level is 100-(% of control response when mixing neonatal or CFA-treated spleen cells). Regression analysis yields the following values : in postnatal hosts, y= 88-0.97x, r=-0.94, n=4 and in CFA-treated adults, y=103-0.49x, r=-0.95, n=5.

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CHAPTER IV: host natural suppressor activity regulates hemopoietic engraftment in antibody-conditioned recipient mice.

Bone marrow transplantation is greatly enhanced by the administration of anti-host MHC antibody in otherwise untreated semi-allogeneic or syngeneic mice (1-3). Under these conditions, donor stem cell engraftment is facilitated by a single injection of anti-host class I monoclonal antibody, resulting eventually in complete chimerism in peripheral blood and bone marrow, depending on the antibody and donor marrow cell doses. This conditioning regimen is not lethal, merely creating a transient and limited medullary hypoplasia. We have recently suggested that natural suppressor activity in the host at the time of transplantation played a role in hemopoietic engraftment in antibody-conditioned recipient mice (4). Natural suppressor activity is defined as an antigen non-specific, non-H-2-restricted regulatory function assayed by *in vitro* immunological suppression (5,6). In two independent situations where recipient natural suppressor activity was increased, bone marrow transplantation resulted in reduced levels of long term peripheral donor blood chimerism. These findings suggested that natural resistance to engraftment, including that seen in syngeneic donor-host combinations, may be mediated in part by natural suppressor cells present in the hemopoietic microenvironment. To directly evaluate the relevance of natural suppressor activity to bone marrow engraftment, we have now examined the kinetics of donor engraftment within the host marrow cavity and analyzed its relationship to natural suppressor activity on a daily basis in the post-transplant period. To accomplish this, we have developed an assay which tracks the marrow cavity engraftment of donor cells by individually phenotyping CFU-GM progenitor cells. In this assay, individual marrow granulocyte-macrophage colonies are analyzed for their isozyme phenotype, thereby distinguishing donor CFU-GM from host CFU-GM, at various times after

transplantation. Results presented here indicate that the rate of donor hematopoietic engraftment in the marrow cavity was inversely correlated with the level of host natural suppressor activity. There was no correlation between donor engraftment kinetics and the number of host CFU-GM present in the marrow cavity at the time of marrow infusion. The adoptive transfer of Thy1-negative spleen cells with natural suppressor activity into unirradiated hosts resulted in delayed marrow cavity engraftment, suggesting that regulatory cells which display natural suppressor activity confer resistance to hemopoietic engraftment.

MATERIALS AND METHODS

Mice. Adult BALB/cCr, (BALB/cCr x C3H/HeJ)F_1 (noted $CC3F_1$) mice were purchased from the University of Alberta animal breeding facility. (BALB/cJ x C57BL/6J)F_1, as well as glucose-6-phosphate isomerase (GPI) congenic strains on a C57BL/6J background (3,4) were bred in our animal facility. Two to three month old mice were used in all experiments and maintained under standard housing conditions.

Bone Marrow Transplantation. The antibody-facilitation protocol has been extensively described (2,3,4), particularly in the BALB/cCr→$CC3F_1$ combination (2,4). Briefly, twenty million nucleated bone marrow cells from BALB/cCr donors were administered intravenously to unirradiated age-matched, sex-matched F_1 hybrid hosts. Conditioning for transplantation consisted of a single injection of 500μg of anti-host MHC monoclonal antibody, either 16-3-1N (anti-H-$2K^k$ mouse IgG2a, ref. 7) or 28-8-6S (anti-H-$2K^b$ mouse IgG2a, ref. 8). Preparation of these antibodies from ascites and their quantitation has been described (4). Administration of an equal amount of irrelevant

antibody or saline has been shown not to facilitate engraftment, or affect bone marrow cellularity (3,4). In some experiments, recipient mice were conditioned for transplantation by an intraperitoneal injection of methotrexate, 100mg/kg (MTX, Horner, Montreal, PQ) or cyclophosphamide. 50 or 200mg/kg (CY, Horner, Montreal, PQ). In adoptive transfer experiments, spleens from age-matched, sex-matched CC3F_1 mice were harvested eight days after intraperitoneal administration of either 100μl of complete Freund's adjuvant (Difco Laboratories, Detroit, MC) or 100μl of saline. After washing single cell suspensions once in balanced salt solution, sixty million cells were injected intravenously within 30 minutes of bone marrow infusion, and, in certain recipients, again at 48 and 96 hours after transplantation. In some experiments, adoptively transferred spleen cells were irradiated or depleted of Thy1-positive cells as described below.

Chimerism assays. The determination of chimerism based on glucose-6-phosphate isomerase (GPI) isozyme analysis has been described (4). Briefly, peripheral blood samples were lysed and run on starch gel electrophoresis. The ratio of donor to host isozymes was determined by a colorimetric reaction quantified via densitometry scanning. Chimerism levels were read from a curve constructed from isozyme measurements of artificial mixes of varying donor-host composition. The sensitivity of this method has been described (4,9). The small amount of GPI isozyme necessary for electrophoretic separation and staining of the different bands allows the correct phenotyping of as few as 30 cells, such as would be found in single bone marrow colonies grown *in vitro* in semi-solid media. This assay has been used to show that peripheral blood chimerism is accurately reflected in the bone marrow in steady-state chimeras (9). To determine the donor or host origin of CFU-GM progenitor cells in the marrow at different times after transplantation, bone marrow cells were cultured in a methyl-cellulose medium (Fisher Scientific, Ottawa,

ON) containing 20% fetal bovine serum (Flow Laboratories, McLean, VA), 20% IMDM (Gibco Laboratories, Grand Island, NY), 10% Wehi-3 supernatant-conditioned medium (kindly provided by D. Branch) and $5x10^{-5}$M 2-mercaptoethanol. One hundred thousand bone marrow cells per ml were cultured at 37ºC in a humidified 5% CO_2-containing incubator for 6-8 days at 1 ml per plate in Lux-R plates (Miles Scientific, Naperville, IL). Colonies were individually picked under the microscope, lysed by freeze-thaw and individually loaded onto the gel for electrophoresis. About 70 colonies per animal were phenotyped, thus allowing an accurate measurement of the degree of donor representation within the granulocyte-macrophage progenitor pool at any given time point after transplantation.

Plaque Forming Cell Assay. This assay has been described (4). Briefly, 10 million (BALB/cJ x C57BL/6J)F_1 spleen cells were cultured for 5 days in triplicate cultures in 24-well plates (Flow Laboratories, McLean, VA) with twenty million sheep red blood cells. Each well contained 1ml RPMI-1640 medium supplemented with 10% prescreened fetal bovine serum (Flow), 1mM sodium pyruvate (Gibco Laboratories, Chagrin Falls, OH), 2mM L-glutamine (Flow) and $5x10^{-5}$M 2-mercaptoethanol. Plaques were enumerated in Cunningham slides in the presence of fresh sheep red blood cells and guinea pig complement. Syngeneic bone marrow cells, spleen cells from 5 day old animals or CFA-treated adults were added at the beginning of the culture to assess their suppressive capacity, which is measured by the reduction of the number of plaques counted in each well. Titration of bone marrow cells in the primary sensitizing cultures has been reported in C57BL/6J and (BALB/cJ x C57BL/6J)F_1 mice (4).

Natural Killer Assay. This assay has been described (4). Briefly, normal and CFA-treated adult spleen cells, 5 day post-natal spleen cells and adult bone marrow cells were washed twice, and incubated with ^{51}Cr-labelled YAC-1 target cells in a standard chromium-release assay. One million cells were added to 10^4 targets in 96-well, V-bottom Linbro plates and incubated for 4 hours at 37ºC. Lysis was measured by counting the gamma emission from 100µl of supernatant from each well. Specific lysis was calculated using the formula (counts in test well - background release) x (maximum release - background release)$^{-1}$, where background release is the radioactivity spontaneously released from 10^4 labelled target cells incubated in medium alone and maximum release is obtained in the presence of 1% Nonidet P-40 (Sigma, Saint Louis, MO). Each group was set up in quadruplicate wells. Natural killer activity in adult spleen after CFA administration and in the untreated neonatal spleen has been described, using different effector : target ratios, in unseparated cells and Percoll gradient fractions enriched for natural killer activity (4).

Cellular phenotyping. The presence of the Thy1 cell surface marker was assessed by *in vitro* complement-mediated antibody depletion. Single cell suspensions of spleen or bone marrow were diluted after washing in balanced salt solution containing 0.2% bovine serum albumin (pH 7.2) at 10^7 cells/ml and incubated on ice for 40 minutes, in the presence of the Thy1.2 IgM monoclonal antibody (NEN, Boston, MA), diluted at 1:500, or without antibody. After one wash, the cells were suspended in rabbit low toxicity complement (Cedarlane, Hornby, ON) diluted in the same solution at 1:10 and incubated for 40mn in a 37ºC water-bath. Cells were then washed twice before usage. Treatment with Thy1.2 monoclonal antibody and complement abrogated the response of splenocytes to Concanavalin A *in vitro* , whereas complement treatment alone had no effect (data not

shown). For *in vitro* studies or adoptive transfer, cells were resuspended in the same volume whether Thy1-depleted or not. Cellular adherence to nylon wool was assessed by incubating 50 million spleen or bone marrow cells for 1 hour in a 5ml nylon wool column in culture medium containing 5% fetal bovine serum in a 37°C incubator. Non-adherent cells were collected by washing each column with 20ml medium and represented 20 to 30% of the loaded cells. After washing, the non-adherent cells were resuspended proportionately to initial cell counts, thus avoiding enrichment for nonadherent cells. Radiosensitivity was assessed by irradiating the harvested cells *in vitro* or irradiating the donor 2 hours before sacrifice using a ^{137}Cs source (Gammacell, AEC, Ottawa, ON) which delivers 80cGy/mn. The cells were then washed once and immediately put in culture or administered intravenously.

RESULTS

Hemopoietic engraftment in the marrow cavity is more rapid in hosts given facilitating antibody one week prior to transplantation than in those given antibody and marrow simultaneously.

We have previously shown that the degree of long term peripheral blood chimerism in antibody-facilitated chimeras is closely correlated with the chimerism of the bone marrow CFU-GM compartment (9). As a necessary background to assessing the role of host regulatory activities in hemopoietic engraftment kinetics, we studied the early phases of engraftment in the marrow cavity by monitoring the chimerism of CFU-GM progenitor cells in the immediate post-transplant period. Adult F_1 hybrid mice were given 20 million parental bone marrow cells, either 7 days after or at the same time as intravenous administration of 500 μg of facilitating antibody, as described previously (4). Bone

marrow was harvested at 48 hour intervals following transplantation and immediately cultured in IL-3-containing methylcellulose. Six to eight days later, chimerism analysis was performed by harvesting individual hemopoietic colonies under the microscope and determining whether they were derived from the donor or the host by electrophoretic analysis of their GPI isozyme type. As shown in figure 1, mice given facilitating antibody at the same time as bone marrow had 14 ± 3% donor granulocyte-macrophage colonies 48 hours after transplantation. In mice given antibody 7 days prior to transplantation, donor representation accounted for 38 ± 9% of the colonies at the 48 hour time point. Donor representation increased to 90 ± 4% after 4 days in the latter group, whereas the same level was achieved only by day 8 in mice given antibody along with donor bone marrow. In control mice given irrelevant antibody, donor-derived colonies accounted for less than 2% of the colonies. Thus, donor hemopoietic engraftment is faster in mice pretreated with facilitating antibody, suggesting that the conditions within the host marrow are more favorable for incoming donor cells 7 days after antibody administration. Based on our earlier report that long term chimerism levels appear to correlate with NS activity in the host at the time of transplantation rather than total marrow cellularity (4), we next examined in parallel the kinetics of these two parameters in the host bone marrow during the immediate post-transplant period.

Injection of facilitating antibody reduces bone marrow natural suppressor activity, which reaches a minimum seven days thereafter.

Bone marrow NS activity was monitored by mixing titered amounts of bone marrow cells with syngeneic spleen cells in an *in vitro* plaque forming cell (PFC) assay, as previously described (4). The suppressor activity was quantified by the degree of reduction of the control PFC response. To assess the kinetics of the reduction in NS activity which

results from facilitating antibody administration, mice given 500 μg of anti-H-2K antibody alone were sacrificed at different time points after injection and their bone marrow tested for NS activity. Administration of irrelevant antibody has previously been shown not to affect NS levels (4). Bone marrow NS activity is shown in figure 2, where three separate experiments or more are averaged for each time point and normalized with respect to control responses (without bone marrow) obtained in each experiment. Addition of the same number of spleen cells ($2.5x10^5$) instead of bone marrow cells did not alter the PFC response (data not shown). The level of bone marrow natural suppressor activity was maximally reduced by day 7.

These findings suggest that the correlation between resistance to engraftment and level of host NS activity can be extended to engraftment kinetics, since engraftment appears to be accelerated when incoming donor stem cells reach a marrow cavity in which NS activity is maximally reduced, which is the case 7 days after administration of facilitating antibody. We next examined the alternative possibility that variations in host hemopoietic cellularity may determine donor engraftment kinetics.

Injection of faciltating antibody partially and reversibly reduces bone marrow cellularity and CFU-GM content.

To evaluate the effect of a single injection of anti-host MHC class I monoclonal antibody on numbers of total bone marrow cells and CFU-GM, marrow cells were harvested from mice at different time points after antibody administration, counted, and evaluated for granulocyte-macrophage colony-forming ability. The effect of antibody treatment on total bone marrow cellularity and CFU-GM is shown in figure 3. Adult (BALB/cJ x C57BL/6J)F_1 mice were given 500 μg of anti-H-$2K^b$ monoclonal antibody on day 0 in a single intravenous injection. Total cell counts (obtained from tibial and femoral

medullary flushing) were maximally reduced by day 5 (79 ± 13% of control) and returned to normal between days 7 and 10 post-injection. CFU-GM (determined in an *in vitro* colony assay, counted on day 7) were lowest between days 2 and 5 (reduced by about 75%), returned to normal levels by day 7, and were slightly above normal by day 10. These results established that under these conditions, total cellularity and committed progenitors of the CFU-GM type were partially and reversibly reduced by antibody conditioning. This corroborates our previous report showing that adult C57BL/6J mice given 1mg of anti-H-2K^b also displayed a partial and reversible reduction of CFU-S_{14} content (88 ± 7% reduction by day 1 and 36 ± 14% by day 7, ref.3).

By comparing the data shown in figures 1 and 2, it is apparent that the kinetics of the effects of antibody treatment on hemopoietic and natural suppressor activity reached their nadir at different times. The comparison of day 0 and day 7 time points is of greatest interest here. By day 7, CFU-GM content (figure 3) was comparable to that of day 0 animals, but NS activity was at it's lowest point (figure 2). Total host marrow cellularity decreased slightly after day 0, and did not significantly differ from control levels on day 7. By following the kinetics of engraftment in the bone marrow cavity after transplanting at these two contrasting time points (figure 1), we conclude that donor engraftment is accelerated when incoming donor stem cells reach a marrow cavity in which NS activity is reduced to a minimum, irrespective of the total number of host bone marrow cells or CFU-GM progenitor cells. We next investigated whether host NS activity correlates with marrow engraftment in conventional chimeras.

Hemopoietic engraftment induced by irradiation or chemotherapy correlates with the reduction of bone marrow natural suppressor activity.

Since we found that antibody conditioning abrogated resistance to engraftment in close correlation with reduced bone marrow NS activity, we next asked whether the reduction of this regulatory activity is also associated with hemopoietic engraftment enhanced by conventional conditioning agents. We therefore evaluated the effect of Y-irradiation, methotrexate and cyclophosphamide on bone marrow cellularity, NS activity and the induction of chimerism.

Normal F_1 hosts were exposed to 3Gy, 6Gy or 9 Gy Y-irradiation, which resulted in varying degrees of bone marrow NS activity reduction (table 1). Two hours after irradiation mice were given 20 million parental (BALB/cCr) bone marrow cells depleted of Thy1-positive cells by treatment with anti-Thy1.2 antibody and complement. Chimerism levels achieved 6 months after transplantation were inversely related to NS activity measured in bone marrow from mice sacrificed 2 hours after irradiation. No such relation was found with respect to total marrow cellularity at the time of marrow infusion, which was unchanged at that time, but did decrease at later time points in a radiation dose-dependent fashion (table 1). We next used cytotoxic drugs which have a more restricted range of action than Y-radiation. The effect of the anti-metabolite methotrexate (100mg/kg) or the alkylating agent cyclophosphamide (50 or 200mg/kg) on promoting chimerism was evaluated, as well as the impact on host bone marrow total cellularity and on NS activity at the time of donor marrow infusion. The latter effects were measured 24 hours after a single intraperitoneal administration of the drug, as the maximum reduction of stem cell activity occurs between 6 and 24 hours after administration of cyclophoshamide (10). As shown in table 1, methotrexate and low-dose cyclophosphamide failed to facilitate engraftment of T cell-depleted parental bone marrow or reduce bone marrow-associated NS activity. On the

other hand, cyclophosphamide given in higher dosage (200mg/kg) led to stable donor chimerism and reduced NS activity. Since all three treatments significantly reduced total bone marrow cellularity at the time of marrow infusion, these results once again suggested that the inactivation of selective bone marrow cell populations is more relevant to facilitating engraftment than is pancytoreduction. Reduction of host bone marrow NS activity appeared to have a better predictive value for engraftment than the non-specific elimination of host bone marrow cells. Administration of 500µg of facilitating antibody, cyclophosphamide at 200ug/kg or exposure to 6Gy, all of which induced significant hemopoietic chimerism, resulted in comparable decreases in NS activity (3.8-, 3.6- and 4.0-fold, respectively). Overall, these results establish that conditioning agents which reduce host bone marrow NS activity promote engraftment, while conditioning agents that do not reduce this activity fail to promote engraftment, regardless of their cytoreductive effect. Our next goal was to begin characterizing the effector cells mediating NS activity *in vitro* , as a prerequisite to the adoptive transfer of this activity in order to more directly evaluate its impact on the engraftment process.

Natural suppressor activity is mediated by Thy1-negative cells present in normal bone marrow, neonatal spleen and spleen from adjuvant-treated adults.

Given the above results, which suggest a direct relationship between host NS activity and resistance to engraftment in unirradiated hosts, we used the *in vitro* NS assay as a means to tentatively identify the regulatory cells, and then test whether these cells control resistance to hemopoietic engraftment. Normal bone marrow, neonatal spleen and spleen in adjuvant-treated adult mice were examined, as resistance to engraftment correlated with increased NS activity in these tissues (4). As shown in figure 4, complement-mediated

lysis of Thy1-positive cells did not reduce NS activity in any of the three tissues, as measured by the PFC assay. When subjected to nylon wool column passage, most of the NS activity was found in the non-adherent fraction, although to a lesser degree in the spleen of adjuvant-treated adult animals, suggesting that adherent cells could contribute to the total suppressor activity. Exposure to Y-irradiation, either *in vitro* or *in vivo* , revealed a dose-dependent radiosensitivity (figure 5), suggesting the coexistence of a radiosensitive component (reduction of the inhibitory activity by about a third after 5 Gy) and a radioresistant component (reduction of about two thirds of the activity after 20 Gy), which may both contribute to the total inhibitory activity measured in the unirradiated tissue. A comparable radiosensitivity profile was found in neonatal spleen and adjuvant-treated adult spleen (data not shown). The three tissues with NS activity, adult bone marrow, neonatal spleen and spleen in adjuvant-treated adult mice, had characteristically low natural killer activity (figure 4), as expected for cells with natural suppressor activity (5,6).

Medullary engraftment is delayed by adoptive transfer of spleen cells from adjuvant-treated donors.

Since engraftment was accelerated when host NS activity was reduced, we next asked whether, conversely, an increase in host NS activity would have the opposite effect, as determined by directly assessing engraftment in the marrow cavity. We therefore asked whether adoptively transferring spleen cells with NS activity elicited by prior adjuvant administration (4) could limit the engraftment process. This was examined directly in the marrow cavity in antibody-facilitated chimeras using the CFU-GM colony assay. Spleen cells from adjuvant- or saline-treated syngeneic F_1 mice were harvested 10 days after CFA inoculation, a time at which resistance to engraftment and splenic NS activity are maximal

(4). To prevent the F_1 hemopoietic cells from repopulating the host[3] , the splenic inocula were exposed to 8 Gy irradiation, a dose that prevents any detectable hemopoietic reconstitution in antibody-conditioned hosts (data not shown), while only partially reducing NS activity (see figure 5). The irradiated spleen cells were injected into adult F_1 hosts, which then received parental bone marrow and facilitating antibody (day 0). No detectable reduction of peripheral blood chimerism was observed following a single injection of 60 million irradiated spleen cells, which also confirms the inability of irradiated cells to engraft (figure 6A). After 3 injections of irradiated spleen cells from CFA-treated donors on days 0, 2 and 4, a significant reduction of chimerism at 4 weeks post-transplant was observed, compared to control spleen-injected recipients, but no long term effect was evident (figure 6A). The kinetic analysis of CFU-GM chimerism in the marrow cavity confirmed the delay in productive donor engraftment in hosts given spleen cells from adjuvant-treated animals (containing NS activity), but not control spleen (without NS activity) (figure 6B). Five days after transplantation, $50 \pm 8\%$ of the CFU-GM were of donor origin in animals given control F_1 spleen cells, but only $10 \pm 9\%$ in those given spleen cells from CFA-treated donors. By day 10, however, donor representation was comparable in both groups, suggesting that the transferred activity was very short-lived and not sufficiently sustained in the transfer protocol to bear on long term engraftment. The short-lived nature of this effect was possibly due to the irradiation of the transferred spleen

[3] In this protocol, parental marrow engraftment could in theory be reduced by hemopoietic competition between parental stem cells and F_1 stem cells present in the splenic inoculum. We evaluated this possibility by using recombinant-inbred C57BL/6J mice carrying a substitution at the *gpi* locus (3). When transplanting parental C57BL/6J-*gpi a* (GPI AA) to (C57BL/6J x C3H/HeJ)F_1 (GPI BB) hybrids and adoptively transferring spleen cells from (C57BL/6J-*gpi a* x C3H/HeJ)F_1 (GPI AB) hybrids, a reduction of parental marrow was indeed observed, accompanied by donor F_1 engraftment (as reflected by peripheral blood chimerism 6 months after transplantation; data not shown). Thus stem cell competition versus other possible mechanisms of downregulating parental engraftment could not be distinguished due to the hemopoietic reconstitutive capacity of the spleen inoculum. Irradiation of the F_1 spleen cell inoculum eliminates this capacity (see text).

cells. Complement-mediated depletion of Thy1-positive cells in the spleen cell inoculum did not abrogate the transfer or resistance to engraftment (figure 6A). Thus we have established that administration of CFA, which increases host NS activity and resistance to hemopoietic engraftment (4), induces a Thy1-negative cell population(s) in the spleen that can mediate adoptive transfer of resistance to marrow engraftment.

DISCUSSION

The general goal of our current studies is to evaluate the role of the bone marrow microenvironment on hemopoietic engraftment in unirradiated hosts. In this report, medullary engraftment kinetics are analyzed with respect to a bone marrow-associated inhibitory function broadly termed natural suppressor activity. We previously reported that long term engraftment is decreased in hosts with elevated NS activity at the time of transplantation (4). This regulatory activity, normally present in neonatal spleen and adult bone marrow, is increased in bone marrow and induced in spleen following administration of complete Freund's adjuvant to adult mice. In these studies, hosts are conditioned by facilitating anti-MHC class I monoclonal antibodies, thus yielding complete, long term engraftment without host irradiation, which enables us to study engraftment in an environment which is not altered like that found after lethal irradiation. Our current results indicate that engraftment kinetics closely correlate with host NS regulatory function rather than total marrow cell numbers or CFU-GM content in the host marrow cavity at the time of transplantation.

The detailed findings reported here can be summarized as follows: 1. donor hemopoietic engraftment, measured as the percentage of total CFU-GM of donor origin at early time points after transplantation, is accelerated when the medullary cavity shows

reduced NS activity; 2. single exposure to conventional conditioning agents induces chimerism in animals in which bone marrow NS activity is reduced, but fails to do so in animals in which NS activity is not reduced, despite a marked cytoreductive effect in the marrow; 3. there is no correlation between the number of host CFU-GM (or total cellularity) in the medullary cavity at the time of transplantation and the engraftment rate of donor CFU-GM; 4. adoptive transfer of irradiated spleen cells with NS activity delays the rate of engraftment; and 5. *in vivo* and *in vitro* data concur to suggest that cells responsible for conferring resistance to engraftment and NS activity are Thy1-negative, radiosensitive cells.

To directly measure the rate of engraftment of donor hemopoietic cells in the marrow cavity, we have developed a technique to determine the genetic origin of individual cells composing the functional hemopoietic pool at any given time. After harvesting bone marrow cells, *in vitro* colonies are generated, using the appropriate growth factors for the lineage of interest. Individually picked colonies are then typed for their GPI isozyme by electrophoresis to determine whether they are of donor or host origin. This simple and accurate technique should be of interest for analysis of engraftment kinetics and differential engraftment between hemopoietic lineages, as well as for evaluation of the effects of different conditioning regimens and the administration *in vivo* of growth factors on the process of engraftment. In order to correctly interpret the data, however, a number of assumptions will have to be defined : that growth in the *in vitro* colony assays reflects hemopoietic output *in vivo* ; that CFU-GM measurements (the lineage chosen here) are indicative of general phenomena bearing on the engraftment process which affect other lineages, including primitive stem cells; and that the early influx of donor hemopoietic cells is somehow related, in a predictable manner, to long term, stable stem cell engraftment. Indeed, the progenitors measured in colony assays in the immediate post-transplant period

are unlikely to be derived from recently engrafted totipotent stem cells. It is interesting to note that in control animals which were not administered facilitating antibody, there was no detectable long term donor chimerism, nor were there appreciable numbers of donor-derived colonies in the early post-transplant period, suggesting that early post-transplant events may, in some cases, be predictive of long term peripheral blood chimerism.

Using this assay, we were able to measure the rate of donor hemopoietic engraftment in the marrow cavity itself as a function of host bone marrow cellularity and host NS activity in the time period immediately after marrow infusion. Using unirradiated antibody-conditioned bone marrow recipients, we found that marrow engraftment was accelerated in hosts transplanted when their NS activity was reduced, without any relation to the status of host bone marrow cellularity. These results were extended to other conditioning agents. Thus we have found that exposure to doses of radiation that effectively condition the host for stable donor engraftment also reduce host bone marrow NS activity. Similarly, exposure to cytotoxic drugs facilitated engraftment only in animals in which NS activity was reduced, irrespective of the cytoreductive effect. Despite substantial reductions in the recipient's marrow cellularity following treatment with methotrexate and low-dose cyclophosphamide, donor engraftment was, at best, only marginally increased (table 1). Conversely, we had shown earlier that administration of complete Freund's adjuvant induces NS activity in the spleen (4), as reported by others with different adjuvants (11-14), and increases resistance to engraftment (4). We report here that this activity is mediated by Thy1-negative spleen cells (figure 4). The adoptive transfer data reported herein indicate that there are cells present in the spleen following adjuvant administration that delay engraftment. These cells are Thy1-negative, as is the case for the cells mediating natural suppression *in vitro* . On the other hand, the adoptive transfer of normal spleen did not confer resistance to engraftment, and NS activity was not

present in normal adult spleen. The partial inactivation of NS function by *in vitro* exposure to 8 Gy (figure 5) may explain the need for repeated administration of the cells and the rapid waning of the transferred activity after the last injection (figure 6). To further understand the mechanisms controlling engraftment in this model, transfer of purified cell populations will be necessary. Based on the extensive correlations between natural suppressor activity and resistance to engraftment, natural suppressor assays appear to provide a valuable technique for purifying the cells that mediate resistance to engraftment in the syngeneic and semi-allogeneic host.

Natural suppressor activity refers to antigen non-specific, non-H-2-restricted suppression and has been detected by the PFC assay (15-17), the mixed leukocyte reaction (5, 18-20) and the mitogen response (6). It is not known whether NS activity is mediated by the same cells in fetal liver (20), neonatal spleen (21-24) and adult bone marrow (15-18, 25), which share the common feature of being the dominant sites of hemopoiesis at each stage of development. There are at least two types of cells that can mediate NS activity, one related to monocytes (26) and the other of unknown lineage (5, 6). Despite some discrepancies, a consensus phenotype has emerged for the latter, namely $Thy1^-$, $CD4^-$, $CD8^-$, surface Ig-negative, non-adherent cells of low density which do not kill YAC-1 cells. Nearly all lineages have been proposed, including myeloid (23, 24, 27, 28), lymphoid (18, 29), NK-related (5, 30) or primitive stem cells (31), as well as a direct lineage relationship between both types of cells mediating natural suppression (23, 28). The effect of NS cells on hemopoiesis is still unknown, although their presence is physiologically restricted to sites of ongoing hemopoiesis. Based on the observations reported here, we suggest that cells mediating this inhibitory activity are capable of controlling seeding and/or proliferation of hemopoietic cells entering the unirradiated host marrow cavity. This raises the question of whether it is indispensible to administer

cytotoxic therapy to "create space" in the marrow in order to secure good donor representation. Successful hemopoietic stem cell engraftment, in a number of different situations, has been linked to conditions that create space in the host bone marrow (32). This is achieved by administering cytoreductive conditioning regimens to the recipient prior to marrow infusion, using either Y-radiation (33, 34) or chemotherapeutic agents (10, 35-37). The current results indicate that it is unnecessary to induce medullary aplasia to achieve substantial donor representation. Rather, engraftment can be achieved by selectively abrogating a critical host regulatory activity present in the bone marrow (38). Our data suggest in fact that reduction of host NS activity as well as pancytoreduction are achieved by standard conditioning regimens, which therefore do not allow to distinguish between these two types of mechanisms. Based on the correlation we observed between promoting engraftment and reducing bone marrow NS activity when administering methotrexate and cyclophosphamide, it is interesting to speculate that the reduction *in vivo* of bone marrow NS activity could serve to predict the value of any other drug as an effective conditioning agent. In another model using congenitally anemic mice, the facility to engraft such animals was suggested to reflect the competition between normal donor and defective host stem cells and, as well, did not correlate with host bone marrow cellularity (39, 40). NS regulatory control might also apply to endogenous repopulation following bone marrow transplantation, the eventual stimulation of host regulatory activities leading to the prevention of effective donor stem cell engraftment. A regulatory control of hemopoietic engraftment is supported by the resistance to engraftment which follows *in vivo* recombinant granulocyte/macrophage-colony stimulating factor (GM-CSF) administration (41), which is possibly due to hemopoietic negative feedback inhibition. Indeed, GM-CSF has been shown to increase NS activity *in vitro* (42) and *in vivo* (M.S., unpublished observations).

While the current findings support the regulatory model of control of stem cell engraftment, the distinction between this and the "space" hypothesis disappears if this regulatory activity directly reflects the size of the is cycling stem cell pool (31). But whatever the effector cell, a physiological role for NS activity in controlling hemopoiesis, and thereby hemopoietic engraftment, is clearly suggested. In this model, NS regulatory activity, produced either by cycling hemopoietic stem cells or some specialized progeny, maintains other stem cells in a slowly proliferating or quiescent state. Such inhibitory activity is therefore expected to apply to blood-borne hemopoietic cells as well, which results in interference with hemopoietic engraftment in the unirradiated host. Negative hemopoietic regulation has been suggested in radiation chimeras as well (43). In these studies, lethally irradiated recipient mice were reconstituted with two small consecutive, isozyme-marked bone marrow inocula, spaced 8 days apart. The poor engraftment of the second inoculum could not be explained by lack of space or competition for growth factors, suggesting that the first inoculum inhibited engraftment of the second. Another role proposed for NS cells is based on their immunosuppressive properties *in vitro* and the association of their presence in spleen with the induction of allogeneic tolerance in certain radiation chimeras (5, 44). A role in autoimmunity has been suggested as well (45, 46). While it is possible that NS activity as it relates to stem cell control and tolerance induction could have a different cellular and/or molecular basis, it is interesting to note that there are molecules, such as TGF-ß, that suppress both lymphocytic responses (47) and hemopoietic stem cell proliferation (48). To address these issues, we are currently investigating how anti-MHC antibody treatment decreases NS activity *in vivo* , as well as methods of more specifically boosting or depleting bone marrow NS activity *in vivo* . The purification of the cells mediating NS activity in the bone marrow will allow us to determine whether they can be distinguished from primitive hemopoietic stem cells. With a better understanding of the

mechanisms controlling hemopoietic stem cell engraftment, we hope that more specific and thus more innocuous conditioning regimens can be designed, permitting the extension of bone marrow transplantation to applications such as gene therapy.

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Conditioning [a]	Bone Marrow Cellularity[b]	NS Activity[c]	Donor Chimerism[d]
None	100 ± 8%	1.0	<4%
3Gy	78 ± 3%	3.2	81 ± 3%
6Gy	64 ± 4%	4.0	98 ± 2%
9Gy	37 ±5%	4.8	99 ± 2%
MTX	64 ± 4%	1.0	4 ± 4%
low CY	69 ± 8%	1.0	<4%
high CY	31 ± 14%	3.6	31 ± 21%
anti-H-2	83 ± 9%	3.8	97 ± 4%

Table 1. *In vivo* effect of *Y*-radiation, methotrexate, cyclophosphamide and facilitating antibody on bone marrow total cellularity, natural suppresor activity and induction of donor hemopoietic chimerism.

[a] Adult F_1 hybrid mice were either sacrificed to harvest their bone marrow or administered intravenously 20 million Thy1-depleted parental marrow cells after receiving the following treatments: exposure to 3Gy, 6Gy or 9Gy *Y*-radiation; administration of 1mg MTX i.p., 1 or 4 mg CY i.p., or 500μg of facilitating antibody i.v.

[b] Bone marrow cells from both hindlimbs were harvested and nucleated cells counted using trypan blue exclusion. 100 ± 10% represents 32 ± 3.3% million cells (n=3 to 6 mice). These numbers represent host bone marrow cellularity 12 hours after irradiation, 24 hours after drug administration and 7 days after antibody administration.

[c] NS activity was assayed in the primary *in vitro* PFC assay by titrating the bone marrow in each culture well. Bone marrow cell numbers required to suppress 50% of the control response (SD_{50}) are shown, where 1.0 represents about $1.5x10^5$ normal bone marrow cells. Data are calculated from one representative experiment of three; s.d. are less than

20% and not shown. These numbers represent host bone marrow NS activity at the time of donor marrow infusion (2 hours after irradiation, 24 hours after drug administration, 7 days after antibody administration).

[d] Chimerism levels in the periphereal blood either 6 months (radiation-induced and antibody-facilitated chimerism) or 3 months (drug-induced chimerism) after transplantation are shown as mean donor representation (%) ± s.d. (n=6 to 10 mice per group).

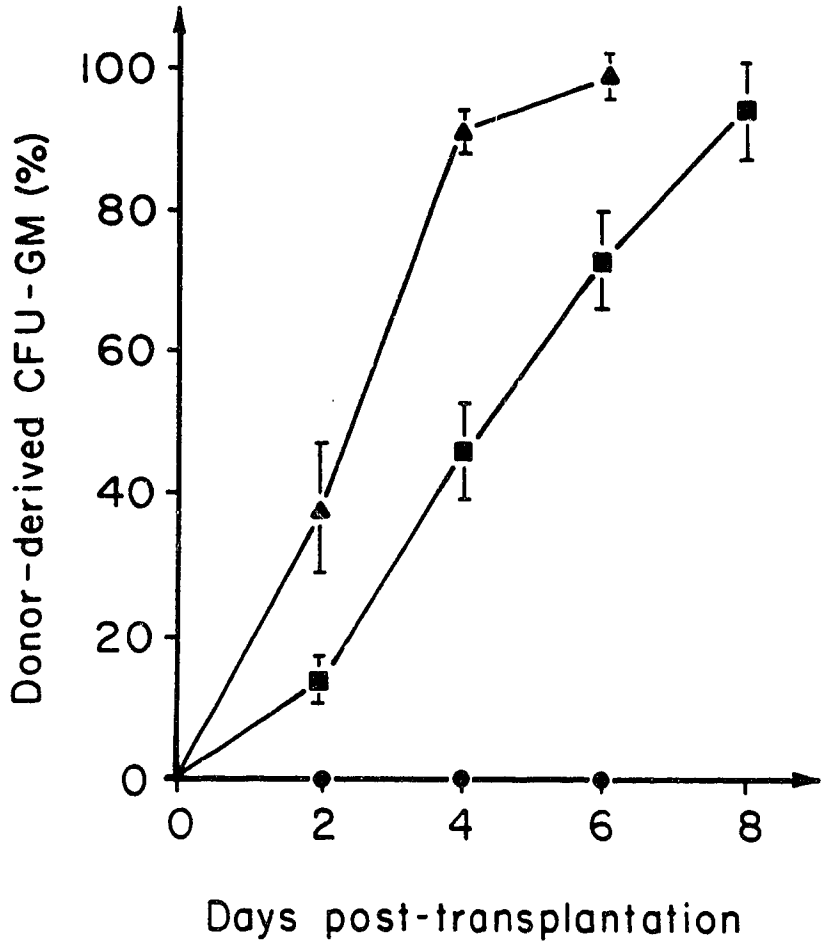

Figure 1. Engraftment in the marrow cavity is accelerated in recipients given facilitating antibody 7 days prior to transplantation (▲) when compared to recipients given bone marrow cells and facilitating antibody together (■). In the absence of antibody, donor representation does not exceed 2% (●). Chimerism is expressed as the percentage of CFU-GM colonies of donor origin, based on isozyme content. Each data point represents 4 or 5 mice, expressed as the mean ± s.d.

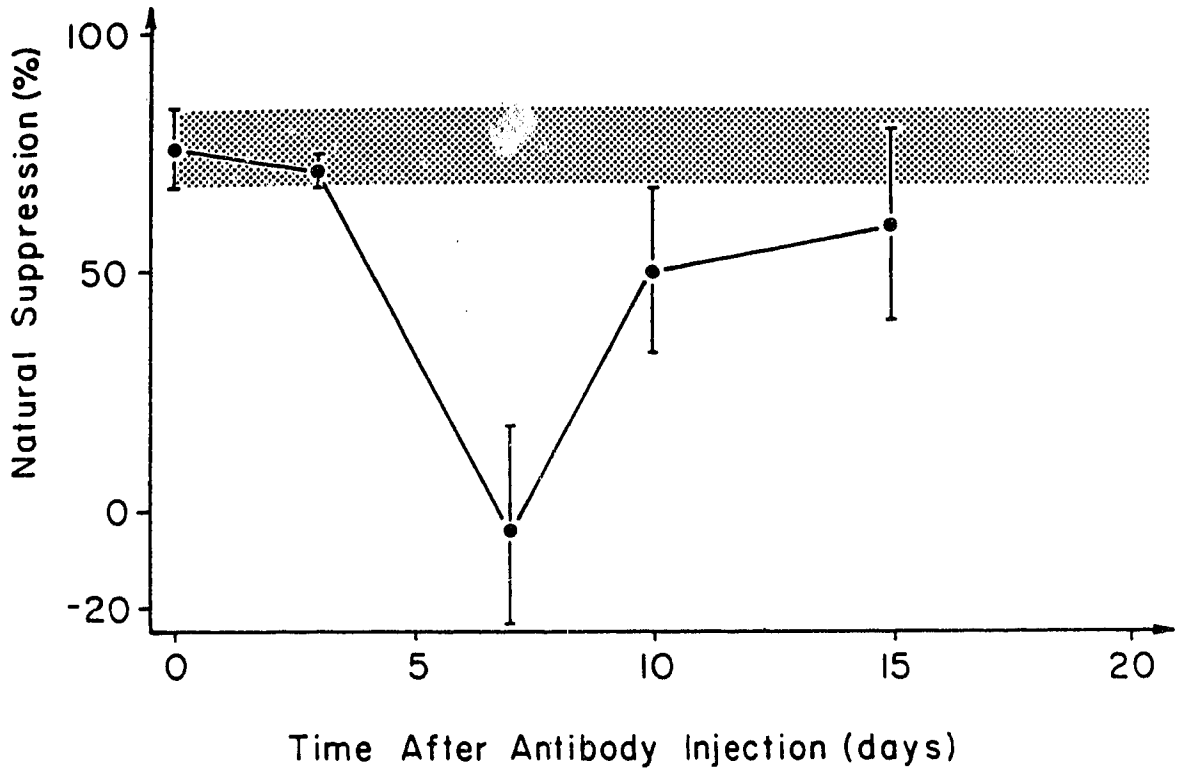

Figure 2. Kinetics of bone marrow-associated natural suppressor activity after administration of facilitating antibody. The suppressor activity is expressed as the percentage suppression of the control response in the primary *in vitro* PFC assay. 2.5×10^5 bone marrow cells, harvested at different time points after antibody administration, are added to each culture well (1:40 bone marrow : spleen cell ratio). Each data point is averaged from 3 to 7 experiments, each normalized to the control response of that experiment (the control response, or 0% suppression, representing 200 to 500 PFC/million spleen cells in different experiments). Results are expressed as mean ± s.d. (which is shaded to indicate suppressor activity in the absence of antibody administration).

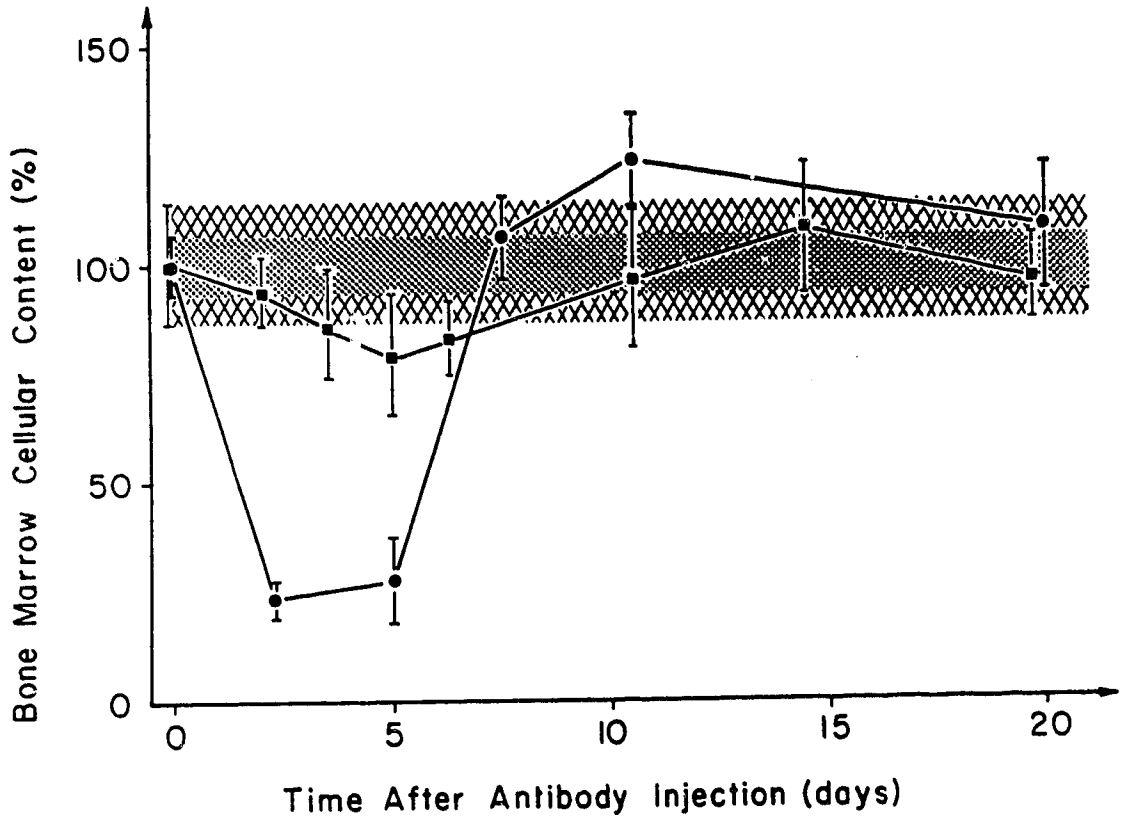

Figure 3. Kinetics of total bone marrow cellularity (■) and bone marrow CFU-GM content (●) after administration of facilitating antibody. Each data point represents 4 to 7 animals (■) or 4 animals (●), expressed as the mean ± s.d., where 100 ± 14% (lightly shaded area) represents 17.5 ± 2.5 million nucleated bone marrow cells and 100 ± 8% (densely shaded area) represents 25 ± 2 thousand CFU-GM per hindlimb.

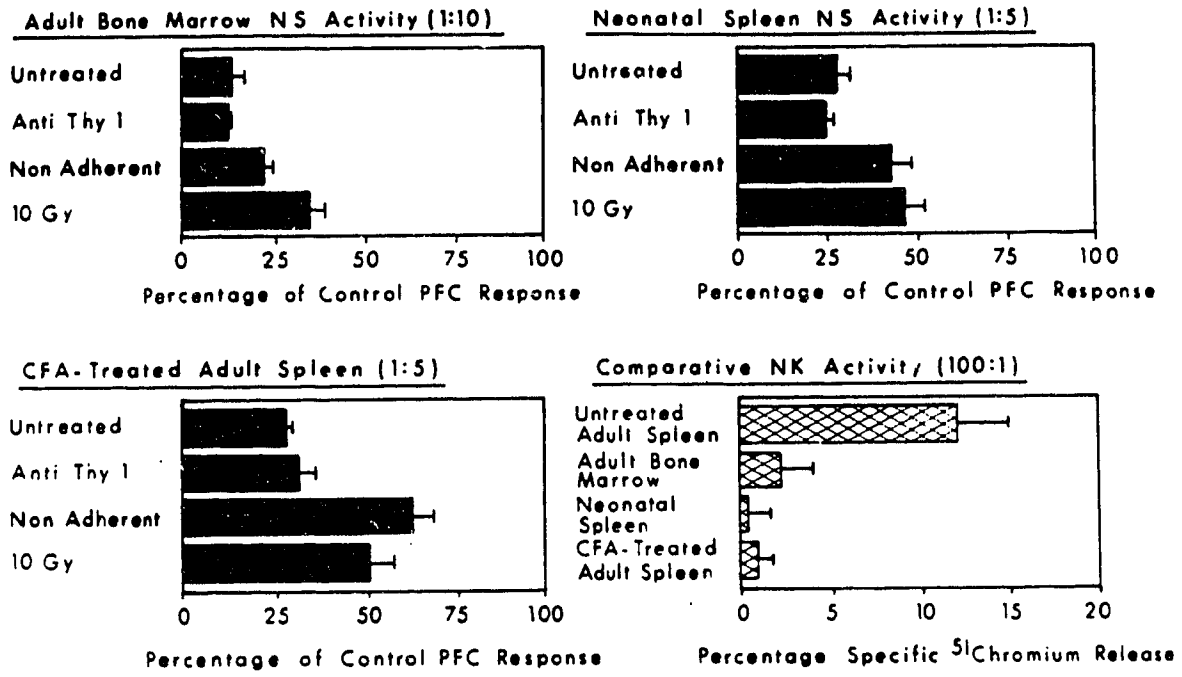

Figure 4. Comparison of natural suppressor activity (first three panels) and natural killer activity (lower right panel) in adult bone marrow, spleen from 5 day old mice and CFA-treated adults. Suppression is expressed as the percentage of control response measured after mixing in the source of suppressor cells in the PFC cultures (the suppressor : target cell ratio is indicated for each panel). "Untreated" represents an untreated source of suppressor cells, "Anti-Thy1" anti-Thy1.2 + complement-treated cells, "Non-Adherent" nylon wool non-adherent cells, and "10Gy" cells irradiated 10 Gy *in vitro* . Natural killer activity is expressed as percentage specific ^{51}Cr release for each tissue at the 100:1 effector : target ratio. Each data point is expressed as the mean ± s.d., each panel representing one of three comparable experiments.

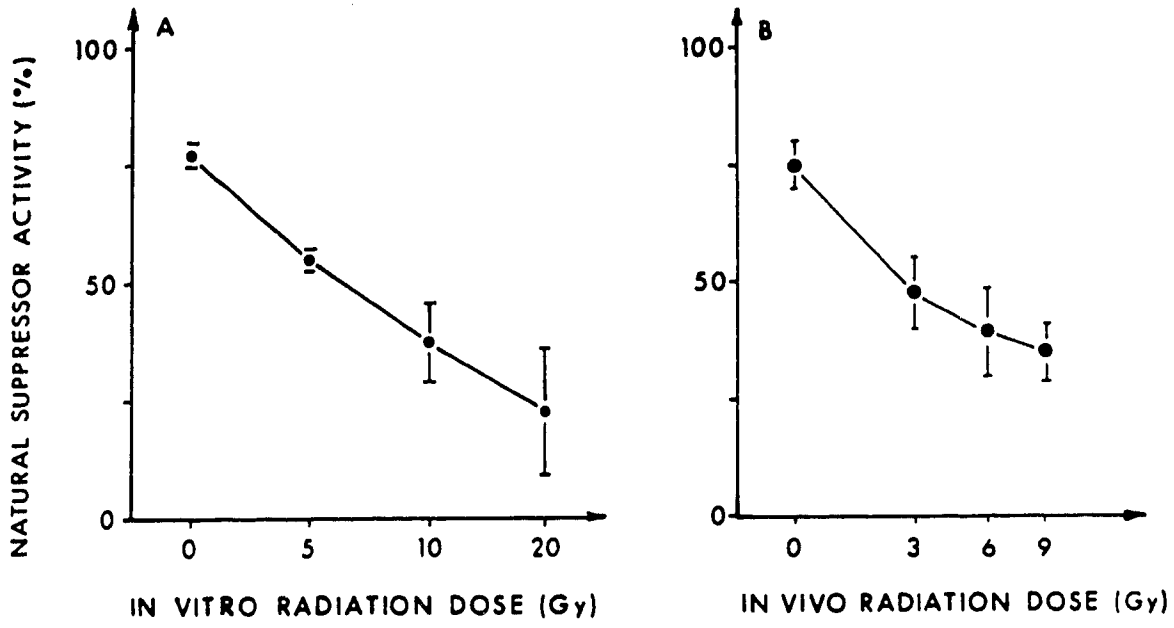

Figure 5. Effect of *Y*-radiation on bone marrow natural suppressor activity, exposed either *in vitro* (A) or *in vivo* (B), 2 hours prior to harvesting bone marrow. Radiation dose is plotted on a logarithmic scale. Percentage suppression, at the 1:40 bone marrow : spleen cell ratio, is plotted relative to the control response (100 ± 11% in the absence of bone marrow cells), as mean ± s.d. (n=3) for each data point from one representative experiment of two.

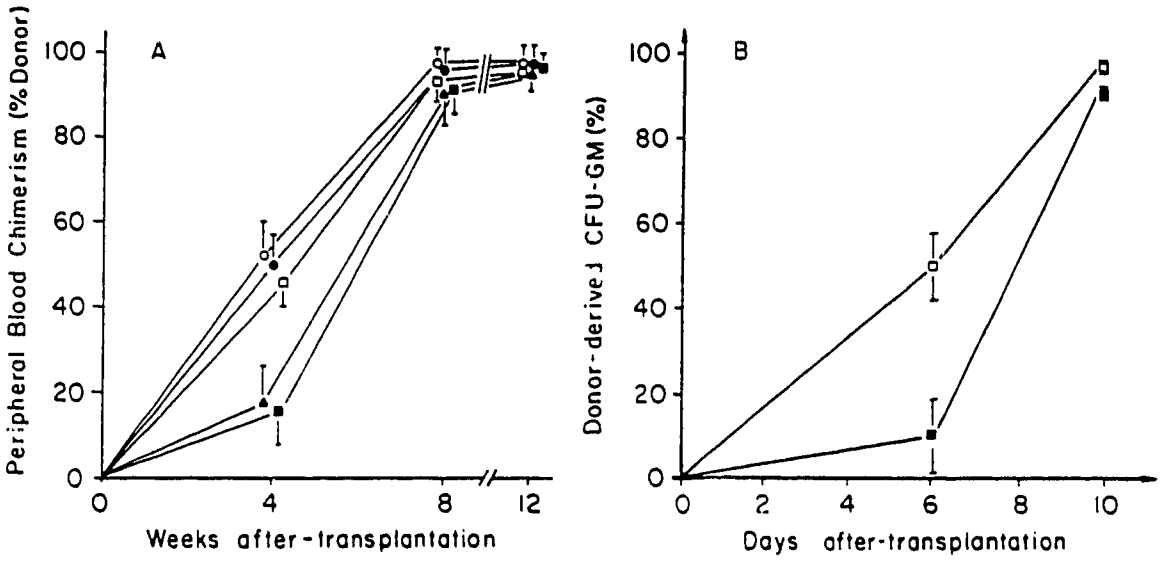

Figure 6. Adoptive transfer of natural suppressor activity delays engraftment kinetics, determined in the peripheral blood (A) and in the marrow cavity (B). In addition to receiving 20 million parental bone marrow cells and 500μg facilitating antibody on day 0, hosts were given either no additional treatment (0)(n=12), 60 million 8Gy-irradiated spleen cells from CFA-treated donors on day 0 (●)(n=9), 60 million 8Gy-irradiated spleen cells from saline-treated donors on days 0, 2 and 4 (▫)(n=13), 60 million 8Gy-irradiated spleen cells from CFA-treated donors on days 0, 2 and 4 (■)(n=10) or the equivalent of the latter inoculum after depletion of Thy1-positive cells (▲)(n=9). Each data point represents the mean ± s.d. for n animals (indicated above for panel A) and for 5 animals in panel B.

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of activity of any given THSC is regulated by the degree of activity of neighbouring THSC, the sphere of influence of one active THSC defining a domain in which other host or transplanted THSC would be inhibited and kept in reserve.

There are at least two major consequences of the NS-regulated model of engraftment, one relating to the physiological function of NS activity, and the other bearing on the concepts which dictate approaches to bone marrow transplantation. Firstly, NS cells may be the cells controlling stem cell activity, possibly as part of a negative feedback loop regulating stem cell proliferation, and hence hemopoietic engraftment in the unirradiated host. These predictions can be readily tested in *in vitro* hemopoietic assays and then *in vivo* , once effector molecules mediating NS activity are purified. Furthermore, the links between hemopoietic activity and NS activity suggest conditions under which NS effector cells may be propagated *in vitro* . One might even speculate further that NS cell dysregulation plays a direct role in hematologic disorders such as aplasia or polycythemia vera. Secondly, in the context of bone marrow transplantation, we suggest that new approaches to host conditioning may be designed based on the regulatory model of engraftment. Their rationale would be to specifically interfere with the inhibitory signals which downregulate engraftment, by using monoclonal antibodies which target NS cells or their effector molecules *in vivo* . The justification for such approaches is to avoid creating an aplastic state prior to transplantation, thus abrogating the use of lethal conditioning regimens and avoiding unnecessary non-specific toxicity.

2. Role of NS activity in immunological tolerance.

In the context of immunological tolerance and immunosuppression, in contrast to hemopoiesis, there is litereature dealing with roles of NS activity *in vivo* . NS activity has

been linked with tolerance to soluble antigen (20) and alloantigen (1,21), and graft-vs-host disease (GVHD) (2,22). The role of NS activity has been extensively reviewed by several groups (1,2,22). In the context of tolerance, Strober and collaborators have investigated the mechanisms by which total lymphoid irradiation (TLI) appears to decrease host-vs-graft and graft-vs-host reactions and improve survival after allogeneic transplantation, at least in mice. Shortly after administering TLI, a surge in NS activity takes place in spleen. This influx has been linked to tolerance induction in allogeneic chimeras. These investigators suggest that, by some undefined mechanism, the development of a mixed leukocyte reaction in the presence of NS cells results in the generation of suppressor T cells specific for alloantigen, rather than alloreactive cytotoxic T cells. Such long-lived antigen-specific suppressor T cells, which survive long after the splenic NS activity has regressed, then maintain tolerance in vivo . This model, despite the absence of any molecular basis, is supported by the *in vivo* observation that the adoptive transfer of interleukin-2-dependent cloned cells with NS activity decreases the incidence of GVHD in radiation chimeras (23). Similarly, findings in double chimeras (syn + allo → syn) support a role for NS cells in the induction of tolerance in radiation chimeras (21). In these studies, the addition to the allogeneic inoculum of syngeneic marrow cells, which, in our view, act like the endogenous marrow cells present after treatment with TLI, increases splenic NS activity and enhances survival of the animals. No other molecular or cellular basis has been proposed by Sachs and colleagues to explain this observation. In GVHD associated with bone marrow transplantation across minor histocompatibility barriers in radiation chimeras, Claman and colleagues have argued that the state of immunosuppression is largely due to the presence of NS activity in spleen (2,22).

Thus, in this context, our findings in diabetic animals appear to extend the association between elevated NS activity and tolerance to soluble antigen and alloantigen to

self antigen. With respect to autoimmunity, there is no available literature linking NS activity and tolerance to self, except for one report from S. K. Singhal's laboratory. These investigators have found an *in vitro* defect in NS activity and a reduced production of the inhibitory lipidic factor they described in NZB mice, thus suggesting a link between defective NS activity and autoimmunity (24). In our studies, we have shown that the boosting of NS activity in diabetes-prone animals at an early age, that is before insulitis develops, is associated with decreased disease incidence and increased survival. While the boosting of NS activity was not specific and the reported studies fall short of demonstrating a cause-effect relationship between NS activity and autoimmunity, we believe our hypothesis is sensible in the context of the aforementioned literature.

3. NS activity: a link between hemopoiesis and immunological tolerance?

One final question may be raised: can the two functions proposed for NS cells, one pertaining to hemopoietic engraftment and stem cell regulation, the other to immunological tolerance, be related? It should first be recalled that the cell population(s) and putative molecules mediating natural suppression are not yet clearly defined, and it is therefore possible that different functions are simply mediated by different effectors. Although the effector cells are defined as Thy1-negative, surface immunoglobulin-negative, plastic non-adherent, radiosensitive cells, it is obvious that these characteristics apply to many different cell types, possibly including more than one cell type with NS activity. It is nonetheless tempting to speculate on the existence of a single common NS cell mediating these apparently diverse functions. This proposed cell could therefore establish a direct relationship between hemopoiesis and immunological tolerance.

The physiological relevance of such a relationship, however, is a questionable

matter, because the tissue distribution of NS activity is so restricted. Indeed, while a potent inhibitory function might affect the establishment of tolerance in radiation chimeras, immunodeficiency in radiation-induced GVHD or tolerance in adjuvant-treated autoimmune animals, none of these situations is strictly physiological. In the extreme, these situations may only represent the fortuitous effect of one function (splenic hemopoiesis) achieving extraneous goals (T cell tolerization). It is more difficult to accomodate a role for NS activity with respect to tolerance in the normal adult animal. Indeed, while NS activity may downregulate mature T lymphocyte functions, bone marrow-associated T cells are only a small population in the mouse. Moreover, tolerization of T cells takes place in a separate organ, the thymus, where there is no NS activity (see tables I-1 and I-2). But this is not the case with regard to B cells, which therefore may be the physiological target of NS activity. In this scenario, an effect on T cells may only represent a side-effect. An alternative possibility, incorporating the capacity to suppress both types of lymphocytes, is that the lymphoid precursor cell is critically regulated by NS activity within the bone marrow. In this case, the overall production of lymphocytes may be regulated in a quantitative aspect, by limiting the proliferation of the common precursor and, perhaps, for the T cell lineage, by preventing differentiation in the marrow cavity, an occurence properly managed in the thymus only. It is then not surprising that the NS effector cell retains its ability to suppress the mature progeny of the lymphoid precursor cells, that is T and B lymphocytes. Finally, it is possible that the true function of NS activity lies only in the control of non-lymphoid hemopoietic cells, yet happens to also suppress mature lymphocytes, an event of little physiological importance whether it is acting on the lymphocytes themselves, their ontogenic precursors or by preventing macrophage-dependent activation, but which provides us with an easy way of monitoring this activity *in vitro* . Resolution of these issues will require a far greater understanding of the molecular basis of NS activity and the

development of animal models in which NS activity can be ablated or increased throughout ontogeny.

In conclusion, we have achieved the proposed objective of defining animal models in which the role of natural suppression, a potent inhibitory immunoregulatory activity, can be analyzed. Our results indicate that NS cells may play a role in regulating hemopoietic engraftment in bone marrow transplant recipients, and support a role in immunological tolerance in the context of autoimmune diabetes.

Table 1: Antibody-facilitated engraftment in genetically NK-deficient mice.

n[a]	host strain[b]	antibody[c]	chimerism[d]
5	C3B6	none	<1%
5	C3B6-*bg*^{2}/*bg*	none	<1%
5	C3B6	400μg	26±9%
6	C3B6-*bg*^{2}/*bg*	400μg	22±11%
4	C3B6	900μg	53±13%
6	C3B6-*bg*^{2}/*bg*	900μg	57±8%

[a] number of mice per group

[b] strain of host mice: (C3H.HeJ x C57BL/6J)F_1 or (C3H/HeJ-*bg*^{2} x C57BL/6J-*bg*)F_1 hybrids

[c] antibody dose (16-3-1N) co-administered with 2x10^7 C57BL/6J-*gpi1*^{a} bone marrow cells, following the standard antibody facilitation protocol

[d] peripheral blood chimerism 6 months after transplantation. This data was also referred to in the discussion of chapter III

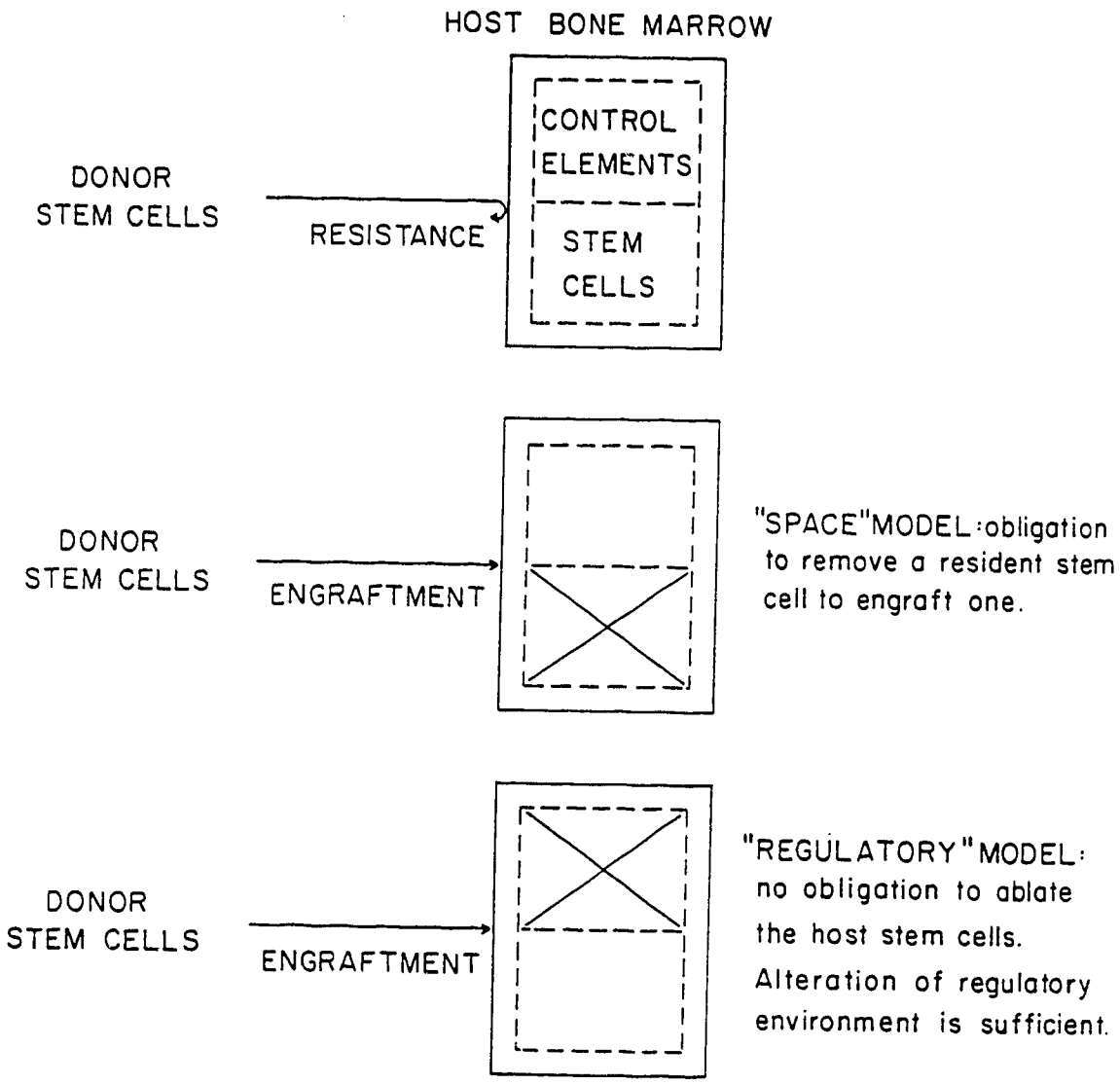

Figure 1: space vs regulation.

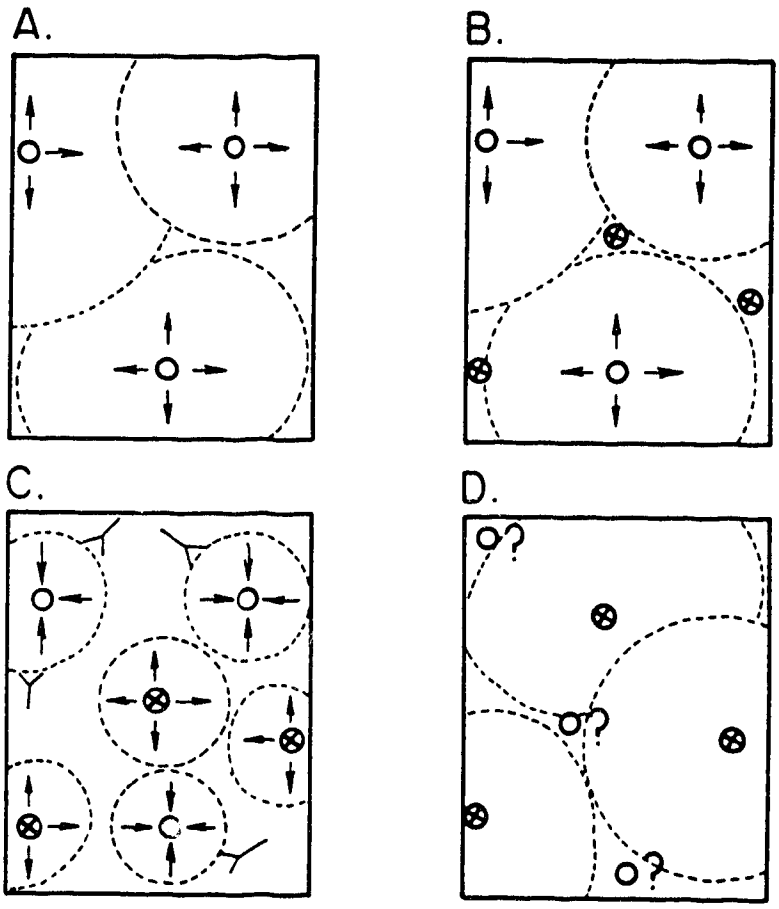

Figure 2. Delayed administration of facilitating antibody in the P→F_1 transplantation model unleaches proliferation of parked parental precursor cells. A. Host stem cells are symbolically represented by one cell surrounded by a halo containing its dividing progeny. B. Administration of isozyme-marked parental cells results in homing to the bone marrow but not proliferation of the donor precursor cells. C. Delayed anti-host antibody administration induces the involution of the host-type hemopoietic foci and permits proliferation and differenciation of donor precursor cells. D. The marrow is eventually reconstituted by donor stem cells.

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APPENDIX I: Operational definition of NS activity *in vitro* .

The first objective of the work presented in this thesis was to set up a dependable assay to monitor NS activity *in vitro* . In the absence of specific markers for the NS effector cells, the cells cannot be easily isolated or even quantified in any given tissue sample. It is therefore necessary to resort to biological assays to measure activity. While it is clear from the literature on NS cells that the activity can be semi-quantified, it is also evident that a number of limitations apply to these functional assays, because the measured function is not exactly defined. Firstly, there is possibly more than one kind of NS cell, and maybe more than one inhibitory mediator for each cell type, which may or may not be independently regulated. Secondly, the relationship between "NS cells" from different sources (bone marrow, neonatal spleen and pharmacologically-induced) is unknown. Thirdly, the actual effector cells may be different in each *in vitro* assay. Finally, it should be borne in mind that the use of inbred strains of animals may introduce hitherto unknown peculiarities pertaining to NS activity and its regulation in one strain versus another.

We therefore first compared three conventional assays used to monitor NS activity. (i) The primary in vitro antibody response was measured after a five day Mishell-Dutton culture of spleen cells in the presence of prescreened sheep red blood cells (SRBC) and 10% fetal bovine serum (FBS). The response was quantified by directly enumerating plaque forming cells (PFC) in the presence of fresh SRBC and guinea pig complement, under the microscope using Cunninghan slides. (ii) The mitogen-induced proliferative response of spleen cells was measured by the incorporation of tritiated-thymidine (^{3}H-TdR) over 12 hours after 72-hour culture in the presence of either concanavalin A (ConA, 2μg/ml) or lipopolysaccharide (LPS, 10μg/ml) in 5% FBS-containing medium. (iii) Proliferation to alloantigen was measured by the incorporation of ^{3}H-TdR over a 24 hour

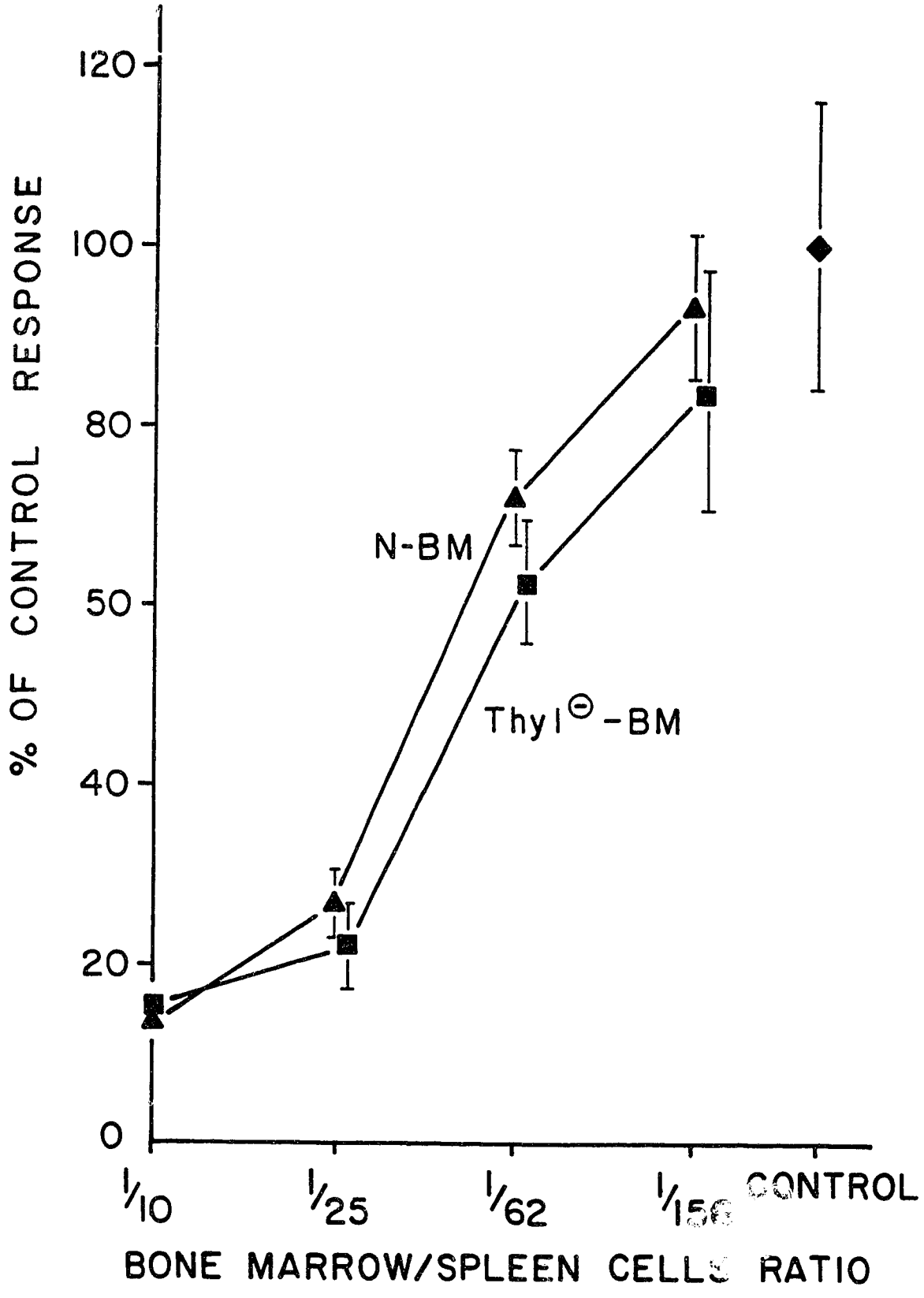
period following a 96 hour culture in the presence of irradiated (20Gy) allogeneic (C3H/HeJ) spleen cells in 8% FBS-containing medium. For all three assays, bone marrow cells were harvested from tibias and femurs from 7-10 week old adult C57BL/6J (B6) or (BALB/cJ x C57BL/6J)F_1 (CB6) mice. After washing the cells, titrated amounts of bone marrow cells were added to syngeneic responder spleen cells at the onset of culture. The following results were obtained. In the PFC assay, plaque generation was reduced by 20 to 100% following the addition of bone marrow cells in ratios of 1:100 to 1:10 (bone marrow : spleen cell ratio at the onset of culture). In the mitogen-induced responses, ^{3}H counts were reduced by 20 to 95% by the addition of bone marrow cells in ratios of 1:4 to 1:1 (bone marrow : spleen ratio) in the LPS response; in the ConA response, the reduction varied between -50% and 25% for the same ratios. In the mixed leukocyte reaction (MLR), ^{3}H counts were reduced from 20 to 60% by the addition of bone marrow cells in ratios of 1:4 to 1:1 (bone marrow : spleen ratio).

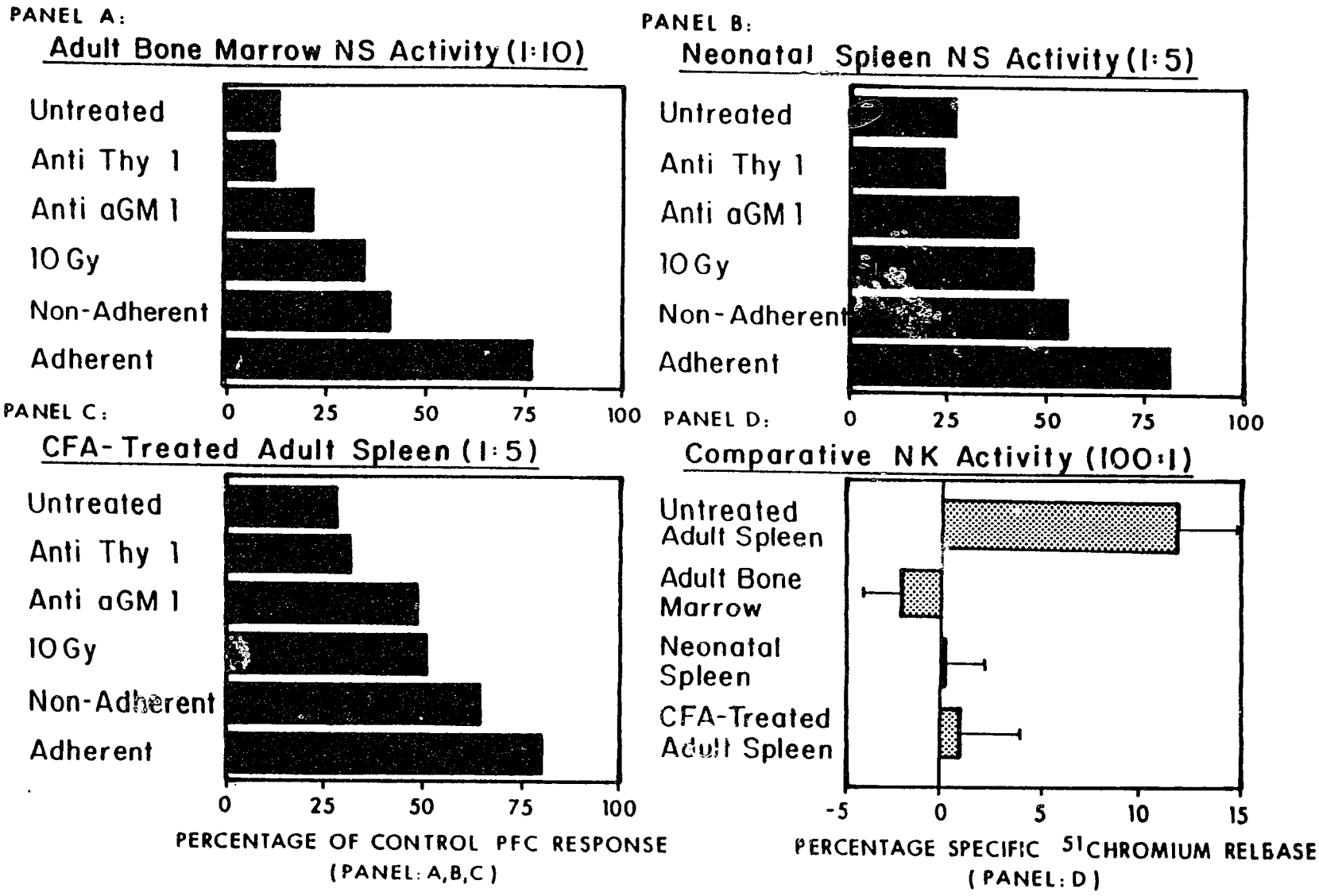
The PFC assay was selected as the reference assay because of its greater susceptibility to suppression and a wider range over which bone marrow cells were inhibitory, covering a 10-fold variation in marrow cell dose. As a secondary assay, the proliferative response to LPS was chosen over the MLR because of its greater susceptibility to suppression, and because it is a shorter and more consistent assay. The response to ConA was only marginally reduced within the same range of bone marrow : spleen ratios and was not investigated further. An example of suppression of the PFC response is shown in figure 1. This assay can be used to determine the phenotype of the NS cell. As indicated in figure 1, the lack of effect on NS activity after treating the marrow with anti-Thy1.2 monoclonal antibody and complement indicates that the effector cells are Thy1-negative. Since the phenotype of the cells mediating natural suppression in the PFC assay is described and discussed more thoroughly in chapters IV and V, the phenotype will only

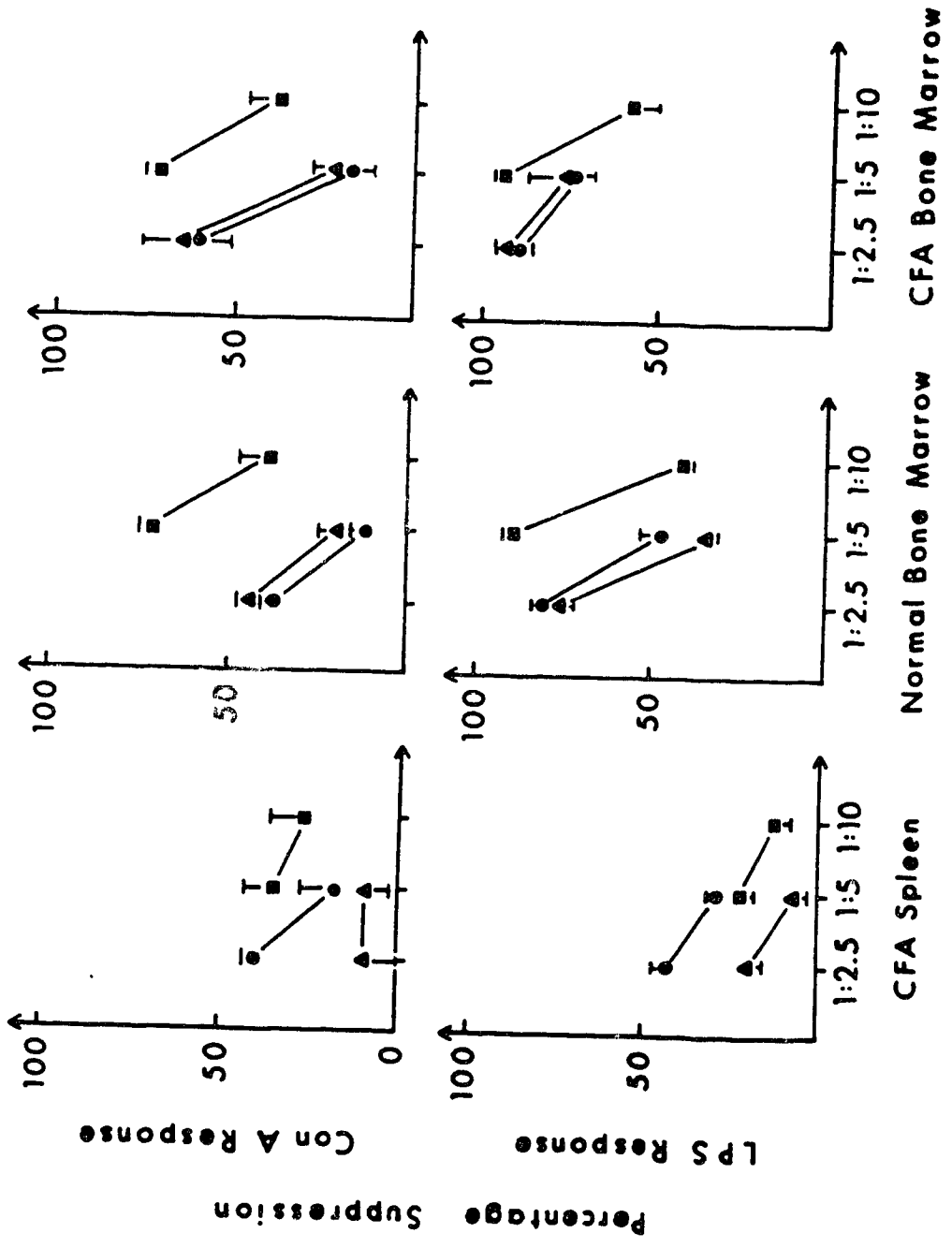
be briefly reviewed here. As shown in figure 2, NS activity, but little or no NK activity, is found in the adult marrow and, to a lesser degree, in the spleen of 5 day old animals and in the spleen of adult mice given 100µl of complete Freund's adjuvant (CFA) 8 days earlier. The 1:10 and 1:5 ratios indicated above each panel refer to the bone marrow/test spleen : responder spleen ratios used in the PFC assay. In all three tissues, suppression is mediated by Thy1-negative, partially asialo-GM1-positive cells mostly present in the plastic non-adherent fraction, although some suppressive function is present in the adherent fraction as well (more so in the CFA-treated adult spleen than in the two other tissues). Exposure to gamma-radiation (10Gy) reduced the activity by about half. In order not to duplicate information reported in chapter V, the following phenotypic characteristics will be described in the proliferative response assays (mostly the response to LPS unless indicated otherwise). The adherence properties of NS effector cells are summarized in figure 3, in which the response in experimental wells is expressed as the percentage of the response obtained in contol wells by adding equal numbers of adherent or non-adherent spleen cells prepared from normal adult spleen. Unseparated cells from each tissue are represented by●, plastic non-adherent cells by▲, and adherent cells by■. These data, averaged from three separate experiments, indicate that there are two effector cell populations, one adherent and one not. While the adherent cells appear to be more suppressive on a cellular basis, most of the total suppressor activity resides in the non-adherent fraction. As indicated in figure 4, the radiosensitivity profile of marrow cells is shown: the level of suppression achieved by normal marrow (●) is reduced by about half following *in vitro* exposure to 5Gy (◧), and by half again after exposure to 10Gy (▲) or 20Gy (◆). Addition of equal numbers of unirradiated, syngeneic adult spleen cells does not significantly affect the response (o). Addition of indomethacin (10-7M,o) at the beginning of the culture reduces by about half the suppressor activity as compared to suppressor activity in the presence of the ethanol-

containing solvant used to disolve indomethacin (●) (figure 5). As shown in figure 6, cellular fractionation on the basis of cell size conducted by staput separation on a continuous FBS gradient reveals that most of the suppressor activity resides in the large cell fractions of bone marrow. Distribution of bone marrow cells in ten fractions (the percentage of the total marrow present in each fraction is indicated on the y axis) is represented by the dotted line, the cell size decreasing from fraction 1 to 10. A constant number of cells from each fraction was tested for its inhibitory activity. The amount of activity for a fixed number of cells is represented by the solid line on an arbitrary scale on the y axis. Comparison to the activity present in total marrow (unfractionated cells), represented as fraction T, indicates that cells mediating NS activity are enriche for in fractions 4 and 5 and against in all other fractions, thus suggesting NS cells are large cells.

Having developed and characterized assays to monitor NS activity *in vitro* , we next sought means of varying levels of NS activity in the live animal.


120
100
80
50
40
20
0
% OF CONTROL RESPONSE
N-BM
Thy1⊖-BM
1/10
1/25
1/62
1/156
CONTROL
BONE MARROW/SPLEEN CELLS RATIO


PANEL A:
Adult Bone Marrow NS Activity (1:10)
Untreated
Anti Thy 1
Anti aGM 1
10 Gy
Non-Adherent
Adherent
0
25
50
75
100
PANEL B:
Neonatal Spleen NS Activity (1:5)
Untreated
Anti Thy 1
Anti aGM 1
10 Gy
Non-Adherent
Adherent
0
25
50
75
100
PANEL C:
CFA-Treated Adult Spleen (1:5)
Untreated
Anti Thy 1
Anti aGM 1
10 Gy
Non-Adherent
Adherent
0
25
50
75
100
PERCENTAGE OF CONTROL PFC RESPONSE
(PANEL: A,B,C)
PANEL D:
Comparative NK Activity (100:1)
Untreated Adult Spleen
Adult Bone Marrow
Neonatal Spleen
CFA-Treated Adult Spleen
-5
0
5
10
15
PERCENTAGE SPECIFIC 51CHROMIUM RELEASE
(PANEL: D)


Percentage Suppression
Con A Response
LPS Response
100
50
0
1:2.5 1:5 1:10
CFA Spleen
Normal Bone Marrow
CFA Bone Marrow
Suppressor Responder Cell Ratio

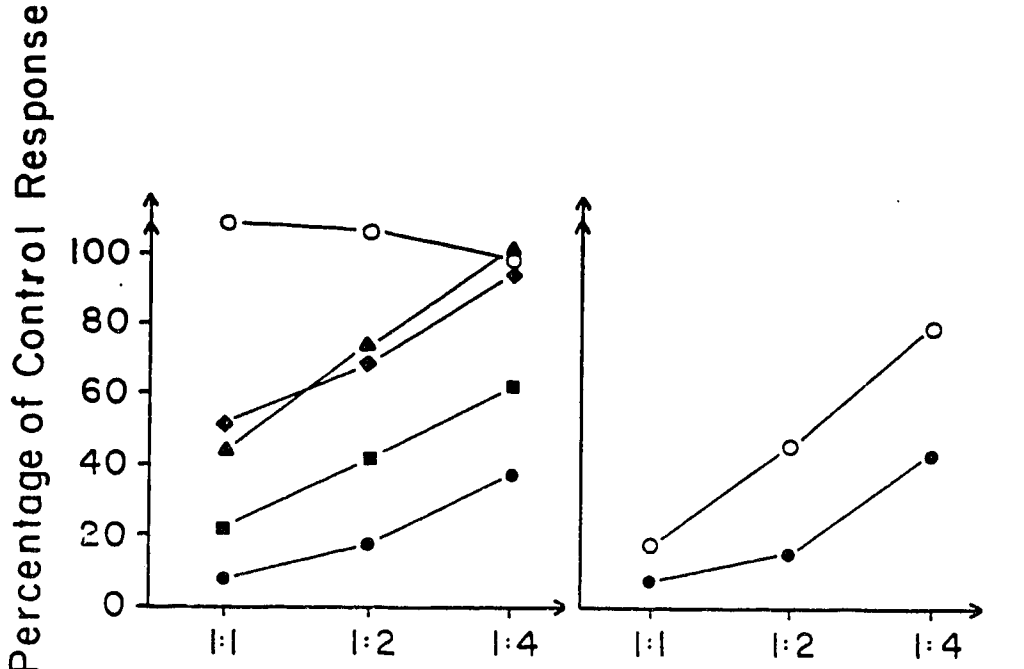


Figure 4

Figure 5

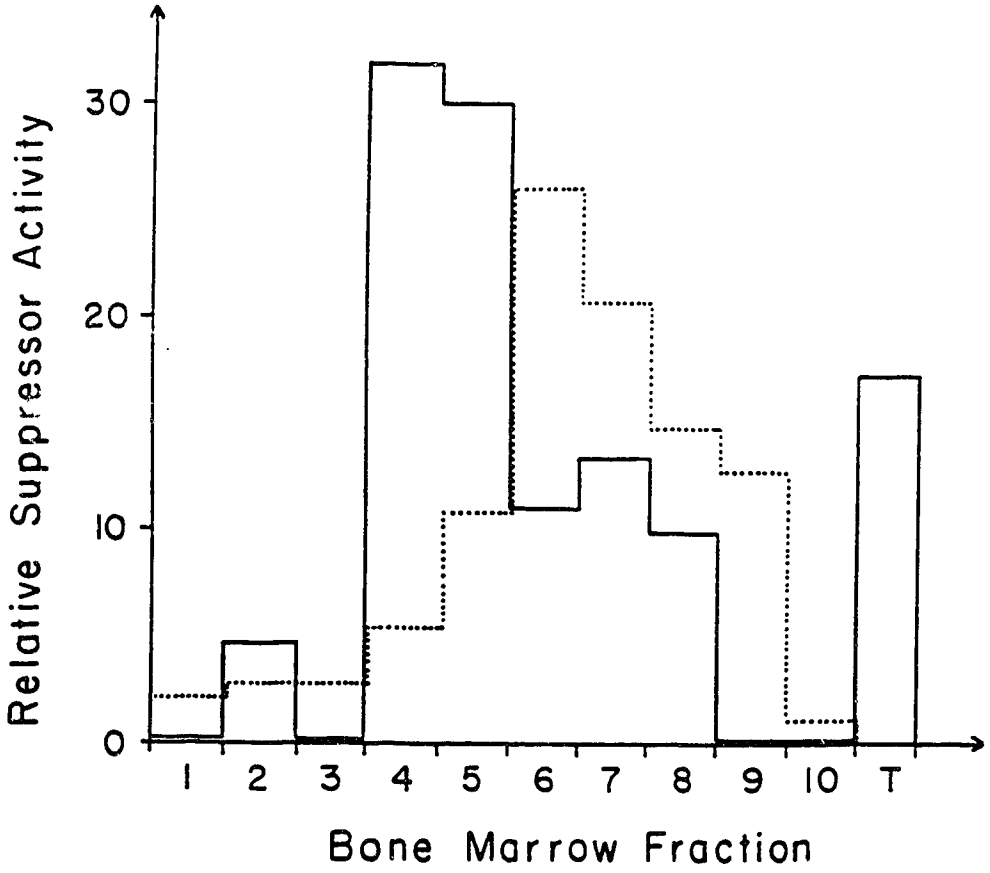


Figure 6

APPENDIX II: modulation of NS activity *in vivo* .

Our second prerequisite objective was to define conditions in which the level of NS activity in the live animal can be controlled. The protocols we have devised will be briefly reviewed here as an introduction to the result sections, using the proliferative response to LPS as the indicator assay. A detailed description and discussion of each protocol are given in the relevant sections of chapters IV to VI.

Three protocols were found to effectively reduce bone marrow NS activity. Whole body irradiation of the animal reduced NS activity in a dose-dependent fashion, on a scale comparable to *in vitro* irradiation, as depicted in figure 7. Bone marrow cells were harvested 2 hours after exposure to 3Gy (■), 6Gy (▲) or 9Gy (◆) and compared for NS activity to bone marrow from unirradiated donors (●). Administration of cytotoxic drugs was also found to reduce bone marrow NS activity (figure 8). High dose administration of cyclophosphamide (CY) or 5-fluoruracil (5FU) led to a four-fold decrease of NS activity for a given, constant number of bone marrow cells. In figure 8, normal bone marrow is represented by●, and treated marrow, harvested 24 hours after CY administration or 72 hours after 5FU, by■for 1mg CY,◆for 4mg CY,▲for 1mg 5FU and○for 3mg 5FU. Administration of anti-host MHC class I antibody also decreased bone marrow NS activity (figure 9). Bone marrow cells (○) were tested for NS activity seven days after the intravenous administration of 500µg of the anti-H-$2K^b$ monoclonal antibody 28-8-6S and compared to bone marrow cells from anti-H-$2K^k$-treated donors (●). NS activity was decreased by about one half in CB6 recipients treated with the relevant antibody.

To increase NS activity, adult mice were given a single 100µl intraperitoneal injection of complete Freund's adjuvant. NS activity was approximately doubled in the

marrow of C57BL/6J recipients (figure 10) and (BALB/cJ x C57Bl/6J)F_1 recipients (figure 11) 8 days after injection (⊘), compared to saline-injected donors (●). A comparable increase was observed after administering 50ng of rGM-CSF twice daily over five consecutive days, either subcutaneously or intraperitoneally (⊘) (figure 12). Marrow was harvested 24 hours after the last injection of the growth factor, and compared for NS activity to marrow from saline-injected animals (●). It should be noted that the induction of NS activity in spleen and its increase in bone marrow following administration of CFA does not depart from the general concept that NS activity is associated with increased hemopoietic activity, as reflected by the increase in CFU-GM content in both these tissues (spleen, ▲, and bone marrow, ●) following CFA administration (figure 13).

Finally, selection of untreated hosts of different age or genetic background could also serve as a basis to study variations of NS activity *in vivo* . As shown in figure 2, neonatal animals have spontaneously elevated splenic NS activity. A comparison of different inbred strains of mice revealed quite significant variations in NS activity levels between strains. One must however be very careful in comparing different strains. Obviously, age-matched, sex-matched animals kept under the same conditions in one animal facility must be used. With regard to the assay itself, it is probably not valid to use syngeneic bone marrow-spleen combinations for each tested strain, because the number of cells actually responding in each spleen aliquot may vary considerably between strains. It is therefore necessary to compare each marrow on the same responder spleen population. This is possible practically by virtue of the lack of genetic restriction of this suppressor activity. There are two ways which minimize the possible reciprocal and complex reactions which may develop in cultures of mixed allogeneic cells. First, each of two different parental marrow cells can be tested independently on the corresponding F1 hybrid spleen cell response, as shown in figures 14 A-C. Second, using MHC-congenic strains, each

marrow can be tested on each spleen in a circular permutation pattern, and the levels of suppression compared for each responder spleen. The PFC assay, despite its greater sensitivity, cannot be used because (i) some strains are very low responders to SRBC and (ii) any degree of MLR reactivity, even across minor histocompatibility barriers, would likely disrupt the culture system beyond interpretation. By using the proliferative response to LPS as the indicator assay, the strains can be compared as follows:

A: B6 (●) = CB3 (▲) > C (■)

B: B6 (▲) ≥ C3B6 (●) >> C3 (◆)

C: C (■) > CC3 (●) > C3 (◆)

D: C (■) > AKR (□) = CBA (○) > MRL-*lpr/lpr* (◆) ≥ C3 (△)*

E: B6 (◆) = B10 (▲) = B10.BR (◇) >> CBA (○)

F: DBA/2 (●) = B10.D2 (◇) > C (■)

(* in another experiment, NS activity levels in MRL-*lpr/lpr* mice were closer to those detected in CBA/J mice)

In conclusion, we have developed the two necessary tools to explore the function of NS activity *in vivo* : a valid assay system *in vitro* , described in appendix I, and means of selecting mice with increased or decreased NS activity *in vivo* , summarized in appendix II. In chapters IV and V, the relationship between NS activity and hemopoiesis is examined, in the context of a bone marrow transplantation model in unirradiated recipient mice. In chapter VI, the relationship of natural suppression to immunological tolerance is addressed, in the context of a murine model of genetically determined autoimmune diabetes.

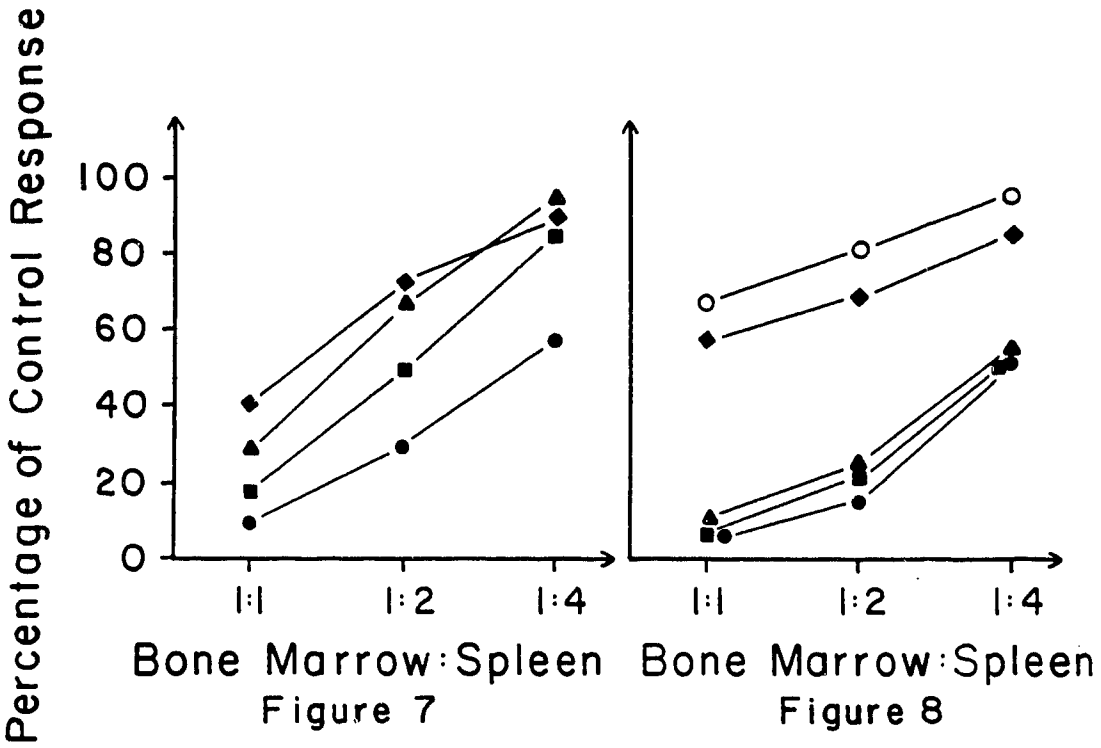


Figure 7

Figure 8

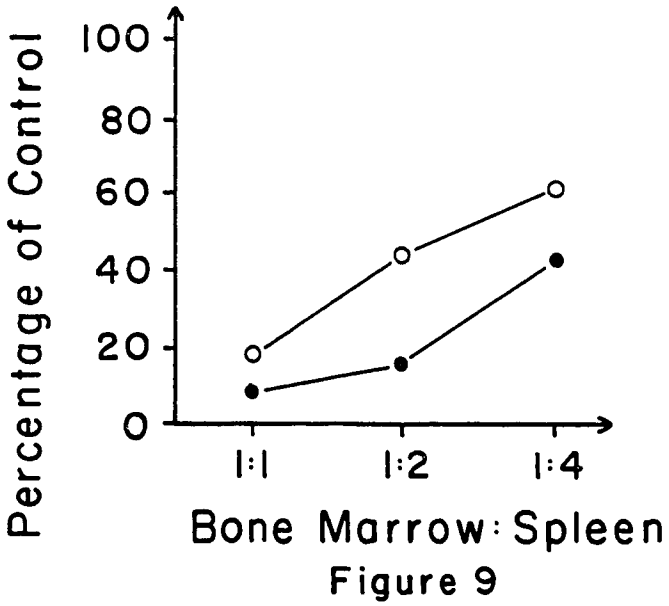


Figure 9

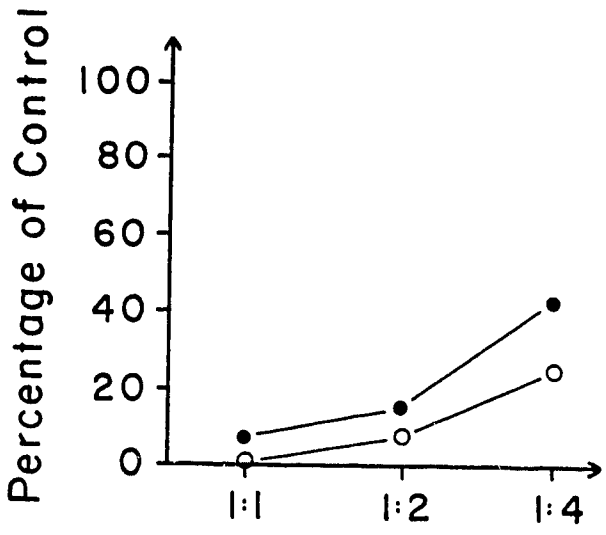


Figure 10

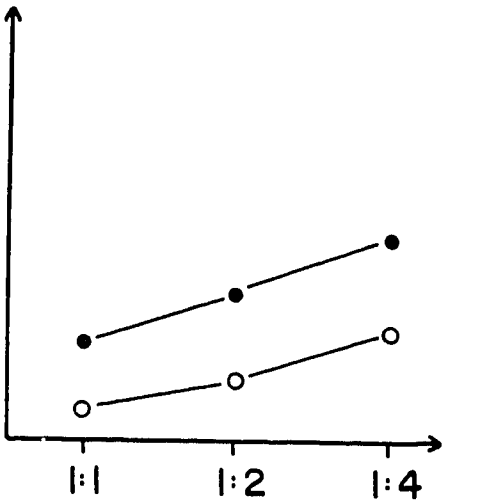


Figure 11

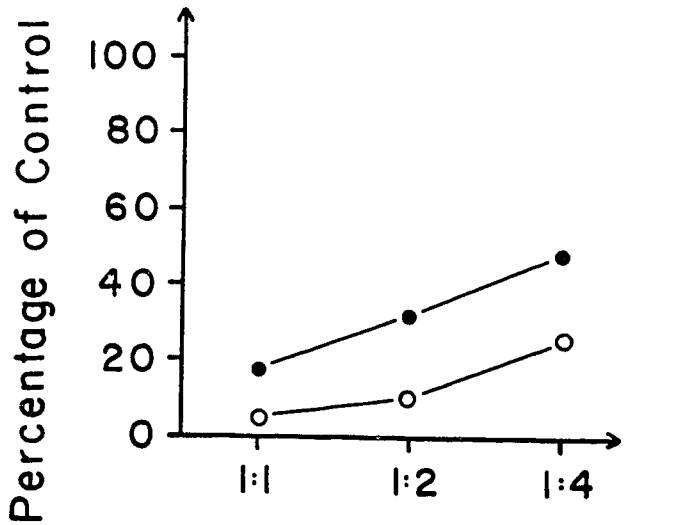


Figure 12

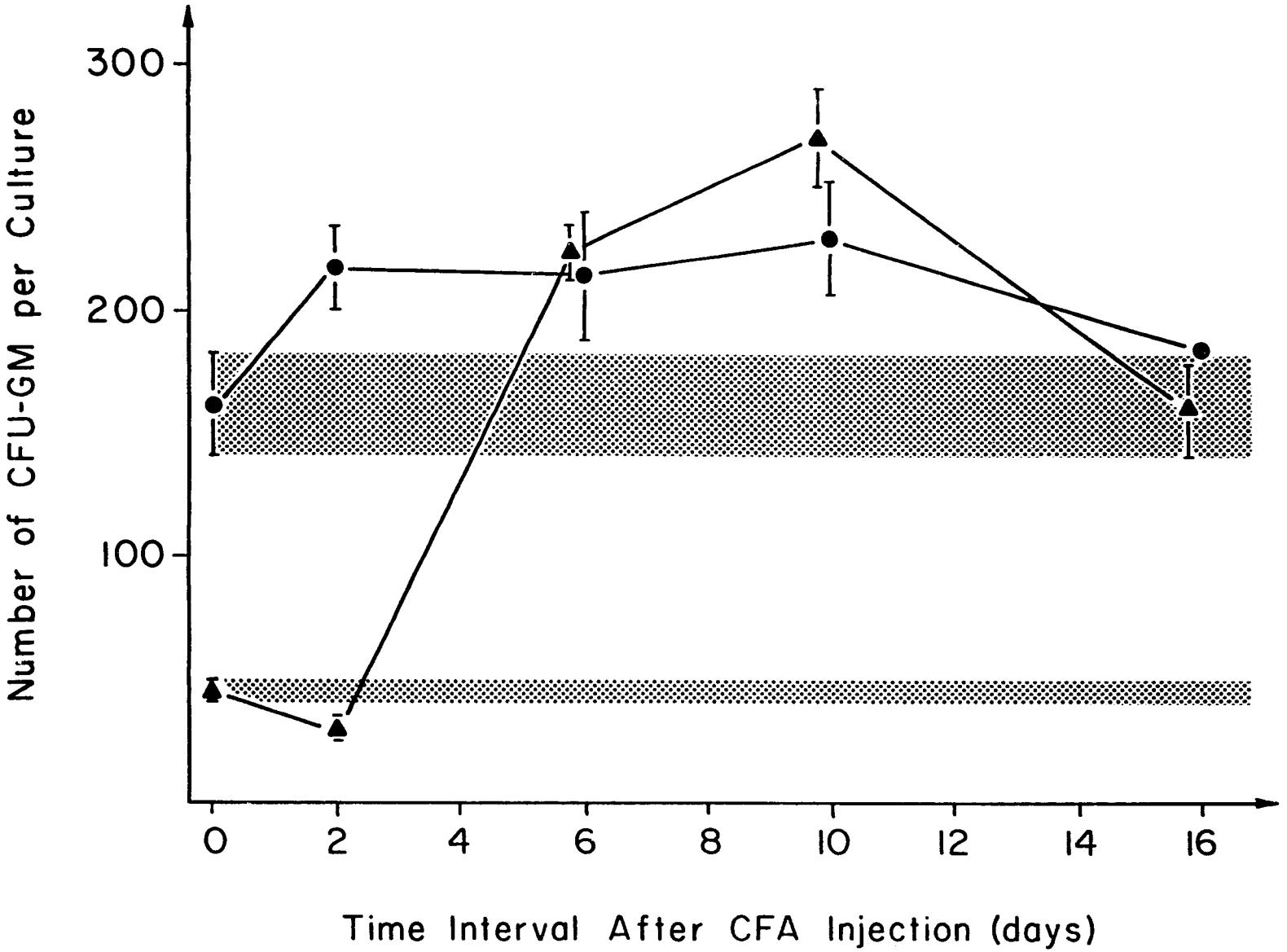
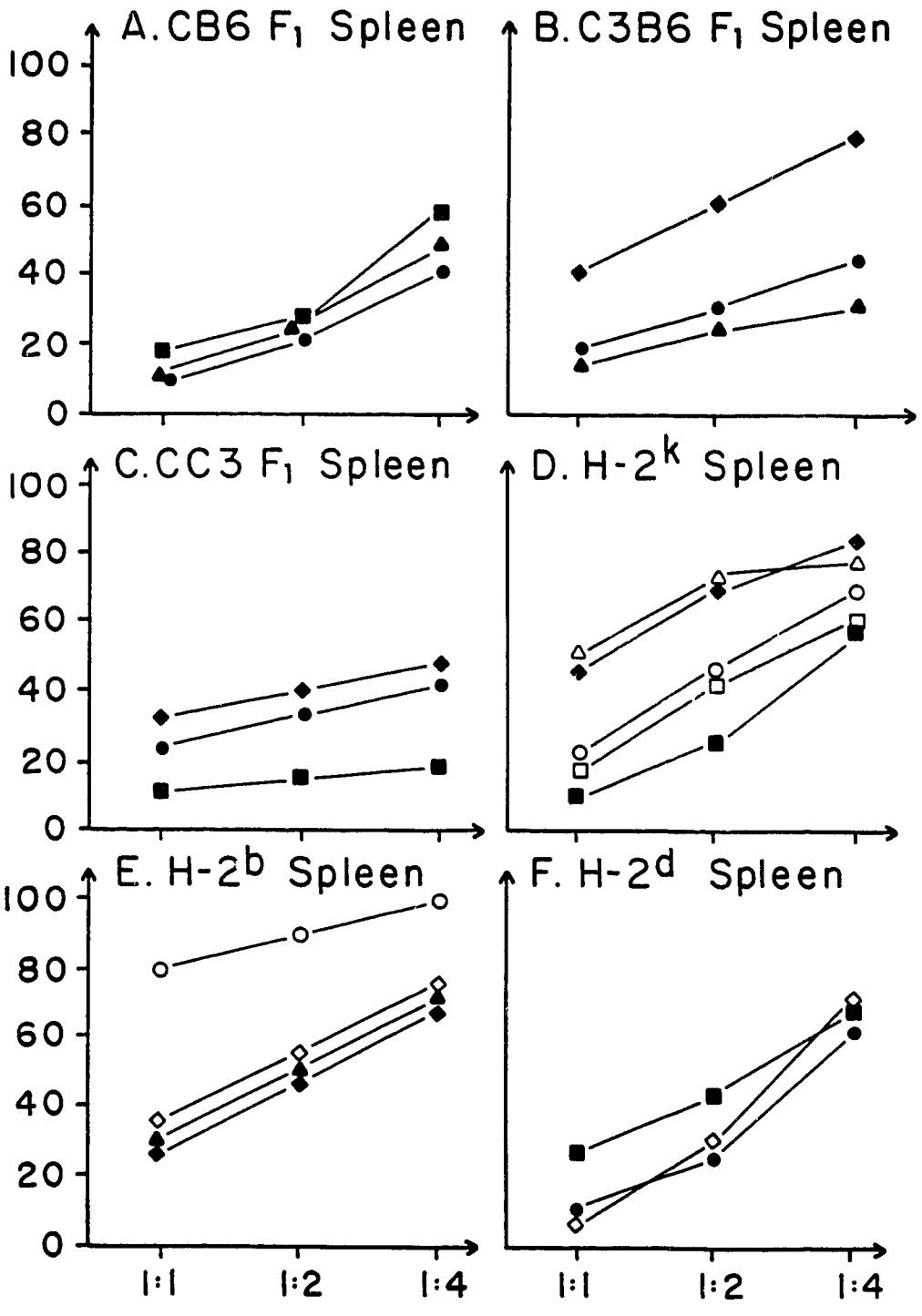

300
200
100
Number of CFU-GM per Culture
0
2
4
6
8
10
12
14
16
Time Interval After CFA Injection (days)

Figure 14


A. CB6 F1 Spleen
B. C3B6 F1 Spleen
C. CC3 F1 Spleen
D. H-2k Spleen
E. H-2b Spleen
F. H-2d Spleen
100
80
60
40
20
0
1:1
1:2
1:4
Bone Marrow: Spleen

APPENDIX III: chimerism analysis based on GPI analysis.

1. Molecular and genetic background.

Glucose phosphate isomerase (GPI) is an enzyme which catalyzes the isomerization of D-glucose-6-phosphate to D-fructose-6-phosphate and is present in all mammalian cells. It is composed of two chains, both encoded by the *Gpi-1* gene located on chromosome 7 in the mouse, which has two alleles, *Gpi-1a* and *Gpi-1b* . Since it is a dimer, there are three possible isozymes: GPI-AA, GPI-BB and GPI-AB. Each of these displays a different migration pattern in starch electrophoresis at pH = 6,2. The AA band is slowest migrating, BB fastest, and the band formed in heterozygous cells is intermediate. These structural properties are retained when cells which posessing different isozymes are mixed, thus permitting the determination of the proportion of each cell population from the analysis of the ratio of one isozyme to another present in a given tissue sample.

Inbred strains of mice have the following *Gpi-1* alleles:

BALB/c, DBA/2, A/J, AKR, NOD, NZB, C57BL/6J-Gpi-1a : *Gpi-1a* .

C57BL/6J, C57BL/10J, C3H/HeJ, CBA/J, CBA/CaJ: *Gpi-1b* .

2. Electrophoretic separation of the different isozymes.

The GPI isozymes are easily and unequivocally separated by starch gel electrophoresis. The quantitation of the different bands makes use of the enzymatic properties of the GPI molecule, which can be visualized by a colorimetric reaction. By transferring the separated bands to a filter, thus precluding further displacement or

diffusion, a permanent record of each run can be made and eventually densitometrically scanned.

The gel is obtained from 15g potato starch (no. S-4501, Sigma, Saint Louis, MO) + 95ml dH_20 +5ml of a buffer consisting of 24,2g Trizma base + 26,5g sodium citrate + dH_20 q.s.p. 1 liter, adjusted to pH = 6,2 by adding about 18ml 12N HCl. After polymerization over a burner, the gel is degased and poured into plastic molds and allowed to settle overnight. Thin slabs (about 1-2mm) are sliced and placed between 2 layers of Mylar. Slots are made by applying a razor blade.

Tissue samples are prepared as single cell suspensions from spleen, bone marrow, thymus, etc. Peripheral blood, cell lines, bone marrow colonies grown in semi-solid medium are used without any preliminary manipulation. The tissue sample is lysed by freeze-thawing and a minute amount loaded with an elongated glass capillary (10µl disposable micro-pipets, no. 21-164-2C, Fisher Scientific Company, Pittsburgh, PA) into the slots.

The electrophoresis is performed for 4 to 5 hours at 200V (DC) at 4ºC. In the buffer tanks, the platinum electrodes and gel slab extremeties are immersed in the same Trizma base + sodium citrate, pH = 6,2 buffer.

Prior to staining, the portion of the gel within 3cm of the loading slots are cut out and applied to a nitrocellulose filter (0,45µ HA filter, no. HAWP 047 30, Millipore Corporation, Bedford, MA). The transfer is efficient within 10mn and does not require additional manipulation. All gels within one experiment should however be treated in parallel under identical conditions because the rate of transfer between slow and fast band is different. The staining buffer is a freshly prepared Tris-HCl buffer made by adding to a 0,3M Tris-HCl solution (4,74g Tris-Hcl + dH_2O q.s.p. for 100ml) a 0,3M Tris-base solution (4,74g Tris-base + dH_2O q.s.p. for 100ml) until pH = 8,0. The resulting solution

CHAPTER V: prevention of type I diabetes in non-obese diabetic (NOD) mice by adjuvant immunotherapy.

The non-obese diabetic (NOD) mouse spontaneously develops an insulin-dependent diabetes mellitus that has many immunological and pathological similarities to human type I diabetes (1,2). The autoimmune nature of the disease is suggested by the lymphocytic infiltration of the Langerhans islets, preceding the destruction of insulin-producing ß cells (3). By 3-5 weeks of age, mononuclear cells infiltrate periductal and perivascular spaces leading to insulitis and diabetes within 3-6 months of age, and by 8 months, most animals are affected. As in man, diabetes in NOD mice is multigenic and possibly controlled by three recessive loci, including one that is linked to the major histocompatibility complex (4-6). The genetically determined diabetes and insulitis in NOD mice are dependent on the presence of NOD hematopoietic stem cells, based on data from radiation chimeras (7,8). The disease can be transferred with splenocytes from NOD mice and both $CD4^+$ and $CD8^+$ T cell subsets are necessary for this transfer (9-11). Since T cells may determine the development of diabetes, we have investigated the effect of treatments that affect the immune response on the course of disease. We report here that adjuvant immunotherapy, consisting of a single injection of complete Freund's adjuvant given at an early age, prevents the appearance of diabetes and confers normal lifespans to NOD mice without any additional treatment. Examination of *in vitro* lymphocyte proliferative responses to alloantigen and mitogen revealed profound modifications in the response of adjuvant-treated animals. Both splenic T and B cell responses were significantly reduced. This reduction was associated with the detection of an antigen non-specific suppressor activity in the spleen and and an increase in natural suppressor activity in the bone marrow.

MATERIALS AND METHODS

Mice. NOD, BALB/cJ, C57BL/6J, (BALB/cJ x C57BL/6J)F_1 mice were bred in our facility at the University of Alberta. NOD mice were kindly provided by Dr. E. Leiter, Jackson Laboratories, Bar Harbor, ME. The incidence of diabetes in both males and females in our NOD colony, established by brother-sister mating, is near 100% by 250 days (50% of the mice are hyperglycemic by 150 days of age). All mice are kept under standard housing conditions. Glycemia was monitored weekly using a Glucoscan 2000 glucometer. Complete Freund's adjuvant was purchased from Difco Laboratories, Detroit, MI.

Mixed Lymphocyte Reaction. The primary *in vitro* allogeneic MLR was studied by culturing $2x10^5$ responder splenocytes with $8x10^5$ stimulator splenocytes irradiated with 30 Gy from C3H/HeJ adult mice. After 96 hours in 8% fetal bovine serum-containing RPMI medium (Flow Laboratories, McLean, VA), each well was pulsed with tritiated thymidine (^{3}H-TdR) for 24 hours before harvesting. All cultures were set up in quadruplicate wells in flat bottom 96 well Linbro plates. In cell mixing experiments, $2x10^5$ spleen or bone marrow cells were added at the onset of culture (1:1 cell mixing ratio).

Mitogen Response. Two hundred thousand splenocytes per well were cultured for 72 hours in the presence of 2.5 ug/ml Concanavalin A (Calbiochem, La Jolla, CA), 10 ug/ml *E. coli*, phenol-extracted Lipopolysaccharide (Sigma, Saint Louis, MO) or medium alone to measure background thymidine incorporation, followed by a 12 hour pulse with tritiated thymidine. Cultures were carried out in medium containing 5% fetal bovine serum, in flat bottom 96 well Linbro plates, each culture set up in quadruplicates. In cell mixing

experiments, $2x10^5$ spleen or bone marrow cells were added at the onset of culture (1:1 mixing ratio), and eventually titrated (by adding 10^5 or $5x10^4$ bone marrow cells to $2x10^5$ responder spleen cells, defining 1:2 and 1:4 bone marrow : spleen mixing ratios, respectively).

RESULTS

A single injection of complete Freund's adjuvant in 5 week old NOD mice prevents development of diabetes.

To investigate the effect of adjuvant on the development of the T cell-dependent diabetes in NOD mice, 5 week old prediabetic NOD mice were administered 50 µl of complete Freund's adjuvant (CFA) in the footpad. As shown in Figure 1, a single injection of CFA prevented the development of hyperglycemia. Serum glucose levels in CFA-treated mice remained under 180 mg/dl (10 mmol/l) up to 11 months after administration of the adjuvant, whereas in control, saline-injected mice, hyperglycemia developed as early as 3 months after birth, usually rising to 500 mg/dl (27.8 mmol/l) or more within 2-3 weeks of onset. All 5 control mice monitored weekly for blood glucose died by 5 months of age, as is generally the case in our colony, whereas all CFA-treated mice (n=13) were still alive one year after birth (Figure 2). Histological examination of the pancreas revealed intact islets and insulin-staining B cells in NOD mice 8 months after CFA administration, whereas virtually all islets showed varying degrees of mononuclear infiltration (insulitis) and islet destruction in untreated NOD mice after 4 months of age (data not shown). Islets exhibiting insulitis, but not destruction, were also observed. Studies are in progress to assess whether earlier administration of CFA can prevent insulitis as well as diabetes. Because of the autoimmune nature of diabetes in NOD mice,

and the apparent limitation of the destructive potential of the insulitis resulting from CFA administration, we next investigated the effect of adjuvant administration on the proliferative responses of lymphocytes.

Administration of CFA results in decreased splenic lymphocyte proliferative responses.

T cell-mediated responses were examined *in vitro* in the primary allogeneic mixed lymphocyte reaction (MLR) and mitogen-induced lymphoproliferative responses. The responses of splenocytes from adjuvant-treated mice were compared to responses of control (saline-injected) age-matched, sex-matched animals. As shown in Figure 3A, the primary allogeneic MLR, studied 8 days after CFA administration, was reduced in both NOD mice and age-matched, sex-matched, non-diabetic (BALB/cJ x C57BL/6J)F_1 mice. In one representative experiment of three, including 3 separately treated mice per group, the lymphocyte proliferative response dropped from 63520 ± 4638 cpm to 48568 ± 2650 cpm in NOD mice and from 62991 ± 1116 cpm to 26958 ± 2867 cpm in the non-diabetic mice. Comparable results were obtained in the mitogen-induced proliferation assays, using either Concanavalin A (Con A) or lipopolysaccharide (LPS) to stimulate the lymphocytes (Figure 3B). To investigate the mechanism of this reduction, cell mixing experiments were performed to find out whether regulatory cells elicited by adjuvant administration might account for this effect.

Antigen non-specific inhibitory cells are elicited in spleen and increased in bone marrow following CFA administration.

To investigate the presence of regulatory cells, spleen cells from CFA-treated or control animals were mixed with syngeneic responder spleen cells from 2 month old,

untreated donors. The data shown in Figure 4 indicate that cells with an antigen non-specific suppressor activity are elicited in the spleen following adjuvant administration. Thus, addition of splenocytes from CFA-treated but not saline-injected animals reduced the primary allogeneic MLR and the mitogen-induced proliferative responses. Cell mixing experiments are shown at one responder spleen : test spleen cellular ratio (1:1). At this mixing ratio, a comparable reduction of the proliferative response in the MLR and the response to LPS was achieved , and a lesser reduction in the Con A response of NOD spleen cells than that of CB6 cells. Taken together, these data establish that an inhibitory cell population(s) is present in spleen following CFA administration, notwithstanding the need for increased cell mixing ratios to reduce the Con A response in NOD mice (cf Discussion). Because non-specific suppressor cells have been suggested to originate in the bone marrow and migrate to the spleen following adjuvant administration (12-14), we also examined the regulatory potential of bone marrow cells. As shown in Figure 5, a naturally occuring suppressor activity in the bone marrow inhibits both the MLR and the proliferative response to LPS, and is augmented by CFA administration. While this increase is apparent in both diabetic and non-diabetic strains, this activity, at the basal level, is less in NOD mice than in the (BALB/cJ x C57BL/6J)F_1 strain. The data shown in figure 5B indicate that about $20x10^4$ NOD bone marrow cells are necessary to reduce the control lymphoproliferative response by 50%, while only $9x10^4$ cells are required 8 days after CFA administration. In non-diabetic (BALB/cJ x C57BL/6J)F_1 mice, $8x10^4$ marrow cells from untreated animals are necessary to reduce the response by half, and only $4x10^4$ cells after CFA administration. Thus, we conclude from these mixing experiments that a natural suppressor activity is increased in bone marrow and elicited in spleen following adjuvant administration.

DISCUSSION

We report here that a single injection of complete Freund's adjuvant given at an early prediabetic stage in NOD mice can prevent the development of hyperglycemia and death. Whereas this was associated with conservation of insulin-producing ß cells, infiltration of the islets with mononuclear cells was not completely eradicated, suggesting that adjuvant therapy acted by reducing B cell destruction by the mononuclear infiltrate. Prevention of diabetes in these animals was accompanied by the global reduction of lymphocyte proliferative responses, the relevance of which is suggested by the ability of T cells to transfer the disease (9-11). Cell mixing experiments indicate that this reduction may be attributed, at least in part, to the induction of regulatory cells in the spleen following adjuvant administration. A number of protocols that inhibit T cell function have been shown to block the progression of disease with prolonged therapy (15-21). The results presented here differ, however, in one major respect : disease prevention was achieved by a single injection, without further therapy, thus establishing that this early immunotherapy can affect pathogenic events over a prolonged period. We propose this effect is associated with the activation of endogenous regulatory cells that can prevent the initiation, amplification and/or effect of the autoimmune response.

Our data indicate that the reduction of lymphocytic proliferative responses in adjuvant-treated NOD mice is associated with the presence in the spleen of antigen non-specific inhibitory cells. A number of reports have established that adjuvant administration activates a natural suppressor activity, ascribed either to suppressor macrophages or to non-adherent, Thy 1-negative natural suppressor cells (reviewed in references 22-24). Natural suppressor (NS) activity is antigen non-specific and non-H-2-restricted, and is physiologically present in neonatal spleen (25-29) and adult bone marrow (12-14,30-33).

It has been suggested that cells mediating NS activity migrate from bone marrow to spleen following adjuvant administration (12-14). We have shown elsewhere that this activity is increased in bone marrow and elicited in spleen following administration of CFA to C57BL/6J and (BALB/cJ x C57BL/6J)F_1 mice, reaching a maximum about 10 days after adjuvant administration, and is mediated by Thy1-negative, plastic non-adherent cells in the bone marrow (34). The data shown here do not exclude that adjuvant administration may independently act through other mechanisms as well, such as cytokine or growth factor production by T cells or by affecting antigenic expression on target cells. The data do not exclude either that some NOD lymphocytes may be refractory to NS inhibitory signal(s), since proliferative responses in NOD mice were less suppressed in CFA-treated NOD mice than in the CFA-treated non-diabetic mice we studied in parallel. Furthermore, a spontaneous reduction of endogenous NS activity could be taken to support a relationship between this immunoregulatory activity and the control of the diabetic phenotype. In the NZB autoimmune mouse strain, a profound deficiency in bone marrow NS activity *in vitro* has been reported and suggested to play a role in the autoimmune response (35).

Natural suppressor cells have been suggested to play an important role in the induction of self tolerance in neonatal mice, in the regulation of immune responses, and in the success of allogeneic bone marrow transplantation following total lymphoid irradiation (reviewed in reference 23). The data presented here are compatible with the idea that a deficient endogenous NS activity may be associated with the development of autoimmunity. In this scheme, the administration of adjuvant early in life (or the eventual influence of environmental factors that increase NS activity) would extend the post-natal "window of tolerance " (23) by boosting NS activity, otherwise spontaneously elevated in the neonatal period (25-29, 34), and thereby enhancing the establishment of immunological tolerance. The adoptive transfer of NS activity has been generally unsuccessful, other than

in local assays or in a graft-versus-host disease model in irradiated recipients (36). Since this suppressor activity is present in bone marrow but not thymus in adult mice (23,24,31), our conclusions also support radiation chimera data indicating that diabetes in NOD mice may be controlled by hematopoietic stem cells in the bone marrow (7,8). Thus, NS cells may have a physiological role in extrathymic mechanisms of tolerance induction to self antigens.

Whatever the mechanism of the prevention of diabetes in NOD mice following administration of CFA, this approach provides a simple method for maintaining NOD mice in a disease-free state. Our results establish that early non-specific immunotherapy in a murine model can prevent the development of diabetes in genetically predisposed individuals. It is hoped that this approach might also serve as a basis for strategies of preventive therapy in human type I diabetes mellitus and perhaps other autoimmune diseases.

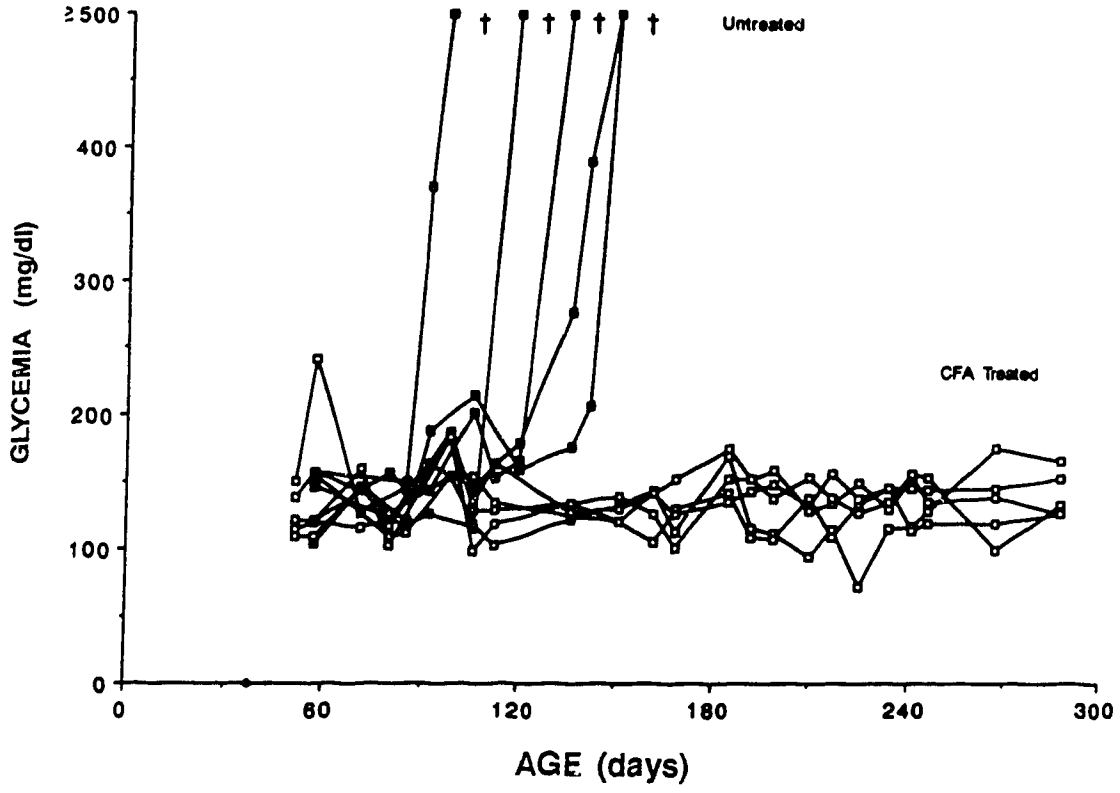


Figure 1. Early adjuvant administration prevents the development of hyperglycemia in NOD mice. NOD mice were given 50 ul of CFA (open squares, n=5) or an equal volume of saline (closed squares, n=5) once only at age 35 days and their serum glucose levels were monitored weekly for 10 months. Animals with a glycemia greater than 500 mg/dl were sacrificed (†).

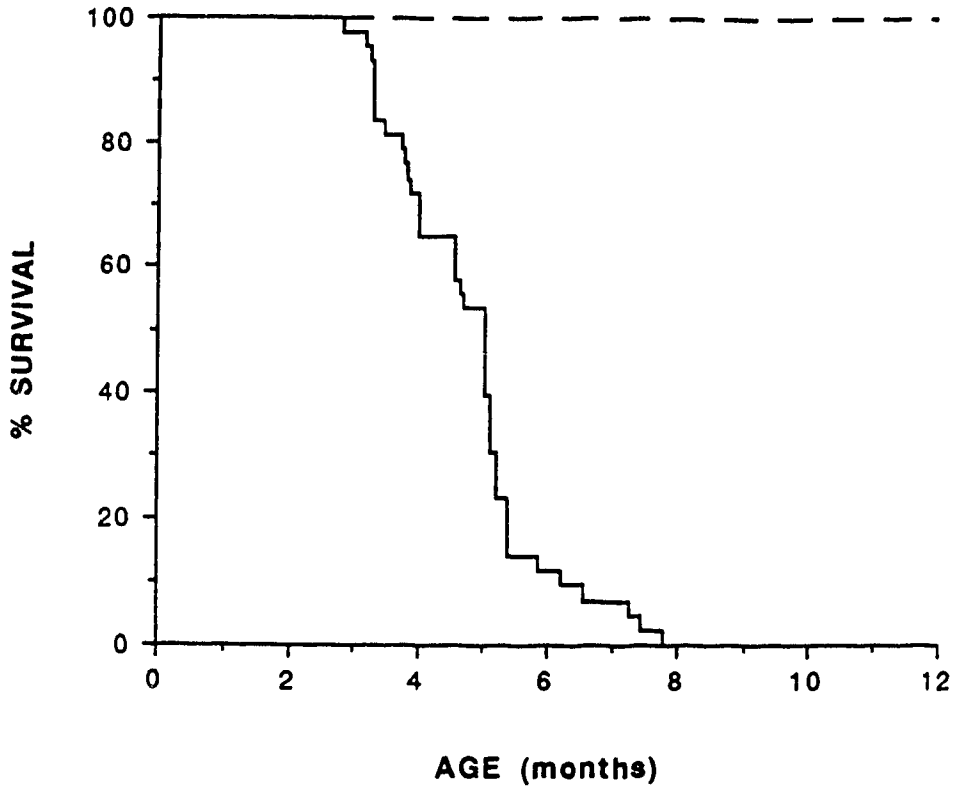


Figure 2. Early adjuvant administration extends the lifespan of NOD mice. Five week old mice were given 50 ul of CFA (n=13), or an equal volume of saline (n=5), or no treatment at all (n=38). The percentage survival is calculated as 100 x (number of surviving animals) x (initial number of experimental animals)$^{-1}$. The solid line represents the survival of pooled saline-injected and untreated animals, and the broken line that of mice given adjuvant. All mice that died had confirmed hyperglycemia.

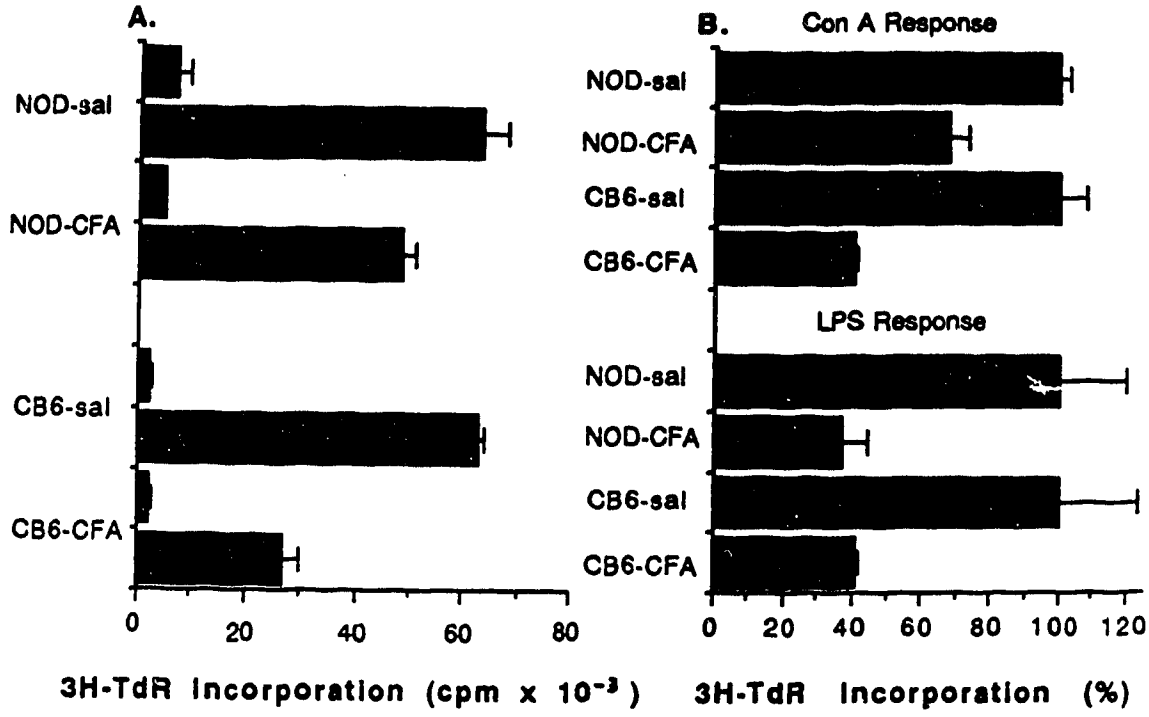


Figure 3. Adjuvant administration results in reduced *in vitro* splenic proliferative responses to alloantigen (A) and mitogen (B). Control, saline-injected animals are represented by -sal and CFA-treated animals by -CFA. For each group of animals in the MLR, syngeneic proliferative responses are plotted above the anti-C3H/HeJ allogeneic response. Mitogen responses are normalized to facilitate comparisons between treatments and mouse strains (NOD mice and (BALB/cJ x C57BL/6J)F_1 mice, abbreviated CB6). Control responses (100%) are 130280 ± 4140 cpm and 68267 ± 5769 cpm in the Con A response and 11629 ± 2209 cpm and 35694 ± 8923 cpm in the LPS response, in NOD and CB6 animals, respectively. Data from one representative experiment,are shown as mean ± s.d. (three mice per group).

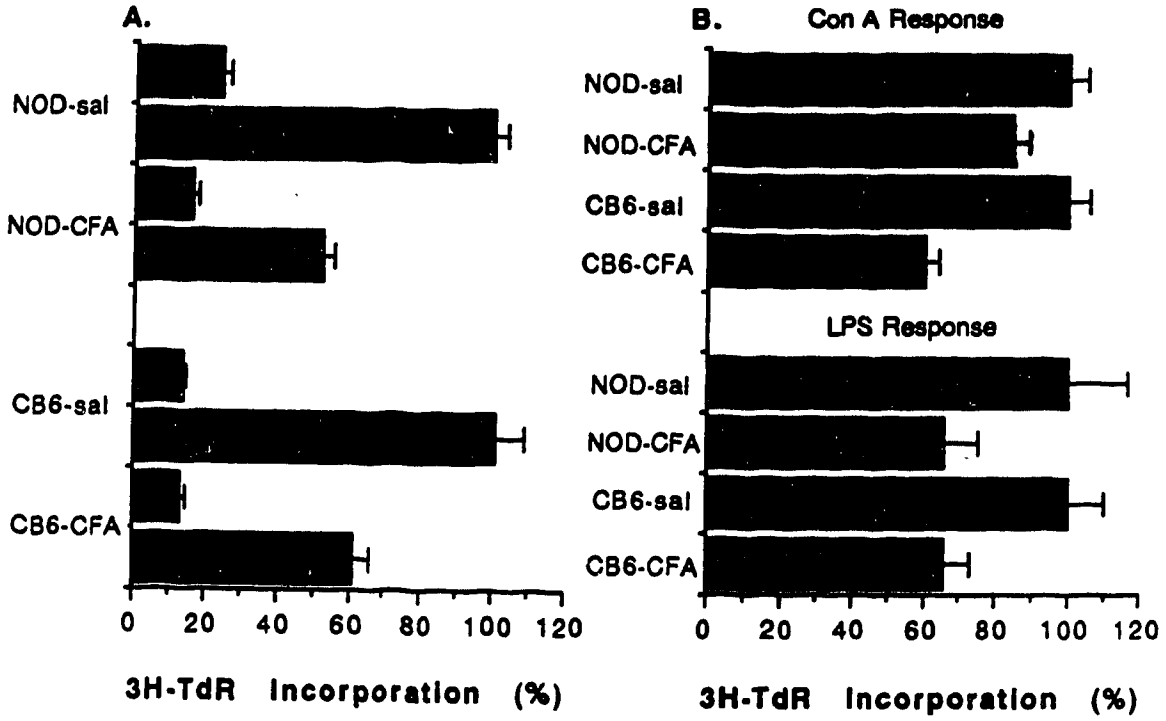


Figure 4. Cells that downregulate the MLR (A) and the proliferative response to mitogen (B) are present in the spleen following adjuvant administration. In these cell mixing experiments, 2x10^5 responder spleen cells were mixed with 2x10^5 syngeneic spleen cells from CFA-treated or control amimals in each culture well (1:1 cell mixing ratio). The addition of spleen cells from CFA-treated animals resulted in a reduction of the proliferative response, indicating that a natural suppressor activity was present in the spleen 8 days after adjuvant administration. Data are normalized to facilitate comparisons. Control responses (100%) are 46409 ± 1213 cpm and 32681 ± 2419 cpm in the MLR, in NOD and CB6 mice, respectively, which is not significantly different from the response of responder spleen cells alone (data not shown). Responses to Con A are 113860 ± 4966 cpm and 84651 ± 5079 cpm, and responses to LPS 14979 ± 1178 cpm and 28664 ± 1251 cpm, in NOD and CB6 mice, respectively. Data from one experiment with three mice per group are plotted as mean ± s.d.

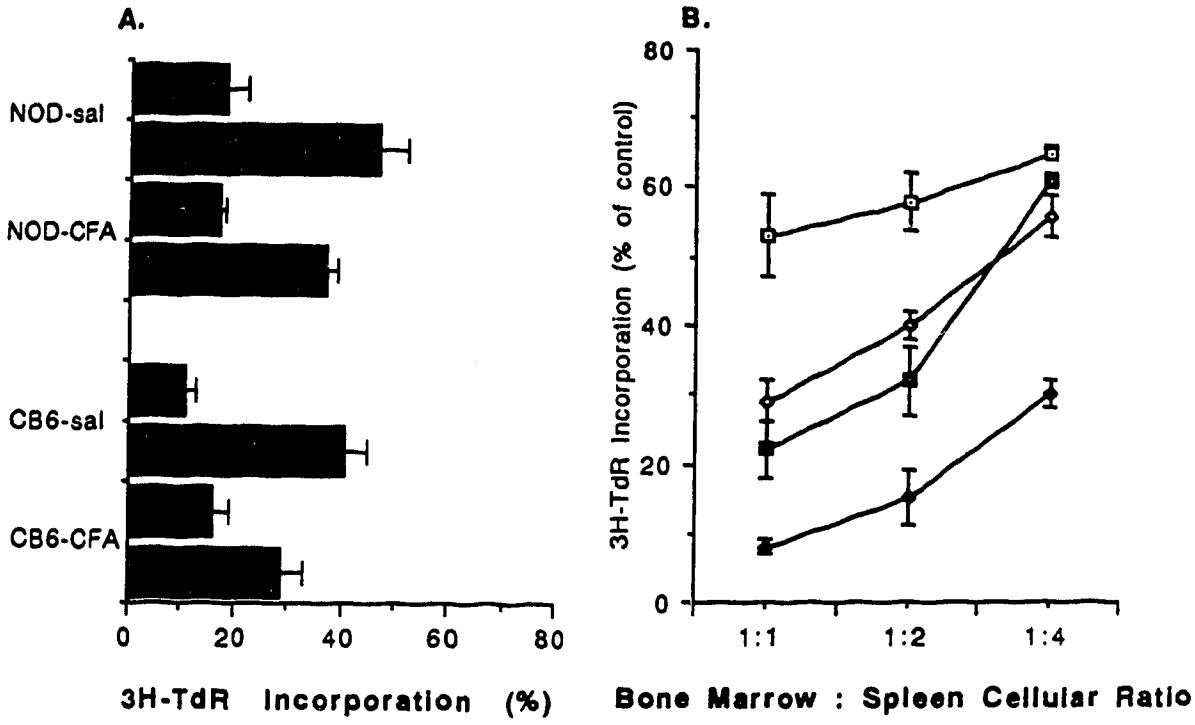


Figure 5. Constitutive bone marrow natural suppressor activity is increased following adjuvant administration. In the MLF (A), at the 1:1 bone marrow : spleen cellular ratio, the proliferative response is reduced from 47 ± 5% to 37 ± 2% when mixed with bone marrow from saline-treated and CFA-treated NOD donors, respectively, and 41 ± 4% to 29 ± 4% in the CB6 counterparts. In the proliferative response to LPS (B), bone marrow dilutions are shown, to permit a semi-quantitative appreciation of the increase in natural suppressor activity. Data in NOD mice are represented by squares, and CB6 mice by diamonds; filled symbols indicate CFA treatment. Data are shown as mean ± s.d. (n=3 for each group).

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CHAPTER VI: protection against diabetes by complete Freund's adjuvant in the BB rat.

Prevention of hyperglycemia and diabetes-related mortality by the administration of complete Freund's adjuvant (CFA) to young non-obese diabetic (NOD) mice is described in the previous section. NOD mice usually develop pancreatic insulitis 3 months after birth and succumb to diabetes 2 to 5 months thereafter. The preventive therapy consists of a single intraperitoneal or subcutaneous injection of CFA in five week old mice. One question raised by this observation is whether this treatment is effective in the prevention of other autoimmune diseases and whether it is applicable in species other than mouse. We have begun to examine this question in another animal model of diabetes, the BB rat.

The BB rat is an inbred strain of rat which spontaneously develops autoimmune insulin-dependent diabetes mellitus. Pancreatic insulitis starts by day 40 after birth, leading to ß cell destruction, and glycosuria usually begins by day 65-80. Glycosuric rats die earlier than NOD mice, often within days of the onset of glycosuria. The disease is distinct from diabetes in NOD mice in that it is associated with marked lymphopenia and autoimmune thyroiditis. Unlike the case for NOD mice, which appear to bear unique MHC class II determinants, there is an inbred MHC-congenic rat which is diabetes-resistant. Like NOD mice, however, diabetes is conferred by the transplantation of hemopoietic stem cells from diabetes-prone BB rats to diabetes-resistant animals.

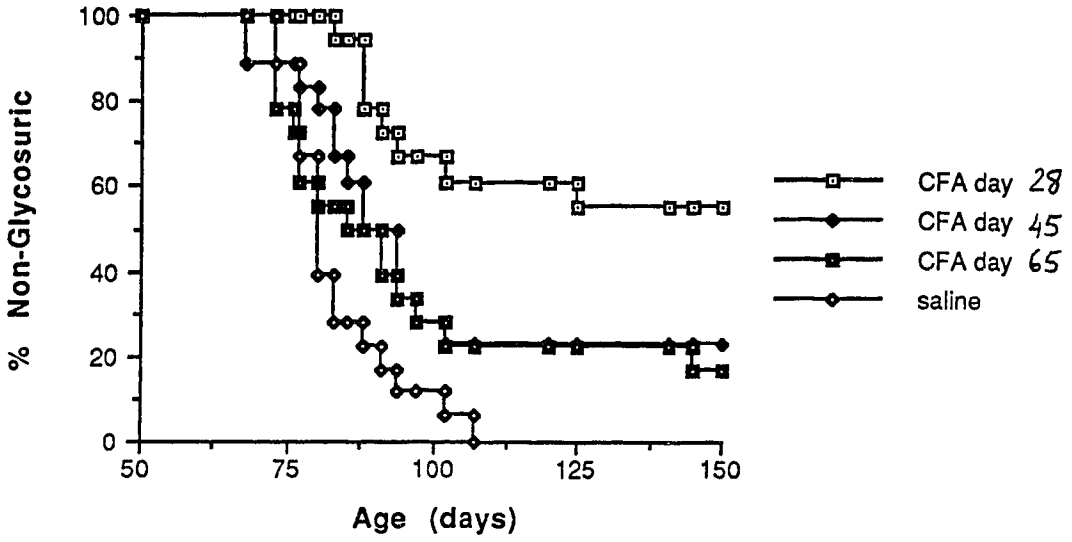
We first investigated whether administration of CFA to diabetes-prone BB rats affects the expression of the diabetic phenotype. Seventy-two age-matched rats were randomly divided into 4 groups, each comprising 10 males and 8 females. In three groups, the animals were inoculated intraperitoneally 200µl of CFA (DIFCO Laboratories, Detroit, MI), administered at 28 days of age (pre-insulitis stage), 45 days (early insulitis) or 65

days (established insulitis), respectively. In the control group, animals received 200µl of saline i.p. at 28 days of age. The rats were monitored for glycosuria, using a glucose-oxydase colorimetric tape (Tes-tape, Eli Lilly Canada Inc., Toronto, ON), and survival, three times a week from day 55 to 95 and twice a week thereafter. The onset of diabetes was recorded as the first of two consecutive days with positive urine samples.

Morbidity and mortality curves indicate that early administration of CFA indeed affects the development of diabetes in the BB rat. Morbidity is 100% by day 105 in the control group and 44% by day 150 in the earliest treatment group (CFA administered on day 28). Morbidity is slightly reduced in the two other groups, 83% and 78% respectively. Mortality by day 150 is reduced from 83% in the control group to 17% in the earliest treatment group, and to 44% and 61% in the two other treatment groups. A study to determine whether CFA administration before day 28 is of more effect is in progress.

Two conclusions can be drawn from this study. First, prevention of diabetes by early adjuvant immunotherapy is extended to another animal species. The disease process is at the least delayed, resulting in a five-fold increase in the proportion of surviving animals by day 150. Furthermore, insofar as diabetes in the BB rat may be considered a different disease by virtue of the important characteristics which distinguish it from NOD mouse diabetes, prevention is virtually extended to another disease, although it also involves ß cell antigens. Second, disease prevention by CFA administration is clearly age-dependent, mostly effective when administered before the onset of detectable insulitis.

These findings are encouraging in that they suggest that adjuvant-mediated prevention of autoimmunity may apply to more than one autoimmune disease in more than one animal species.


% Non-Glycosuric
100
80
60
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20
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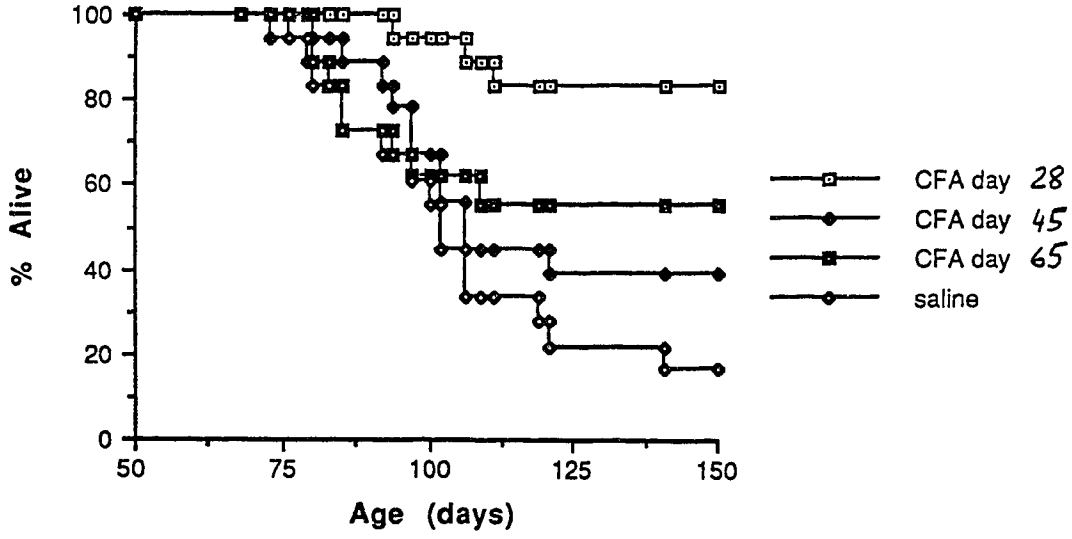
75
100
125
150
Age (days)
CFA day 28
CFA day 45
CFA day 65
saline


% Alive
100
80
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40
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0
50
75
100
125
150
Age (days)
CFA day 28
CFA day 45
CFA day 65
saline

CHAPTER VII: GENERAL DISCUSSION: the role of NS activity *in vivo* .

La preuve, le mal hereditaire de l'homme.

Elias Canetti, 1973, Le territoire de l'homme.

The experiments described in this thesis aim at improving our understanding of the role of natural suppressor (NS) activity in the live animal. Possible physiological functions were explored by seeking correlations between quantitative variations in NS activity *in vivo* and functions thought to be affected by this inhibitory activity. We examined the role of NS activity in relation to hemopoietic engraftment in a murine model of bone marrow transplantation (chapters II to IV). In another set of experiments, in animal models of autoimmune diabetes mellitus, we uncovered a possible role for NS activity in disease prevention and, by extension, in immunological tolerance (chapters V and VI). By implicating NS activity in the control of hemopoietic engraftment and in the expression of genetically determined autoimmunity, we have realized the objective of developing dependable *in vivo* models in which the role of NS activity may be properly addressed. Since the data have previously been discussed in each chapters, we will avoid duplicating the respective dicussions, and rather focus on our current understanding of the physiological role of NS activity. Based on our data, we will introduce models which attempt to integrate all the available information. At first, the role of natural suppression in hemopoiesis and immunological tolerance will be considered separately.

1. Role of NS activity in hemopoietic regulation.

To our knowledge, there is no literature dealing with the role of NS activity in

hemopoiesis. It has been suggested by many authors, however, that such a role may exist, based on the consistent occurrence of NS activity at sites of ongoing hemopoiesis (reviewed in refs. 1, 2). We have investigated the role of NS activity in resistance to hemopoietic engraftment for the following reasons: (a) it is present in the bone marrow of normal adult recipients (and there only in the adult), and has been suggested to play a role in hemopoietic regulation, as it is also found in fetal liver and neonatal spleen; (b) it is apparently not antigen-specific and is not H-2-restricted in its function, which is compatible with an effect on semi-syngeneic targets (the parental marrow in the P-->F_1 model) and syngeneic targets (donor marrow in the syngeneic model); and (c) these characteristics meet the criteria for natural resistance to hemopoietic engraftment ("a cytostatic effect exerted by the host environment on transplanted cells after the latter have settled into sites of hemopoiesis", see chapter I). Natural suppression differs from the functions usually referred to under the heading of natural resistance, which includes various cytotoxic effector cells (reviewed in chapter I), in that it does not entail the lysis of the target cells, resulting in their definitive elimination, but their inhibition or suppression (by an undefined molecular mechanism which is reviewed in chapter I). The conventional explanations for natural resistance, however, did not fit *a priori* our observations regarding graft failure in unirradiated F_1 hybrid recipients given a large parental marrow graft (chapter II). In those studies, we found that, in the absence of any conditioning, the administration of $15x10^6$ parental marrow cells depleted of mature T lymphocytes did not induce any chimerism in peripheral blood (table II-1) or bone marrow (figure II-1). The donor hemopoietic stem cells, however, were not eliminated, since they could be activated and induced to take over the hemopoietic compartments by the delayed administration of a specific host conditioning which targets the host cells and spares parental marrow (an anti-H-2 monoclonal antibody directed to the H-2K molecule not shared by the donor and host). The survival of donor

precursor cells up to 30 days after marrow infusion was thereby established, and argued against their complete elimination by lytic mechanisms. Moreover, secondary transfer experiments indicated that the donor precursor cells were parked in the host marrow (figure II-2 and table II-2), but incapable of generating CFU-GM or peripheral blood cells.

The evidence supporting a role for NS activity in resistance to hemopoietic engraftment can be summarized as follows: unirradiated adult hosts pretreated with complete Freund's adjuvant (3) exhibit increased resistance to hemopoietic engraftment , in both $P \rightarrow F_1$ and syngeneic donor-host combinations (figure III-1); neonatal hosts are resistant as well, becoming gradually more susceptible to antibody-facilitated engraftment in the first weeks after birth (figure III-5). In both cases, levels of NS activity are increased in the host as compared to the normal untreated adult, and the variable degree of resistance observed at different time points after CFA administration or birth closely parallels the level of NS activity in the host at the time of transplantation (figures III-2, III-6 and III-9). Conversely, the administration of facilitating antibody decreases bone marrow NS activity (figure VIIIA), by a factor of 4 to 10 as determined by NS monitoring using the *in vitro* plaque forming cell assay. When examining engraftment kinetics, it was found that the replacement of the host CFU-GM pool by donor-derived CFU-GM was accelerated when transplanting donor marrow at the time host NS activity is lowest (figures IV-1 and IV-2). Conversely, the adoptive transfer of spleen cells with NS activity (harvested from adjuvant-treated animals) decreased engraftment kinetics, while the transfer of spleen cells devoid of NS activity (harvested from saline-injected control animals) did not (figure IV-6).

These correlations and the more direct evidence provided by the adoptive transfer experiments are supported by two other sets of experiments, one arguing against plausible alternative explanations for resistance to engraftment, the other suggesting that the reduction of host NS activity may play a role in engraftment induced by conditioning agents

other than anti-host antibody. Two mechanisms controlling the hemopoietic engraftment process which readily come to mind are NK-mediated resistance mechanisms and the availability of "space" in the host marrow for incoming donor stem cells. The arguments against a role for NK cells are the following: (a) the adjuvant treatment which increases resistance to hemopoietic engraftment reduces NK activity in the host (figure III-3B); (b) neonatal hosts, which are resistant to engraftment, are devoid of NK activity (figure III-6B); (c) NK-deficient animals are not more susceptible to antibody-facilitated engraftment than their wild-type congenics (table VII-1, this chapter); (d) all these studies were performed using relatively high marrow doses which are known to override hybrid resistance mechanisms. Conversely, a role for NK cells in facilitating engraftment is not suggested because: (a) antibody-facilitated engraftment increases in the post-natal period (figure III-5) while NK activity remains undetectable (figure III-6B); (b) NK-deficient animals are not less susceptible to antibody-facilitated engraftment than the wild-type congenics (table VII-1); (c) administration of facilitating antibody reduces host NK activity (figure III-8B), while enhancing engraftment. The arguments against creating "space" in the marrow as the key to facilitating engraftment are the following: (a) after administration of CFA, the increase in host marrow cellularity is marginal (figure III-4) and is therefore unlikely to account for the increased resistance to engraftment; (b) after administration of facilitating antibody, the decrease in marrow cellularity is marginal (figure IV-3) and is therefore unlikely to account for the decreased resistance to engraftment; (c) furthermore, the recovery of NS activity in the bone marrow after administration of facilitating antibody is deferred compared to the cellular replenishment, and is therefore compatible with the prolonged effect of antibody observed in the syngeneic transplantation model, in which facilitation of engraftment was still observed 4 weeks after antibody administration (4); (d) the reduction of host marrow cellularity induced by different cytotoxic drugs does not

correlate with the engraftment which is permitted by some but not others (table IV-1). In the same experiments, a role for host NS activity in the control of hemopoietic engraftment is suggested by the anti-NS effect of other conditioning agents (table IV-1). Whole body irradiation with 6Gy reduced bone marrow NS activity more than did 3Gy, which was reflected in the resulting degree of chimerism. Cyclophosphamide (200mg/kg) induced chimerism whereas cyclophosphamide at 50mg/kg and methotrexate (100mg/kg) failed to do so. Although all these conditioning regimens reduced host marrow cellularity, only those that significantly reduced NS activity induced chimerism.

I have therefore proposed a model in which hemopoietic engraftment is controlled by a bone marrow-associated inhibitory regulatory activity. This controlling activity is either mediated by NS effector cells themselves or a cell population which is very closely associated with NS cells. The latter distinction can only be resolved once highly purified NS effector cells become available. In the regulatory model of engraftment, it is not necessary to destroy host hemopoietic tissue in order to create "space" for incoming donor cells. While the latter, a more radical form of conditioning, is effective, it is in fact sufficient to shut off the controlling regulatory population which prevents successful donor engraftment. The two opposing models, based either on space or regulation, are represented in figure 1. Although many investigators believe that creating space is the key to successful marrow transplantation, there is literature supporting the regulatory model. In congenitally anemic *W* mice, donor engraftment can be achieved in the absence of any host conditioning (5,6). Resistance to engraftment has been shown not to be a direct function of host cellularity, which varies in different variants of the *W* series. Since NS activity in the host has been found to vary with the activity of cycling CFU-S (7), our model predicts that *W* mice, which have defective stem cell activity, have low NS activity and are therefore more susceptible to donor hemopoietic engraftment. In another context, decreased

engraftment was observed in lethally irradiated mice treated with rGM-CSF (8). In those experiments, lethally irradiated mice were given T cell-depleted allogeneic marrow grafts under conditions which permit stable hemopoietic chimerism. But in hosts treated with rGM-CSF from day 0 till day 10 post-transplant, chimerism levels were significantly decreased, although survival was enhanced, a finding the authors could not easily explain. Since rGM-CSF increases NS activity (*in vitro* , ref. 9, and *in vivo* , annex II), we suggest the growth factor increased the recovery of endogenous hemopoiesis, thus resulting in increased NS activity and decreased donor engraftment. In reconstituted syngeneic radiation chimeras, Diener and colleagues have directly shown that the established proliferating pool can inhibit incoming stem cells, ruling out lack of space in the recipient and exhaustion of available endogenous growth factors (10). Our own data also support such a regulatory control exerted by cycling hemopoietic stem cells on quiescent precursors in the apparent graft failures in unconditioned semi-allogeneic chimeras (chapter II), which is represented in figure 2. In this model, as certain totipotent hemopoietic stem cells (THSC) enter the active hemopoietic pool to generate CFU-S and lineage-specific progenitors after a possible transition from a quiescent state (G_0) to a cycling state (G_1 and beyond), three primary events would simultaneously take place. First, the proliferation and differentiation of the cycling THSC, which is accompanied by the production of negative feedback signals (which can be monitored as NS activity *in vitro*). Second, the other stem cells are maintained in a quiescent state by the negative signals emanating from the cycling THSC, which thereby regulates the number of active stem cells at any given time in the marrow volume which is within the range of action of such negative feedback inhibition. Third, the proliferating clone itself must become resistant to the inhibitory activity. This may be achieved if the activation of the THSC is coupled with the induction of a refractory state and the loss of responsiveness to the negative signal within the progeny. This latter

event may find precedence in the natural suppression of the *in vitro* plaque forming cell response, where the IgM response appears to be abrogated by the addition of bone marrow cells to the culture but not the IgG response (11), as well as in the necessity to add the bone marrow cells within 24 hours of the onset of the culture to observe any suppression (11,12). Both observations concur to suggest that beyond a certain state of activation and/or differentiation the lymphocyte may evade the inhibitory signals.

Other existing models which account for resistance to stable hemopoietic engraftment, excluding the elimination of donor cells by natural or acquired immunity, are based on the idea of avilability of "space" in a broad sense, defined as the access to priviliged sites which preserve stem cells and/or are capable of supporting their proliferation and differentiation. The first intramedullary obstacle for the THSC to reach such a site is the blood-bone marrow barrier (13). Blood cells are produced in the extravascular spaces of the bone marrow, and are subjected to transcellular migration to enter the blood compartment. The medullary endothelium is selective for erythrocytes vs granulocytes and might therefore be conceived as regulating the entry and exit of hemopoietic stem cells. Its function in marrow graft resistance, if any, may be abrogated by the endothelial dammage inflicted by irradiation, cytotoxic drugs or anti-host H-2 antibody. The experiments involving secondary transfer of bone marrow from primary, unconditioned marrow recipients (figure II-2 and table II-2) suggest however that, even in the unirradiated host, the blood-bone marrow barrier is not a major obstacle to engraftment.

Whatever the destination of the donor hemopoietic stem cells, competition between stem cells may result in graft failure if host stem cells are somehow favored over those of donor origin, either by virtue of a numerical advantage or of intrinsic properties of individual stem cells. The existence of such competition is well established: in reconstitution experiments in syngeneic radiation chimeras, the co-administration of fetal

liver and adult bone marrow will eventually result, in the long term, in repopulation by cells of fetal origin, despite the administration of fewer fetal CFU-S than adult CFU-S (14). This may be explained by some intrinsic property of the fetal THSC, in terms of self-renewing capacity, or by a quicker doubling time of the fetal cell, which after a time lapse dominates the active hemopoietic pool (15). Another case possibly illustrating such "dominance" is the engraftment of *W* anemic mice by wild-type, syngeneic marrow cells in the absence of any conditioning (6). But a general downfall of competition experiments is the difficulty to distinguish whether the reconstitution by few clonal precursors is caused by few cells reaching the appropriate site or by many doing so but few proceeding to proliferate (14,16). It is difficult to assess whether BALB/c and (BALB/c x C3H/HeJ)F_1 marrow cells, which were used in most experiments reported here, differ in such a respect. Undoubtedly, in the absence of a drastic conditioning regimen such as lethal whole body irradiation, which likely nullifies the numerical advantage of host stem cells, competition is a realistic explanation for resistance to engraftment. It is however unlikely to explain the resistance to engraftment we observed in adoptive transfer experiments (figure IV-6). In those experiments, the administration of equal numbers of nucleated spleen cells with or without NS activity, which were irradiated *in vitro* (8Gy) to abrogate stem cell function, resulted in decreased engraftment only when adding spleen cells containing NS cells.

The niche concept is the best defined model clearly defining the space stem cells may be competing for (17). In this model, the hemopoietic stem cell, which is more primitive than the CFU-S, is essentially a fixed tissue cell whose maturation is prevented. Its progeny, unless they occupy a similar stem cell niche, are first generation colony forming cells, which proliferate and mature to acquire a high probability of differentiation. When the THSC divides, only one of the two daughter cells can be retained in the niche and the other becomes the first generation colony-forming cell. If this cell could find a

niche, it would become a fixed stem cell. In this event, the facilitating antibody would somehow have to create space in the niche to allow for definitive engraftment to occur (the antibody is sufficient to ensure engraftment since it is effective in the syngeneic donor-host combination (4) and in the absence of T cell-mediated graft-vs-host reactions in the $P \rightarrow F_1$ model, see annex IV). This is however unlikely to be the case given that antibody-conditioning is much milder than lethal irradiation (which sometimes is followed by endogenous repopulation) and minimally reduces total host marrow cellularity. To be effective, antibody-conditioning would therefore have to preferentially target the THSC amongst all marrow cells, which is unlikely because this conditioning is never lethal.

Others have proposed a medullary organisation based on functional volumes (18,19), as opposed to specific anatomic sites such as niches, which are functionally structured around one THSC. The partitioning of bone marrow into localized functional units is based on dose-response (CFU-S content) curves in reconstitution experiments using unirradiated *W* recipient mice, which indicate that bone marrow seeding occurs in random sites, but that the limitation of CFU-S proliferation which ensues is independent of the number of CFU-S initially seeded in a given volume, thus suggesting that it is the hemopoietic proliferation which is controlled rather than the successful seeding of a privileged site (18). This is consistant with a putative regulatory role of short range interactions. This partitioning in regulatory domains is also supported by massive transfusion experiments ($4x10^7$ bone marrow cells administered each of 5 consecutive days) which allow for significant permanent donor engraftment (about 20%). These observations argue against obligatory sites such as niches and for the existence of more sites than those utilized by the normal, healthy animal (19). Our findings are consistant with such a model in which functional sites may be authorized or suppressed by neighbouring active stem cells, via local negative feedback signals. In this way, the degree

is stable and may be stored at 4ºC. The staining buffer is made by disolving 66µl G6PD (Glucose-6-phosphate-dehydrogenase, 1mg/ml, no. 127 035, Boehringer-Mannheim), 20mg F6P (Fructose-6-phosphate disodium salt, no. 104 850, Boehringer-Mannhein), 10mg NADP (NADP disodium salt, no. 128 040, Boehringer-Mannheim), 4mg PMS (Phenazine Methosulfate, no. P-9625, Sigma) and 10mg NBT (Nitro blue tetrazolium, no. N-6876, Sigma) in 53 ml of the staining buffer. Each filter is then incubated with the staining solution in small Petri dishes in a 37ºC incubator. Purple bands are visible within 10 to 30 min, depending on the amount of enzyme loaded. The reaction is terminated by rinsing the filter under the tap.

To fix the stained filter, filters are soaked overnight in a fixing solution made up of 20ml dH_2O + 20ml methanol + 4ml glacial acetic acid. Gels are eventually scanned using a colorimetric densitometer (CAMAG electrophoresis scanner), set up with a Hewlett-Packard integrator which prints the density profile of each band and integrates the surface under each peak, thus permitting a comparison of the relative presence of each band in the sample.

Summary of the required solutions:

Tank buffer: 24,2g Trizma base
25,5g sodium citrate
dH_20 q.s.p. 1l
add ~18ml 12N HCl till pH = 6,2

Stain buffer: 0,3M Tris HCl (4,74g in 100ml dH2O)
0,3M Tris base (4,74g in 100ml dH20)
bring Tris HCl to pH = 8,0 with Tris base

Staining solution: 53ml stain buffer

65µl G6PD

20mg F6P

10mg NADP

04mg PMS

10mg NBT

Fixing solution: 20ml dH_2O

20ml methanol

04ml glacial acetic acid.

3. Chimerism determination in mixed cell populations.

The value of GPI isozyme analysis resides in its exquisite sensitivity, which permits the determination with great precision of the percentage of each band in any sample. This is of particular interest in the analysis of the chimerism level after bone marrow transplantation in different tissues, such as bone marrow, blood, spleen, thymus and lymph node.

Single cell suspensions are lysed by freeze-thawing and loaded onto the starch gel for electrophoresis. After electrophoretic separation, isozymes are transferred to n[illegible]llulose filters and fixed. Each sample is then scanned by densitometry, as illustrated [illegible]gure. The ratio of one band to another is then determined and calculated from a curve constructed by mixing peripheral blood of donor and host origin in known proportions. Examples of such curves are shown in the figure, wherein each curve was constructed from numerous GPI analyses of mixes of blood from C57BL/6J and C57BL/6J-*gpi1-a* or BALB/cCr and (BALB/cCr x C3H/HeJ)F1 mice.

4. Bone marrow colony phenotyping.

Another advantage of GPI isozyme analysis also accrues from its sensitivity, permitting the determination of isozyme ratios present in very small cell samples. This method has been used to determine the genetic origin of single large cells such as an oocyte. It has also been applied to the analysis of bone marrow colonies grown in vitro in semi-solid medium. At the end of the culture, colonies (at least 30 cells) are individually picked under the microscope by aspiration into very thin capillary pipets and frozen at -20ºC. Colonies are individually loaded, usually 50-70 per animal, and stained. In this discrete assay, colonies are either of donor or host origin.

CFU-GM and CFU-mix colonies were grown in the following way. Bone marrow cells were washed once and diluted at 10^5/ml in the semi-solid medium described below. One ml of medium was then dispensed in Lux-R plates (Miles Scientific, Naperville, IL) which were then placed in a 5% CO_2 atmosphere in a humidified incubator for 6-8 days (CFU-GM) or 10-12 days (CFU-mix).

10ml methylcellulose (Fisher Scientific, Ottawa, ON)

20% fetal bovine serum (Flow Laboratories, McLean, VA)

20% IMDM (Gibco Laboratories, Grand Island, NY)

10% WEHI-3-conditioned culture supernatant.

5x10-5M 2-mercaptoethanol

2U/ml purified erythropoietin for CFU-mix cultures.

Resultant colonies are then individually typed for *gpi* expression.

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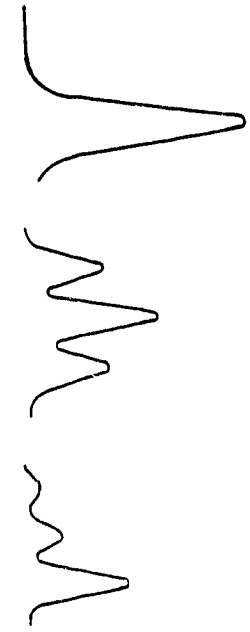

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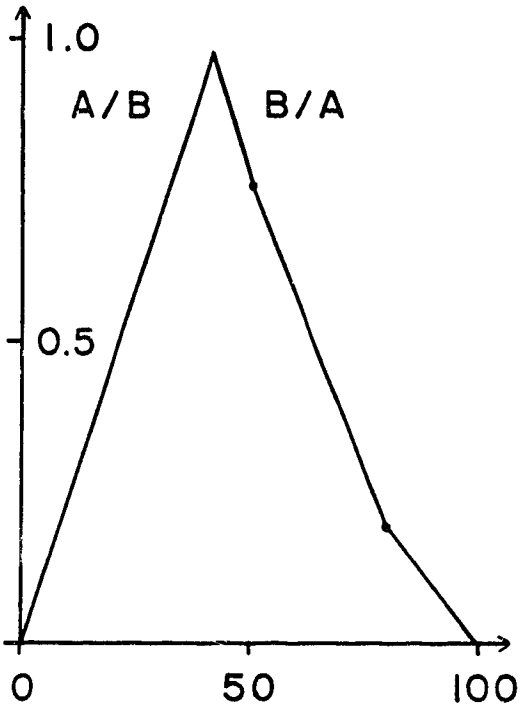

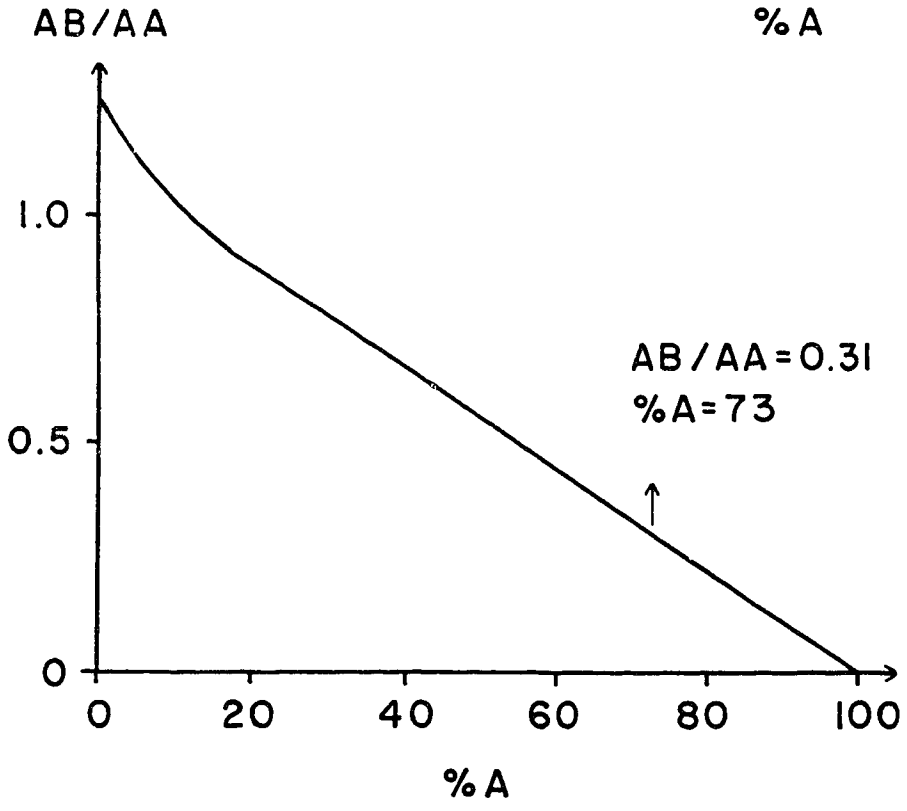

APPENDIX IV: donor T cells are not necessary for antibody-facilitated bone marrow engraftment.

Bone marrow graft recipients receive a conditioning regimen which is designed to promote donor stem cell engraftment. Ideally, this should lead to tolerance of donor antigens by the host while allowing stable and reasonably complete donor takeover of central and peripheral hemopoietic tissues. This is currently achieved through various treatments aimed at diminishing host cells which mediate host resistance to donor stem cells. The bone marrow graft, however, is not just a source of stem cells, since it's resident T cells appear to play an active role in promoting engraftment. This is indicated by reports of the failure of T cell-depleted bone marrow to engraft when compared to unmanipulated bone marrow (1-4). It appears that the reduction of engraftment following such manipulations is not due to direct damage inflicted upon the hemopoietic stem cells (5). This suggests that the preparative regimen does not allow the host to be completely engrafted, but rather creates conditions whereby a donor T cell-dependent pathway leading to engraftment is operational. If true, this approach runs contrary to current concepts of graft-versus- host disease (GVHD) prophylaxis, the essence of which is efficient donor T cell depletion (6,7). This dilemma demands a reevaluation of GVHD prophylaxis, including further understanding of it's pathogenesis, as well as a search for new approaches to host conditioning.

We previously described the production of bone marrow chimeras in mice in which conditioning is mediated by administering anti-host antibody *in vivo* , in the absence of host irradiation or cytoreductive drug therapy (8,9). Recipient F_1 hybrid mice are injected with parental bone marrow and anti-host major histocompatibility complex (MHC) class I monoclonal antibody, a procedure that can lead to virtually complete donor

engraftment without the GVHD potentiation that results from host irradiation. Recently, this approach has been successfully applied to syngeneic engraftment (10,11). Engraftment is antibody-dependent, because control hosts show only marginal or no donor stem cell engraftment (9-11). Although the antibody is essential for creating conditions for successful engraftment by parental stem cells, we wanted to determine whether donor T cells contributed to engraftment in this model. This question was also prompted by the observation that engraftment is more efficient in P→F_1 than syngeneic donor-host combinations, all parameters other than donor genotype being identical (10). We therefore adressed the role of graft-associated T cells by studying the capacity of T cell-depleted or host-tolerant donor marrow to engraft unirradiated, antibody-conditioned hosts. We report here that optimal (complete) antibody-dependent donor stem cell engraftment is unaffected either by donor T cell depletion or inducing tolerance to the host. This suggests that antibody-facilitation may be a useful alternative to methods of bone marrow transplantation that are hindered by a T cell requirement for engraftment in the face of GVHD.

MATERIALS AND METHODS.

Mice. BALB/cCr and (BALB/cCr x C3H/HeJ)F_1 hybrid mice were bred at the University of Alberta. Glucose-6-Phosphate Isomerase (Gpi) recombinant mice (C57BL/6J, *Gpi-1b* , and recombinant inbred C57BL/6J-*Gpi-1a*) have been described (11). Age-matched, sex-matched adult mice (2-3 months old) were used for transplantation and housed under standard conditions.

Antibodies. 16-3-1N is a mouse anti-H-2K^k IgG2a antibody (12), prepared from ascites fluid. Purification and quantification have been described (11). GK1.5 (a rat anti-L3T4

IgG2b, ref. 13) and 2.43 (a rat anti-Ly2.2 IgG2b, ref. 14) were used after 45% ammonium salt precipitation of culture supernatants at a concentration of 2 mg/ml and 3 mg/ml respectively based on absorption at 280 nm. These antibodies were acquired from the American Type Culture Collection, Rockville. MD. Thy1.2 is a mouse IgM directed against the Thy1.2 molecule, purchased from NEN, Boston, MA.

***In Vitro* Complement-Mediated Antibody-Depletion.** Single cell suspensions of spleen or bone marrow cells were diluted in balanced salt solution (BSS) containing 0.2% bovine serum albumin (pH 7.2) at 10^7 cell/ml and incubated on ice for 45 mn, washed once then resupended in rabbit low toxicity complement (Cedarlane, Hornby, ON) at a 1:10 final dilution in the same solution for 45 mn in a 37ºC water-bath. Antibodies were used at 1:100 final dilution for GK1.5 and 2.43 and 1:500 final dilution for Thy1.2. This procedure was repeated a second time. Cells were washed once and resuspended in BSS for injection or culture medium for proliferation assays.

Transplantation Procedure and Chimerism Measurement. Donor mice were sacrificed by cervical dislocation and had their tibias and femurs removed. Bone marrow plugs were flushed out with 2-3 ml cold BSS, washed once and resuspended for injection. Conditioning consisted of a single intravenous or intraperitoneal injection of anti-K^k monoclonal antibody. Control animals receiving no antibody, normal mouse serum or isotype-matched monoclonal antibodies specific for another H-2 haplotype display the same low level of engraftment or none (9-11).The chimerism measurement has been previously described (15). Briefly, peripheral blood samples were lysed and run on starch gel electrophoresis followed by quantitative determination of donor and host isozymes based on a colorimetric reaction quantified by densitometry. Chimerism levels were read from a

curve constructed from measurements of artificial cell mixes of known donor-host proportions. This method is very sensitive and reproducible, and is discussed elsewhere 3. Antibody-facilitated engraftment is stable after 8-10 weeks post transplant throughout the recipient's lifespan and achieves comparable chimerism in erythroid and myeloid lineages (15), suggesting stem cell engraftment, as further corroborated by transplantation to secondary hosts, shown later in this paper.

***In vitro* Proliferation Assays.** Conventional 72 hour cultures were set up in 96 well plates (Costar, Cambridge, MA), containing 2.5 10^5 spleen cells or 10^6 bone marrow cells each. The T cell specific mitogen Concanavalin A (Calbiochem, LaJolla, CA) was used at a final concentration of 2 μg/ml, while E. coli phenol-extracted lipopolisaccharide (Sigma, Saint Louis, MO) was used to measure T- independant proliferation at a final concentration of 10 μg/ml. Proliferation was then measured as the cellular incorporartion of ^{3}H-thymidine (NEN, Boston MA) over an 8 hour period as determined by a LKB liquid B-scintillation counter.

***In vivo* monitoring of GVHD.** Two to three month old BALB/cCrxC3H/HeJ females were lethally irradiated from a ^{137}Cs source (Gammacell, AEC, Ottawa, ON), receiving 8.0 Gy at 80 cGy/mn. Animals were injected intravenously with 10 million spleen cells (preferred to bone marrow in order to get a vigorous graft-versus -host rection) from BALB/cCr-->(BALB/cCr x C3H/HeJ)F_1 antibody-facilitated chimeras or age-matched normal parental BALB/cCr females. Animals were maintained on oral tetracycline for the first week and monitored daily for survival and weekly for weight. Results are expressed as actuarial survival and relative weight variations, compared to that of 8 animals receiving

syngeneic F_1 spleen cells and calculated as (weight day n - weight day 0) in allogeneic recipients divided by the mean of (weight day n - weight day 0) in syngeneic recipients.

Stem Cell Assays. The bone marrow stem cell content was assessed by two methods. 1) CFU-S were determined by enumerating subcortical nodules in the spleens of lethally irradiated (9 Gy, 80 cGY/mn) syngeneic BALB/cCr females, 12 days after inoculation (17). One hundred thousand bone marrow cells from pooled BALB/cCr-->(BALB/cCr x C3H/HeJ)F_1 or parental donors were injected intravenously into 5 or 6 recipient mice, 4 hours after irradiation. These inocula were derived from the bone marrow used for antibody-facilitated transplantation experiments. 2) CFU-GM progenitor cells were enumerated in an *in vitro* colony assay. Bone marrow cells were cultured for 6 days at 10^5 cells/ml in a semi-solid medium containing 50% methyl cellulose (Fisher Scientific, Ottowa, ON), 20% fetal calf serum (Flow Laboratories, McLean, VA), 20% IMDM (Gibco Laboratories, Grand Island, NY), 10% WEHI-3 supernatant-conditioned medium and 5.10^{-5}M 2-mercaptoethanol, at 1 ml/plate in Lux-R plates (Miles Scientific, Naperville, IL). Cultures were set up in triplicate and counted under the microscope on day 6.

RESULTS

Engraftment of T cell-depleted bone marrow is not different from that of non-depleted marrow.

In order to evaluate the role of donor T cells in P→F1 antibody-facilitated bone marrow transplantation, the engraftment potential of parental marrow was assessed after double antibody and complement-mediated T cell depletion of the donor inoculum (see Materials and Methods). In Table 1, one experiment representative of five is reported,

showing the reduction of the Concanavalin A-induced proliferative response achieved in spleen and bone marrow by two consecutive antibody-mediated T cell depletion steps. Specific T cell subset (anti-L3T4 and anti-Ly2) treatment as well as anti-Thy1 depletion reduced the Con A response close to background levels, while the response to LPS was not reduced. T cell depletions are shown on spleen cells as well as bone marrow cells because the induced T cell proliferation of the latter is much less due to a lower T cell content and the presence of cells with natural suppressor activity (17). The Thy1.2 depletion was consistently found to be slightly more efficient, reducing proliferation to background (stimulation index equal to or less than one). To compare the engraftment capability of untreated, complement-treated or T cell-depleted bone marrow, adult F1 mice were transplanted with 15 million donor bone marrow cells and co-injected with 500 ug of anti-Kk monoclonal antibody, a protocol which achieves virtually complete long term donor representation in the peripheral blood (Figure 1). When using 10 million donor bone marrow cells, only mixed chimerism is achieved (data not shown). No difference in final engraftment levels (at 12 weeks) or engraftment kinetics (peripheral blood donor representation at 4 weeks) was found between untreated, complement-treated and T cell-depleted marrow (Figure 1). The possibility exists, however, that minimal specific anti-host reactions are still operative. To evaluate this in an independent manner, we compared the engraftment capacity of parental marrow from host-tolerant donor mice to that of normal (alloreactive) donors.

The engraftment kinetics of host-unresponsive donor marrow is identical to that of normal alloreactive marrow in antibody-conditioned hosts.

It is possible to assess the role of donor T cells in P→F1 engraftment using host-tolerant donors because a careful analysis of stable, long-term antibody-facilitated P→F1

chimeras in the same strain combination has shown that these chimeras are generally immunocompetent and specifically host-tolerant by the criterion of anergy in *in vitro* assays. Limiting-dilution analysis revealed very low frequencies of anti-F_1 pre-cytotoxic T lymphocytes and failed to detect any suppressor activity maintaining this unresponsiveness (18). In order to test whether this apparent state of tolerance withstands *in vivo* transfer, an adoptive transfer in a GVHD model was set up. Adult F_1 mice were irradiated (8 Gy) and then injected with 20 million spleen cells from either long term, stable, completely engrafted, antibody-facilitated P→F_1 chimeras (9 months post-transplant) or age-matched parental donors. Survival and weight loss were monitored, and compared to those of a third group of mice receiving 20 million spleen cells from age-matched F_1 mice. As shown in Figure 2 (top section), half the mice receiving parental spleen cells had succumbed to GVHD by the fourth week, whereas in mice receiving chimeric spleen cells no deaths were observed at 10 weeks (nor were there deaths in syngeneic recipients, data not shown). No clinical signs of GVHD or weight loss were detected (Figure 2B). These data establish that parental T cells from these P→F_1 chimeras injected into F_1 hosts are incapable of inducing the classical signs of secondary disease. There was no significant difference in day 12 CFU-S and CFU-GM content between the two types of bone marrow donors, suggesting that both were hemopoietically equivalent (Table 2). The engraftment achieved with bone marrow from completely engrafted P→F_1 chimeras was compared to that achieved with marrow from untreated, age-matched parental donors (Table 2).Transplantation with grafts from alloreactive or allotolerant donors resulted in the same final level of antibody-facilitated engraftment, as well as similar engraftment kinetics based on peripheral blood chimerism levels 4 weeks after transplantation. Thus engraftment of parental stem cells in the absence of demonstrable T cell alloreactivity was not impaired, a finding that is in accord with the previous data involving *in vitro* donor T cell depleted marrow.

DISCUSSION

The role of donor T cells in promoting engraftment of inoculated stem cells is a controversial issue in clinical bone marrow transplantation. This role has been highlighted by the description of increased graft failure or leukemic relapse rates after transplantation of T cell depleted bone marrow (1-4). As a consequence, many groups have interrupted their T cell depletion programmes , at the cost of increased GVHD incidence and severity. Alternative approaches to the retaining of all or selected T cell subsets present in the graft depend on the development of new models of host conditioning that bypass T cell dependency. In a murine model, we have reported that resistance to engraftment of donor stem cells in unirradiated adult hosts can be abrogated by anti-host antibody treatment, allowing for complete donor engraftment in $P \rightarrow F_1$ and syngeneic donor-host combinations (8-11). This is achieved following a single intravenous injection of anti-host MHC class I antibody as the sole conditioning regimen. We investigated the role of T cells in the donor inoculum on the level of donor hemopoietic takeover because, as we reported earlier, engraftment levels achieved in a given F1 host for a given dose of donor marrow cells and anti-host MHC antibody are greater in the $P \rightarrow F_1$ than the syngeneic $F_1 \rightarrow F_1$ combination (for example, 58 ± 12% (mean ± standard deviation) in the $P \rightarrow F_1$ versus 21 ± 7% in the syngeneic $F_1 \rightarrow F_1$ in (C3H/HeJ x C57BL/6J)F_1 animals receiving 1.2 mg anti-K^k monoclonal antibody and 20 million donor marrow cells while animals receiving irrelevant control antibody show no detectable donor engraftment in 9-10 mice per group. See also reference 10).

The irrelevance of donor T cells for overcoming resistance to transplantation in antibody-dependent engraftment is indicated by two sets of observations : first, efficient depletion of

donor T lymphocytes by anti-T cell antibodies and complement did not result in reduced engraftment levels ; second, marrow from specifically host allotolerant parental donors did not show reduced capacity of engraftment when compared to marrow from normal parental donors. Both observations point to an engraftment process which is not affected by removal of graft-associated T cells, thus suggesting that in this model the anti-host activity provided by the conditioning antibody is sufficient to eventually overcome host resistance effectors. Other mechanisms can account for the greater efficiency of semiallogeneic engraftment compared to syngeneic engraftment : non-T cell-mediated graft- versus- host reactions that promote engraftment (19)-which is not ruled out by the observations reported here-, intrinsic hemopoietic advantage of parental stem cells in this strain combination, residual anti-donor stem cell reactivity of the antibody in the syngeneic situation or a combination of the above.

Various approaches have been taken to remedy T cell depletion-associated graft failures and leukemic relapses. Pan-T cell depletion has been either abandoned, limited by retaining reduced levels of T cells or replaced by subset-specific T cell depletion (the latter assuming that one can predict which T cell subset predominantly induces GVHD in a given donor-host combination). Based on the findings reported here, we propose that anti-host antibodies could be used to promote donor engraftment, as well as to reduce the use of non-specific and deleterious agents in the conditioning regimen, because anti-host antibodies can promote engraftment in the normal, immunocompetent engraftment- resistant adult and because this effect is not impaired by T cell depletion of the donor inoculum. Others have now started to use monoclonal antibodies to reduce the incidence of graft failure in particular conditions and in association with other combined therapeutic agents (20,21). Our goal is to identify the critical targets which are affected by the anti-host MHC antibodies and eventually produce specific, efficacious reagents that would allow

successful bone marrow transplantation in unirradiated syngeneic and allogeneic recipients.

TABLE 1

	Bone marrow	Spleen	
Cell treatment*	Con A response#	Con A response	LPS response#
None	2.5	24.7	14.6
Complement (C')	2.7	21.0	17.3
Anti-L3T4,Ly2 + C'	1.4	1.9	28.7
Anti-Thy1.2 + C'	0.9	0.8	27.4

* In vitro treatment with monoclonal antibodies and complement is described in Materials and Methods.

Proliferative responses to mitogens (Con A, 2 μg/ml, and LPS, 10 μg/ml) are expressed as stimulation indices. Background thymidine incorporation is 2735±249 cpm/well and 1890±114 cpm/well for bone marrow and spleen, respectively. Standard deviations are less than 8% and not shown.

TABLE 2

A.Bone marrow inoculum*	BALB/c donor@	(BALB/c→CC3) donor@
% BALB/c chimerism	100%	98±4%
CFU-S content	28.5±4.5	26.5±2.5
CFU-GM content	336±36	386±4
B.Chimerism level#		
At 4 weeks	49±8%	52±6%
At 12 weeks	96±5%	98±4%

@ Marrow from stable P→F_1 chimeras or age- and sex-matched parental mice are compared in their hemopoietic content (section A of table) and engraftment capacity (section B of table).

* Chimerism (expressed as % parental isozyme representation in peripheral blood lysates), CFU-S (expressed as the number of subcortical nodules enumerated 12 days after injection of 10^5 bone marrow cells) and CFU-GM (expressed as the number of colonies per 10^5 bone marrow cells counted after a 6 day culture) are described in Materials and Methods.

Engraftment levels of marrow from alloreactive and allotolerant donors in antibody-conditioned $CC3F_1$ hosts is determined in peripheral blood lysates 4 and 12 weeks after transplantation.

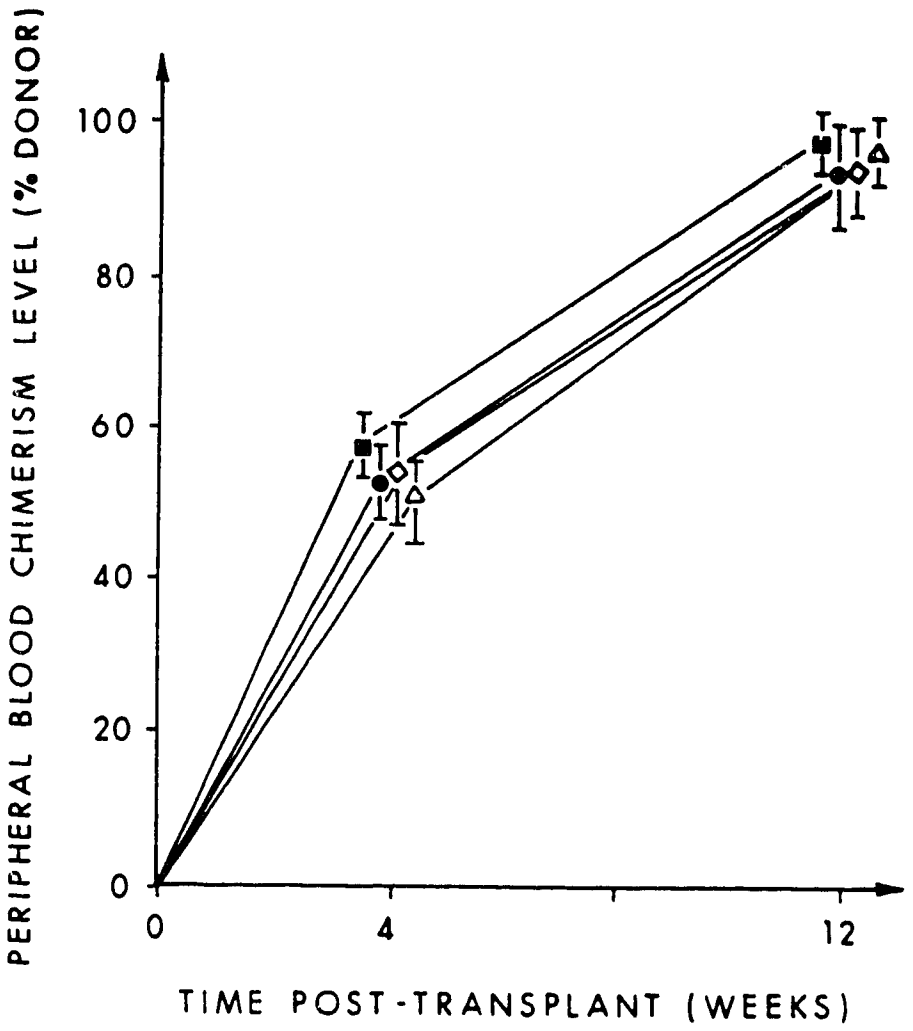

Figure 1. Engraftment kinetics determined by peripheral blood analysis in antibody-conditioned F_1 hybrid hosts. Parental donor marrow is untreated (●), complement-treated (Δ), anti-L3T4, anti-Ly2 and complement-treated (◇) or anti-Thy1.2 and complement-treated (■).

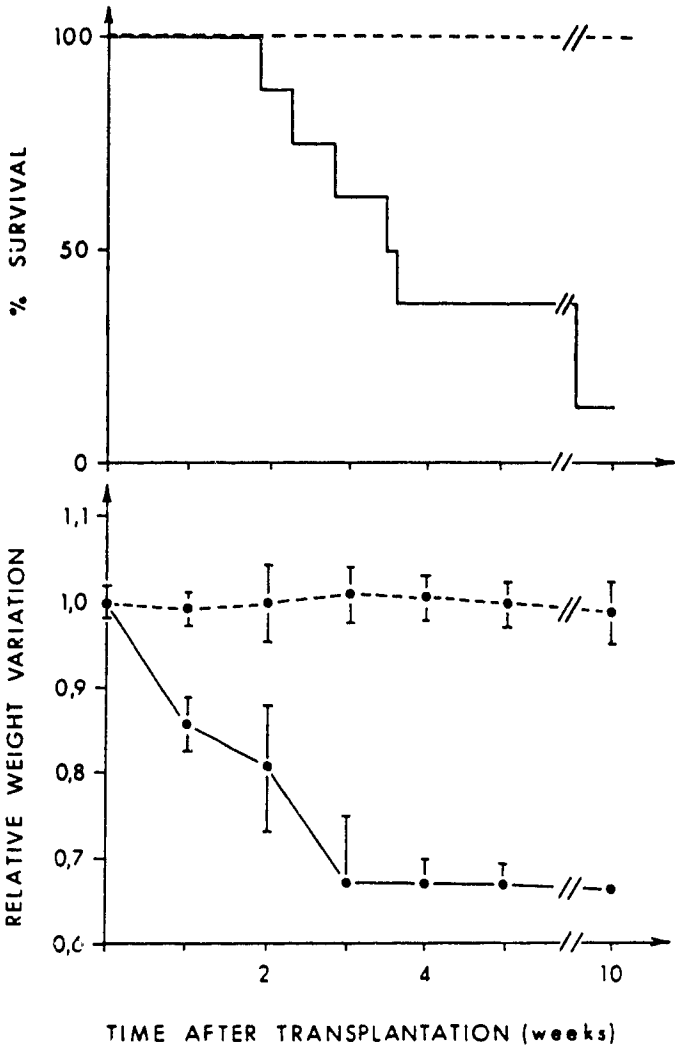

Figure 2. (top) Actuarial survival in irradiated F_1 hyrid recipients given 20 million spleen cells from stable ($P \rightarrow F_1$) chimeras (--) or from age- and sex-matched parental donors (–). (bottom) Relative weight variation in the same animals, compared to F_1 spleen cell recipients. See Materials and Methods for calculation of index.

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