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PARENTAL BONE MARROW GROWTH
IN YOUNG HYBRID MICE

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ABSTRACT

When bone marrow is transplanted from certain inbred mouse strains to F₁ hybrids of that strain, the graft often fails to proliferate. It has been reported that this phenomenon, known as Poor Growth, is not demonstrable in recipients less than three weeks of age. The purpose of the present study was to investigate some of the parameters involved in this phenomenon and its sudden appearance at three weeks of age. By employing ¹²⁵IUdR uptake and hemopoietic colony assays following transplantation of marrow to mice of various ages and treatment groups, the following conclusions were drawn. 1) Parental marrow grew equally well in both parental strain and F₁ hybrid recipients less than three weeks old; 2) The observed growth of hemopoietic tissue was not due to endogenous stem cell proliferation; 3) Changes in radiation sensitivity did not account for the fluctuations of hemopoiesis seen in mice from one to five weeks of age. 4) Factors in milk, either specific or non-specific did not influence the outcome of parental marrow grafts; 5) Neither stimulator cells in mice less than three weeks of age nor graft destroying cells in older mice could be demonstrated; 6) Natural Killer cells were unable to effect in vitro cytolysis of bone marrow or the hemopoietic stem cell compartment thereof.

Two mechanistic models of Poor Growth are presented and discussed and a new model is proposed.

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CHAPTER I

INTRODUCTION

I. TRANSPLANTATION IMMUNOLOGY

One of the major barriers to tissue transplantation has been the ability of the immune system of the recipient to recognize the grafted tissue as "foreign" and mount a response against it which may result in the "rejection" of the graft. Turn of the century reports by Loeb and his collaborators were among the first to demonstrate this (51, 52). They found that a tumor of Japanese waltzing mouse origin was transplantable to nearly 100% of these mice, yet failed to grow in an unrelated strain. Although neither the nature of the genes responsible nor the means by which they acted were known, the conclusion that susceptibility to transplanted tumors is genetically controlled was inescapable.

The discovery pointed the way for other investigators who, during the course of the next 40 years, worked to define and interpret the genetics of tissue transplantation. By 1941 Little had established a set of "laws," based on his own observations as well as those of others, which predicted the outcome of tissue grafts (50). These were summarized by Snell and Stimpfling (77) as follows:

1. Grafts within inbred strains are successful; or stated in more general terms, isografts succeed.
2. Grafts between inbred strains are not successful; or stated in more general terms, allografts fail.

3. Grafts from either inbred parent strain to the F_1 hybrid succeed, but grafts in the reverse direction fail.
4. Grafts from F_2 or subsequent generations also grow in all F_1 mice.
5. Grafts from either inbred parent strain are accepted by some members of an F_2 generation, but rejected by others.

From these and other observations, two basic principles of transplantation genetics have emerged. The first is that the success or failure of a tissue transplant is dictated by the presence of transplantation antigens, which are the products of a set of genes designated histocompatibility genes. In the mouse, the major transplantation antigens are controlled by the genes in a complex locus termed the H-2 locus. When the prospective donor and host are identical with respect to these genes, grafts will be successful when exchanged between them. However, disparity at the H-2 locus (or any of the other histocompatibility loci) will result in the eventual failure of the graft (7).

The second principle is that the family of genes or alleles which may occupy any given histocompatibility locus are inherited and expressed codominantly (7). Thus a cross between an individual that is genotypically a/a for a given histocompatibility locus and one that is of a b/b genotype would produce offspring both genotypically and phenotypically a/b . It is for this reason that, as noted above, grafts from inbred parental strains are accepted by F_1 hybrid recipients. Such grafts present no new histocompatibility antigens not already expressed in the F_1 recipients.

II. BONE MARROW TRANSPLANTATION

Bone marrow consists of a variety of mature blood cells as well as numerous precursors of such cells. In addition, bone marrow contains a population of hemopoietic stem cells which, by definition, maintain their own numbers while continuing to supply the body's demand for mature blood elements (86). It is the presence of these stem cells which gives bone marrow its sustained blood forming ability.

Because of its hemopoietic capacity, bone marrow transplanted in the form of dissociated cells, was first employed as a treatment of the deleterious effects of ionizing radiations on hemopoiesis. Supralethal doses of radiation have been shown to result in anemia, leukopenia and thrombocytopenia as well as damage to lymphatic tissues and bone marrow aplasia (4, 75). In mice, death after such exposures usually occurred within two weeks. In 1951 Lorenz and his coworkers demonstrated that the survival of irradiated mice could be significantly prolonged by the injection of bone marrow cells (58). Later reports (11, 22, 59, 65) have been established that the radioprotective capacity of marrow results from the reconstitution of the hemopoietic compartment of the host by donor cells.

Perhaps foremost among the reconstituting cells is the multi-potential hemopoietic stem cell originally detected by Till and McCulloch (79) and commonly referred to as a colony-forming unit (CFU). When transplanted into an irradiated host, these cells give rise to discrete hemopoietic foci in the spleen of the recipient. These foci or colonies can be erythrocytic, granulocytic or megakaryocytic in nature, however lymphoid colonies do not arise from these cells. There is considerable

evidence that a truly pluripotent stem cell exists which is capable of lymphoid progeny as well (1, 80) however its relationship to CFUs remains at this time unclear (1). Nevertheless, the ability of transplanted bone marrow cells to repopulate all hemopoietic compartments of radiation injured recipients has been amply demonstrated (1, 80).

Bone marrow, as a tissue graft, has several unique features which distinguish it from other types of grafts. First of all, it is typically transplanted by intravenous injection in the form of a suspension of dissociated cells. Secondly, removal of the recipient's bone marrow in preparation for a marrow transplant is accomplished by either large doses of radiation or by very powerful cytotoxic drugs rather than by surgery. Third and most important, the immunologic consequences of bone marrow transplantation are often the reverse of those encountered with other types of tissue grafts. Because the classical immunity of the host is abolished by the destruction of the host marrow, rejection of a marrow transplant is unlikely. However, the graft itself is capable of reconstituting in the recipient an immune system of the donor type. In histoincompatible donor-host combinations, this gives rise to a phenomenon called graft-versus-host (GVH), in which the transplant, in effect, attempts to reject the host. At the present time, GVH remains one of the largest obstacles to routine clinical bone marrow transplantation (9).

III. POOR GROWTH

The Poor Growth phenomenon represents an additional complication to the genetics of bone marrow transplantation. Since the mid-1960's, it has been well established that bone marrow grafts from certain inbred

mouse strains fail to proliferate as well in lethally irradiated F_1 hybrid recipients as they do in syngeneic mice (13, 15, 24, 38, 39). This phenomenon is inexplicable in terms of "classical" transplantation immunology for at least two reasons. First, as noted above, all of the histocompatibility antigens of both parents should be expressed in an F_1 hybrid. Second, even in the presence of foreign transplantation antigens, the immune system of the recipient should be unable to respond to such antigens following lethal irradiation (60).

Poor Growth (also called hybrid resistance (16), hemopoietic resistance (17), genetic resistance (56), allogeneic inhibition (39), syngeneic preference (38) and CFU repression (61) shall be defined here as the sub-optimal proliferation of hemopoietic tissue grafts in lethally irradiated recipients. This definition includes failures of parental grafts in hybrid recipients as well as grafts from allogeneic and xenogeneic donors (82).

Although several theories have been put forth as to the nature of these graft failures, no completely satisfying explanation of the mechanisms involved has been advanced. To date, the most widely accepted of these hypotheses involves the view that Poor Growth is the result of a host-derived immunity (13). According to this hypothesis, the failure of parental marrow grafts in F_1 hybrid recipients results from an immune reaction on the part of the F_1 hybrid against an antigen (or antigens) expressed by the hemopoietic tissues of the parental strain which are absent in the F_1 .

At the heart of this hypothesis are two assumptions which are radically different from what is known about "classical" transplantation immunology. The first is that a cell mediated immune reaction can take

place in a recipient after radiation exposures known to abrogate other immune responses (2, 46). Secondly, that certain transplantation antigens, termed hemopoietic histocompatibility (Hh) antigens, are not codominantly expressed (56). Although extensive research has been carried out in this field over the last 15 years, the validity of these two assumptions has not been confirmed. (See Chapter VIII for further discussion).

IV. POOR GROWTH AND YOUNG MICE

Cudkowicz (16) has reported that, in contrast to the Poor Growth seen in adult F_1 mice, hemopoiesis in young F_1 recipients of parental marrow was comparable to that seen in syngeneic hosts when the mice were less than 21 days old at the time of transplantation. This observation has been interpreted as a reflection of the "late maturation" of the cellular mechanism responsible for the rejection of bone marrow grafts (16, 18). If true, such a late maturation would represent yet another unique feature of the "effector cells" of Poor Growth, in that other immunocompetent cell types are either already functional at birth or become so within 10 days thereafter (78). Subsequent reports (82) have shown that transplantation of otherwise poorly growing allogenic and xenogenic bone marrow result in high levels of hemopoietic reconstitution in very young recipient mice.

However, reports of parental bone marrow growth in young hybrid recipients have failed to address themselves to the question of whether the observed hemopoietic activity resulted from the ability of these recipients to support parental bone marrow growth. The extent of host stem cell proliferation following radiation exposure was only nominally

investigated in these reports (16, 18), even though changes in both the CFU content and radiation sensitivity are characteristic of very young mice (12, 23, 63). Such information is basic to an accurate interpretation of these data. Moreover, such an interpretation requires data, as yet unavailable in the literature, regarding the magnitude of hemopoiesis in both F_1 and syngeneic recipients of parental marrow at the time of "maturation" of Poor Growth.

V. PURPOSE OF RESEARCH

The purpose of the present research was to investigate the cellular basis of hemopoietic recovery in very young F_1 hybrid recipients of parental bone marrow with the expectation that such research might shed some light on the mechanism by which stem cells are regulated in this system. In Chapter II of this volume, the routine materials and methods employed in this research are described. Chapter III consists of a confirmation of hemopoietic recovery in young F_1 recipients of parental marrow and an exploration of the hemopoietic background of these recipients. The role of both specific and non-specific milk factors in this phenomenon is reported in Chapter IV. Chapter V shows the results of experiments designed to study the ability of lymphoid cells from young F_1 donors to influence the outcome of parental marrow grafts in older F_1 recipients. In Chapter VI, the presence of a cell type in adult F_1 mice, capable of diminishing parental bone marrow growth in young recipients, was sought. The ability of a putative "effector" cell, the Natural Killer cell, to lyse parental bone marrow or the stem cell compartment of that marrow in vitro was tested and the data are

presented in Chapter VII. In Chapter VIII, two previously proposed mechanistic models of Poor Growth are presented and discussed. In addition, a new model for the regulation of hemopoietic stem cell growth is advanced in this Chapter, which is based on the data presented here as well as those of others.

CHAPTER II

GENERAL MATERIALS AND METHODS

I. INTRODUCTION

Many procedures, reagents and mouse strains were employed during the course of the research described in this volume. Of those, the materials and methods used routinely are reported in this chapter. Other techniques or variations of the materials and methods described here, which were employed in only a few, specific experiments, are reported in the chapters to which they are applicable.

II. ANIMALS

Adult (12-22 weeks of age), inbred mice of the C57BL/6 (B6) strain as well as (C57BL/6 x DBA/2F₁ (BDF₁) hybrid mice of both sexes were obtained from Jackson Laboratories, Bar Harbor, Maine. Adult DBA/2 and congenitally athymic Balb/c nu/nu mice as well as young (1-42 days of age) BDF₁, B6 and (DBA/2 x C57BL/6)F₁ (called DBF₁) mice of both sexes were bred and maintained in the Biology Division of the Oak Ridge National Laboratory, Oak Ridge Tennessee, from stock originally obtained from Jackson Laboratories.

Stock animals were generally housed ten per cage in the Biology Division animal facility. They were maintained at 72°F with a 12 hour alternating dark/light cycle. Food (Purina Mouse Chow) and water were given ad libitum. Experimental animals were housed in a separate room from that of the stock animals under identical conditions, with the exception that experimental animals were generally housed five per cage.

III. IRRADIATION

Whole body x-irradiation of mice was performed using either a Philips x-ray machine or a G.E. Maxitron 300 under the following conditions:

The Philips machine was operated at 250 kV and 15 mA with 1.0 mm Al added filtration and an HVL of 0.5 mm Cu. A target to object distance of 60 cm was used, and at this distance the exposure rate ranged from 140 to 170 R/min. as measured in air. The G.E. Maxitron 300 was operated at 300 kV and 20 mA at a target to object distance of 70 cm. Two mm Al added filtration was used to give an HVL of 0.5 mm Cu. Under these conditions the exposure rate ranged from 180 to 205 R/min. measured in air.

For whole body irradiation, mice were placed in a circular lucite holder with a maximum capacity of 12 mice. The holder was then placed on a turntable which rotated during irradiation to assure uniformity of radiation exposure.

IV. CELL SUSPENSIONS AND DOSES

Bone marrow cell donors were sacrificed by decapitation, exsanguinated, and the bones to be used (femora, tibia and/or humeri) were removed. Marrow plugs were expelled into cold, sterile phosphate buffered saline (PBS: 15.3g NaCl; 1.45g Na_2HPO_4 ; 0.424g KH_2PO_4 in twice distilled water to a final volume of 2.0 liters), pH 7.2 to 7.4, by aspiration of the marrow cavity with a 22 gauge needle. The marrow cavities were subsequently flushed with additional cold PBS to remove adhering cells. The cells thus obtained were passed several times

through first a 22 gauge and then a 27 gauge needle and finally filtered through 80 mesh nylon to obtain a single cell suspension.

Spleen and thymus cell donors were also sacrificed by decapitation and exsanguinated. Cell suspensions from these organs were prepared by gentle disruption of the organ in a thick walled Sorval centrifuge tube with a teflon pestle in a small amount (1.0 to 2.0 ml) of PBS. The cells were then passed through 80 mesh nylon to remove debris and cell clumps. Unless otherwise noted, all cell suspensions employed in the present work were obtained from a pool of at least three animals to reduce the effects of individual variability.

The number of nucleated cells in the suspensions so obtained was assessed with a Coulter particle counter. In preparation for counting, the cells were diluted in a solution of 0.01% saponin in 0.15 molar saline to lyse red blood cells. The percentage of viable cells in these suspensions (based on their ability to exclude Eosin Y; Matheson, Coleman and Bell) was determined in a hemacytometer under 240X magnification.

Unless otherwise stated, cell doses reported in this volume are expressed in terms of viable nucleated cells.

V. TRANSPLANTATION

In general, dissociated spleen or bone marrow cells were transplanted within 24 hours after lethal irradiation of the recipients. Lethal radiation doses given were 900R for hybrid mice and 800R for inbred strains. Transplantation was accomplished by intravenous injection of the cellular inoculum in a lateral tail vein. The size of the inoculum varied considerably depending on the genetic make up of the donor and recipient, the particular growth assay employed and the objectives of the experiment.

VI. $^{125}\text{IUdR}$ UPTAKE ASSAY

Cellular proliferation in the spleen following irradiation and transplantation was assessed by the $^{125}\text{IUdR}$ (5- ^{125}I do 2'-deoxyuridine) assay developed by Cudkowicz (14). Five days after inoculation of irradiated recipients with 0.5 to 1.0×10^6 viable nucleated bone marrow cells, 10^{-7}M FUdR (5-Fluoro 2'-deoxyuridine) in physiologic saline was administered to each recipient intraperitoneally in a volume of 0.2 ml. This was done to inhibit the endogenous synthesis of thymidine nucleotides and maximize the incorporation of $^{125}\text{IUdR}$ into DNA. Thirty minutes following FUdR administration, the recipient mice were given 0.5 Ci of $^{125}\text{IUdR}$, (Amersham Searle) intraperitoneally in a volume of 0.2 ml. Eighteen hours later, the mice were killed by cervical dislocation and their spleens removed. These spleens were routinely fixed in Tellyesniczky's solution (1:1:20 of formalin:glacial acetic acid: 70% ethanol) and their radioactivity counted in a Packard Auto Gamma counter.

For each experiment in which this assay was employed, a standard, consisting of 1% of the injected dose, was also prepared and counted. Background counts were made periodically, and these values were taken into account in the final expression of the results. Counts were usually expressed as a percentage of the injected dose and were determined as follows:

$$\frac{\text{Counts per minute (Experimental)} - \text{Counts per minute (Background)}}{\text{Counts per minute (Standard)}}$$

Percent Injected Dose

VII. MACROSCOPIC SPLEEN COLONY ASSAY

The spleen colony assay of Till and McCulloch (71) was used both to determine the stem cell content of spleen and marrow inocula and to assess the proliferation of these stem cells following transplantation. Spleen or bone marrow cells were given to irradiated mice in a dose estimated (by prior experience or pilot experiment) to yield a countable number of splenic colonies (15-25 being ideal). Eight days after transplantation, the mice were killed by cervical dislocation and their spleens removed and fixed in Tellyesniczky's solution. Gross colonies were enumerated under a dissecting microscope at 9X magnification. The results are expressed for a given group as the mean number of colonies per spleen $\pm$ the standard error of the mean.

CHAPTER III

BONE MARROW TRANSPLANTATION IN YOUNG RECIPIENT MICE

I. INTRODUCTION

In 1968 it was reported (16) that, in mice less than three weeks of age, hemopoietic recovery was comparable following x-irradiation and parental strain bone marrow transplantation, regardless of whether the recipients were of the donor strain or an F_1 hybrid of that strain. This effect, which was in direct contrast to the Poor Growth usually observed in these hybrids, (15, 24, 56), persisted until 19 to 22 days of age, during which time a sharp decline in splenic hemopoiesis (with respect to syngeneic recipients) occurred in F_1 hybrid recipients of parental marrow. Although supplementary data regarding this sudden "maturation" of Poor Growth has since been reported (18), much information, most notably the magnitudes of both the observed bone marrow growth and the endogenous stem cell proliferation, has been unavailable in the literature.

Data from preliminary experiments (Goodman and Shimpock - unpublished) suggested that, while the magnitude of hemopoiesis observable in F_1 hybrid recipients of parental bone marrow was greater in mice less than three weeks of age as compared with adult recipients, endogenous stem cell proliferation in these mice was also drastically elevated with respect to adult recipients.

It was therefore essential to undertake a careful, detailed study of bone marrow growth in young parental strain and F_1 hybrid recipients. Inasmuch as the information regarding the hemopoietic stem cell content

(63, 69), radiation sensitivity (12, 23, 71) and the extent of endogenous hemopoietic recovery following irradiation in mice of this age has been sketchy, and because these parameters might affect the levels of hemopoiesis following irradiation and bone marrow transplantation, all were explored in detail. Such information was essential for assessing the proliferative capacity of parental bone marrow grafts in these animals.

Answers to the following questions were sought in the studies presented in this chapter:

1. What is the hemopoietic stem cell content of BDF₁ hybrid mice during the first five weeks of life? Might changes in the stem cell content of these mice at about 21 days of age cause a sudden decline in hemopoietic recovery following irradiation and bone marrow transplantation?
2. How does the radiation sensitivity of these mice vary during the first five weeks? Might these changes account for the apparent good growth of parental bone marrow during the first three weeks of life?
3. Does parental B6 bone marrow indeed grow well in young hybrid recipients? Does Poor Growth suddenly "mature" at 21 days of age?

II. MATERIALS AND METHODS

B6 and BDF₁ hybrid mice were used in these studies. Cell doses used in the determination of CFU content ranged from 5×10^4 to 10^5 bone marrow cells and 5×10^4 to 10^6 spleen cells, depending on the age of the donor. All other procedures were as described in Chapter II.

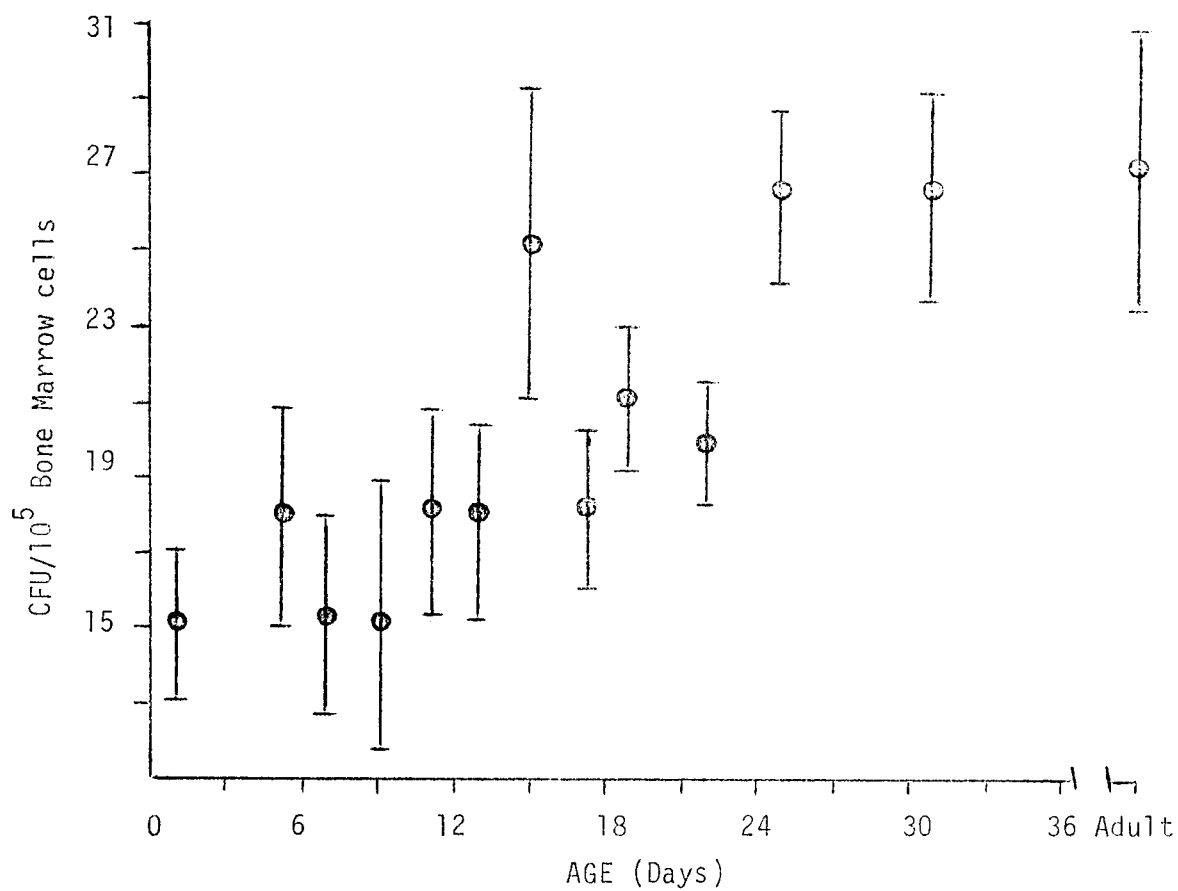
III. ENDOGENOUS COLONY FORMING UNITS

IN YOUNG BDF₁ MICE

In order to assess the hemopoietic stem cell or colony forming unit (CFU) content of BDF₁ hybrid mice, 1 to 32 days of age, spleen and bone marrow cells from donors of this age were transplanted into lethally irradiated (900R) syngeneic recipients 12 to 18 weeks of age, and gross hemopoietic colonies arising in the spleen were enumerated eight days post-transplantation. The CFU contents (expressed as CFU/10⁵ viable nucleated cells) of the bone marrow and spleen of these donors are shown in Figs. III-1 and III-2 respectively.

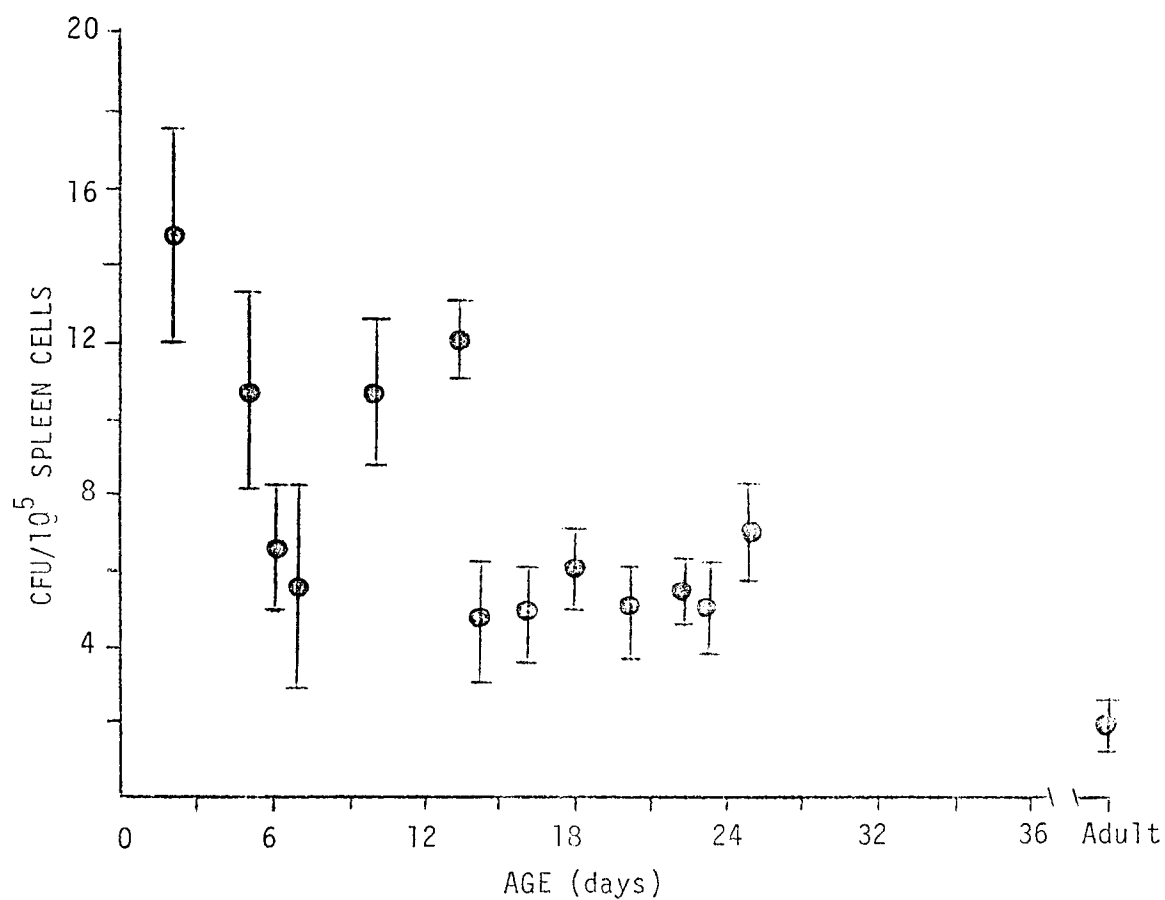
In the bone marrow, the CFU concentration remained relatively constant at a value of 15.1 to 17.0 CFU/10⁵ cells through 13 days of age. This concentration increased during the third and fourth weeks, reaching a level of 26.0 to 27.0 CFU/10⁵ by the end of the fourth week. This was not significantly different ($P=.05$) from the mean adult bone marrow CFU content of 27.1 CFU/10⁵ obtained in these experiments, and was comparable to the values reported by others for adult mice (64, 76).

The stem cell concentration in the spleen (Fig. III-2), on the other hand, shortly after birth appeared to begin a progressive decrease - somewhat erratic in these experiments - which lasted through the first two weeks of life. During the third and fourth weeks, this value appeared to plateau at a level of 4.5 to 6.7 CFU/10⁵ nucleated spleen cells. In contrast to the CFU concentration in the bone marrow at this age, that in the spleen (6.66 CFU/10⁵ nucleated spleen cells) was significantly different ($P<.001$) from the adult level of 1.9 CFU/10⁵ observed in these experiments. To obtain a coherent and comprehensive picture of the CFU



CFU concentration in the bone marrow of BDF₁ mice of both sexes was determined as described in Chapter II. The mean and standard deviation bars are shown for each age.

Figure III-1. CFU in the Bone Marrow of Young Mice.



CFU concentration in the spleens of BDF₁ mice of both sexes was determined as described in Chapter II. The mean and standard deviation bars are shown for each age.

Figure III-2. CFU in the Spleens of Young Mice.

content of the spleen and bone marrow at this age, the data, pooled by weeks are presented in Table III-1.

Because tibial, and in one instance humeral marrow was used in addition to femoral marrow in these experiments, the estimates of femoral cellularity presented in Table III-1 were calculated from an approximation based on data reported by Tyan, that the cellular content of the tibia is equal to 0.3226 that of the femur and that the cellularity of the humerus is 0.8000 that of the tibia. For this reason, these data must be regarded as estimates rather than definitive values.

IV. POST-RADIATION SURVIVAL

Because preliminary data (Goodman and Shinpock - unpublished) suggested that very high levels of spontaneous hemopoietic regeneration occurred in the spleens of mice less than three weeks of age following x-irradiation doses of 700 to 800R without subsequent marrow transplantation, it appeared appropriate to use a higher dose (1000R) as a conditioning agent prior to bone marrow transplantation in these studies. This was done in order to minimize the levels of endogenous hemopoietic recovery which could conceivably interfere with the interpretation of the results of these experiments. It was not possible, however, to complete the studies of bone marrow growth following this exposure dose due to a sharp increase in the radiation sensitivity of mice of this age which became manifest in these experiments at approximately 17 to 19 days of age.

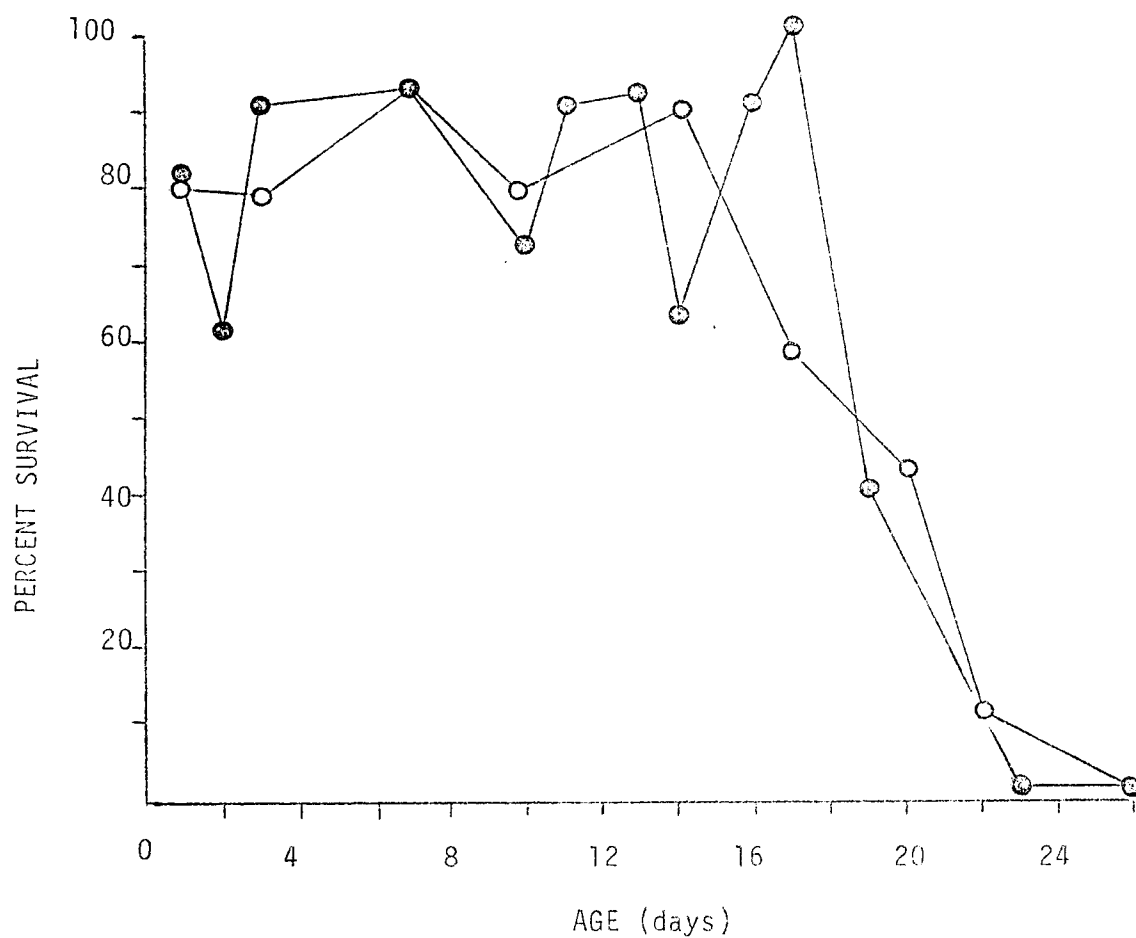
Fig. III-3 shows the percent survival of both B6 and BDF₁ mice six days following 1000R of x-radiation as a function of age at the time

TABLE III-1

ESTIMATES OF CFU CONTENT IN SPLEEN AND BONE MARROW

Age (Weeks)	Spleen		Bone Marrow		
	Nucleated Cells $\times 10^6$ /spleen	CFU/ 10^5	CFU/spleen	Nucleated Cells $\times 10^6$ /femur	CFU/ 10^5 CFU/femur
1	35.92	9.27	3330	1.94	15.96 310
2	43.65	8.97	3915	5.06	18.84 953
3	60.84	5.26	3200	7.27	19.46 1415
4	83.82	5.81	4870	10.22	23.03 2353
12-18 (adult)	173.00	1.94	3356	16.16	27.15 4387

These data represent a pool of the data presented in Figs. III-1 and III-2.



Survival was assessed six days after exposure to 1000R x-radiation. Open circles represent B6 mice, closed circles represent BDF₁.

Figure III-3. Percent Survival after 1000 R.

of exposure. Survival of both B6 and BDF₁ mice from 1 to 16 days of age ranged from 62 to 90% six days post-irradiation. A rapid decline in survival was observed, beginning at 17 days in B6 and 19 days in BDF₁ mice. By 23 days of age, survival of BDF₁ mice had reached zero, with B6 survival at zero by the age of 26 days. Both B6 and BDF₁ mortality remained at the level of 90 to 100% through 42 days of age.

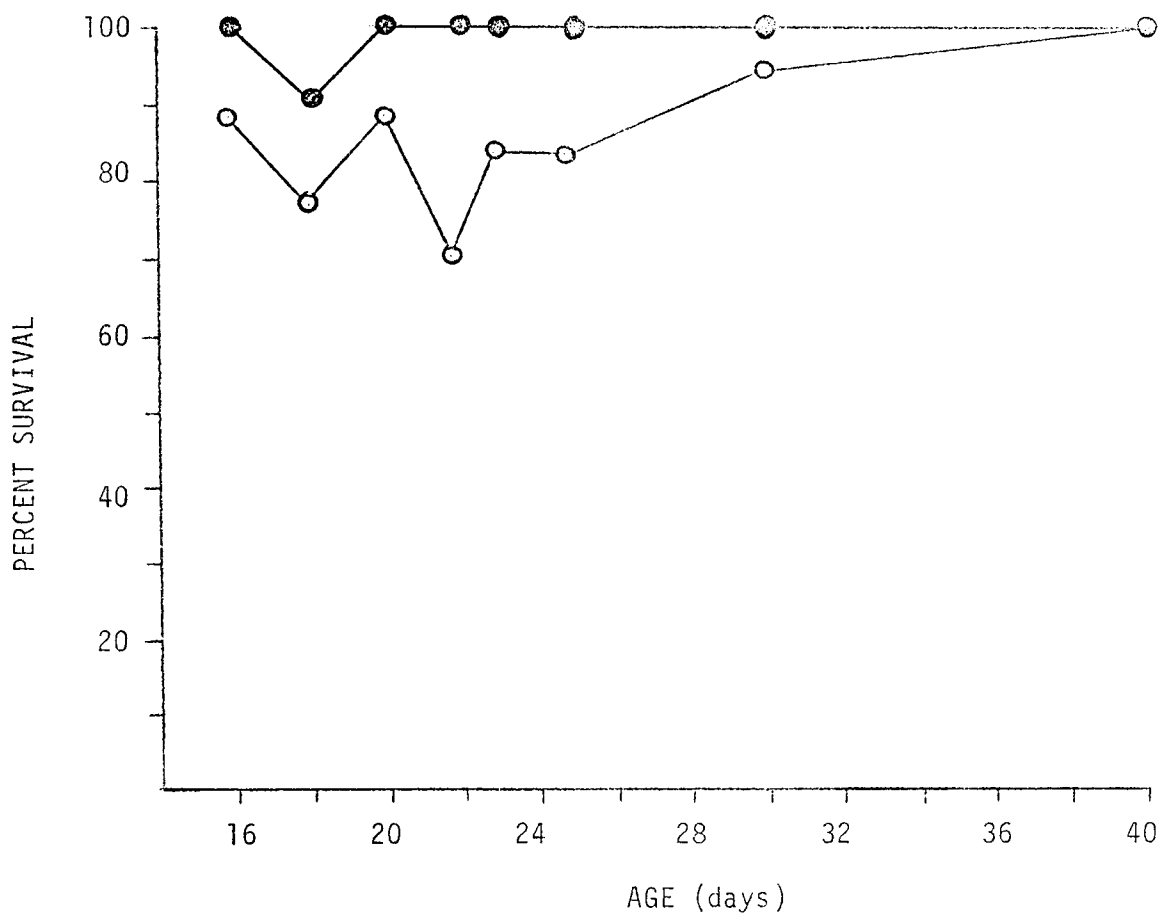
In order to determine the optimal radiation dose which could be used as a treatment for mice of this age, 23 day old B6 mice were exposed to various doses of x-radiation and their survival was assessed six and ten days later. A radiation dose was sought that would give acceptable survival and the lowest possible levels of host derived hemopoietic regeneration. As the assays of bone marrow growth employed in these studies required survival to eight days or less, long term survival was not followed. The results are presented in Table III-2. As expected, survival varied inversely with the exposure dose in these animals. Based on the data in Table III-2, 600R was selected as a routine dose, as it was the highest dose which did not result in unacceptable mortality. Eighty percent survival was observed at six and ten days.

Figure III-4 shows the survival of both B6 and BDF₁ mice six days after irradiation with 600R as a function of age. Although some mortality was observed after this exposure dose, especially in B6 mice (10 to 29%) 14 to 24 days of age, survival of BDF₁ mice was uniformly high throughout this time period. These data were obtained from radiation control animals (irradiated but not bone marrow transplanted) used to determine the extent of endogenous regeneration in the spleen after irradiation (see Section V of this Chapter).

TABLE III-2
SURVIVAL OF 23 DAY OLD B6 MICE AFTER
VARIOUS DOSES OF RADIATION

RADIATION DOSE (R)	SURVIVAL	
	AT 6 DAYS	AT 10 DAYS
1000	0/9	0/9
800	1/7	0/7
700	3/6	1/6
600	8/10	8/10
500	8/10	7/10

Data are expressed as the number of surviving mice/the number exposed.



Survival was assessed six days after exposure to 600R x-radiation. Open circles represent B6 mice; closed circles represent BDF₁.

Figure III-4. Percent Survival after 600R.

V. ENDOGENOUS HEMOPOIESIS FOLLOWING RADIATION

The extent of endogenous hemopoietic recovery of both B6 and BDF₁ mice of both sexes, 1 to 42 days of age was determined by ¹²⁵IUdR assay of the spleens of these mice six days after x-ray doses of 600, 700 and 1000R. These results are presented in Figure III-5. To put these data in perspective, Figure III-5 also shows the ¹²⁵IUdR uptake in the spleens of BDF₁ adults (12 to 15 weeks) following an x-ray exposure of 900R as well as that seen after reconstitution of these adults with 10⁶ syngeneic bone marrow cells. The spleens from adult mice showed no macroscopic colonies after this exposure dose. Those reconstituted with bone marrow had macroscopic colonies too numerous to count.

It is apparent from this figure that very young BDF₁ mice had high levels of hemopoiesis following even 1000R of radiation. Indeed, the levels of hemopoietic recovery observed in mice less than one week of age following exposures of 600 or 700R were comparable to that of bone marrow reconstituted adults.

In all cases, the extent of post-irradiation recovery declined with age. By day 11, the splenic ¹²⁵IUdR uptake of BDF₁ mice was not significantly different ($P=.05$) from that of adult mice following 900R exposures. However following 600 or 700R, the splenic regeneration remained higher ($P<.05$) than that seen in adults until 22 to 23 days of age.

Figure III-5. Endogenous Splenic Hemopoiesis in BDF₁ Mice Following Radiation Exposures.

IUdR uptake was determined as described in Chapter II. Mice were exposed to 1000R (dotted line), 700R (dashed line), or 600R (solid line) five days prior to spleen removal. The upper bar represents the mean $\pm$ standard error of IUdR uptake seen in BDF₁ adult recipients following exposure to 900R and transplantation of 10^6 syngeneic marrow cells. The lower bar shows the IUdR uptake seen in BDF₁ adult radiation (900R) controls.

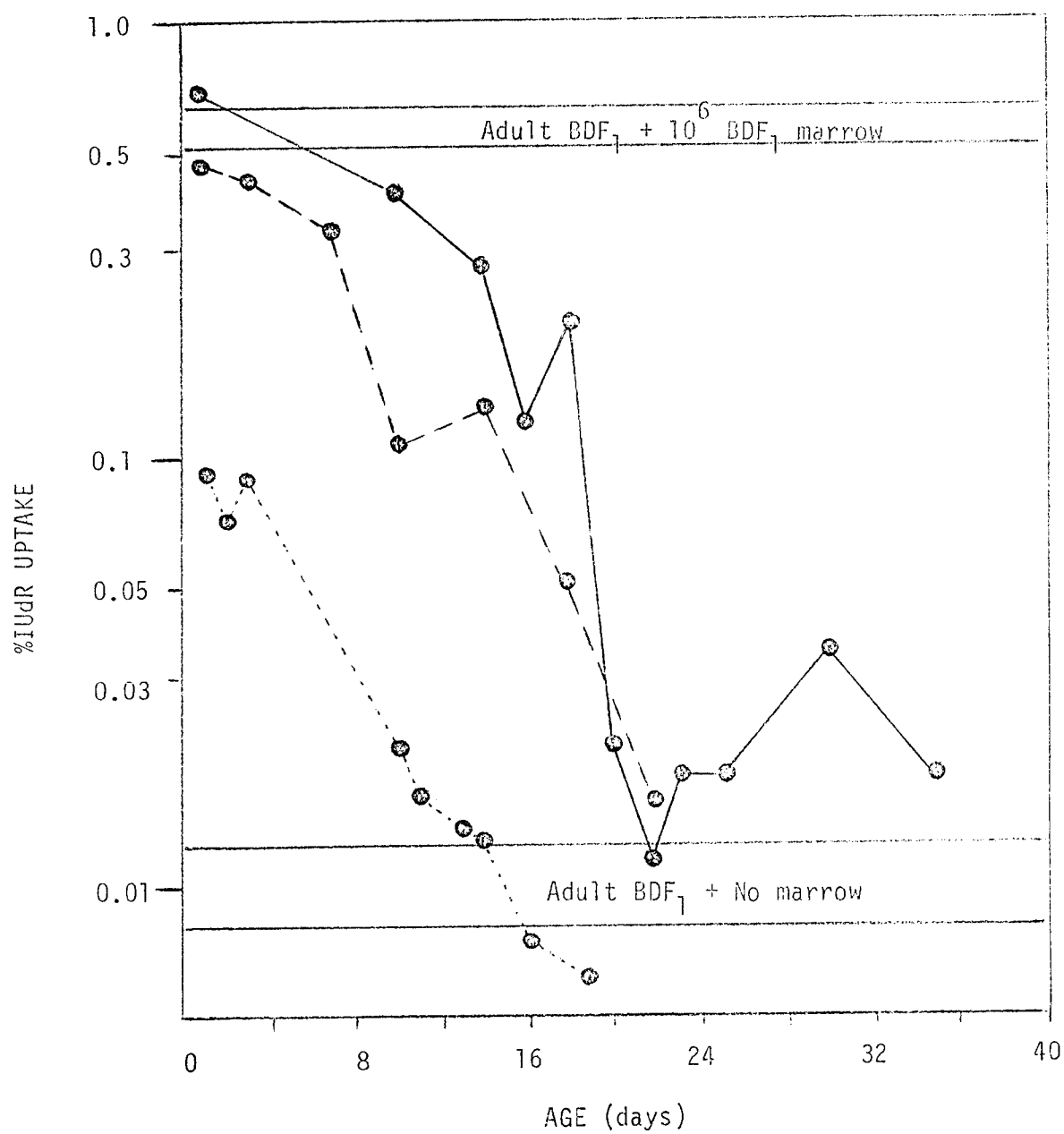


FIGURE III-5

VI. GROWTH OF PARENTAL STRAIN B6 BONE MARROW IN YOUNG B6 AND BDF₁ RECIPIENTS

The magnitude of B6 bone marrow growth in B6 and BDF₁ mice of both sexes 13 to 42 days of age was determined by the ¹²⁵IUdR assay following irradiation and injection of 10⁶ marrow cells. Radiation control animals, irradiated and not transplanted, were littermates of those receiving bone marrow whenever possible. The results of these experiments, employing an x-ray exposure of 1000R are shown in Table III-3. As noted in Section IV of this Chapter, attempts to assess bone marrow growth in these animals between 20 and 42 days of age following 1000R met with excessive mortality, and this study could therefore not be extended past 19 days.

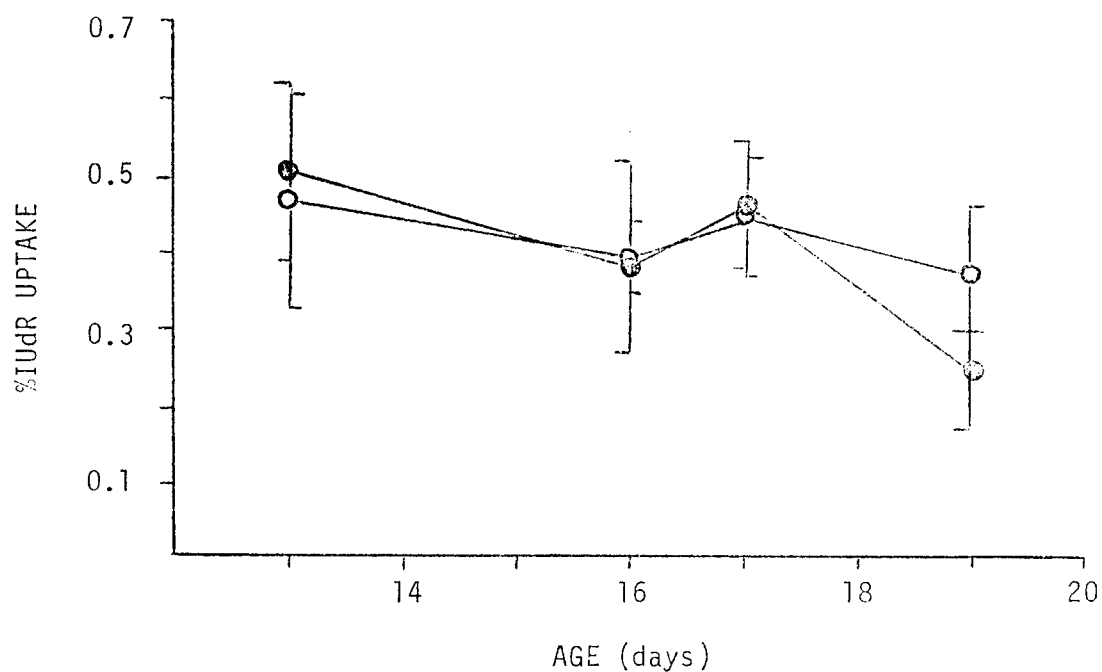
Hemopoietic recovery observed following irradiation and B6 bone marrow transplantation in recipient mice within this limited age range was comparable in both B6 and BDF₁ animals. ¹²⁵IUdR uptake was observed to be significantly higher ($P < .05$) in both B6 and BDF₁ marrow recipients 13 to 17 days of age than that seen in syngeneic adult recipients of B6 marrow.

The amount of regeneration in these recipients attributable to the bone marrow grafts was determined by subtracting the ¹²⁵IUdR uptake seen in non-transplanted controls from that of bone marrow recipients of the same strain and age. These data are presented in Figure III-6. B6 bone marrow displayed approximately equal growth in both B6 and BDF₁ recipients 13 to 19 days of age. This effect contrasted sharply with the failure of this marrow to proliferate in BDF₁ adult recipients.

TABLE III-3
GROWTH OF B6 BONE MARROW IN B6 AND BDF₁
RECIPIENTS FOLLOWING 1000R

RECIPIENT AGE	¹⁰ B6 BONE MARROW	B6 ¹²⁵ IuDR %SPLENIC UPTAKE	N	BDF ₁ ¹²⁵ IuDR %SPLENIC UPTAKE	N
13 Day	+	0.526 [±] .088	10	0.491 [±] .067	19
13 Day	-	0.016 [±] .010	8	0.019 [±] .006	11
16 Day	+	0.399 [±] .096	7	0.400 [±] .029	10
16 Day	-	0.014 [±] .002	7	0.018 [±] .005	7
17 Day	+	0.475 [±] .077	6	0.466 [±] .069	18
17 Day	-	0.017 [±] .003	4	0.012 [±] .001	8
19 Day	+	0.271 [±] .085	4	0.383 [±] .009	11
19 Day	-	0.010 [±] .002	7	0.009 [±] .001	10

¹²⁵IuDR %uptake values were obtained as described in Chapter II. These values are expressed as the means - standard errors.



%IUDR uptake was determined as described in Chapter II. The data represent the uptake seen in animals given 10^6 B6 bone marrow minus that seen in radiation control mice. Open circles represent B6 recipients; closed circles represent BDF₁ mice. Standard error bars are also shown for each point.

Figure III-6. B6 Bone Marrow Growth in Recipients Exposed to 1000R.

In order to study the kinetics of the appearance of Poor Growth in young BDF₁ mice, a series of experiments was carried out in which an exposure dose of 600R was employed prior to bone marrow transplantation. The use of this exposure dose made possible the assessment of B6 bone marrow growth in recipients 14 to 42 days of age. The results of these experiments are summarized in Table III-4. As was the case following exposures of 1000R, relatively high levels of recovery were observed in both B6 and BDF₁ animals, 14 to 20 days of age, following marrow transplantation.

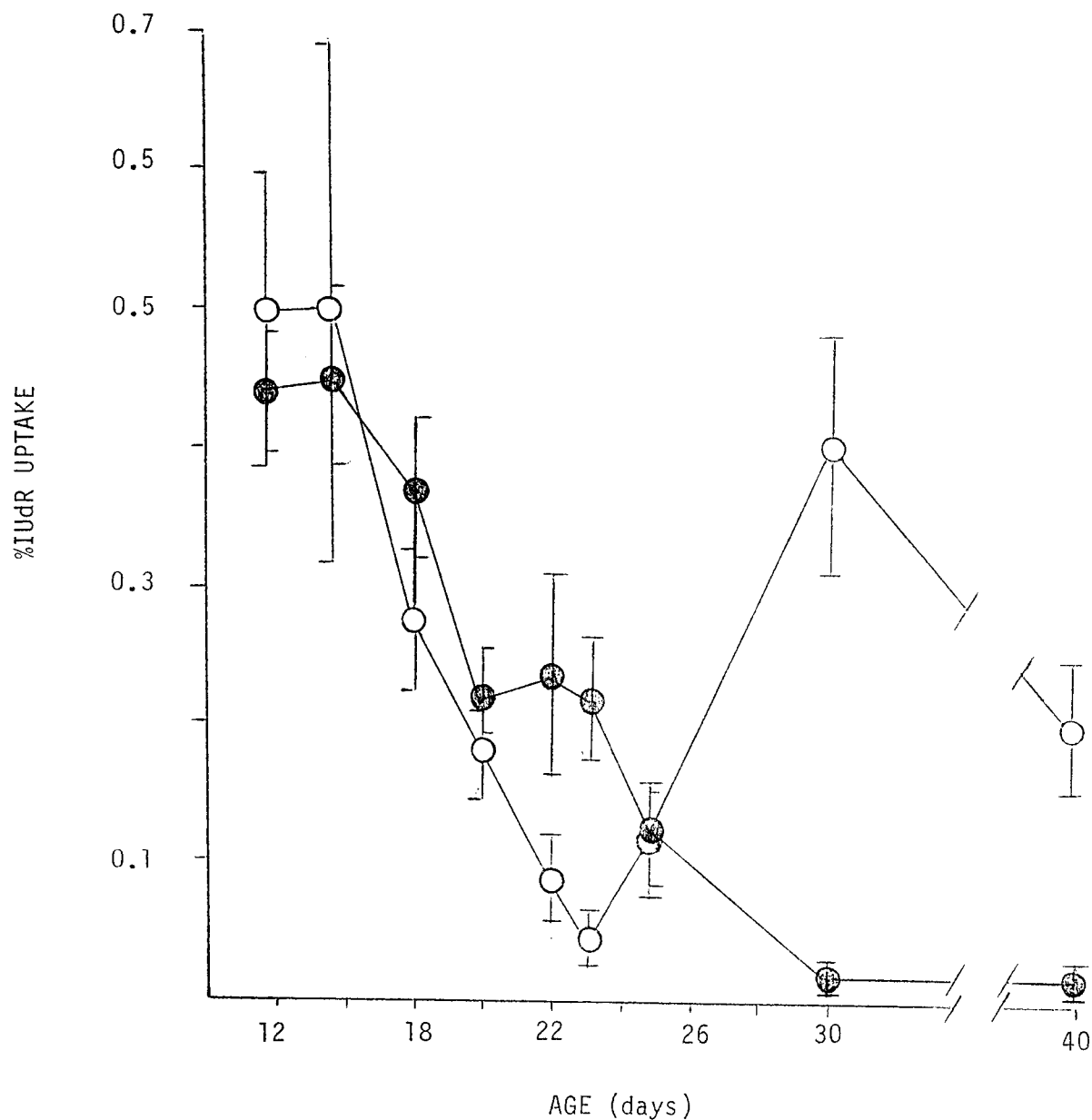
The ¹²⁵IUdR uptake in BDF₁ recipients of parental marrow tended to decrease with age throughout the age range studied. Splenic hemopoiesis in B6 recipients decreased with age in animals 14 to 23 days of age and then recovered to relatively high levels in 25 to 42 day old mice. In both B6 and BDF₁ mice given 600R but no bone marrow, the ¹²⁵IUdR uptake was significantly elevated (P .05) at 14 to 18 days of age over that seen in older, 20 to 42 day old mice.

The difference between the ¹²⁵IUdR uptake of transplanted versus non-transplanted mice, as a function of age following x-ray exposures of 600R is shown in Figure III-7. The same general pattern as that seen in Table III-4 persisted after this correction for endogenous recovery was made. B6 bone marrow appeared to proliferate in BDF₁ recipients as well as, and often better than it did in syngeneic recipients during the 14 to 25 day age span. While the proliferation observed in BDF₁ mice continued to decline in older recipients, it increased in B6 mice after 23 days of age. Thus Poor Growth would appear in these experiments to have "matured" between 25 and 30 days of age. This is somewhat different from the 19 to 22 day age of "maturation" previously reported.

TABLE III-4
GROWTH OF B6 BONE MARROW IN B6 AND BDF₁
RECIPIENTS FOLLOWING 600R

RECIPIENT AGE	10 ⁶ B6 BONE MARROW	B6		BDF ₁	
		¹²⁵ IUdR %SPLENIC UPTAKE	N	¹²⁵ IUdR %SPLENIC UPTAKE	N
14 Day	+	0.588 ⁺ .099	10	0.704 ⁺ .062	11
	-	0.695 ⁺ .014	9	0.265 ⁺ .038	11
16 Day	+	0.596 ⁺ .182	10	0.577 ⁺ .072	12
	-	0.083 ⁺ .026	8	0.125 ⁺ .022	13
18 Day	+	0.340 ⁺ .049	13	0.571 ⁺ .050	11
	-	0.070 ⁺ .011	8	0.211 ⁺ .046	10
20 Day	+	0.191 ⁺ .030	8	0.238 ⁺ .039	13
	-	0.033 ⁺ .005	9	0.024 ⁺ .005	11
22 Day	+	0.091 ⁺ .026	11	0.242 ⁺ .063	14
	-	0.011 ⁺ .002	10	0.012 ⁺ .002	12
23 Day	+	0.057 ⁺ .017	13	0.240 ⁺ .037	13
	-	0.011 ⁺ .002	11	0.018 ⁺ .005	12
25 Day	+	0.131 ⁺ .044	14	0.132 ⁺ .035	16
	-	0.023 ⁺ .011	11	0.018 ⁺ .004	13
30 Day	+	0.404 ⁺ .105	8	0.052 ⁺ .009	13
	-	0.019 ⁺ .003	11	0.034 ⁺ .007	12
40-42 Day	+	0.197 ⁺ .037	12	0.031 ⁺ .004	17
	-	0.010 ⁺ .001	10	0.019 ⁺ .002	13

¹²⁵IUdR %uptake values were obtained as described in Chapter II.
These values are expressed as means - standard errors.



%IuDR uptake was determined as described in Chapter II. The data represent the uptake seen in animals given 10^6 B6 bone marrow minus that seen in radiation control mice. Open circles represent B6 recipients; closed circles represent BDF₁ mice. Standard error bars are also shown for each point.

Figure III-7. B6 Bone Marrow Growth in Recipients Exposed to 600R.

VII. IRRADIATED BONE MARROW DONORS

To preclude the possibility that the presence of B6 bone marrow cells following transplantation was in some manner inducing proliferations of host type hemopoietic cells, 16 day old BDF₁ mice were irradiated (600R) and given B6 bone marrow from either normal donors or donors irradiated (1000R) immediately prior to the collection of their marrow. The pre-irradiation of donors was carried out to abolish the proliferative capacity of the stem cells in this marrow. The results are shown in Table III-5.

Although BDF₁ recipients of normal B6 bone marrow showed greatly increased ¹²⁵IUdR uptake over that seen in non-transplanted mice, bone marrow from irradiated recipients had no effect on hemopoietic regeneration in these mice. The efficacy of prior irradiation of bone marrow donors in preventing proliferation of these grafts was demonstrated by the lack of significant splenic regeneration in syngeneic adult recipients of marrow from these donors.

VIII. DISCUSSION

The hemopoietic regeneration observed in these experiments after irradiation and B6 bone marrow transplantation in both B6 and BDF₁ animals confirmed earlier reports that Poor Growth was not detectable in hybrid mice during the first three weeks of life (16, 18). ¹²⁵IUdR uptake following parental bone marrow transplantation in BDF₁ mice, conditioned with either 600 or 1000R, was equivalent to, and sometimes greater than that seen in age-matched syngeneic B6 recipients during the first 25 days of life. The age at which Poor Growth appeared to

TABLE III-5
EFFECT OF BONE MARROW FROM 1000R IRRADIATED DONORS
ON ^{125}I UdR UPTAKE

RECIPIENTS STRAIN	AGE	RADIATION DOSE (R)	10^6 B6 MARROW DONOR	^{125}I UdR %UPTAKE	N
BDF ₁	16 Day	600	Normal	0.473 [±] .108	9
BDF ₁	16 Day	600	Irradiated	0.139 [±] .032	10
BDF ₁	16 Day	600	None	0.154 [±] .029	8
B6	15 Weeks	800	Normal	0.372 [±] .029	15
B6	15 Weeks	800	Irradiated	0.017 [±] .003	15
B6	15 Weeks	800	None	0.015 [±] .003	10

^{125}I UdR %uptake values were obtained as described in Chapter II.
These values are expressed as means - the stand errors.

"mature," taken by others to be the time at which parental bone marrow appeared to proliferate less well in hybrid recipients than in syngeneic ones (16), was somewhat older (25^+ days) in these experiments than that (21 days) previously reported (16, 18).

The earlier reports however, expressed growth of parental marrow in the hybrid only as a percentage of the growth observed in parental strain recipients. Poor Growth is indeed a failure of hemopoietic cells of a given strain (in this case B6) to proliferate well relative to its capacity to do so in syngeneic recipients. However, as the ability of syngeneic mice to support such proliferation changed radically with age in these experiments, expression of parental bone marrow growth in hybrid recipients only as a percentage of its proliferation in syngeneic recipients would tend to be both misleading and mask the underlying kinetics involved. In fact, the "maturation" of Poor Growth in these experiments was not due to a sudden drop in the ability of hybrid recipients to support parental bone marrow growth as might be assumed to be the case from earlier reports (16, 18). Parental bone marrow grew progressively less well with age in BDF₁ mice from 16 to 42 days old in these experiments. The growth of this marrow in B6 recipients, 16 to 23 days old, also declined with age. It was only after three and one-half weeks that bone marrow growth recovered from the decline in B6 mice and thus outpaced that seen in F₁ animals. Poor Growth thus "matured" rather abruptly in BDF₁ hybrids during the fourth week of life when this growth was considered relative to that seen in age matched parental strain recipients. This "maturation" was in fact a much more gradual process when the growth of parental marrow in hybrid

recipients was considered relative to the growth potential of that marrow. It is clear from the data presented in Section III of this Chapter that endogenous CFU in the spleens of these mice prior to 21 days of age did not account for the hemopoiesis detected in these experiments after irradiation and parental bone marrow transplantation. Although post natal changes in the CFU concentration of the spleen did occur in this hybrid, the critical time period (18 to 26 days) was marked by a plateau of CFU concentration. Moreover, in spite of the early high concentration of splenic CFU and its subsequent decline, the total number of CFU in these spleens remained relatively constant because of increased splenic cellularity.

The possibility that excess bone marrow CFU, available for recruitment to the spleen prior to 26 days of age, might account for the observed improved hemopoietic growth appeared unlikely not only because of their relatively low total numbers, but also the low concentration of CFU at this site as well. Thus endogenous CFU, not undergoing a radical decline in either concentration or absolute numbers during the critical third to fourth week time period, could not account for the hemopoietic regeneration of F_1 hybrids following irradiation and parental bone marrow transplantation.

Whether changes in the radiation sensitivity of these mice played a role in the regeneration of hemopoiesis could not be as easily dismissed. There was indeed a parallel following irradiation and parental bone marrow transplantation between splenic recovery and radiation sensitivity, both in terms of general sensitivity as assessed by survival and the sensitivity of endogenous hemopoiesis to irradiation. That significant endogenous regeneration took place

in 14 to 18 day old animals following 600R is in disagreement with Cudkowicz and Bennett (18), who reported no such regeneration following 600 to 700R in mice of this age. As neither the specific data nor the methods used to obtain this result were reported, it is not possible to reconcile the conflict.

It is true that due to the relative insensitivity to radiation of host hemopoietic cells in mice 14 to 18 days of age, some recovery in the spleens of these mice arose spontaneously in the absence of transplanted bone marrow. This endogenous growth however, could not account for most of the regeneration seen following parental bone marrow transplantation. This is based on the following several observations.

After taking the $^{125}\text{IUdR}$ uptake observed in animals given no bone marrow into account, significant hemopoiesis was observed (Figures III-6 and III-7). Recipient mice exposed to 1000R at 13 to 19 days of age, in which little or no recovery was detected in the absence of marrow grafts, still displayed high levels of $^{125}\text{IUdR}$ uptake following parental bone marrow transplantation. This growth, minus that seen in non-transplanted controls was comparable to that seen in BDF_1 recipients of the same age range following 600R and marrow transplantation after the same correction was made. Finally, injection of irradiated B6 bone marrow, incapable of proliferation, into BDF_1 mice in which endogenous recovery was observed, did not result in an increase in endogenous proliferation (Table IV-5). Such an increase would have been expected if the presence of parental cells induced the growth of host type stem cells.

It was concluded therefore that much hemopoietic recovery did take place in the spleens of BDF₁ mice following B6 bone marrow transplantation when the transplant was given during the first three to four weeks of recipient age. Most of the observed regeneration was attributable to proliferation of the graft and not to endogenous recovery. This effect diminished with age and was absent in BDF₁ mice 30 days of age and older.

CHAPTER IV

WEANING AND BONE MARROW GROWTH IN YOUNG MICE

I. INTRODUCTION

It was noted at the outset of the present research that the time at which the divergence in parental bone marrow growth patterns between parental and F_1 hybrid recipients occurred coincided precisely with the age at which laboratory mice are routinely weaned (62). Indeed, in both the experiments reported in Chapter III and those reported by others (16, 18), mice receiving transplants before 21 days of age were caged with their mothers throughout the duration of the experiment. However those mice transplanted at 21 days of age or older were weaned and caged separately from their mothers prior to bone marrow transplantation. Thus mice 20 days of age at the time of transplantation, 25 days old at the termination of the experiment, were nursing and receiving a substantial portion of their nourishment in the form of milk, whereas mice 21 days of age at transplantation were deprived of milk throughout the duration of the experiment. The possibility that this dietary discrepancy between experimental groups played a role in the eventual fate of the bone marrow grafts in these mice was therefore considered.

Although these two phenomena (weaning and bone marrow engraftment) appear superficially unrelated, there were several reasons to believe that the presence of milk in the diet of these mice, or lack of it, might indeed influence bone marrow cell engraftment. It has been demonstrated that the milk of several species, including mice, contains both immunoglobulins (10, 34) and immunocompetent cells (5, 6, 21, 66, 84) of maternal origin, which are ingested by suckling mice. It has further

been demonstrated the i.v. introduction of parental strain T cells into adult F_1 hybrids at the time of transplantation has a profound effect on marrow cell engraftment, virtually abolishing Poor Growth (4, 27-33, 20). It was reasoned therefore, that such cells, introduced through the milk, might account in part for the absence of poor growth in nursing mice. As all experiments reported in Chapter III employed BDF_1 hybrid mice, the mothers of which were syngeneic with the bone marrow donors, the possibility that a B6 specific milk factor influenced the engraftment of transplanted B6 bone marrow in the suckling hybrids, appeared to be a tenable one.

Because the mechanism by which Poor Growth is effected has yet to be determined, the possible influence of other cellular or soluble factors in milk on the growth of transplanted bone marrow could not be ignored. A series of experiments was therefore carried out to determine a) whether a factor or factors, present in the milk of mice syngeneic to the given bone marrow graft and specific for that strain, was responsible for the absence of Poor Growth observed in mice consuming that milk, or b) whether the consumption of milk exerted a non-specific influence on the growth of transplanted marrow. This was accomplished by two types of experiments. The first employed the use of both reciprocal hybrids (BDF_1 and DBF_1) as well as BDF_1 mice fostered by D2 mothers. The second utilized mice kept on an exclusive milk diet well beyond the usual 21 day weaning age.

II. MATERIALS AND METHODS

Bone marrow transplantation and $^{125}\text{IUdR}$ assays were carried out as detailed in Chapter II. BDF_1 , DBF_1 and B6 mice were used as recipients.

Bone marrow donors were B6 mice of both sexes, 14 to 17 weeks of age.

Fostered Mice

In some experiments, litters of BDF₁ mice were removed from their mothers within 24 hours of birth and caged with a lactating D2 female. The litter and foster mother were closely observed for three to six hours to determine whether or not proper "adoption" had taken place as assessed by free nursing on the part of the litter. If no nursing was observed during this time, the litter was then removed to a cage containing a second lactating D2 female and observed once more. "Adoption" by either the first or second female invariably occurred.

The litters were then maintained with their surrogate mothers for at least two weeks prior to irradiation and bone marrow transplantation and remained so throughout the experiments.

Delayed Weaning

In experiments designed to test the possible effects of non-specific milk factors on bone marrow growth, prospective recipients were kept on a diet which consisted exclusively of milk and water for periods of time ranging up to 28 days of age. This was accomplished by the use of surrogate mothers as follows:

At 12 to 14 days of age, litters of mice were removed to pans which were not supplied with solid food. The mother was separated from the litter and housed in a feeding cage containing food and water. A lactating B6 female was then placed in the pan containing the litter and kept there until the litter had been "adopted" (three to six hours). Real and surrogate mothers were then alternated between cages containing the nursing litter and the feeding pans. Such alternations were performed

every three to four hours for the duration of the experiment, usually 7 to 17 days. In every case, only mothers of BDF₁ litters were used as surrogates for BDF₁ litters and mothers of B6 litters were used as surrogates for B6 litters.

III. MARROW GROWTH IN RECIPROCAL HYBRIDS AND FOSTERED MICE

Having established that BDF₁ mice exhibit growth of transplanted bone marrow comparable to that of syngeneic recipients during the first three weeks of life, both the DBF₁ (D2 x B6) reciprocal hybrid of BDF₁ and BDF₁ mice fostered by D2 females were used to test whether a diet of milk from a source syngeneic with the bone marrow graft, enhanced the growth of that graft. It was reasoned that if the absence of Poor Growth in these young mice was due to the presence of a strain specific soluble or cellular factor(s) introduced through the milk, then hybrid mice, raised on milk from a source allogeneic with respect to the transplanted bone marrow, should exhibit hematopoietic regeneration significantly less than that seen in mice receiving B6 milk.

Tables IV-1 and IV-2 show the extent of hemopoietic recovery in the spleen five days after transplantation of parental marrow. Recipient mice were 16 days old in all cases and had previously been irradiated with 1000R (Table IV-1) or 600R (Table IV-2). In all cases, no significant differences ($P=.05$) among B6, BDF₁, DBF₁ and BDF₁ fostered recipients of B6 bone marrow were observed. It should be noted that the BDF₁ fostered recipients had access to B6 milk during the first 24 hours of life and the DBF₁ recipients had no access whatever. The data in Table IV-1 represent two experiments, whereas those in Table IV-2 represent three.

TABLE IV-1

GROWTH OF B6 BONE MARROW IN BDF₁ FOSTERED AND DBF₁ MICE
FOLLOWING 1000R IRRADIATION

RECIPIENT	10 ⁶ B6 BONE MARROW	125 IUDR - SPLEEN			N
		%UPTAKE	Δ UPTAKE	INDEX	
BDF ₁	+	0.340 ⁺ .029	0.322	84.3	10
BDF ₁	-	0.018 ⁺ .005			8
BDF ₁ (fostered)	+	0.331 ⁺ .043	0.312	81.7	8
BDF ₁ (fostered)	-	0.019 ⁺ .007			5
DBF ₁	+	0.320 ⁺ .008	0.313	81.9	8
DBF ₁	-	0.007 ⁺ .002			5
B6	+	0.399 ⁺ .096	0.382	100.0	9
B6	-	0.017 ⁺ .009			5

%uptake values were obtained as described in Chapter II. Δ uptake is the difference in the uptake in mice given bone marrow minus that of radiation controls. The index values are a percentage of uptake as compared to syngeneic recipients.

Recipients were 16 days old and of both sexes in all cases.

TABLE IV-2
GROWTH OF B6 BONE MARROW IN BDF₁ FOSTERED AND DBF₁ MICE
FOLLOWING 600R IRRADIATION

RECIPIENT	10 ⁶ B6 BONE MARROW	125 IUDR - SPLEEN			N
		%UPTAKE	Δ UPTAKE	INDEX	
BDF ₁	+	0.577 [±] .072	0.453	90.1	12
BDF ₁	-	0.125 [±] .022			13
BDF ₁ (fostered)	+	0.607 [±] .117	0.463	92.0	11
BDF ₁ (fostered)	-	0.144 [±] .040			10
DBF ₁	+	0.589 [±] .126	0.489	97.2	13
DBF ₁	-	0.100 [±] .017			8
B6	+	0.596 [±] .182	0.503	100.0	10
B6	-	0.083 [±] .026			8

%uptake values were obtained as described in Chapter II. Δ uptake is the difference in the uptake of mice given bone marrow minus that of the radiation controls. The index is the percentage of uptake as compared to syngeneic recipients.

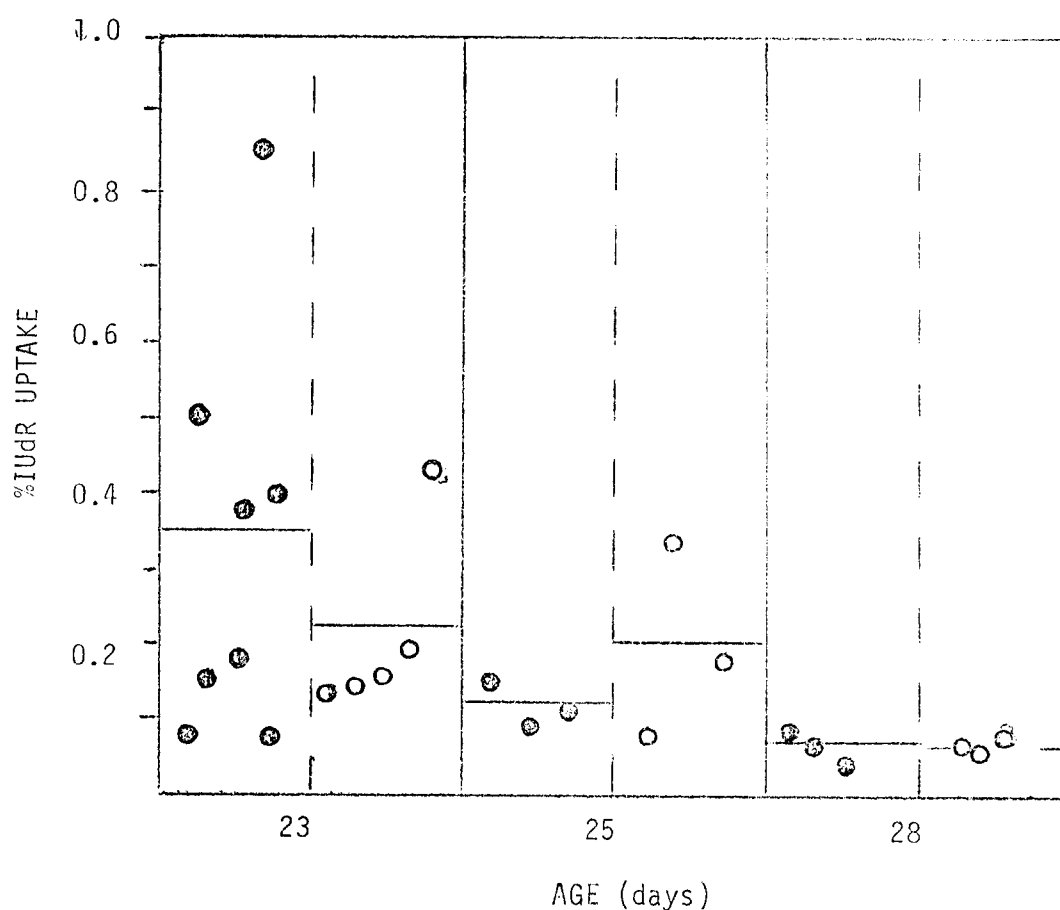
Recipients were 16 day old mice of both sexes in all experimental groups.

IV. DELAYED WEANING

In order to determine whether the presence of milk in the diet of young hybrid mice played a more general role in the growth of transplanted parental marrow, the age at which BDF₁ mice were weaned was extended from 21 days to 23, 25 or 28 days of age. As mice of this age take an increasing portion of their diet in the form of solid food, precautions were taken to ensure that the diets of the mice used in these experiments consisted entirely of milk and water prior to irradiation and bone marrow transplantation.

The growth of B6 bone marrow in BDF₁ hybrids maintained on milk versus that seen in mice weaned at 21 days of age is shown in Figure IV-1. In recipients 23 days of age, the mean splenic uptake of ¹²⁵IUDR in mice maintained on milk was nearly twice that observed in mice weaned at 21 days of age. Because of the variability of bone marrow growth which occurred among different litters within experimental groups, these mean values were not significantly different from each other. It was noted however, that the mice in two out of five litters maintained on milk exhibited hemopoietic recovery significantly ($P < .05$) higher than the mean recovery observed in mice weaned at 21 days of age.

At 25 days of age, bone marrow recipients continued to display considerable variability. However the growth of bone marrow in nursing mice tended to be lower than that seen in normally weaned animals. Twenty-eight day old recipients showed virtually no differences, either between litters within experimental groups or between litters maintained on milk versus those consuming solid food. The growth of transplanted marrow in all BDF₁ mice of this age was typical of the Poor Growth seen throughout adulthood.



IuDR uptake was determined as described in Chapter II. Each point represents the mean value for a given litter (3-7 mice). Closed circles represent litters maintained on milk; open circles represent litters weaned at 21 days of age. The overall mean of each group is indicated by a horizontal bar.

Figure IV-1. Effect of Weaning on Bone Marrow Growth.

V. GENERAL HEALTH AND BONE MARROW GROWTH

Because the ages (23 to 26 days) of the recipients in much of this work corresponds to the time of maximum radiation sensitivity in these animals (Chapter III), the state of health of experimental mice was noted at the time of sacrifice. A striking correlation between the general state of health of bone marrow transplant recipients and the proliferation of those grafts was observed. Table IV-3 shows a comparison between the growth of B6 marrow in both BDF₁ and B6 recipients found to be in either relative good health or listless and moribund upon gross examination at the time of sacrifice. In both BDF₁ as well as syngeneic recipients, the grafts showed profoundly reduced growth in mice found to be in apparently poor health as compared to healthy recipients. In many cases these recipients showed no better hemopoietic recovery than that seen in animals given no bone marrow following irradiation (radiation controls).

VI. DISCUSSION

Whether Poor Growth is viewed as an anti-parent immune reaction (13, 17, 18) or as a failure of transplanted parental bone marrow to proliferate in the absence of some inducing factor of parental origin (71), it is not difficult to conceive of mechanisms by which milk of parental strain origin might affect this phenomenon. If an anti-parent immune response were the mechanism by which Poor Growth is effected, cells or cell associated antigens in the milk might induce a temporary tolerance of sorts in nursing mice. Cells capable of suppressing such an anti-parent response in the milk could account for the proliferation

TABLE IV-3

B6 BONE MARROW GROWTH IN 23 DAY OLD RECIPIENTS APPEARING
LISTLESS FIVE DAYS AFTER TRANSPLANTATION

RECIPIENT	GENERAL APPEARANCE	WEIGHT (grams)	%UPTAKE $^{125}\text{IUdR}$	N
BDF ₁	Good	12.3 ± 0.9	$0.240 \pm .037$	12
BDF ₁	Listless- Moribund	7.8 ± 0.9	$0.038 \pm .017^{\text{a}}$	5
B6	Good	11.8 ± 1.1	$0.057 \pm .017$	13
B6	Listless- Moribund	8.1 ± 0.9	$0.019 \pm .002^{\text{a}}$	6

^aUptake significantly ($P < .01$) less than that seen in healthy recipients.

%Uptake values are expressed as means $\pm$ standard errors of those means.

Mice found to be listless in all other experiments at the time of sacrifice were eliminated from the data pool.

observed. Alternatively, parental antigens might effect an "over-load" of the immune mechanism involved as has been proposed for parental thymocyte augmentation of bone marrow grafts in adult hybrids (82). If Poor Growth is the result of a missing factor of some type, necessary for the proliferation of B6 bone marrow, the presence of such a factor in milk might explain bone marrow growth observed in nursing hybrids.

However there appears to be no requirement for B6 milk in order for B6 bone marrow to proliferate in BDF₁ hybrid recipients of nursing age. Both BDF₁ mice fostered by D2 females as well as DBF₁ mice nursed by their natural D2 mothers exhibited bone marrow growth comparable to that seen in BDF₁ recipients nursed by their B6 mothers. Thus parental B6 bone marrow growth in F₁ hybrid recipients less than three weeks of age is not dependent on a strain specific milk factor.

The data involving the delayed weaning of BDF₁ mice speak against the involvement of nonspecific milk factors effecting parental bone marrow growth in hybrid recipients. No significant difference between the growth of B6 bone marrow in hybrid recipients maintained on milk and hybrids weaned at 21 days of age was detected in these experiments. It was unfortunately not possible to obtain data regarding the growth of marrow in mice weaned prior to 21 days of age, because there was almost 100% mortality in such animals following irradiation and bone marrow transplantation. However, that the extension of the nursing period was not accompanied by a similar extension of good growth indicates that milk factors are not responsible for supporting hemopoietic grafts in nursing mice.

Significantly increased bone marrow growth over that seen in mice weaned at 21 days occurred in two litters whose nursing period was

extended. It is highly unlikely that this was the result of growth-enhancing milk factors. Improved growth was observed in 23 day old animals only and not in 25 or 28 day old mice. Further, even in 23 day old mice, significantly increased bone marrow growth was a relatively rare occurrence (two out of eight litters).

It has been suggested that the increase in radiation sensitivity at three weeks of age is due to a sudden, temporary increase in the radiation sensitivity of the intestine (23). It has further been suggested that this sensitivity is the result of developmental changes in the intestinal mucosa in preparation for the digestion of solid food (23). It is possible that these developmental changes might be somewhat altered or delayed in mice whose dietary intake consists solely of liquids, thus increasing their general resistance to total body irradiation. As it is clear that the general state of health of bone marrow transplant recipients following irradiation can influence the proliferation of the transplant (Table IV-3), it is conceivable that the exceptional hemopoietic growth observed in some litters of 23 day old nursing mice is due to a delay in the onset of extreme radiation sensitivity. This, coupled with the biological variability inherent in mice of this age, would account for the occasional high levels of parental marrow growth seen in 23 day old nursing mice.

CHAPTER V

STIMULATOR CELLS AND BONE MARROW

GROWTH IN YOUNG BDF₁ MICE

I. INTRODUCTION

There are several lines of evidence which suggest that the proliferation of hemopoietic stem cells in the mouse may be regulated in vivo by interactions with lymphoid cells. The growth of parental bone marrow in F₁ hybrid recipients can be greatly enhanced by treatment of recipients with parental strain spleen or lymph node cells two or more weeks prior to irradiation and transplantation (24, 27). Treatment of F₁ recipients with parental cells from a variety of tissue sources at the time of transplantation has also been shown to augment the growth of parental marrow (28-33). It has further been reported that, in syngeneic transplantation situations, T-cells are capable of exerting a stimulatory influence on CFU growth (53, 85). However, the administration of F₁ hybrid cells has repeatedly failed to dramatically stimulate the growth of parental marrow in F₁ recipients when given either prior to (24, 27) or at the time of (28, 29, 30) transplantation. As the donors of F₁ cells were themselves incapable of properly supporting parental bone marrow growth, the inability of their cells to stimulate such growth is not surprising.

In this Chapter, an attempt was made to determine whether BDF₁ spleen or thymus cells from donors less than three weeks old, themselves capable of supporting B6 bone marrow growth, could augment such growth in young adult BDF₁ recipient animals.

This was done for two reasons. First, such experiments would determine whether the mechanism by which parental bone marrow is able to proliferate in these recipients involves a stimulatory cell and second, they would shed light on the question of whether the inability of adult F_1 donors to stimulate parental marrow growth indicates a genetic restriction governing this type of cellular interaction.

II. MATERIALS AND METHODS

Bone marrow transplantation and ^{125}I UdR assays were performed as described in Chapter II. BDF_1 recipients of both sexes were 12 to 17 weeks old at the time of irradiation. B6 recipients of both sexes were 13 to 17 weeks of age. Bone marrow was transplanted within 24 hours after x-ray exposure (900R - BDF_1 and 800R - B6) unless otherwise noted.

III. EFFECT OF BDF_1 THYMUS CELLS ON B6 BONE MARROW GROWTH IN BDF_1 RECIPIENTS

One to four hours prior to the transplantation of B6 bone marrow cells, BDF_1 hybrids were given an intravenous dose of 4×10^7 thymus cells from either young (14 to 17 days) or adult (12 to 15 weeks) BDF_1 or B6 donors. This protocol was chosen because it was known to result in the augmentation of B6 marrow growth when B6 thymocytes were used (28, 31 70). Augmentation was confirmed in these experiments (Table V-1). Thymocytes from adult B6 donors increased the splenic uptake of ^{125}I UdR following B6 bone marrow transplantation from 0.031% seen in recipients of marrow only to 0.314% in recipients of both B6 marrow and thymocytes. These thymocytes, when given alone, did not increase the ^{125}I UdR uptake over that seen in radiation control animals. B6

TABLE V-1

EFFECT OF BDF₁ AND B6 THYMUS CELLS FROM YOUNG AND ADULT DONORS
ON THE GROWTH OF B6 BONE MARROW IN BDF₁ ADULT RECIPIENTS

RECIPIENT STRAIN	4 X 10 ⁷ THYMUS CELL DONORS	10 ⁶ B6 BONE MARROW	¹²⁵ IUdR %UPTAKE	N
BDF ₁	B6 - Adult	+	0.314 ⁺ .029	20
BDF ₁	B6 - Adult	-	0.020 ⁺ .001	15
BDF ₁	B6 - Young	+	0.291 ⁺ .049	20
BDF ₁	B6 - Young	-	0.033 ⁺ .010	14
BDF ₁	BDF ₁ - Adult	+	0.035 ⁺ .012	19
BDF ₁	BDF ₁ - Adult	-	0.029 ⁺ .007	15
BDF ₁	BDF ₁ - Young	+	0.040 ⁺ .010	25
BDF ₁	BDF ₁ - Young	-	0.031 ⁺ .013	13
BDF ₁	-	+	0.031 ⁺ .005	20
BDF ₁	-	-	0.019 ⁺ .002	15
B6	-	+	0.375 ⁺ .073	18
B6	-	-	0.019 ⁺ .003	12

BDF₁ and B6 adult recipients and thymus cell donors were 12 to 15 week old males. Young thymus cell donors were 14 to 17 day old animals of both sexes. %Uptake of ¹²⁵IUdR is expressed as means $\pm$ standard errors.

thymus cells from young donors proved equally effective in abrogating Poor Growth in these experiments.

Thymocytes from adult BDF₁ donors on the other hand, proved inconsequential to the growth of parental marrow in BDF₁ recipients. This finding was in agreement with earlier reports of such failure (28, 30). Thymus cells obtained from young BDF₁ donors were equally ineffective in stimulating the growth of B6 bone marrow in hybrid hosts, as no significant differences were seen between recipients of these cells plus marrow and recipients of marrow only.

The magnitude of hemopoiesis observed in syngeneic recipients of B6 marrow is also shown in Table V-1. Although the uptake of ¹²⁵IUdR tended to be somewhat higher (0.375%) in these animals than that seen in BDF₁ mice given marrow plus adult B6 thymocytes (0.314%) or young B6 thymocytes (0.291%), there were no significant differences (P=.05) between these groups.

IV. EFFECT OF BDF₁ SPLEEN CELLS ON B6 BONE MARROW GROWTH IN BDF₁ RECIPIENTS

Because parental bone marrow growth in young F₁ hybrid animals was routinely assayed in terms of splenic hemopoiesis, it was reasoned that any stimulator cells might logically be sought within the spleen itself. To that end, spleen cells from young and adult BDF₁ donors were administered to prospective BDF₁ donors by the same protocol used with thymus cells described in Section III of this chapter. The results of these experiments are presented in Table V-2.

In distinction to the thymus cell preparations, the spleen cells used in these experiments contained large numbers of CFU (see Chapter III,

TABLE V-2

EFFECT OF BDF₁ SPLEEN CELLS FROM YOUNG AND ADULT DONORS
ON THE GROWTH OF B6 BONE MARROW IN BDF₁ ADULT RECIPIENTS

RECIPIENT STRAIN	4 X 10 ⁷ BDF ₁ SPLEEN CELL DONORS	10 ⁶ B6 BONE MARROW	¹²⁵ IUDR %UPTAKE	N
BDF ₁	Young	+	0.039 ⁺ .013	18
BDF ₁	Young	-	0.019 ⁺ .001	20
BDF ₁	Adult	+	0.028 ⁺ .010	16
BDF ₁	Adult	-	0.020 ⁺ .009	10
BDF ₁	-	+	0.031 ⁺ .003	17
BDF ₁	-	-	0.018 ⁺ .044	10
B6	-	+	0.527 ⁺ .044	14
B6	-	-	0.029 ⁺ .005	10

Spleen cell donors were irradiated (1000R) immediately prior to spleen removal. BDF₁ adult recipients and spleen cell donors were 13 weeks old males, as were B6 recipients. Bone marrow donors were B6 males, 13-15 weeks old. Recipients were irradiated (900R-BDF₁ and 800R-B6) approximately 18 hours prior to transplantation. Young spleen cell donors were 13 to 15 days old.

%Uptake of ¹²⁵IUDR in the spleens of recipients was determined as described in Chapter II. These values are expressed as means - the standard errors.

These data represent two experiments.

Section III). In order to prevent the proliferation of stem cells from the spleen cell preparations, which would be indistinguishable from the proliferation of B6 stem cells in the bone marrow inoculum, donors were exposed to 1000R immediately prior to spleen removal.

As expected, BDF₁ spleen cells from adult donors had no effect on the growth of parental marrow in hybrid recipients. A similar marrow graft failure was observed in recipients of marrow plus young BDF₁ spleen cells. No significant differences ($P=.05$) among recipients of marrow only and recipients of marrow plus adult spleen or marrow plus young spleen were observed in these experiments. The proliferative capacity of the marrow inocula employed was assessed in syngeneic recipients (Table V-2). The uptake of $^{125}\text{IUdR}$ in the spleens of syngeneic recipients (0.527%) was more than ten times higher than that observed in BDF₁ recipients in all experimental and control groups.

The results of these experiments, although unequivocal, could not completely rule out the presence of cells, stimulatory to parental bone marrow growth, in the spleens of F₁ hybrids less than three weeks old. Although thymus cells could be administered without prior irradiation of the donors, spleen cells could not. Even though irradiated B6 spleen cells have been shown to enhance parental bone marrow growth in hybrid mice, the possibility existed that similar cells of F₁ origin might not be able to settle properly in the spleens of syngeneic recipients, and their ability to stimulate hemopoietic activity could thereby be impaired.

In an attempt to circumvent this problem, a series of experiments was carried out in which prospective BDF₁ recipients were sub-lethally (500R) irradiated and given spleen cells from normal, unirradiated

donors. Two days after this treatment, when cells could be presumed to have settled normally, recipient mice were irradiated (900R) and given B6 bone marrow as usual. This protocol provided the viable spleen cells with both an irradiated splenic environment in which to settle and an adequate time to do so. The results of these experiments are summarized in Table V-3. Once again spleen cells, at a dose of 5×10^7 , from young and adult BDF₁ donors failed to enhance the growth of B6 bone marrow. No significant differences were seen among recipients of B6 bone marrow plus adult spleen, bone marrow plus young spleen, and recipients of marrow only.

V. PRETREATMENT OF BDF₁ RECIPIENTS WITH BDF₁ SPLEEN CELLS

The ability of parental strain spleen cells to alleviate Poor Growth in hybrid recipients, when the cells are administered intraperitoneally to prospective marrow recipients two weeks or more prior to irradiation and transplantation has been well documented (24, 27). Although the mechanism by which parental spleen cells function in this situation remains unclear, such pretreatment of prospective recipients could not be excluded as a potential means of detecting cells that might be capable of stimulating parental bone marrow growth in very young hybrid mice. Consequently, spleen cells from both B6 and BDF₁ donors were administered intraperitoneally to prospective BDF₁ recipients 14 days prior to irradiation and bone marrow transplantation. Young donors in these experiments were less than seven days old so that their chronological age would be less than three weeks at the time of transplantation. Spleen cells from adult D2 donors were also

TABLE V-3

EFFECT OF VIABLE BDF₁ SPLEEN CELLS FROM YOUNG AND ADULT DONORS
ON THE GROWTH OF B6 BONE MARROW IN BDF₁ ADULT RECIPIENTS

RECIPIENT STRAIN	5 X 10 ⁷ BDF ₁ SPLEEN CELL DONORS	10 ⁶ B6 BONE MARROW	¹²⁵ IUdR %UPTAKE	N
BDF ₁	Young	+	0.029 ⁺ .003	29
BDF ₁	Young	-	0.008 ⁺ .001	24
BDF ₁	Adult	+	0.032 ⁺ .005	30
BDF ₁	Adult	-	0.009 ⁺ .001	22
BDF ₁	-	+	0.030 ⁺ .005	30
BDF ₁	-	-	0.009 ⁺ .002	22
B6	-	+	0.352 ⁺ .051	21
B6	-	-	0.007 ⁺ .001	24

BDF₁ adult recipients and spleen cell donors were 15 to 17 week old females. BDF₁ young spleen cell donors were 14 to 18 day old animals of both sexes. B6 recipients and marrow donors were 12 to 13 week old females.

Two days prior to irradiation (900R-BDF₁ and 800R-B6) and bone marrow transplantation, all recipients were sub-lethally irradiated (500R) and viable spleen cells were administered to appropriate groups immediately thereafter.

%Uptake of ¹²⁵IUdR in the spleens of recipients was determined as described in Chapter II. These values are expressed as means - the standard errors.

These data represent three individual experiments.

given to one group of recipients as an additional control. These data are shown in Table V-4.

The ability of adult B6 spleen cells to enhance the subsequent growth of B6 marrow in hybrid recipients was clearly indicated by the increased splenic uptake of $^{125}\text{IUdR}$ in animals pretreated with such cells (0.193%) over that seen in normal recipients (0.015%). The $^{125}\text{IUdR}$ uptake in animals pretreated with young B6 spleen cells (0.162) was not significantly different ($P=.05$) from that seen following pretreatment with adult cells. Spleen cells from D2 donors had no effect on the outcome of marrow grafts in these experiments. BDF_1 spleen cells from either young or old donors were equally ineffective in stimulating parental marrow growth when given in this manner.

Because the mechanism by which parental strain spleen cells stimulate bone marrow growth is not understood, the possibility was considered that a graft-versus-host reaction (GVH), initiated by B6 spleen cells, might underlie the effect. If true, BDF_1 stimulator cells might not have been detected in these experiments simply because no GVH would arise from pretreatment of recipients with syngeneic BDF_1 spleen cells. In an attempt to resolve this question, three experiments were carried out in which prospective BDF_1 recipients were given D2 spleen cells five days prior to pretreatment with BDF_1 spleen in order to initiate a GVH reaction. D2 spleen was chosen because it had been established that these cells did not alter the Poor Growth of B6 marrow in these hybrids (Table V-4). Table V-5 shows the results of these experiments. Recipient mice, pretreated with B6 spleen, again displayed an enhanced capacity to support the growth of B6 bone marrow grafts. This capacity was not altered by the additional treatment of

TABLE V-4
 PRETREATMENT OF BDF₁ RECIPIENTS WITH SPLEEN CELLS
 FROM B6, D2 AND BDF₁ DONORS

RECIPIENT STRAIN	3 X 10 ⁷ SPLEEN CELL DONORS	10 ⁶ B6 BONE MARROW	¹²⁵ IUdR %UPTAKE	N
BDF ₁	B6 - Adult	+	0.193 ⁺ .022	19
BDF ₁	B6 - Young	+	0.162 ⁺ .038	20
BDF ₁	D2 - Adult	+	0.013 ⁺ .001	19
BDF ₁	BDF ₁ - Adult	+	0.013 ⁺ .001	20
BDF ₁	BDF ₁ - Young	+	0.012 ⁺ .001	20
BDF ₁	-	+	0.015 ⁺ .002	20
BDF ₁	-	-	0.012 ⁺ .001	15
B6	-	+	0.300 ⁺ .206	20
B6	-	-	0.012 ⁺ .001	14

BDF₁ and B6 spleen cell donors were males, 14 and 16 weeks old respectively. D2 spleen donors were 13 week old females. Bone marrow donors were 18 week old males. Both B6 and BDF₁ recipients were males, 15 to 16 weeks old at the time of irradiation (900R - BDF₁ and 800R - B6). Mice were given spleen cells intraperitoneally 14 days prior to irradiation and transplantation of marrow.

%Uptake of ¹²⁵IUdR in the spleen was determined as described in Chapter II and are expressed as means - the standard errors.

These data represent two experiments.

TABLE V-5

EFFECT OF D2 SPLEEN CELLS ON THE PRETREATMENT OF
BDF₁ RECIPIENTS WITH SPLEEN CELLS FROM
B6, D2 AND BDF₁ DONORS

RECIPIENT STRAIN	3 X 10 ⁷ SPLEEN CELL DONORS		10 ⁶ B6 BONE MARROW	125 IudR %UPTAKE	N
	DAY-19	DAY-14			
BDF ₁	D2-Adult	B6-Adult	+	0.179 ⁺ .036	20
BDF ₁	D2-Adult	D2-Adult	+	0.021 ⁺ .001	19
BDF ₁	D2-Adult	BDF ₁ -Adult	+	0.019 ⁺ .002	29
BDF ₁	D2-Adult	BDF ₁ -Young	+	0.027 ⁺ .003	30
BDF ₁	-	B6-Adult	+	0.220 ⁺ .029	30
BDF ₁	-	-	+	0.029 ⁺ .001	28
BDF ₁	-	-	-	0.019 ⁺ .001	23
B6	-	-	+	0.357 ⁺ .053	25
B6	-	-	-	0.020 ⁺ .002	21

BDF₁, B6 and D2 spleen cell donors were 14 to 17 week old males. B6 bone marrow donors were 14 week old females. B6 recipients were 16 week old males. BDF₁ recipients were 12 to 16 week old females.

%Uptake of ¹²⁵IudR in the spleen was determined as described in Chapter II and are expressed as the means ± standard errors.

These data represent three experiments.

these recipients with D2 spleen. All other experimental groups, including recipients of young BDF₁ plus D2 spleen cells, failed to show any significant ($P=.05$) augmentation of marrow growth above that seen in D2 treated or normal recipients.

VI. HUMORAL STIMULATORS

A limited number of experiments was carried out to test for the presence of a factor or factors in the serum of young hybrid mice that might be capable of stimulating the growth of parental marrow in these animals. These experiments were severely restricted in both size and scope because of the small amounts of serum obtainable from mice less than three weeks of age.

Adult BDF₁ recipients were given intravenous injections of serum in doses ranging from 0.1 to 1.0 ml immediately following marrow transplantation. The serum used was obtained from 12 to 18 day old BDF₁ mice of both sexes. Both serum previously stored for one to five months at -40° C. as well as serum collected on the day of transplantation was used. In two experiments bone marrow was incubated with serum for one hour at 37° C. at a concentration of 10^7 cells/ml undiluted serum. A summary of these experiments is given in Table V-6. Neither preincubation of bone marrow with serum nor treatment of recipients with serum resulted in any detectable increase of parental bone marrow growth in these recipients.

VII. DISCUSSION

There now exists a considerable body of evidence strongly suggesting that the growth of hemopoietic stem cells can be regulated by

TABLE V-6

LACK OF STIMULATION OF B6 BONE MARROW GROWTH IN BDF₁
 RECIPIENTS BY SERA FROM YOUNG BDF₁ HYBRID DONORS

SERUM TYPE	VOLUME (ML)	10 ⁶ B6 BONE MARROW	¹²⁵ IUdR %UPTAKE	N
Stored	0.1	Normal	0.024 ⁺ .002	6
Stored	0.2	Normal	0.022 ⁺ .001	3
Stored	0.5	Normal	0.024 ⁺ .001	4
Stored	1.0	Normal	0.027 ⁺ .004	3
Stored	1.0	Serum-Incubated	0.020 ⁺ .003	3
Fresh	0.5	Normal	0.019 ⁺ .002	5
Fresh	1.0	Serum-Incubated	0.020 ⁺ .002	3
None	-	Normal	0.027 ⁺ .005	43
None	-	-	0.016 ⁺ .001	31

Recipients were BDF₁ males and females 12 to 14 weeks old. Serum donors were BDF₁ animals of both sexes 12 to 18 days old. B6 bone marrow donors were 13 to 17 week old males. Stored serum was kept at -40° C. for up to five months before use. Fresh serum was obtained on the day of transplantation. Serum was administered intravenously within two hours of marrow transplantation. Serum-incubated marrow was treated with either fresh or stored serum for one hour at 37° C. at a concentration of 10⁶ cells/ml serum.

These data were obtained from five separate experiments and we expressed as means ± standard errors.

interactions with lymphoid cells. In the parent into F_1 transplant situation, there are compelling data that marrow growth can be augmented by lymphoid cells of parental origin (28-33). As there is disagreement as to the nature of the mechanisms involved in Poor Growth, there is also considerable debate as to the mechanism by which this alleviation of Poor Growth is effected. Two explanations have thus far been offered. On the one hand it has been claimed that lymphoid cells, possessing the putative Hh antigens of the parental type, overload the immune effector cell mechanism of the recipient and allow parental CFU to go undetected as foreign. Proliferation of parental CFU can therefore succeed under these circumstances (82). On the other hand it has been argued that parental lymphoid cells function not as a source of antigen but as a direct stimulant of parental hemopoietic stem cells (70). Demonstrations of the ability of thymic lymphocytes to stimulate hemopoietic stem cell proliferation in vitro and in syngeneic transplant situations in vivo (53, 85) support this hypothesis. In addition, considerable strength has been given this argument by the recent finding by Goodman et. al. (33) that augmentation of parental bone marrow growth by parental thymocytes does not occur in F_1 recipients which have been extensively depleted of T-cells. If parental thymocytes acted solely as a source of antigen under such circumstances, depletion of host T-cells would not effect their ability to overload the host immunity to them.

It was therefore quite reasonable to suppose that the proliferation of parental marrow in very young hybrid mice might be mediated by a stimulatory lymphoid cell and that such a cell would be detectable by its transplantation into adult F_1 recipients. Tables V-1 through V-5

clearly show however, that this was not the case. None of the transplantation protocols, employed in these studies, resulted in the increase of splenic hemopoiesis in recipients of young BDF₁ lymphoid cells plus B6 marrow over that seen in recipients of marrow only. It must be concluded therefore, that transplantable stimulator cells, of the type presumed to be present in B6 thymus and spleen did not mediate the improved parental marrow growth seen in young BDF₁ hybrids. This finding indicates that the failure of adult BDF₁ cells to stimulate parental bone marrow growth did not stem from inability of the donors to support such growth, but probably from a genetic restriction of this type of interaction.

An additional reason to suspect that young BDF₁ spleen cells might enhance the growth of B6 marrow in hybrid recipients was suggested by a study of Natural Killer (NK) cells (49). There appears to exist in mouse spleen a cell type capable of cytotoxicity against a variety of both malignant (35, 40-43) and normal (42) cells. Because this response does not require previous specific exposure or priming of these cells to their targets, they have been designated Natural Killers (48). Recently, NK cells have been proposed as the cells responsible for "immunity" to marrow cell grafts (43, 49, 54, 55, 57).

In an attempt to draw a parallel between NK activity and Poor Growth, Lotzova and Savary (56, 57) showed that spleen cells from mice less than three weeks of age were capable of suppressing NK activity in vitro. This finding was used to explain why parental marrow grew well in F₁ recipients of this age. If the explanation by analogy is valid, it would be anticipated that young hybrid spleen cells, capable of suppressing NK activity, would enhance parental bone marrow growth

in adult recipients. The failure of such cells to do so in the experiments presented here suggests 1) suppression of NK activity in vitro has no in vivo analogue, 2) NK reactivity is not the mechanism by which Poor Growth is mediated.

The studies involving stimulation of marrow growth by serum factors, although very limited in scope, strongly indicated that such factors could not abrogate Poor Growth in adult hybrid recipients. These studies did not, however, preclude the existence of such factors nor their possible detection by other means. Further studies would have required a prohibitive number of young serum donors and were therefore not pursued.

CHAPTER VI

"EFFECTOR" CELLS AND BONE MARROW GROWTH IN YOUNG MICE

I. INTRODUCTION

It was established in Chapter III of this report that the transplantation of B6 bone marrow into BDF₁ recipients less than three weeks old resulted in hemopoietic recovery comparable to that seen in age matched B6 mice. It was further demonstrated that neither fluctuations in the endogenous stem cell pools of the hosts nor changes in radiation sensitivity characteristic of mice of this age could account for this recovery or its subsequent disappearance in older BDF₁ recipients. Thus the hemopoiesis observed in young BDF₁ recipients of B6 marrow was ascribed to the ability of these mice to support the proliferation of B6 hemopoietic stem cells which contrasted sharply with the inability of older BDF₁ animals to do so. The influence of exogenous factors, either specific or non-specific, in the milk consumed by suckling mice on the outcome of marrow grafts was discounted in Chapter IV.

It has been suggested that Poor Growth is the result of an F₁ anti-parent immune response to putative Hh antigens expressed on the surface of hemopoietic stem cells in the donor marrow (17, 56). It has further been suggested that treatments which abrogate Poor Growth do so either by eliminating the cellular mechanism responsible for the "rejection" of marrow stem cells (19, 82) or by rendering them dysfunctional (56). Early reports of parental bone marrow growth in young F₁ hybrids therefore claimed that the lack of Poor Growth in mice of this age was due to

the "late maturation" of the "effector" cell mechanism responsible for the destruction of parental marrow grafts (16, 18).

The experiments presented in this Chapter were designed to determine whether this hypothesis would be borne out experimentally, with the use of young hybrid recipients as a test model. It was reasoned that if the growth of parental marrow in young hybrids was due to the lack of "effector" cells, then introduction of such cells into young hybrid recipients would decrease the ability of these hybrids to support parental marrow growth. To this end, young BDF₁ hybrids were given spleen cells from adult BDF₁ donors either at the time of B6 marrow transplantation or at various intervals prior to marrow grafting. The subsequent outcome of these grafts was determined five days post-transplantation by the ¹²⁵IUdR assay.

II. MATERIALS AND METHODS

Bone marrow transplantation and ¹²⁵IUdR assays were performed as described in Chapter II. Recipients were BDF₁ mice of both sexes, 18 to 19 days old at the time of irradiation. In experiments where spleen cells were given after irradiation of the recipients, the donors of the spleen cells were themselves irradiated (1000R) immediately prior to spleen removal. Spleen cell donors were either age-matched with recipient mice (young) or 14 to 17 weeks old (adult). Recipient mice were exposed to 600R of x-rays prior to marrow transplantation.

III. POST-IRRADIATION SPLEEN CELL TREATMENT

A series of experiments was carried out in which graded doses of spleen cells from adult BDF₁ donors were administered to young BDF₁

recipients one to three hours before B6 bone marrow transplantation. The spleen cell donors were exposed to 1000R of x-radiation immediately prior to sacrifice and spleen removal to inhibit the proliferation of splenic CFU from these donors to the young recipients. As "effectors" of Poor Growth must necessarily be resistant to radiation of this magnitude (72), it was believed that such exposure should not impair the ability of these cells to carry out their function after transplantation into young recipients. The recipients in these experiments were 18 to 19 days old. Mice of this age were chosen because they were still within the age range capable of supporting parental bone marrow growth and yet large enough so that marrow could be transplanted with relative ease and accuracy.

The combined results of four such experiments are presented in Table VI-1. Treatment of recipients with spleen cells from adult donors in doses ranging from 10^7 to 4×10^7 cells appeared to have no suppressive effect on the outcome of parental bone marrow grafts as no significant differences ($P=.05$) were observed between the $^{125}\text{IUdR}$ uptake of recipients of marrow only and that of recipients of marrow plus spleen. Nor was any trend in that direction observed with increasing spleen cell dose. As was expected, spleen cells from young (14 to 18 day old) BDF_1 donors did not effect the growth of parental marrow grafts.

Spleen cells from irradiated young or adult hybrid donors were rendered incapable of proliferation in these hosts as none of the recipient groups of spleen only showed an $^{125}\text{IUdR}$ uptake significantly ($P=.05$) above that seen in radiation control animals. This finding also provides additional confirmation of the contention (Chapter III,

TABLE VI-1

EFFECT OF BDF₁ SPLEEN CELLS GIVEN AFTER IRRADIATION ON THE GROWTH OF B6 MARROW IN YOUNG BDF₁ RECIPIENTS

BONE MARROW	CELL DOSE		¹²⁵ IuDR %UPTAKE	N
	ADULT SPLEEN	YOUNG SPLEEN		
10 ⁶ B6	10 ⁷	-	0.306 ⁺ .039	9
	2 x 10 ⁷	-	0.289 ⁺ .040	12
	3 x 10 ⁷	-	0.331 ⁺ .024	14
	4 x 10 ⁷	-	0.291 ⁺ .031	13
	-	10 ⁷	0.329 ⁺ .022	8
	-	2 x 10 ⁷	0.319 ⁺ .053	10
	-	3 x 10 ⁷	0.292 ⁺ .015	9
	-	4 x 10 ⁷	0.325 ⁺ .033	11
	-	-	0.340 ⁺ .029	31
	-	-	-	-
	-	-	-	-
	-	-	-	-
None	10 ⁷	-	0.161 ⁺ .023	8
	2 x 10 ⁷	-	0.179 ⁺ .030	9
	3 x 10 ⁷	-	N.D.	-
	4 x 10 ⁷	-	0.139 ⁺ .041	10
	-	10 ⁷	0.173 ⁺ .028	11
	-	2 x 10 ⁷	0.150 ⁺ .034	7
	-	3 x 10 ⁷	N.D.	-
	-	4 x 10 ⁷	0.157 ⁺ .019	9
	-	-	0.140 ⁺ .035	22
	-	-	-	-
	-	-	-	-
	-	-	-	-

Adult spleen cell donors were BDF₁ females 14 to 16 weeks old. Young spleen donors were BDF₁ animals of both sexes, 14 to 18 days of age. All spleen cell donors were irradiated (1000R) immediately prior to spleen removal. Marrow donors were B6 mice of both sexes, 14 to 15 weeks old. Recipients were BDF₁ animals of both sexes, 18 to 19 days old at the time of irradiation (600R).

%Uptake of ¹²⁵IuDR was determined as described in Chapter II. The results are expressed as means ± the standard errors.

These data represent four experiments.

Section VII) that the mere presence of additional transplanted cells does not promote the proliferation of host-type hemopoietic stem cells in young mice.

IV. SPLEEN CELL PRETREATMENT

Because adult F_1 spleen cell treatment at the time of parental bone marrow transplantation failed to cause a decrease in the growth of these marrow grafts, a second series of experiments was undertaken to determine whether spleen cells, given prior to irradiation could effect such a suppression. In these experiments, 3×10^7 spleen cells from adult BDF_1 donors were administered to young BDF_1 recipients at various intervals before irradiation and B6 bone marrow transplantation. As the recipients were irradiated (600R) after spleen cell treatment, there was no need to compromise the proliferative capacity of these cells by irradiation of the donors. Recipients in these experiments were again 18 to 19 day old BDF_1 mice of both sexes. The results are presented in Table VI-2.

Pretreatment of prospective F_1 recipients at intervals of from 2 to 15 days prior to irradiation with spleen cells from adult F_1 donors again did not affect the outcome of subsequent parental-strain bone marrow grafts. No significant differences ($P=.05$) in the ^{125}I UdR uptake of spleen cell treated versus non-treated bone marrow recipients could be detected in these experiments. Included as an additional control were mice which were pretreated with young F_1 spleen cells (from age-matched donors). These animals too showed no change in ^{125}I UdR uptake with respect to non-pretreated controls.

TABLE VI-2

EFFECT OF BDF₁ SPLEEN CELL PRETREATMENT ON THE
GROWTH OF B6¹ MARROW IN YOUNG BDF₁ RECIPIENTS

DAY OF SPLEEN CELL TREATMENT	10 ⁶ B6 BONE MARROW	PRETREATMENT - SPLEEN TYPE					
		ADULT		YOUNG		NONE	
		¹²⁵ IUdR %UPTAKE	N	¹²⁵ IUdR %UPTAKE	N	¹²⁵ IUdR %UPTAKE	N
-2	+	0.599 [±] .049	10	0.587 [±] .035	9	0.562 [±] .079	11
	-	0.093 [±] .020	8	0.080 [±] .015	9	0.087 [±] .026	7
-5	+	0.611 [±] .092	8	0.623 [±] .050	10	0.621 [±] .072	9
	-	0.124 [±] .022	7	0.137 [±] .014	6	0.120 [±] .029	10
-10	+	0.543 [±] .043	12	0.529 [±] .063	9	0.559 [±] .050	12
	-	0.099 [±] .010	13	0.093 [±] .029	10	0.085 [±] .009	9
-15	+	0.576 [±] .079	7	0.591 [±] .053	6	0.587 [±] .048	6
	-	0.086 [±] .012	5	0.091 [±] .005	4	0.070 [±] .019	5

Adult spleen cell donors were BDF₁ females 14 to 17 weeks old. Young spleen donors were BDF₁ mice of both sexes 4 to 15 days of age (age-matched with recipients at the time of pretreatment). Marrow donors were B6 females 12 to 16 weeks old. Recipient mice were BDF₁ animals of both sexes, 18 to 19 days old at the time of irradiation (600R).

Recipient mice were pretreated intraperitoneally with 3×10^7 spleen cells on the day indicated (day of irradiation = day 0).

%Uptake of ¹²⁵IUdR was determined as described in Chapter II. The results are expressed as means ± the standard errors.

These data represent four experiments.

V. DISCUSSION

Although the existence of an "effector" cell type, capable of killing transplanted hemopoietic stem cells has been postulated for several years (17, 18, 19), no direct test of this hypothesis has been reported in the literature regarding Poor Growth. The experiments presented in this Chapter represent one such test, the results of which do not speak in favor of an "effector" cell mechanism. Doses of irradiated adult F_1 spleen cells of up to 4×10^7 cells, administered at the time of parental marrow transplantation, were unable to suppress the growth of marrow transplants in young hybrid recipients, in spite of claims that "effector" cells constitute a sub-population of adult F_1 spleen cells (19). Normal hybrid spleen cells were equally ineffective at inhibiting parental marrow growth in young F_1 mice when administered from two to 15 days prior to transplantation.

One possible reason for the failure of adult F_1 spleen cells to suppress parental bone marrow growth in young F_1 hybrid recipients is that such cells simply do not exist. However, other explanations are also possible. It is conceivable for instance, that though they exist, "effector" cells are unable to function after transplantation to a new host. If true however, this would suggest a cellular basis of Poor Growth somewhat more complicated than the direct stem cell killing now postulated (19). It is also possible that "effector" cells were not transplanted in sufficient numbers to be effective in these experiments. However, although complete abrogation of bone marrow growth might be an unreasonable expectation, the administration of 4×10^7 cells, roughly the equivalent of 1/3 of an adult spleen, should have resulted in at least a marginal decrease in parental bone marrow growth

if direct graft destruction were their mode of action in this system. Thus the data presented here suggest that if they do exist, "effector" cells probably do not act simply as stem cell killers, but may be involved in the regulation of stem cell growth by some other, less direct means. One such mechanism is postulated in Chapter VIII, Section III of this volume.

CHAPTER VII

NATURAL KILLER CELLS AND POOR GROWTH

I. INTRODUCTION

Cell mediated immune responses against tumor cell lines in vitro have been widely investigated employing numerous experimental systems and assays. In 1973, Herberman et al (40) published a report regarding one such system involving T-cell dependent cytotoxicity. Their system was basically as follows: Two weeks following immunization of mice with murine sarcoma virus (MSV), spleen cells from these mice were tested for their ability to lyse RBL-5 tumor cells (a Raucher virus-induced leukemic cell line) when cultured together for four hours. The assay employed in these experiments was a ^{51}Cr release assay in which the target (tumor) cells were labeled with ^{51}Cr prior to culturing with attacker (spleen) cells. The cytolytic effect of the attacker cells was determined by the percentage of isotope released by the target cells into the culture medium after co-culturing of target and attacker cells.

As part of this study, the investigators included control tests employing spleen cells from normal, non-immunized mice as the attacker cell population. They found that when cultured with these "control" cells, the tumor cells released low but significant amounts of ^{51}Cr (1.7 to 7.2%) above spontaneous release levels. This apparent cytotoxicity was of interest to them not only because it appeared to arise without the usual requirement of prior antigenic exposure, but because in contrast to the immunized cells, the cytotoxicity of normal cells

did not appear to be thymic dependent, as treatment of these cells with anti- θ serum (a T-cell specific antiserum) and complement did not reduce their cytotoxic potential.

The discovery of a thymic independent, naturally occurring and rapidly reacting immune mechanism against tumor cells caught the interest of several investigators. The possibility that such a mechanism could be of major importance, not only in autoimmunity but in immunosurveillance against tumors, did not long go unnoticed (35, 45, 65). Further credence to this possibility was provided by reports of such natural killer (NK) activity in both guinea pigs (3) and humans (45, 67, 73). Noting similarities between in vitro NK activity and Poor Growth in vivo, several groups studying experimental bone marrow transplantation have also been drawn into the investigation of NK cells.

Results of investigations directed at determining whether or not NK activity and Poor Growth are mediated by the same mechanism have shown a parallel between the response of both NK activity and Poor Growth to a variety of treatments. On the basis of some positive correlations derived from such studies, it has been proposed that the cells responsible for NK cytotoxicity in vitro are the same as those responsible for "rejection" of parental bone marrow grafts in vivo (49, 54, 55, 57). Direct attempts to establish such a relationship have not been forthcoming. Missing from the literature regarding this subject are reports in which NK cells of hybrid origin were tested for their ability to lyse parental strain bone marrow cells. This Chapter reports one such study designed to determine whether NK cells of F_1 hybrid origin exert a cytotoxic effect on parental hemopoietic cells.

I. MATERIALS AND METHODS

Culture Medium

RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units/ml) and streptomycin (50 ug/ml) was used in all experiments as a diluent and tissue culture medium.

Target Cells

Two tumor cell lines were used as targets for NK lysis, EL-4, a tumor of C57Bl (H-2^b) origin was maintained in an ascites by serial passage in B6 hosts. L-1210, of D2 (H-2^d) origin, was also maintained as an ascites tumor in D2 mice. In addition, bone marrow from both B6 and BDF₁ mice were also employed as target cells.

Labeling of Target Cells

Prior to labeling with ⁵¹Cr, target cells were washed in medium, counted and resuspended to a concentration of 10⁷ cells/ml. ⁵¹Cr, in the form of sodium chromate, was added (100 uCi/10⁷ cells) and the cells were then incubated at 37 C for 45 minutes. After incubation the cells were washed three times in medium, resuspended and allowed to stand for 30 minutes on ice. The cells were then washed once again, counted and adjusted to the desired concentration.

NK Cells

B6, BDF₁ and Balb/c nu/nu (nude) mice six to ten weeks old, bred and maintained at the Biology Division of the Oak Ridge National Laboratory, were used as donors of NK cells. Spleen cell suspensions were prepared as described in Chapter II with the exception that culture medium was used in place of PBS. After initial preparation, spleen

cells were washed once in medium, counted and resuspended to the desired concentrations.

⁵¹Cr Release Assay

⁵¹Cr-labeled target cells were diluted to 10^6 cells/ml and 0.05 ml quantities of these suspensions were added to glass tubes (11 X 75 mm) giving a final concentration of 5×10^4 cells/tube. 0.10 ml of spleen cells was added to the tubes in concentrations of 12.5-100 $\times 10^6$ cells/ml to give final concentrations of 1.25-10 $\times 10^6$ cells/tube. Target and effector (spleen) cells were incubated for four hours at 37°C in a humidified incubator in an atmosphere of 5% CO₂ in air. At the end of the incubation period, 1.0 ml of medium was added to each tube and the cells were pelleted by centrifugation. The supernatant from each tube was decanted and counted in a Packard Auto-Gamma counter. The levels of spontaneous release were determined by incubation of labeled target cells without added spleen. Maximum release in these experiments was considered to be the release seen after four to five cycles of freeze-thawing of labeled target cells by alternation between an ethanol-dry ice bath and a 37°C water bath. Results are expressed as % lysis as determined by the following formula:

$$\% \text{ lysis} = \frac{\text{Counts per minute (test)} - \text{Counts per minute (spontaneous)}}{\text{Counts per minute (maximum)}} \times 100$$

All tests reported here were carried out in triplicate.

CFU Assay

In a manner identical to that used in the ⁵¹Cr release assay, unlabeled bone marrow cells were diluted and incubated with spleen cells

from donors which were irradiated (1000R) immediately prior to spleen removal. Twelve tubes of each bone marrow type were prepared and after incubation the cells from these tubes were pooled, washed in medium and diluted to 12 ml. 1.0 ml of this suspension was injected into lethally irradiated recipients, syngeneic to the bone marrow in the inoculum. This was equal to a theoretical dose of 5×10^4 marrow cells, however this could not be directly determined by counting because of the spleen cells in these preparations. The actual dose was, in all probability, somewhat lower than that calculated from the initial values. Recipient mice were sacrificed seven to eight days after transplantation and gross splenic colonies were enumerated as described in Chapter II. In each experiment, the presence of NK activity in the spleen cells used was determined by their ability to lyse ^{51}Cr -labeled L-1210 tumor targets.

III. ANTI-TUMOR NK ACTIVITY

The ability of spleen cells from B6, BDF₁ and Balb/c nude mice to lyse EL-4 and L-1210 tumor targets was tested at effector:target ratios of from 25:1 to 200:1. The results of three such experiments are shown in Table VII-1. EL-4 tumor cells are thought to possess high concentrations of B6 type Hh antigens on their surfaces (74). Their susceptibility to NK lysis by both BDF₁ and Balb/c nude spleen cells was therefore of great interest because B6 bone marrow grows poorly in recipients of both of these strains (19). However EL-4 cells proved to be particularly poor targets of NK lysis in these experiments, confirming similar findings by others (41, 48).

L-1210 tumor cells appeared to be a much more suitable target for the demonstration of NK activity. Both B6 and BDF₁ spleen cells showed

TABLE VII-1
NK ACTIVITY AGAINST EL-4 AND
L-1210 ASCITES TUMORS

EXPERIMENT NUMBER	TARGET	EFFECTOR	% LYSIS EFFECTOR:TARGET RATIO			
			25:1	50:1	100:1	200:1
1	EL-4	B6	-5.2	-6.7	-4.8	-5.9
		BDF ₁	-3.8	-1.0	-2.2	-0.9
		Balb/c Nude	-6.0	-2.7	0.7	1.2
	L-1210	B6	-3.6	2.7	5.6	6.6
		BDF ₁	-2.6	1.9	3.7	5.7
		Balb/c Nude	3.6	9.4	15.8	19.3
2	EL-4	B6	-2.9	-0.8	1.2	-1.2
		BDF ₁	-4.2	-4.4	-0.3	-2.2
		Balb/c Nude	-2.8	-1.6	2.4	2.3
	L-1210	B6	-2.3	1.0	2.4	3.7
		BDF ₁	-3.1	0.3	5.6	7.2
		Balb/c Nude	5.0	9.2	11.3	13.5
3	EL-4	B6	N.D.	-7.9	-6.3	-2.0
		BDF ₁	N.D.	-12.2	-7.8	-1.8
		Balb/c Nude	N.D.	-3.2	-2.4	-0.1
	L-1210	B6	N.D.	-3.6	-1.7	-2.1
		BDF ₁	N.D.	2.3	2.3	4.1
		Balb/c Nude	N.D.	4.2	9.7	10.8

In each experiment effector cells were obtained from a pool of at least three mice of each strain. Spleen cell donors were between six and eight weeks old in all cases except the Balb/c nudes in experiment 3 which were ten weeks of age.

low and approximately equal reactivity against this tumor with a maximum lysis of 6.6% by B6 and 7.2% by BDF₁. Spleen cells from Balb/c nude mice were much more reactive to L-1210 cells, giving rise to a cytotoxicity against these cells which ranged from 10.8% to 19.3% at an effector:target ratio of 200:1. This finding is in agreement with other reports of elevated NK activity in nude mice (41, 42). Because of this finding, L-1210 cells were routinely employed as a control test for the presence of NK activity in all subsequent experiments.

IV. ANTI-BONE MARROW NK ACTIVITY

A working NK anti-tumor system having been established, experiments were next directed toward the detection of anti-bone marrow NK activity. The specific question to which these experiments were addressed was whether B6 bone marrow could be lysed by NK cells from either BDF₁ or Balb/c nude donors, whose inability to support the growth of this marrow in vivo has well been documented (18, 19). To this end, ⁵¹Cr-labeled bone marrow was incubated with spleen cells from B6, BDF₁ and Balb/c nude donors and the release of ⁵¹Cr was assessed after four hours. The ability of these spleen cells to lyse L-1210 tumor targets was also assayed. The results are summarized in Table VII-2.

In experiment 1 of this Table, low levels of NK activity against L-1210 cells were observed in B6 and BDF₁ spleen cells. Relatively high anti-L-1210 NK lysis (21.2%) was again detected in Balb/c nude spleen. However, none of these spleen cell preparations were able to induce the release of ⁵¹Cr by B6 bone marrow cells above the spontaneous release levels. In experiment 2, BDF₁ bone marrow was also included as a control target cell. Although again no lysis of bone marrow cells

TABLE VII-2
NK ACTIVITY AGAINST B6 AND BDF₁
BONE MARROW CELLS

TARGET	EFFECTOR	% LYSIS	
		EXPERIMENT 1	EXPERIMENT 2
B6 Bone Marrow	B6	-2.7	-3.6
	BDF ₁	-5.3	0.3
	Balb/c Nude	-2.4	-5.6
BDF ₁ Bone Marrow	B6	N.D.	-5.6
	BDF ₁	N.D.	-1.3
	Balb/c Nude	N.D.	0.8
L-1210 Tumor	B6	5.4	-2.3
	BDF ₁	9.8	-1.6
	Balb/c Nude	21.2	5.8

In each experiment effector cells were obtained from a pool of at least three mice. Spleen cell donors were six to eight weeks old in all cases.

An effector:target ratio of 200:1 was employed in both experiments.

was observed, both B6 and BDF₁ spleen cells showed no demonstrable lytic activity against the tumor targets, in contrast to the results of the previous Table. Balb/c nude spleen was cytotoxic to L-1210 at a relatively low level (5.8%) compared with other data (Table VII-1), but these cells were also unable to lyse bone marrow cell targets.

V. ANTI-STEM CELL NK ACTIVITY

It has been suggested (81) that the reason F₁ anti-parental strain bone marrow NK activity might not be detected is that the hemopoietic stem cells may be the only population of bone marrow cells that express Hh antigens and thus the only marrow cells subject to NK lysis. As these represent only a very small percentage of adult bone marrow cells (25 to 30/10⁵ total marrow cells; see Chapter III, Section III), the release of ⁵¹Cr by these cells would be completely undetectable in a conventional NK cell assay, even though the stem cells may in fact be killed by NK cells in vitro. In order to test this hypothesis, unlabeled B6 bone marrow was incubated with irradiated spleen cells from B6, BDF₁ and Balb/c nude donors and then transplanted into irradiated B6 recipients. The effect of NK cells on marrow CFU was assessed eight days later by enumeration of gross hemopoietic colonies in the spleens of recipient mice. Included in these experiments were ⁵¹Cr release assessments of anti-bone marrow NK activity as well as activity against L-1210 tumor cells. The results are shown in Table VII-3.

Again, while all three effector cell types were able to lyse L-1210 target cells (confirming the radiation resistance of NK cells (47)), no cytotoxicity to B6 bone marrow was detectable by ⁵¹Cr release. Moreover, upon transplantation of the cell mixtures to irradiated B6

TABLE VII-3
EFFECT OF NK CELLS ON B6 CFU

TARGET	EFFECTOR	%LYSIS	CFU	N
L-1210 Tumor	B6	9.8 [±] 2.7	-	-
	BDF ₁	6.8 [±] 2.3	-	-
	Balb/c Nude	15.5 [±] 3.6	-	-
B6 Bone Marrow	B6	-2.7 [±] 0.7	11.8 [±] 1.7	20
	BDF ₁	-3.6 [±] 1.9	12.4 [±] 1.5	19
	Balb/c Nude	-1.4 [±] 1.4	13.9 [±] 3.2	20
	None	0.0	13.4 [±] 1.2	20
None	B6	-	0.0	15
	BDF ₁	-	0.1 [±] 0.1	15
	Balb/c Nude	-	0.0	14
	None	-	0.0	14

Effector cells were obtained from a pool of at least three mice. Spleen cell donors were six to nine weeks old and were irradiated (1000R) immediately prior to spleen removal. Bone marrow cells were also obtained from a pool of three mice.

These data represent two experiments. %lysis data are expressed as the mean of the values obtained in the two experiments - standard errors. CFU data are expressed as the mean of all the individual recipients - the standard error of that mean. N refers to the total number of recipients of syngeneic marrow.

recipients, no reduction in the number of CFU could be detected in NK treated marrow cells. There were no differences ($P=.05$) between recipients of NK incubated marrow and recipients of marrow incubated in medium alone. That this colony-forming ability was derived from B6 marrow and not the spleen cells present in the inocula was demonstrated by the lack of CFU in recipients of spleen only. Thus NK cells did not selectively attack bone marrow stem cells in these experiments.

VI. DISCUSSION

The relatively recent discovery of NK cells (30) has done much to lend credibility to the hypothesis that Poor Growth is an F_1 anti-parent immune reaction. Indeed one of the biggest drawbacks to such an hypothesis has been the need to postulate an immune cell type of a highly unconventional nature. Among the traits necessarily ascribed to the "effectors" of Poor Growth are radiation resistance (73), rapid reactivity (17), appearance after three weeks of age (16) and ability to respond to cells without prior exposure to their antigens (17). At the time of their postulation, no cell type known possessed these traits. Thus with the later discovery of such a cell type (NK), it was very inviting to claim them to be the long-sought "effectors" of Poor Growth.

However, as tempting as this speculation might be, it is important to keep it in its proper perspective - that of a hypothesis. There are other assumptions of the immune reaction theory that appear not to be borne out by experimental evidence (See Chapter VIII, Section I). Furthermore, the theory predicts that F_1 NK cells should be cytotoxic to parental bone marrow or at least to the stem cell compartment of the marrow. However, the data presented in this Chapter show that this is

not the case. Neither whole parental bone marrow, assayed by ^{51}Cr release nor parental CFU assayed by transplantation were sensitive to NK lysis by either BDF_1 or more highly reactive Balb/c nude spleen cells. Moreover, if Hh antigens are the targets of NK cells in the Poor Growth system, EL-4, believed to express large amounts of these antigens, should make particularly sensitive targets for NK lysis. This also has not been borne out here or by others (41, 48).

Although these data do not preclude an indirect role of NK in the failure of parental marrow grafts to proliferate in F_1 recipients, they certainly speak strongly against NK cells as killers of such grafts.

CHAPTER VIII

DISCUSSION: MECHANISMS OF POOR GROWTH

I. THE IMMUNE RESPONSE/NATURAL KILLER HYPOTHESIS

Since the discovery of Poor Growth, the mechanisms by which this phenomenon is mediated have been the subject of considerable speculation (17, 18, 33, 38, 70). Among the hypotheses put forth to explain the nature of Poor Growth, the most widely accepted has been one originally conceived by Cudkiewicz (13) and since expanded by him (15-19) as well as others (56, 82). This theory holds that Poor Growth is the result of an F_1 anti parent immune reaction specifically directed at parental hemopoietic stem cells.

Central to this hypothesis is the assertion that there exists in the mouse genome unique histocompatibility genes, now designated Hh genes, whose phenotypic expression, in the form of Hh antigens, is limited to hemopoietic cells. The unique quality of these genes stems from their apparent inheritance characteristics. While the gene products of other histocompatibility loci are expressed codominantly in F_1 hybrid mice (77), Hh gene products are not so expressed. F_1 mice, it has been suggested, express an Hh phenotype which may be different from either parental phenotype due simply to heterozygosity at the Hh locus (56) or to interaction of the two parental alleles (15). In cases in which Poor Growth is observed, the difference between the Hh antigens of the hybrid and those of the adult are so great that F_1 animals are able to recognize them as immunologically "foreign" and mount an immune response against them by selective cytotoxicity of the hemopoietic cells

bearing these surface antigens (15). Because of this immunity, bone marrow grafts between Hh incompatible mouse strains would not be expected to survive.

In order to explain how a host is able to mount an immune reaction against Hh antigen bearing cells following radiation exposures known to abrogate classical immunities (60), a new type of immunocompetent cell has been postulated (14, 16-18). This cell type has been ascribed properties which reflect the known facts regarding the Poor Growth phenomenon itself. Because Poor Growth can be detected following lethal radiation exposures of the host, the "effector" cells have been postulated to be radiation resistant (72).

The absence of Poor Growth in mice less than three weeks of age has led to the conclusion that "effector" cells are late in maturing (16).

The cell type responsible for mediating F_1 anti parent reactions has been assumed to be derived in the bone marrow as treatment of prospective recipients with either ^{89}Sr or anti mouse bone marrow serum weakens Poor Growth (56). The persistence of Poor Growth following thymectomy of the recipients (17, 30, 33) and reports of "hyper-resistance" to bone marrow grafts in athymic (nude) mice (8, 19), have led to the conclusion that "effector" cells are not T-cells or in any way dependent on the thymus for their function (17, 56). Abrogation of Poor Growth by anti macrophage agents (18, 56) has given rise to speculation that "effector" cells are either macrophages themselves or are dependent upon accessory macrophages in order to respond to Hh antigens.

Recently, striking parallels have been drawn between "effectors" of Poor Growth and Natural Killer cells, a summary of which is presented below:

1. Both are rapidly reacting mechanisms which do not require prior antigenic exposure in order to function (49, 54).
2. Neither function is apparent in mice until after three weeks of age (45, 54).
3. Both are relatively radiation resistant (45, 54).
4. Both are suppressed by depletion of the host bone marrow with either ^{89}Sr or rabbit anti mouse bone marrow serum (45).
5. Both are reduced by anti macrophage agents (45, 54, 55).

On the basis of such comparisons, it has been suggested that in addition to mediating cellular toxicity to both normal and malignant cells in vitro, NK cells are responsible for rejection of Hh incompatible cells in vivo.

Observations of the ability of parental cells, given after irradiation, to augment parental bone marrow growth have been explained within the context of the immune rejection/Natural Killer hypothesis as an "overload" of the "effector" cell mechanism with Hh incompatible cells (82). It has further been claimed that Hh incompatible cells have the ability to induce "specific unresponsiveness" to Hh antigens. This argument was advanced in order to explain the abrogation of Poor Growth by parental cells when given several days prior to irradiation and bone marrow transplantation (56).

Although this model can be used to explain many of the observations which have been reported regarding Poor Growth, there are several serious drawbacks to this hypothesis that deserve note. First and perhaps most important, the existence of the putative Hh antigens may be called into question. In the 15 or so years since their postulation, and despite vigorous attempts to do so (25, 26), the presence of Hh antigens on the

surface of hemopoietic stem cells has not been demonstrated. As Hh antigens form the basis of the immune rejection/Natural Killer hypothesis, the inability to detect them by serological means is of great concern.

Secondly, although the ability of parental thymocytes to augment bone marrow growth is in itself compatible with the proposed model, recent evidence casts doubt on the suggestion that parental cells "overload" an immune mechanism. A study employing F_1 recipient mice extensively depleted of T-cells showed that parental thymocytes were unable to augment the growth of transplanted parental marrow in these animals (33). This finding is neither predicted by, nor compatible with the hypothesis at hand. Depletion of host T-cells should not render donor thymocytes incapable of augmenting stem cell proliferation if thymocytes acted solely as an antigenic overload.

Thirdly, in spite of the parallels between NK reactivity and the phenomenon of Poor Growth, there are data which suggest that, while there may be some relationship between the two, NK cells probably do not mediate Poor Growth by direct cytotoxicity of hemopoietic stem cells. A comparison of strain responses in both systems shows that the B6 strain is considered a prototype strain with respect to Poor Growth both in terms of the proliferation of B6 marrow in semi-allogeneic and allogeneic recipients and the proliferation of allogeneic marrow grafts in B_6 hosts (14, 17, 80). Yet B6 has often been shown to be unimpressive in terms of its NK activity (41). Tumor targets such as EL-4, of B_6 origin and thought to possess high levels of surface Hh antigens (74) make particularly poor targets in the NK system (41, 48).

Furthermore, there have been no reports of NK activity directed specifically against parental strain marrow cells. The data presented

here (Chapter VII) indicate that neither BDF_1 nor more highly reactive Balb/c nude NK cells show any appreciable cytotoxicity against B6 bone marrow cells above that seen against syngeneic cells. Nor do these NK cells exert a suppressive effect on the proliferative ability of B6 marrow subsequently transplanted into syngeneic recipients. For these reasons, the immune response/Natural Killer model has proven to be an unsatisfying one.

II. STIMULATING FACTOR HYPOTHESIS

Because of some of the discrepancies inherent in the above model and the hypothetical nature of Hh antigens, Pritchard (70) in 1974 proposed an alternative model to explain Poor Growth which did not require the postulation of an F_1 anti parent immune response. It stated, in brief, that the growth of hemopoietic stem cells is stimulated by short-range humeral factors produced by a type of thymocyte which bind to receptors on the surface of stem cells, thus initiating proliferation and/or differentiation. Both the factors and their corresponding receptors, it was suggested, display varying degrees of strain-specificity, with B6 being among the most highly specific. The model further proposed that the genes for the factors and their receptors are expressed codominantly. Thus in a theoretical $(A \times B)F_1$ hybrid, approximately 50% of the factors produced would be of A strain type and the other 50% B strain type.

Poor Growth in this model is a consequence of the low cross-reactivity of B6 receptors with non-B6 factors. In an F_1 hybrid, B6 marrow would be presented with mixture of factors, only 50% of which would be capable of stimulating the proliferation of B6 stem cells, thus giving

rise to a decrease in proliferation of this marrow from that seen in syngeneic recipients. The research which lead to this hypothesis employed (B6 X C3H) F_1 mice which are able to adequately support the growth of C3H marrow, as are B6 recipients. The good growth of C3H marrow, in these hybrids was ascribed to a high cross-reactivity of B6 factors and C3H receptor sites, as evidenced by the good growth of C3H marrow in B6 hosts.

This hypothesis can be seen to have certain distinct advantages over the immune response/Natural Killer model. For instance, Poor Growth can be explained within the context of this model without the necessity of postulate a very non-classical immune system. Nor does this model center on antigens whose existence has defied demonstration. Furthermore, not only is it able to predict the ability of parental thymocytes to augment parental bone marrow growth (production by parental thymocytes of compatible factors), but it is also compatible with the inability of thymocytes to augment growth in T-cell depleted recipients. One need only postulate an accessory cell type, missing in T-cell depleted animals, which is required for factor production by thymocytes.

However, this hypothesis too, is not without its short-comings. The model would seem to predict that B6 bone marrow should grow approximately half as well in F_1 hybrids as in syngeneic recipients, as one half of the factors produced by these mice would be stimulatory to B6 stem cells. B6 bone marrow in fact grew much more poorly than that in the (B6 X C3H) F_1 mice studied (70). In the case of BDF $_1$ mice, the model predicts that D2 marrow should grow as poorly in these animals as does B6 marrow because D2 stem cells do not cross-react with B6 factor, as seen by the Poor Growth of D2 marrow in B6 recipients (70). This

prediction however, is not borne out as D2 marrow grows nearly as well in this hybrid as it does in syngeneic recipients, and far better than does B6 marrow.

III. A NEW MODEL

Because both of the currently proposed models, detailed above, fail in some respects to adequately deal with many of the observations regarding Poor Growth, a new model is proposed. This model can be summarized as follows:

1. Hemopoietic stem cell proliferation is stimulated by short-range humoral factors which are produced by at least one subpopulation of T-cells (and quite possibly other cell types).
2. Initiation of stem cell division is brought about by the binding of these factors to specific receptors on the surface of stem cells.
3. Factors can be of two types: a "strain-specific" factor, produced under most circumstances, which exhibits varying degrees of cross-reactivity with stem cell receptor sites of other strains. This factor will be referred to as "strain-specific hemopoietic factor" (SHF). In situations of great demand for CFU proliferation, a second type of factor is produced which is entirely strain (and species) non-specific. This will be referred to as "non-specific hemopoietic factor" (NHF).
4. Genes for both SHF and their corresponding receptors are closely linked in most mouse strains.
5. In the F_1 heterozygote, both SHF and receptor alleles may

undergo an interaction which results in the phenotypic expression of SHF and receptors that may display varying degrees of cross-reactivity with parental SHF and receptors.

6. Both B6 SHF and B6 receptors tend to have a low affinity for SHF and receptors of non-B6 strains.
7. The production of SHF by T-cells requires the interaction of the SHF producing cell with a second T-cell type. This interaction does not require strain homology between the two interacting cell types.
8. Production of NHP is inhibited by NK cells, possibly by direct cytotoxicity of NHP producing cells. Homology is not required between NK and NHP producing cells in order for this inhibition to be achieved.

The proposed model predicts that since B6 receptors have a low cross-reactivity with respect to non-B6 SHF, B6 stem cells would not be stimulated to proliferate in non-B6 transplant recipients. As F_1 recipients produce SHF of a non-B6 type, marrow transplants from B6 donors would be expected to fail in these recipients, which of course is exactly the case.

The relatively good growth of D2 marrow in BDF_1 recipients can be explained within the context of this model as the result of a high cross-reactivity between D2 receptors and F_1 type SHF.

This model has several clear advantages over either of the two models detailed above. That it explains the growth of D2 marrow in BDF_1 hybrids is one such advantage over the hypothesis put forth by Prichard. In addition, it is able to deal with an expanded definition of Poor Growth which includes bone marrow failures from allogeneic (17)

and xenogeneic (17,82) donors. It has been shown for example, that prior irradiation (500R, ten days prior to lethal irradiation and bone marrow transplantation) abrogates not only the Poor Growth of parental marrow in F_1 hybrids, but the Poor Growth of completely allogeneic and xenogeneic marrow in inbred recipient mouse strains as well (82). Such observations are inexplicable by Pritchard's model, as it states that prior irradiation results only in the increased production of a relatively strain-specific factor (70). The hypothesis presented here indicates that such growth would be the result of the production of NHF, capable of stimulating allogeneic and xenogeneic stem cells, in response to the demand brought on by prior irradiation.

Whereas the inability to demonstrate Hh antigens is a clear liability to the immune response/Natural Killer hypothesis, it is perfectly compatible with the model proposed here as no such antigens are suggested. The abrogation of Poor Growth by lymphoid cells represents, according to this model, the transplantation of SHF producing cells. Thus, when B6 lymphoid cells are given to a recipient of B6 marrow, the production of syngeneic SHF by the transplanted lymphoid cells stimulates the proliferation of the marrow stem cells. The proposed role of an accessory cell in the production of SHF adequately accounts for the failure of parental thymocytes to augment bone marrow growth in T-cell depleted recipients. Future demonstrations of other cell types involved in the regulation of SHF production, while not specifically proposed here, would certainly be compatible with this new model.

Perhaps one of the most intriguing aspects of this model is that it can be used to explain both the observed parallels between Poor Growth and NK activity as well as the discrepancies between the two phenomena.

Because an inhibitory influence of NK cells on the production of NHF is proposed, the model predicts that it should be harder to abrogate Poor Growth in mice that demonstrate high NK reactivity than in mice with moderate or low NK potential. Studies involving the Poor Growth and NK activity of nude mice bear out this prediction. Nude mice have not only been shown to exhibit very high levels of NK activity (41, 42), but Poor Growth in these mice has been more difficult to abolish (8, 19).

The theory further predicts that, aside from the administration of cells which produce SHF compatible with parental marrow receptors, the ability of host pretreatments to abrogate Poor Growth will depend upon their ability to suppress NK activity. Thus the radiation sensitivity of NK cells (49) accounts for the Poor Growth in bone marrow recipients following lethal irradiation (72). Host pretreatments such as ^{89}Sr treatment (49), rabbit anti mouse bone marrow injection (49) and treatment with anti macrophage agents (49, 55, 57) have been shown to reduce NK reactivity and would therefore be predicted to result in the agumentation of parental bone marrow growth, which they do (49, 55, 57).

Although the model ascribes a role to NK cells in the Poor Growth phenomenon, the discrepancies between Poor Growth and NK activity can easily be accounted for within the context of the hypothesis. The apparent inability of F_1 NK cells to specifically lyse parental bone marrow or its stem cells does not weaken the model because the proposed function of NK cells in this system is the inhibition of NHF production rather than a direct assault on parental stem cells. Nor does the model suggest that a correlation exists between NK reactive strains and those

exhibiting Poor Growth. Poor Growth, according to this hypothesis, is the result of a lack of cross-reactivity between the SHF of one strain and the receptors for SHF of another, which leads to a poor proliferation and is not the result of direct cell killing by F_1 NK cells of parental bone marrow. For these reasons, this model is also compatible with the observations that the B6 tumor EL-4 is not susceptible to NK lysis (41, 48).

The absence of detectable NK activity in most mice until after three weeks of age (44) suggests that NHF production would be uninhibited in these animals, thus accounting for their ability to support parental bone marrow grafts. That lymphoid cells from these young mice are not capable of augmenting the growth of B6 marrow in adult BDF $_1$ recipients (Chapter V), is also compatible with the model. Lymphoid cells from young donors would, when transplanted to adult mice, become subject to the inhibitory influences of host NK cells and therefore cease NHF production.

The hypothesis put forth here would, however, predict that parental bone marrow growth in young F_1 mice should be diminished by co-transplantation of adult spleen cells, as the NK cells from adult donors should be able to inhibit NHF production by young mice. In the experiments reported here (Chapter VI), this was not the case. However this failure may have been due to a) the known liability of NK cells, b) too few NK cells in the spleen cell preparations employed or, c) the inability of NK cells to function after transplantation. Indeed, the transplantation of NK activity against tumors has similarly proven difficult. In the only report of success in this area (47), it was found that whole spleen cells were ineffective in preventing tumor growth. Only after

extensive enrichment of NK activity with the use of Ly 5.1 antiserum were transplanted spleen cells able to inhibit tumor growth. Such enrichment may be required for NK cells to reduce the production of NHF in young hybrid mice.

This model makes the rather unorthodox suggestion that NK cells may exert their inhibitory influence on NHF production by direct cytotoxicity of NHF producing cells. This proposal is not unique however as the concept that one cell type may suppress the functions of another by killing it has been proposed before (49). In the present case such a suggestion would mean that while the inability of NK cells to specifically lyse bone marrow stem cells presents no problem, it would be predicted that NK activity against NHF producing cells should be demonstrable. Such a prediction may have already been borne out. It has been reported (36, 37) that both thymus and spleen cells from mice less than three weeks old are susceptible to NK lysis and that the disappearance of this susceptibility parallels the onset of Poor Growth (37). An inverse relationship between susceptibility to NK lysis of thymus cells and the endogenous levels of NK activity has also been reported. These findings further support the proposed model of Poor Growth.

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